Journal Pre-proof

Injectable Contraceptive, Depo-Provera, Produces Erratic Beating Patterns in Patient-Specific Induced Pluripotent Stem Cell-derived Cardiomyocytes with Type 2 Long QT Syndrome

Alexa M. Pinsky, BA, Xiaozhi Gao, BS, Sahej Bains, BS, CS John Kim, PhD, Julien Louradour, PhD, Katja E. Odening, MD, PhD, David J. Tester, BS, John R. Giudicessi, MD, PhD, Michael J. Ackerman, MD, PhD

PII: S1547-5271(23)00218-7

DOI: https://doi.org/10.1016/j.hrthm.2023.03.002

Reference: HRTHM 9732

To appear in: *Heart Rhythm*

Received Date: 2 November 2022

Revised Date: 22 February 2023

Accepted Date: 1 March 2023

Please cite this article as: Pinsky AM, Gao X, Bains S, Kim CJ, Louradour J, Odening KE, Tester DJ, Giudicessi JR, Ackerman MJ, Injectable Contraceptive, Depo-Provera, Produces Erratic Beating Patterns in Patient-Specific Induced Pluripotent Stem Cell-derived Cardiomyocytes with Type 2 Long QT Syndrome, *Heart Rhythm* (2023), doi: https://doi.org/10.1016/j.hrthm.2023.03.002.

Injectable Contraceptive, Depo-Provera, Produces Erratic Beating Patterns in Patient-Specific Induced Pluripotent Stem Cell-derived Cardiomyocytes with Type 2 Long QT Syndrome

Running Title: Depo-Provera Induces Erratic Beating in LQT2 iPSC-CMs

Authors: Alexa M. Pinsky, BA[1], Xiaozhi Gao, BS[1], Sahej Bains, BS[1], CS John Kim, PhD[1], Julien Louradour, PhD[2], Katja E. Odening, MD, PhD[2], David J. Tester, BS[1], John R. Giudicessi, MD, PhD[1], and Michael J. Ackerman, MD, PhD[1]

Institutional affiliations: [1] Departments of Cardiovascular Medicine (Division of Heart Rhythm Services, Windland Smith Rice Genetic Heart Rhythm Clinic), Pediatric and Adolescent Medicine (Division of Pediatric Cardiology), and Molecular Pharmacology & Experimental Therapeutics (Windland Smith Rice Sudden Death Genomics Laboratory), Mayo Clinic, Rochester, MN.

[2] Translational Cardiology, Department of Cardiology and Department of Physiology, University Hospital Bern, University of Bern, Bern, Switzerland

Reprints and correspondence:

Michael J. Ackerman, M.D., Ph.D.

Mayo Clinic Windland Smith Rice Sudden Death Genomics Laboratory

Guggenheim 501, Mayo Clinic, Rochester, MN 55905

ackerman.michael@mayo.edu, @MJAckermanMDPhD

Conflicts of Interest AMP received the Sarnoff Cardiovascular Research Foundation Fellowship as a medical student to support her time while performing this research. MJA is a consultant for Abbott, Boston Scientific, Daiichi Sankyo, Invitae, Medtronic, and Thryv Therapeutics. MJA and Mayo Clinic are involved in an equity/intellectual property/royalty relationship with AliveCor, Anumana, ARMGO Pharma, Pfizer, and UpToDate. However, none of these entities

were involved in this study in any manner. The rest of the authors have no conflicts of interest to disclose.

Word count: 4802 words, 6 figures, 29 references

Abstract

Background: Long QT syndrome type 2 (LQT2) is caused by pathogenic variants in *KCNH2*. LQT2 may manifest as QT prolongation on an ECG and present with arrhythmic syncope/seizures, sudden cardiac arrest/death. Oral progestin-based contraceptives may increase the risk of LQT2-triggered cardiac events in women. We previously reported on a LQT2 woman with recurrent cardiac events temporally related and attributed to the progestin-based contraceptive, medroxyprogesterone acetate ("Depo-Provera", Depo).

Objective: To evaluate the arrhythmic-risk of Depo in a patient-specific induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) model of LQT2.

Methods: An iPSC-CM line was generated from a 40-year-old female with p.G1006Afs*49-KCNH2. A CRISPR/Cas9 gene-edited/variant-corrected, isogenic control (IC) iPSC-CM line was generated. FluoVolt was used to measure the action potential duration (APD) following treatment with 10 µM Depo. Erratic beating patterns characterized as alternating spike amplitudes, alternans, or early after depolarization-like phenomena were assessed using multi-electrode array (MEA) following 10 µM Depo, 1 µM isoproterenol (ISO), or combined Depo + ISO treatment.

Results: Depo treatment shortened the APD-90 of the G1006Afs*49 iPSC-CMs from 394±10 ms to 303±10 ms ($p<0.0001$). Combined Depo and ISO treatment increased the percent of electrodes displaying erratic beating in G1006Afs*49 iPSC-CMs [baseline 18±5% vs. Depo + ISO 54±5% ($p<0.0001$)] but not in IC iPSC-CMs [baseline 0±0% vs. Depo + ISO 10±3% ($p=0.9659$)].

Conclusion: This cell study provides a potential mechanism for the patient's clinically documented Depo-associated episodes of recurrent ventricular fibrillation. This in-vitro data

should prompt a large-scale clinical assessment of Depo's potential pro-arrhythmic effect in women with LQT2.

Key Words: Long QT Syndrome Type 2, Depo-Provera, Arrhythmia, iPSC, Cardiomyocytes

INTRODUCTION

Congenital long QT syndrome (LQTS) is an inherited disorder of cardiac repolarization that affects an estimated 1 in 2,000 individuals. From a cellular electrophysiology perspective, abnormal cardiac ion channel function increases ventricular action potential duration (APD) and may manifest clinically as a prolonged heart-rate corrected QT interval (QTc) on a 12-lead electrocardiogram.[1] Patients with LQTS are at increased risk for syncope, seizures, and even sudden cardiac death particularly following a triggered event associated with exertion, extreme emotion, or auditory stimuli. Three LQTS-causative genes (*KCNQ1*, *KCNH2*, and SCN5A) encoding for cardiac ion channels account for the vast majority of genetically proven cases.[2, 3]

While LQTS is typically inherited in an autosomal dominant pattern, environmental modifiers such as sex hormones affect the substrate of males and females with LQTS differently. Female sex hormones such as estrogen and progesterone play complex roles in modulating the ion channels responsible in LQTS pathogenesis. Postpubertal LQTS women are at an increased risk for cardiac events compared to postpubertal men and recent studies indicate an increased risk of cardiac events with use of progestin-only oral contraceptives, particularly in women with LQT2.[4, 5]

Previously, we reported the case of a woman diagnosed clinically and genetically with LQT2 that experienced recurrent LQT2-triggered cardiac events including several ventricular fibrillation (VF)-terminating therapies from her implantable cardioverter defibrillator (ICD) while taking medroxyprogesterone acetate (Depo-Provera, Depo), an injectable, synthetic progestin commonly used as hormonal contraception.[6] Although endogenous progesterone shortens the QT interval, the effects of Depo have never been studied.[7] Herein, we present the

first-ever evaluation of Depo in an induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) model of LQT2 derived from the woman in our original case report.

METHODS

Patient samples

Peripheral blood mononuclear cells (PBMCs) were collected at the Mayo Clinic, Rochester, MN from a 40-year-old female diagnosed with LQT2 and positive for the p.G1006Afs*49-KCNH2 pathogenic variant following written informed consent in accordance with Mayo Clinic Internal Review Board (IRB number 09-006465) approved study. To prevent the re-identification of the patient included in this study, individual patient data will not be made available to other researchers. The authors declare that all supporting data are available within the article and its online supplementary files. The research reported in this paper adhered to Helsinki Declaration guidelines.

Generation of patient-specific and isogenic control induced pluripotent stem cell derived cardiomyocytes

Generation of patient-specific induced pluripotent stem cells (iPSC) from PBMCs, creation of CRISPR/Cas9 gene-edited/variant-corrected, isogenic control (IC) iPSCs by Applied StemCell (Milipitas CA), cardiomyocyte differentiation and cardiomyocyte dissociation were performed as described previously.[8, 9] Standard iPSC and iPSC-derived cardiomyocyte (iPSC-CM) quality control measures including karyotyping, Sanger sequencing variant confirmation, and immunostaining for pluripotent markers (Tra-1-60, SSEA-3, OCT4 and Nanog) were performed as described previously.[8, 9] A list of primers and antibodies used are presented in **Supplemental Table 1** and **Supplemental Table 2**. Detailed methods are presented in the **Supplement**.

Electrophysiological property and arrhythmia analysis

The iPSC-CMs of p.G1006Afs*49-KCNH2 were assessed for their cardiac cellular electrophysiologic properties before and after administration of 10 µM Depo (medroxyprogesterone acetate). Fluovolt voltage-sensing dye was used for optical action potential duration (APD) measurements. Multi-electrode array (MEA) was used to measure field potential durations (FPD), corrected for beating frequency using the Fridericia formula (FPDc), and for assessing iPSC-CM beating patterns following administration of 10 µM Depo under both baseline and 1 µM isoproterenol (ISO) to mimic adrenergic surge conditions. Each dot in the MEA results figure represents a technical replicate (one electrode). Erratic beating frequency was adjudicated manually from electrical tracings after processing with the Comprehensive *In Vitro* Proarrhythmia Assay (CiPA) tool (Axion BioSystems) and classified as alternating spike amplitudes, alternans, or early after depolarization-like phenomena.

Detailed methods are presented in the **Supplement**.

Statistical analysis

All data points are shown as the mean value and bars represent the standard error of the mean. A Student's t-test, paired t-test, or ANOVA was performed where appropriate using GraphPad Prism 8.3 to determine statistical significance.

RESULTS

ECG evaluation of index patient captures QTc shortening while on Depo

A young woman with LQT2 was referred to Mayo Clinic for evaluation and treatment following an appropriate VF-terminating ICD shock when she was four-weeks postpartum with her first child. We subsequently discovered and reported her pathogenic variant as p.G1006Afs*49-KCNH2.[6] Her clinical case is detailed extensively in our previous 2012 case report and is visually represented in **Figure 1**.[6] At the time of both of her subsequent LQT2-triggered episodes

of torsades de pointes and VF, she was receiving Q3-month injectable subcutaneous Depo-Provera for hormonal birth control.

Electrocardiogram (ECG) data was obtained from the index patient's electronic medical record as well as dates of Depo injections. Her sentinel event was an out-of-hospital cardiac arrest at the age of 16 years, prompting a secondary prevention ICD through which she has had multiple appropriate defibrillations, most in the context of concurrent Depo use (**Figure 1A**). The patient's mean lifetime QTc is 520 ms with her maximum recorded QTc at 610 ms. **Figure 1B** and **Figure 1C** display a representative ECG while off Depo, demonstrating a QTc of 547 ms. This ECG was taken after her appropriate AICD shock while on 50 mg Atenolol (this beta blocker was started elsewhere). A few years later, shortly after another appropriate ICD therapy for VF, her QTc was recorded at 475 ms while receiving Depo injections (**Figure 1B** and **Figure 1C**).

Generation of p.G1006Afs*49-KCNH2 patient-specific LQT2 iPSC-CM line

An iPSC cell line was created from the young woman who has the p.G1006Afs*49-KCNH2 pathogenic variant. A CRISPR/Cas9 gene-edited/variant-corrected, isogenic control (IC) iPSC line was also created. Immunofluorescence for pluripotency markers (Tra-1-60, SSEA-3, OCT4 and Nanog) was confirmed in the p.G1006Afs*49-KCNH2 iPSCs (**Figure 2A**) and iPSC-CMs demonstrated cardiomyocyte markers (**Figure 2B**). Sanger sequencing of both p.G1006Afs*49-KCNH2 and IC iPSCs confirmed the expected variants (**Figure 2C**). Additionally, the patient's iPSCs displayed a normal female karyotype (**Figure 2D**).

Treatment with Depo shortens the cellular action potential duration (APD) and field potential duration (FPD) of iPSC-CM models of LQT2.

To examine whether Depo alters APD measurements in 2-D cell culture, Fluovolt voltage-sensing dye was used to assess p.G1006Afs*49-KCNH2 iPSC-CMs at baseline and after treatment with 10 µM Depo. Representative tracings are displayed in **Figure 3A**. Compared to baseline (394±10 ms, n=35), treatment with 10 µM Depo significantly shortened the APD90 in the p.G1006Afs*49-KCNH2 iPSC-CMs (303±10 ms, n=42, p<0.0001; **Figure 3B, Supplementary Table 3**). Additionally, Depo treatment resulted in shortening of both the APD50 (baseline: 318±8ms versus Depo: 208±4 ms; p<0.0001) and the APD30 (baseline: 275±7 ms versus Depo: 175±3 ms; p<0.0001, **Figure 3B**) in the p.G1006Afs*49-KCNH2 iPSC-CMs.

Similarly, MEA-measured FPD corrected using the Fridericia formula (FPDc) was significantly shorter in p.G1006Afs*49-KCNH2 iPSC-CMs treated with 10 µM Depo (364±9 ms, n=49) compared to untreated p.G1006Afs*49-KCNH2 iPSC-CMs (406±6 ms, n=161, p=0.003, **Figure 4**).

Depo treatment with sympathetic stimulation increases the propensity for arrythmia in p.G1006Afs*49-KCNH2 iPSC-CMs

Erratic beating patterns have been characterized previously in LQT2 human iPSC-CMs using MEA.[10] In our study, there was a marked difference in the frequency of erratic beating between p.G1006Afs*49-KCNH2 (18±5%, n=25) and IC iPSC-CMs (0±0%, n=15, p= 0.0065, **Figure 5, Supplemental Table 4**) under baseline conditions, where each data point represents the sum of electrodes displaying behavior as a percentage of total beating electrodes. However, there was no significant change in the erratic beating patterns of the IC iPSC-CMs (0±0%, n=15) following 10 µM Depo (1±1%, n=10, p= >0.9999), 1 µM ISO (14±5%, n=16, p= 0.8337), or combined 10 µM Depo + 1 µM ISO (10±3%, n=13, p= 0.9659) treatment (**Supplemental Table 3**).

Although there was no significant increase in frequency of erratic beating in the G1006Afs*49 iPSC-CMs (18±5%, n=25) following 10 µM Depo (35±8%, n=15, p= 0.5290) or 1 µM ISO (39±8%, n=21, p= 0.1515) treatment alone, combined 10 µM Depo plus 1 µM ISO treatment increased significantly the percent of electrodes displaying erratic beating in p.G1006Afs*49-KCNH2 iPSC-CMs (54±5%, n=38, p= <0.0001, **Figure 5, Supplemental Table 3**).

Nadolol treatment reduces propensity of arrhythmia in p.G1006Afs*49-KCNH2 iPSC-CMs

Per guideline-directed therapy, our patient's original beta blocker therapy with atenolol was changed to one of the two preferred beta blockers, nadolol. Treatment with 10 µM nadolol reduced the frequency of erratic beating observed following 10 µM Depo plus 1 µM ISO in p.G1006Afs*49-KCNH2 iPSC-CMs from 54±5% (n=38) to 36±6% (n=30, p=0.0292, **Figure 6**).

DISCUSSION

Herein, we describe for the first time a patient-specific, iPSC-CM study to provide a potential mechanism for a LQT2 patient's clinically documented, Depo-associated episodes of recurrent ventricular fibrillation. Sex hormones, particularly estrogen and progesterone, modulate arrhythmogenic risk in LQTS, though exact mechanisms are complex.[7] Hormonal culpability is illustrated clearly for women with LQTS in general and LQT2 in particular. For example, their burden of cardiac events is markedly higher during the 9 months post-partum when compared to the 40 weeks before pregnancy or the 40 weeks of pregnancy.[11] Additionally, the postpartum period is associated with a decrease in progesterone levels compared to levels during pregnancy. Furthermore, progestin-based contraceptives such as the mini-pill and Depo are common forms

of postpartum birth control as they lack the thrombotic risk that estrogen-based contraceptives carry.[12] According to the Center for Disease Control, 24% of women ages 15-49 have used Depo.[13]

A recent large-scale clinical study found that progestin-only oral contraceptive use in women with LQT2 without concomitant beta blocker use increased risk of recurrent cardiac events by 8-fold.[4] Despite these new clinical observations, there is a paucity of studies evaluating the biology of hormonal contraception in LQTS women. Herein, we detail the electrophysical properties of the injectable progestin-based contraceptive, Depo, on a novel patient-derived LQT2 iPSC-CM cell model.

In 2012, we described a young woman diagnosed with LQT2 who experienced multiple episodes of ventricular fibrillation while receiving Q-3 month injections of Depo for hormonal contraception.[6] The patient has an LQT2-causative pathogenic variant in *KCNH2* resulting from a single-nucleotide deletion causing a frameshift mutation and subsequent amino acid C-terminus truncation, annotated as G1006Afs*49. Canonically, frameshift variants such as p.G1006Afs*49-KCNH2 result in haploinsufficiency via non-sense mediated decay. However, recent *in vitro* data suggests that p.G1006Afs*49-KCNH2 does not follow this rule. Interestingly, the work by Zio et al. demonstrates that p.G1006Afs*49-KCNH2 results in a truncated Kv11.1/hERG protein capable of reaching the plasma membrane where it exerts a dominant-negative effect.[14]

The patient's QTc was notably shorter while on Depo. To determine if our cellular model recapitulated this clinical phenotype, we evaluated the patient's iPSC-CMs with Depo treatment. After successful creation of the patient's p.G1006Afs*49-KCNH2 iPSC-CM line, cellular measurements of APD and FPDc were used as a cellular surrogate for the QT interval.

Consistent with the patient's clinical phenotype, Depo treatment shortened both the APD and FPDc in her p.G1006Afs*49-KCNH2 iPSC-CMs.

Until now, the synthetic progestin Depo has not been evaluated for its effects on the cardiac APD. However, endogenous progesterone shortens the QT interval through a variety of mechanisms including activation of slow delayed rectifier potassium currents (I_{Ks}) and inhibition of L-type calcium channels ($I_{Ca\text{-}L}$).[7, 15, 16] Studies using guinea pig ventricular myocytes have demonstrated that these nongenomic actions of progesterone work via a nitric-oxide synthase signal transduction pathway.[17] Furthermore, progesterone binding to sigma-receptors modulates voltage-gated sodium ion current, enhancing its QT-shortening effect.[18] Additionally, LQT2 rabbit studies reveal antiarrhythmic progesterone effects (mainly due to APD-shortening and SERCA activation).[19] Clinical studies corroborate the QT-shortening effect of endogenous progesterone.[20, 21] Furthermore, progesterone was protective against drug-induced QT prolongation in a randomized double-blind placebo study with healthy women.[22]

As Depo is a synthetic progestin, it exerts many progestogenic antiproliferative effects on the uterine endometrium conferring its high efficacy as a contraceptive. However, from a cardiovascular standpoint, the effects of Depo are often opposite from endogenous progesterone, warranting these hormones' individual evaluation on arrhythmogenic risk.[23] Our results of Depo-mediated APD shortening in an iPSC-CM model cast new light on the effect of Depo in LQTS and align its QT-action with that of progesterone. The specific mechanism for this Depo-mediated APD shortening remains elusive and should prompt further mechanistic evaluation, however, its actions may be like endogenous progesterone. With our data, one might expect postpartum use of Depo to shorten the QT interval, but this paradox of increased cardiac events

in our patient and her cardiomyocytes may be due to variable spatial distribution and effect of the hormone leading to transmural heterogeneity.

Genotype-phenotype correlation studies have demonstrated that LQT2 patients are at increased risk of cardiac events with sudden auditory triggers, believed to be mediated through beta-adrenergic stimulation and ameliorated with beta-adrenergic antagonists such as beta blockers (β-blockers). LQT2 women are predisposed to life-threatening arrythmias such as torsades de point, especially at times of sudden arousal and heightened sympathetic activity.[24] Therefore, we used the beta-adrenergic agonist, isoproterenol (ISO), to mimic this adrenergic surge that places LQT2 patients at increased risk of cardiac events.[25] When treated with Depo plus ISO, a 3-D cellular syncytium of p.G1006Afs*49-KCNH2 iPSC-CMs showed a significant increase in erratic beating activity. Interestingly, there was no significant change in the erratic beating patterns of the IC iPSC-CMs following treatment with Depo and ISO. One strength of evaluating the cardiomyocytes in MEA, over other single-cell methods, is the ability to record electrophysiologic properties from a cellular syncytium. Although the canonical mechanism of action potential prolongation digressing into a dangerous arrythmia such as torsades de pointes is via an EAD- or delayed after depolarization (DAD)-mechanism, it is possible that Depo is shortening the APD of individual cells to varying degrees leaving a multi-layered, cellular syncytium vulnerable to heterogeneous refractory periods in the wells. Clinically, this could mean that during sympathetic stimulation, Depo's progesterone-like attenuation of the QT interval is being countered by a pro-arrhythmic accentuation in transmural dispersion of repolarization culminating in the clinical observation of increased LQTS-triggered cardiac events in women using this form of contraception. Adding to the complexity, Depo binds to other steroid receptors, including glucocorticoid and estrogen receptors, leading to another potential

mechanism of Depo's arrhythmogenicity in this patient's cells and a potential source of follow-up studies.[26] As studies in LQT2 and wildtype rabbits revealed that estradiol exerts proarrhythmic effects by increasing $I_{Ca\text{-}L}$ and sodium calcium exchanger NCX activity and expression thereby facilitating EADs[19,16], Depo might exert some of its proarrhythmic effects also via its agonistic binding to estrogen receptors.

Nonselective beta-adrenoreceptor antagonists (β-blockers), such as nadolol, are first-line pharmacologic agents for LQTS in general including LQT2.[27] After each of her recurrent cardiac events, the index patient was briefly on nadolol therapy, which was discontinued due to symptomatic intolerance. Of note, both incidences of the patient's Depo-associated cardiac events happened following β-blocker cessation. Our study demonstrates that in the presence of combined Depo and ISO, nadolol treatment reduces the frequency of erratic beating in p.G1006Afs*49-KCNH2 cardiomyocytes. This is similar to nadolol's ability to ameliorate electrophysiological abnormalities in isoprenaline-treated human iPSC-CMs as measured by MEA.[10]

β-blocker therapy significantly attenuates the risk of cardiac events in women diagnosed with LQTS taking progestin-only oral contraceptives.[4] Given our findings, it is possible that Depo, also a progestin-only form of contraception, might pose similar arrhythmogenic risk to women with LQT2 and β-blocker therapy might mitigate these risks. Since our patient was on Depo without β-blocker coverage, this may have left her with a particularly vulnerable arrhythmogenic risk profile, and we believe that the use of Depo in the setting of adrenergic stimulation in the absence of β-blocker therapy could have precipitated her cardiac events. Furthermore, arrhythmias always occurred during the postpartum period (in which she received the medroxyprogesterone acetate); and therefore, the pro-arrhythmic triggers may be even more

complex as during the postpartum several other potentially pro-arrhythmic hormones are at play, such as oxytocin and prolactin that both block I_{Ks}.[28]

Although the study by Goldenberg et al. did not evaluate Depo, it suggests the formulation of the contraceptives matters, specifically finding progestin-only oral contraceptives to carry the largest risk to LQT2 women.[4] Other studies have determined that oral-contraceptive QT-mediation may depend on the progestin androgenicity, specifically that pro-androgenic first- and second-generation oral contraceptives shorten the QTc, while anti-androgenic fourth-generation oral contraceptives lengthen the QTc.[29] Depo has been shown to be androgenic, while progesterone is antiandrogenic. Our observation of Depo shortening the QTc in our patient and shortening the APD in our cells matches with other observations of pro-androgenic contraceptives, potentially suggesting that Depo's hormonal influence and arrhythmogenic potential may extend beyond its shared-chemical origin with progesterone.[17, 21] The similar and dissimilar characteristics of exogenous progestins as it pertains to channelopathies are complex, and further studies are needed to parse apart the differential action of progestin-based contraceptives, Depo included.

CONCLUSION

This study marks the first-ever 'disease in a dish' model of the injectable contraceptive Depo-Provera (Depo) in LQTS. Although the concentration of Depo used here with iPSC-CM cellular models is significantly higher than the plasma concentrations measured in preclinical animal models, our results indicate that Depo induces erratic beating patterns in human patient-derived re-engineered heart tissue under sympathetic, β-adrenergic stimulation with isoproterenol. β-adrenergic antagonism with nadolol treatment significantly attenuates this erratic beating frequency in LQT2 patient-derived iPSC-CMs treated with Depo and isoproterenol. Further

large-scale prospective clinical evaluations of Depo's pro-arrhythmic potential are needed as well as a detailed mechanistic study of this hormone's pathophysiology as it pertains to LQTS.

Acknowledgements

AMP would like to thank the Sarnoff Cardiovascular Research Foundation for their support and mentorship throughout this research.

Sources of Funding

This work was supported by the Windland Smith Rice Comprehensive Sudden Cardiac Death Program, Rochester, Minnesota (M.J.A.).

REFERENCES

1. Sencen L. Long QT Syndrome. NORD (National Organization for Rare Disorders).

2. Alders M, Bikker H, Christiaans I. Long QT Syndrome. In: Adam MP, Everman DB, Mirzaa GM, Pagon RA, Wallace SE, Bean LJH, Gripp KW, Amemiya A, eds. GeneReviews®. Seattle (WA): University of Washington, Seattle; 1993.

3. Tester DJ, Ackerman MJ. Genetics of long QT syndrome. Methodist DeBakey Cardiovasc J 2014;10:29-33.

4. Goldenberg I, Younis A, Huang DT, Yoruk A, Rosero SZ, Cutter K, et al. Use of oral contraceptives in women with congenital long QT syndrome. Heart Rhythm 2022;19:41-48.

5. Locati EH, Zareba W, Moss AJ, Schwartz PJ, Vincent GM, Lehman MH, et al. Age- and sex-related differences in clinical manifestations in patients with congenital long-QT syndrome: findings from the international LQTS registry. Circulation 1998;97:2237-2244.

6. Giudicessi JR, Brost BC, Traynor KD, Ackerman MJ. Potential depot medroxyprogesterone acetate–triggered torsades de pointes in a case of congenital type 2 long QT syndrome. Heart Rhythm 2012;9:1143-1147.

7. Sedlak T, Shufelt C, Iribarren C, Merz CN. Sex hormones and the QT interval: a review. J Womens Health (Larchmt) 2012;21:933-941.

8. Dotzler SM, Kim CSJ, Gendron WAC, Zhou W, Ye D, Bos JM, et al. Suppression-replacement KCNQ1 gene therapy for type 1 long QT syndrome. Circulation 2021;143:1411-1425.

9. O'Hare BJ, John Kim CS, Hamrick SK, Ye D, Tester DJ, Ackerman MJ. Promise and potential peril with lumacaftor for the trafficking defective type 2 long-QT syndrome-causative variants, p.G604S, p.N633S, and p.R685P, using patient-specific re-engineered cardiomyocytes. Circ Genom Precis Med 2020;13:466-475.

10. Matsa E, Rajamohan D, Dick E, Young L, Mellor I, Staniforth A, et al. Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. Eur Heart J 2011;32:952-962.

11. Seth R, Moss AJ, McNitt S, Zareba W, Andrews ML, Qi M et al. Long QT syndrome and pregnancy. J Am Coll Cardiol 2007;49:1092-1098.

12. US Medical Eligibility Criteria (US MEC) for Contraceptive Use, 2016 | CDC. 2022/05/18/T04:30:30Z 2022.

13. NSFG - Listing C - Key statistics from the national survey of family growth. 2019/11/06/T07:43:29Z 2019.

14. De Zio R, Gerbino A, Forleo C, Pepe M, Milano S, Favale S, et al. Functional study of a KCNH2 mutant: Novel insights on the pathogenesis of the LQT2 syndrome. J Cell Mol Med 2019;23:6331-6342.

15. Grouthier V, Moey MYY, Gandjbakhch E, Waintraub X, Funck-Brentano C, Bachelot A, et al. Sexual dimorphisms, anti-hormonal therapy and cardiac arrhythmias. Int J Mol Sci 2021;22:1464.

16. Odening KE, Koren G. How do sex hormones modify arrhythmogenesis in long QT syndrome? Sex hormone effects on arrhythmogenic substrate and triggered activity. Heart Rhythm 2014;11:2107-2115.

17. Nakamura H, Kurokawa J, Bai C-X, Asada K, Xu J, Oren RV, et al. Progesterone regulates cardiac repolarization through a nongenomic pathway. Circulation 2007;116:2913-2922.

18. Johannessen M, Fontanilla D, Mavlyutov T, Ruoho AE, Jackson MB. Antagonist action of progesterone at σ-receptors in the modulation of voltage-gated sodium channels. Am J Physiol Cell Physiol 2011;300:C328-337.

19. Odening KE, Choi B-R, Liu GX, Hartmann K, Ziv O, Chaves L, Schofield L, Centracchio J, Zehender M, Peng X, Brunner M, Koren G. Estradiol promotes sudden cardiac death in transgenic long QT type 2 rabbits while progesterone is protective. Heart Rhythm 2012/05// 2012;9:823-832.

20. Nakagawa M, Ooie T, Takahashi N, Taniguchi Y, Anan F, Yonemochi H, et al. Influence of menstrual cycle on QT interval dynamics. Pacing Clin Electrophysiol 2006;29:607-613.

21. Odening KE, Koren G, Kirk M. Normalization of QT interval duration in a long QT syndrome patient during pregnancy and the postpartum period due to sex hormone effects on cardiac repolarization. HeartRhythm Case Rep 2016;2:223-227.

22. Tisdale JE, Jaynes HA, Overholser BR, Sowinski KM, Flockhart DA, Kovacs RJ. Influence of oral progesterone administration on drug-induced QT interval lengthening: A randomized, double-blind, placebo-controlled crossover study. JACC Clin Electrophysiol 2016;2:765-774.

23. Hermsmeyer RK, Thompson TL, Pohost GM, Kaski JC. Cardiovascular effects of medroxyprogesterone acetate and progesterone: a case of mistaken identity? Nat Rev Cardiol 2008;5:387-395.

24. Kim JA, Lopes CM, Moss AJ, McNitt S, Barsheshet A, Robinson JL, et al. Trigger-specific risk factors and response to therapy in long QT syndrome type 2. Heart Rhythm 2010;7:1797-1805.

25. Khositseth A, Tester DJ, Will ML, Bell CM, Ackerman MJ. Identification of a common genetic substrate underlying postpartum cardiac events in congenital long QT syndrome. Heart Rhythm 2004;1:60-64.

26. Odening KE, Choi B-R, Koren G. Sex hormones and cardiac arrest in long QT syndrome: does progesterone represent a potential new antiarrhythmic therapy? Heart Rhythm 2012;9:1150-1152.

27. Han L, Liu F, Li Q, Qing T, Zhai Z, Xia Z, et al. The efficacy of beta-blockers in patients with long QT syndrome 1-3 according to individuals' gender, age, and QTc intervals: A network meta-analysis. Front Pharmacol 2020;11:579525.

28. Bodi I, Sorge J, Castiglione A, Glatz SM, Wuelfers EM, Franke G, et al. Postpartum hormones oxytocin and prolactin cause pro-arrhythmic prolongation of cardiac repolarization in long QT syndrome type 2. Europace 2019;21:1126-1138.

29. Sedlak T, Shufelt C, Iribarren C, Lyon LL, Bairey Merz CN. Oral contraceptive use and the ECG: evidence of an adverse QT effect on corrected QT interval. Ann Noninvasive Electrocardiol 2013;18:389-398.

402 **FIGURES**
403
404
405
406 **Figure 1 | LQT2 patient's cardiac timeline and ECG data on and off Depo.** Panel **A** displays
407 a patient's LQTS-related cardiac timeline from the case published by *Giudicessi JR & Ackerman*
408 *MJ et al, 2012, Heart Rhythm*.[6] Panel **B** displays the patient's ECGs on and off Depo. In Panel **C**
409 is the overlay of Lead I tracings from the ECGs in panel B, demonstrating QT-shortening on
410 Depo. Timeline image created using Biorender.com.

411

412

413 **Figure 2 | Generation of patient-specific iPSC-CMs.** In Panel **A** is Sanger sequence
414 confirmation of *KCNH2* Exon 13 G1006Afs*49 iPSCs heterozygous frameshift mutation with
415 isogenic control sequencing data shown for reference. Shown in panel **B** is a normal female
416 karyotype from G1006Afs*49-iPSCs and the isogenic control iPSCs. In panel **C** are
417 representative confocal images of undifferentiated patient-specific variant G1006Afs*49 iPSCs
418 and isogenic control iPSCs demonstrating 4 pluripotency markers (Tra-1-60, NANOG, SSEA4,
419 and OCT4). Panel **D** demonstrates G1006Afs*49 iPSC-CMs and isogenic control iPSC-CMs
420 with mature cardiomyocyte markers [α-actinin and cardiac troponin-T (c-TNT)] following
421 cardiac differentiation.

422

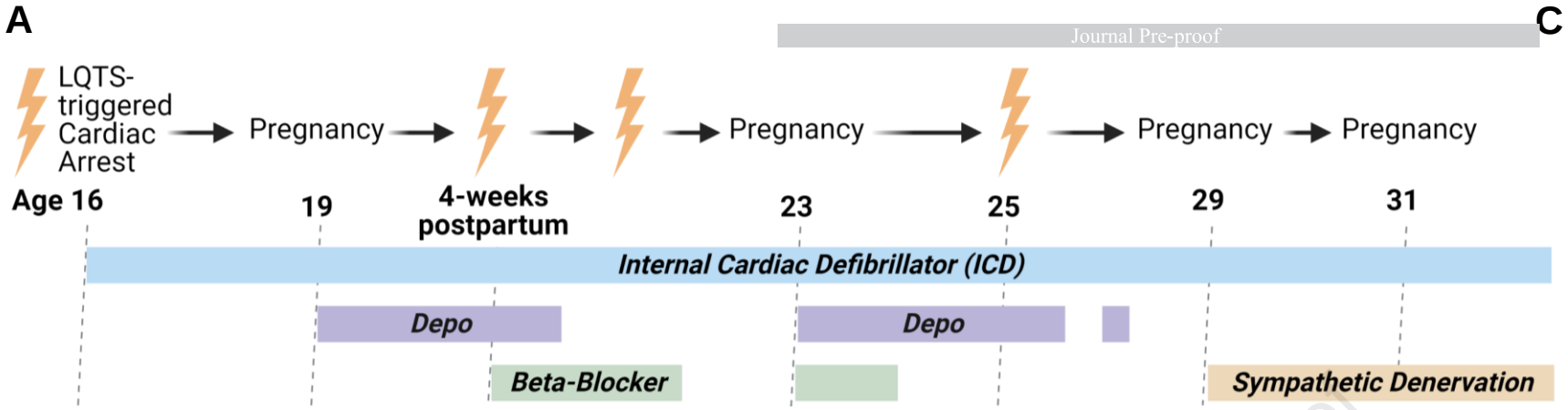
Figure 3 | Depo shortens action potential duration in p.G1006Afs*49-KCNH2 variant iPSC-CMs. Panel **A** shows representative tracings of p.G1006Afs*49-KCNH2 iPSC-CMs using FluoVolt voltage-sensing dye. Baseline is in black and 10 µM Depo treatment is in orange. In panel **B** are quantifications of APD_{90}, APD_{50}, and APD_{30} values at baseline (gray) n=35 and with Depo (orange) n=42. Values above axis represent means, error bars are SEM. Data shown is from 3 independent differentiations & independent experiments. **** = p<0.0001.

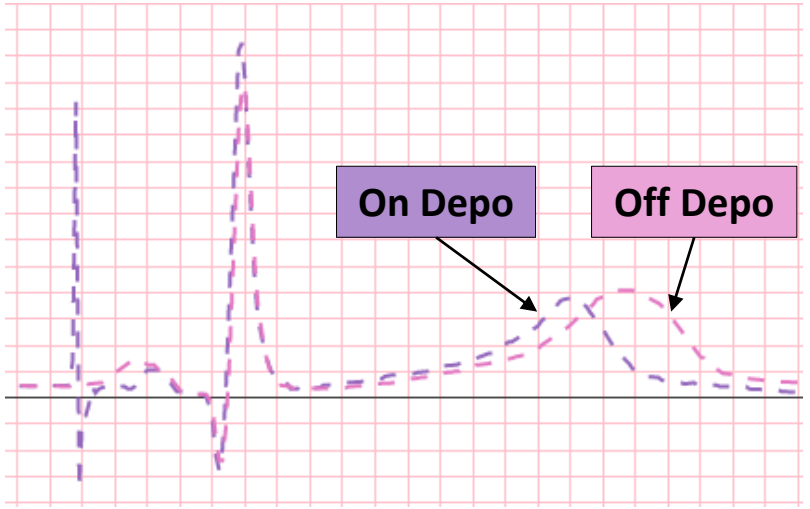
Figure 4 | Depo shortens field potential durations in p.G1006Afs*49-KCNH2 iPSC-CMs. **A.** Representative tracings of raw field potential durations of isogenic control cells at baseline and with Depo treatment. Quantification of FPDc values with **B.** isogenic control cells at baseline (gray) n=103 and with 10 µM Depo treatment (orange) n=93, value above axis represents means. Error bars represent SEM. ns = p>0.05. **C.** Representative tracings of raw field potential durations of G1006A*fs49 cells. **D.** KCNH2-G1006A*fs49 cells at baseline (gray) n=161 and with 10 µM Depo treatment (orange) n=49, value above axis represents means (p=0.0003). Error bars represent SEM. *** = p=0.0003. Each dot in panel B and D represents a technical replicate (one electrode).

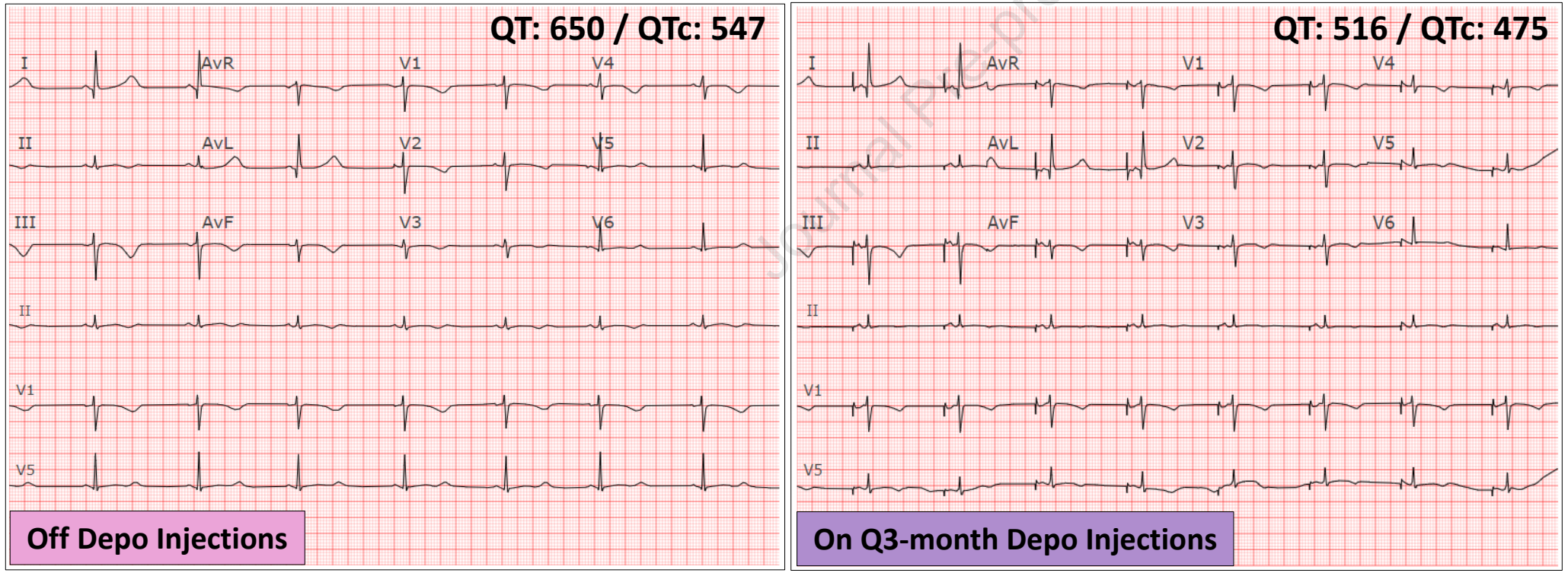
Figure 5 | LQT2 iPSC-CMs with DEPO + ISO demonstrate erratic beating in multi-electrode array (MEA). Panel **A** shows a representative tracing of erratic beating patterns

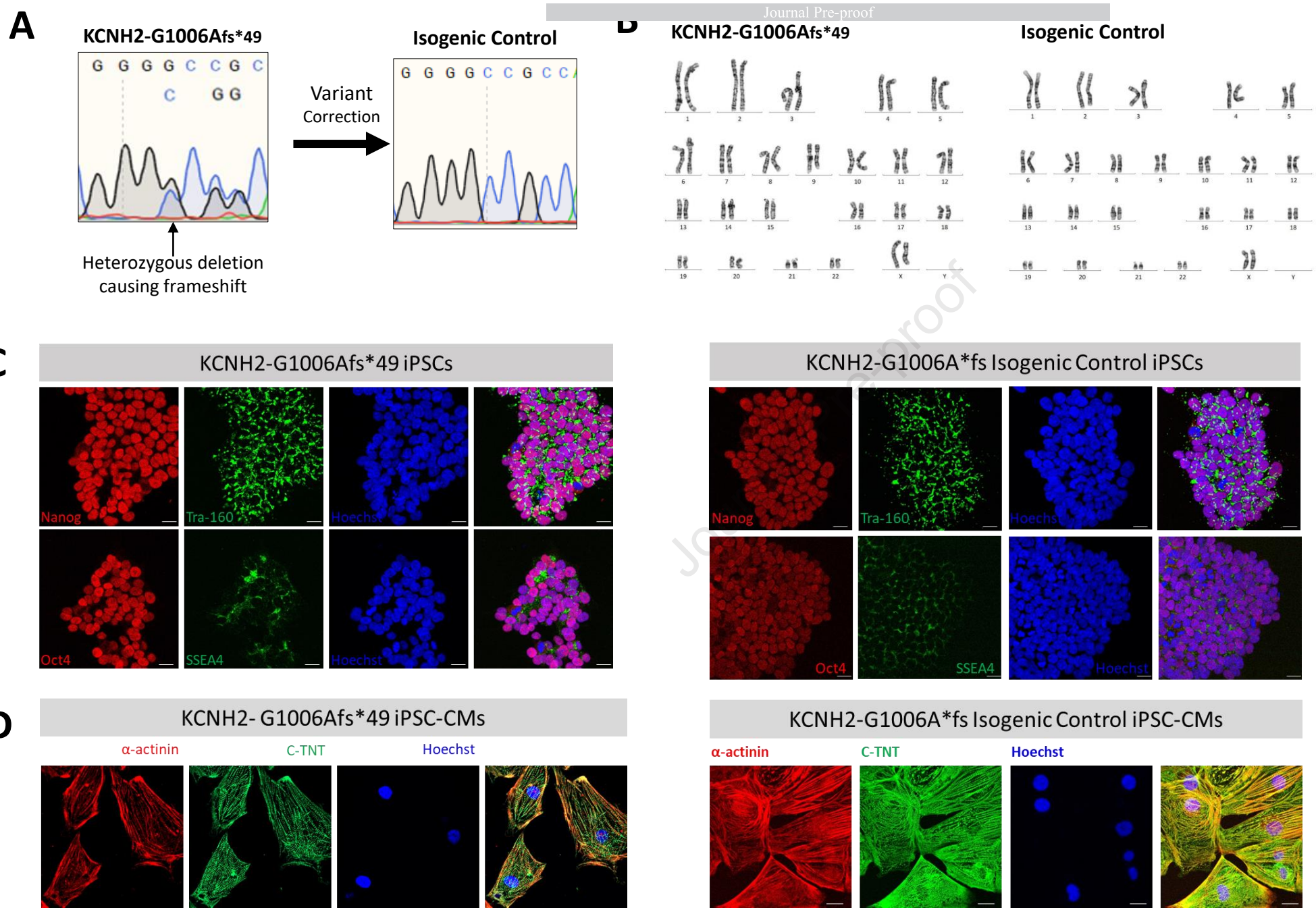
p.G1006Afs*49-KCNH2 cells' electrical activity before (left column, black) and after (right column, purple) addition of 10 µM Depo + 1 µM ISO. Panel **B** demonstrates the beating period before (left column, black) and after (right column, purple) addition of Depo + ISO. Panel **C** shows quantification of proportion of electrodes with erratic beating patterns in isogenic control and p.G1006Afs*49-KCNH2 iPSC-CMs. Conditions include baseline (gray), Depo (orange), combined Depo + Isoproterenol (ISO) treatment (purple), and ISO alone (green). Erratic beating frequency was defined as dropped beats, alternans, or early after depolarization-like phenomena. Error bars represent SEM. p.G1006Afs*49-KCNH2 is the combination of 2 cell line clones, individual clone data as well as replicates and statistical values can be found in the supplemental material.

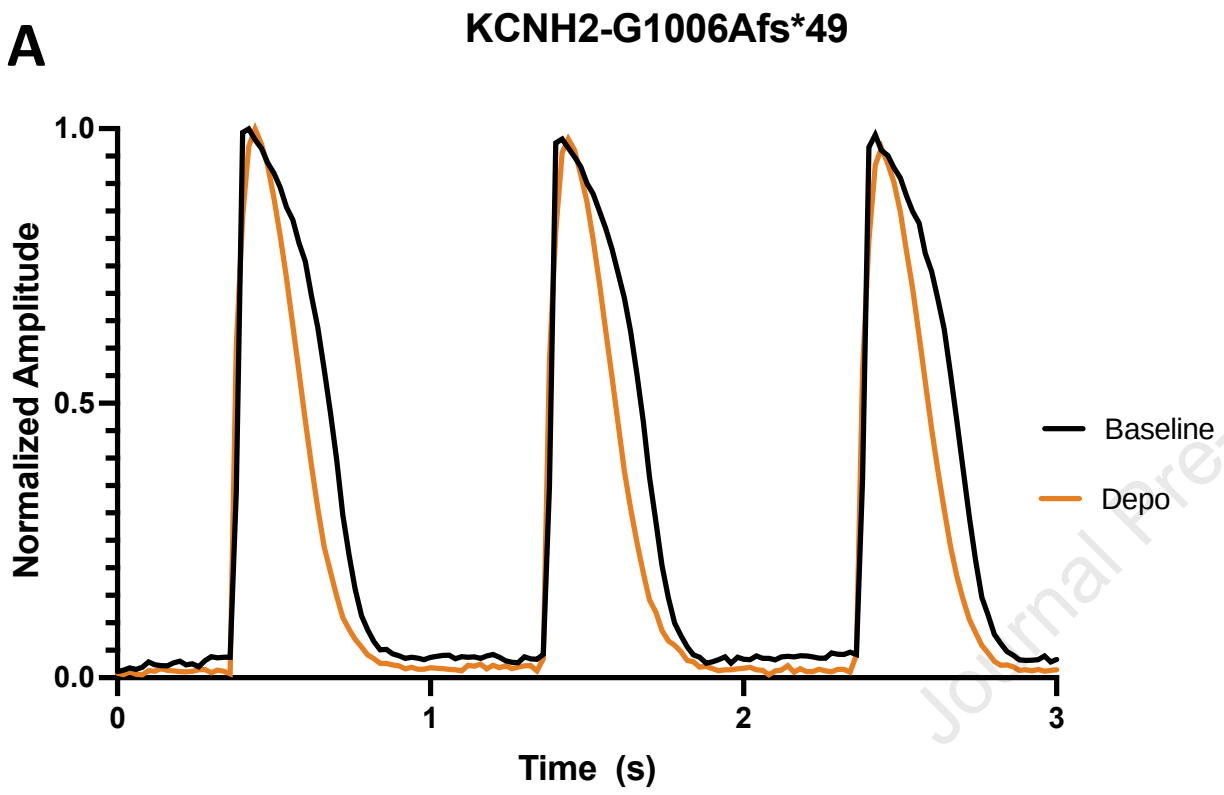
Figure 6 | Nadolol reduces erratic beating in p.G1006Afs*49-KCNH2 iPSC-CMs with Depo + ISO. Panel **A** shows quantification of the proportion of electrodes in each well demonstrating erratic beating with Depo and ISO (purple) and Depo + ISO + 10 µM Nadolol (Blue) in p.G1006Afs*49-KCNH2 iPSC-CMs; p=0.0292. Cells were pretreated with 10 µM Depo for 30 minutes before treatment and incubation with 1 µM ISO ± 10 µM Nadolol for 10-12 minutes at time of measurement. Data represents the combination of 2 cell G1006Afs*49 clones, n=38 (Depo + ISO) and n=30 (Depo + ISO + Nadolol), from 4 independent experiments. Error bars represent SEM.

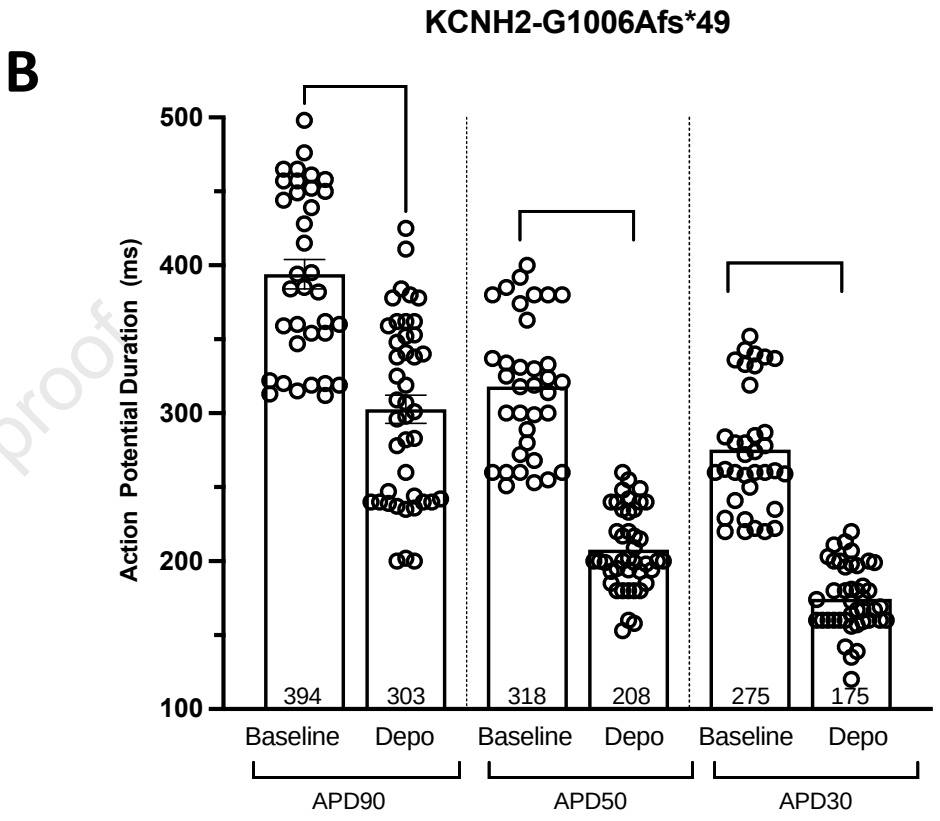


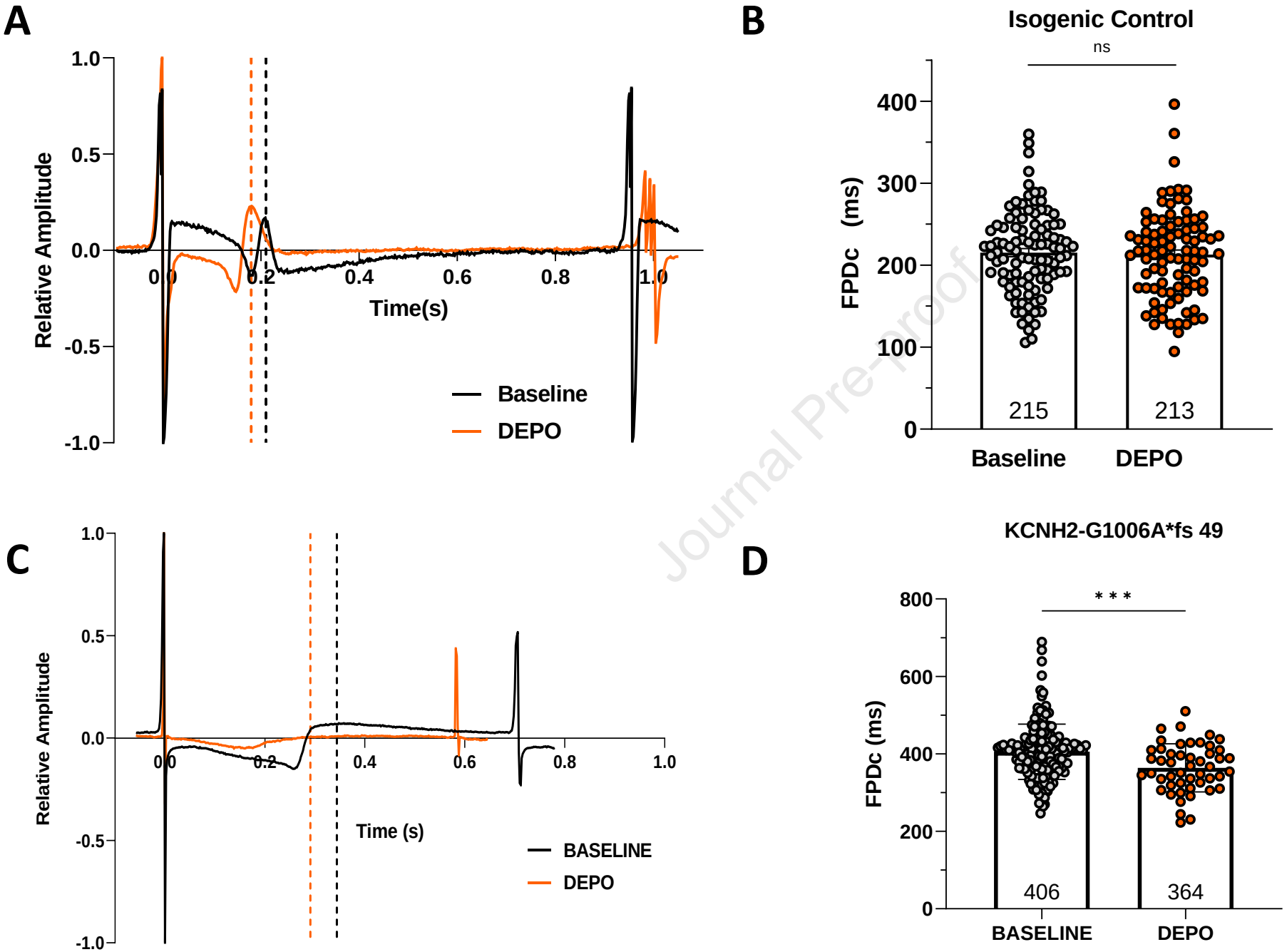


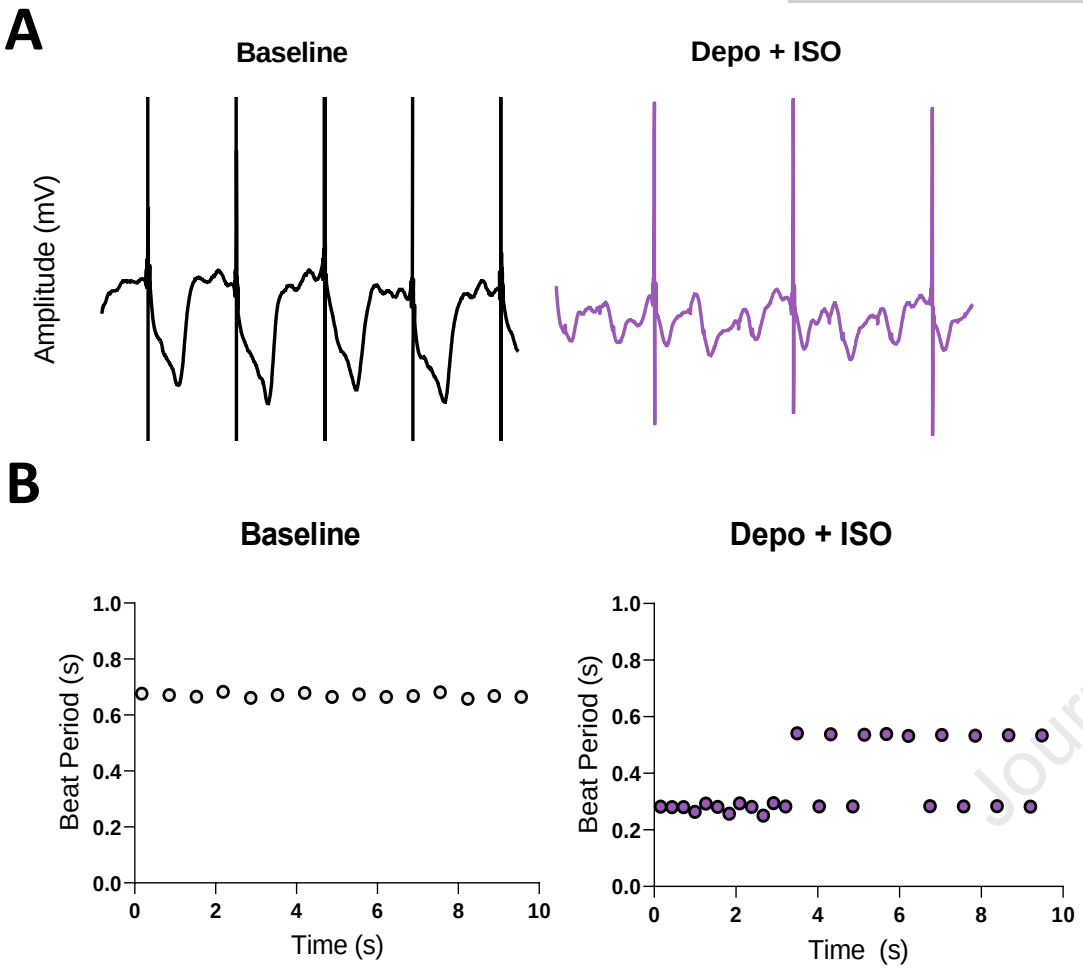


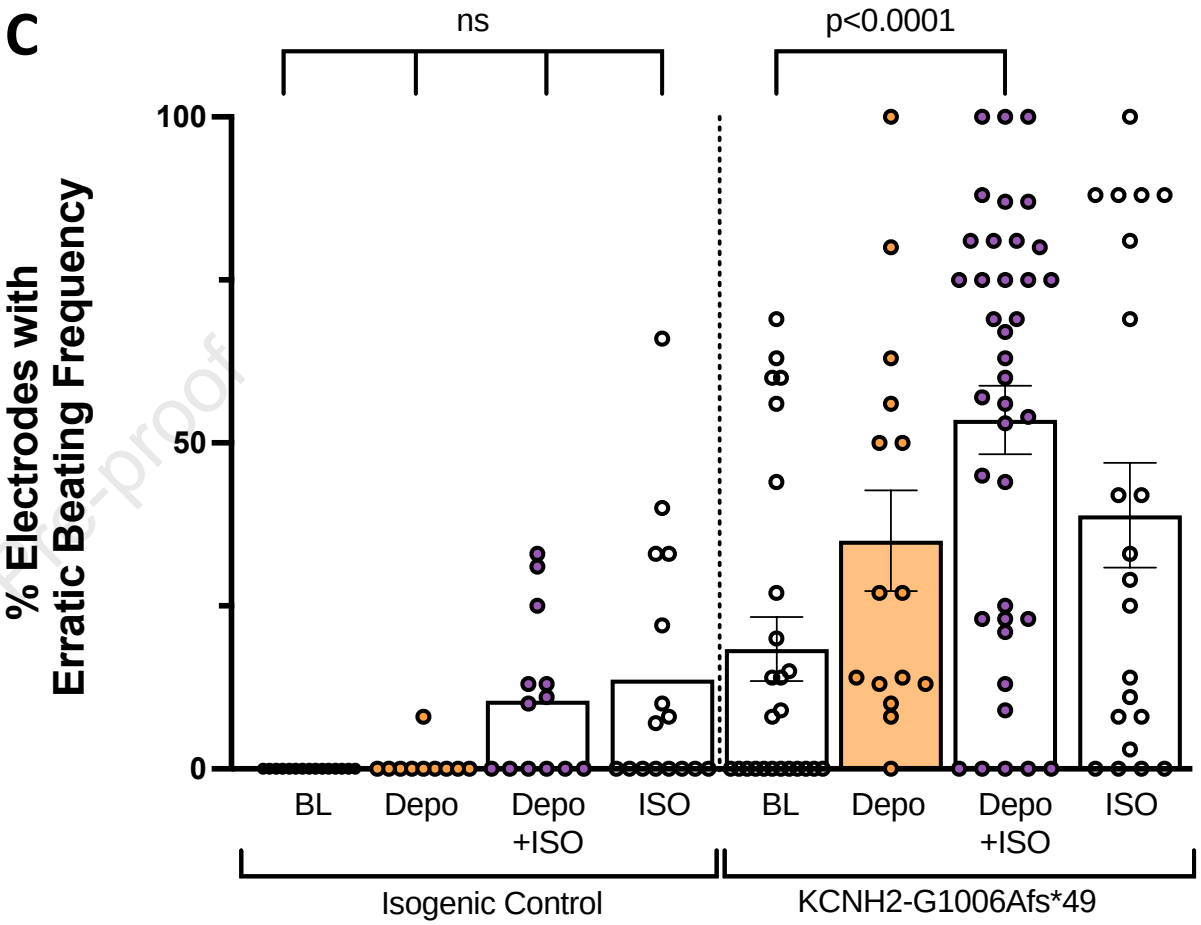


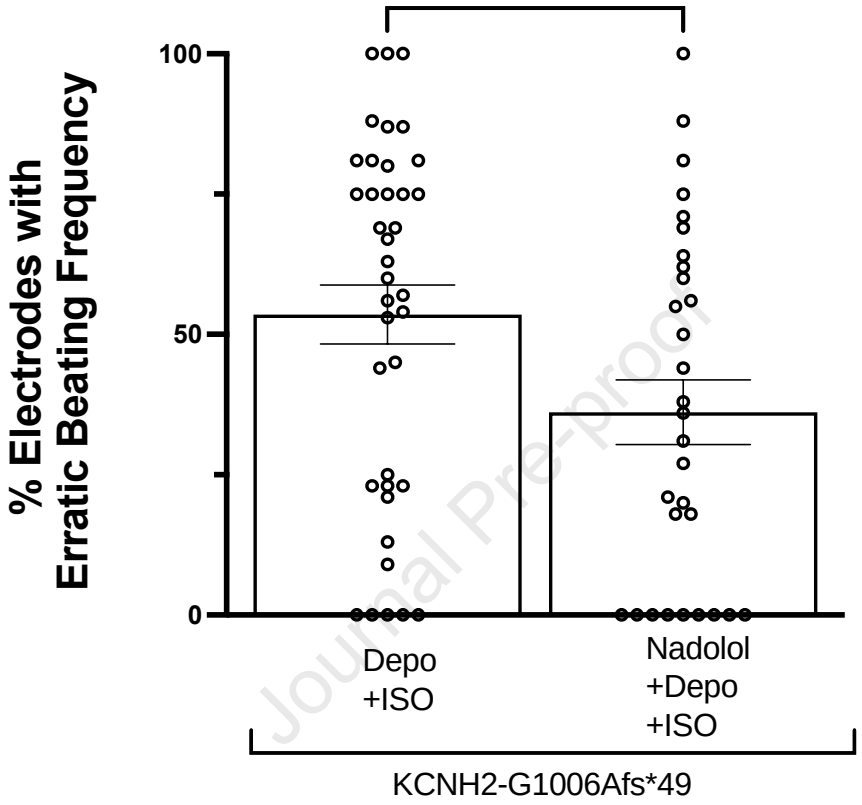












SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Generation, Maintenance, and Quality Control of Reprogrammed iPSCs

Patient PBMCs were processed and stored in liquid nitrogen by the Mayo Clinic Regenerative Medicine Biotrust Facility. Frozen PBMCs were thawed quickly and reprogramed via transduction into induced pluripotent stem cells (iPSCs) using the CytoTune-iPS 2.0 Sendai Reprogramming kit. Colonies were picked 2 weeks after nucleofection with Yamanaka's factors. Following reprogramming and expansion of single colonies, Sanger sequencing in both clones derived from the index patient confirmed the mutation, p.G1006Afs*49-KCNH2, using primer sequences as shown in **Supplemental Table 1**. Immunostaining with pluripotency markers, Nanog, SSEA4, Tra-160, Oct4 showed expression in all clones (**Supplemental Table 2**).

iPSCs were cultured in mTeSR Plus [STEMCELL; Vancouver, Canada] supplemented with 1% antibiotic/antimycotic solution on Matrigel-coated [Corning; Corning, NY] 6-cm culture and 6-well dishes in a 5% CO_2 incubator at 37°C. At 80-90% confluence, iPSCs were passaged using ReLeSR [STEMCELL Technologies, 05872].

Generation of CRISPR-Cas9 Variant Corrected Isogenic Control

Isogenic "variant corrected" control iPSCs were generated using CRISPR-Cas9 technology via contract with Applied Stem Cell [Applied Stem Cell, Milpitas, CA]. Guide RNAs (gRNAs) for this G1006Afs*49 line were designed, validated in vivo, and one candidate gRNA was selected for genome editing the patient iPSC line (**Supplemental Table 1**). A single-stranded oligodeoxynucleotide (SSODN) was designed as a repair template and a silent mutation in the gRNA binding site was created to prevent re-cutting **(Supplemental Table 1).** The

G1006Afs*49 patient line was transfected with the gRNAs and ssODN constructs before undergoing puromycin selection. Single-cell colonies were genotyped and mutation correction clones were expanded for future studies.

iPSC-CM Differentiation, Culture, and Dissociation

As previously published, iPSCs were cultured in 6-well plates and differentiated into cardiomyocytes (CMs) at 85% confluency.[1] On day 0 of differentiation, culture media was changed from mTeSR-Plus to RPMI 1640 GlutaMAX plus 25mM HEPES supplemented with B27-minus insulin (RPMI/B27-ins) [Thermo] containing 5μM CHIR99021 [MilliporeSigma; St. Louis, MO]. On day 2 (following 48 hours), media was changed to RPMI/B27-ins containing 5μM IWP-2 [MilliporeSigma]. On day 4, the medium was changed back to the maintenance medium RPMI/B27-ins. Spontaneous beating began on days 7-10. Once beating, CMs were dissociated enzymatically using STEMdiff cardiomyocyte dissociation kit [STEMCELL] as previously described.[1] After 24 hours, cells were maintained in RPMI/B27+ins medium. Immunostaining of CMs following plating on an 8-well chamber slide demonstrated staining of Cardiac Troponin-T (cTnT) and α-Actinin (**Supplemental Table 2**). Isogenic control cell line data is included in **Supplemental Figure 1.**

Voltage Dye Optical Action Potentials in iPSC-CMs

iPSC-CMs were plated at 400,000 cells/dish on Matragel-coated 35 mm glass-bottom culture dishes. Prior to imaging, CMs were washed with pre-warmed (37°C) HEPES-buffered Tyrode's solution [Alfa Aesar; Haverhill, MA]. Each 35mm glass-bottom dish was incubated at 37°C in the dark for 20min with 0.5μL FluoVolt dye, 5μL PowerLoad, and 0.5mL Tyrode's solution [FluoVolt Membrane Potential kit, Thermo]. Excess dye was rinsed three times with Tyrode's solution, and a final 1.5mL Tyrode's solution was added to the iPSC-CMs for imaging. Depo

was prepared fresh daily at 4X in pre-warmed tyrode solution for a final concentration of 10 µM. During imaging, the dishes were kept in a heated 37°C stage-top chamber [Live Cell Instrument; Seoul, South Korea] with 5% CO_2. Under 40X-water objective magnification, using a Nikon Eclipse Ti light microscope [Nikon; Tokyo, Japan], optical action potentials were recorded in 20s fast time-lapse videos at a rate of 50 frames/sec (fps, 20ms exposure time) with LED illumination at 5% power. iPSC-CMs were paced at 1Hz (9ms pulse duration, 25V) using a MyoPacer field stimulator [Ion Optix; Westwood, MA] to eliminate beat-rate dependent effects on the APD. Small rectangular regions of interest were drawn over flashing areas of single cells for analysis. The NIS-Elements software [Nikon] was used to measure the fluorescence intensity over time within each region of interest, resulting in optical action potential traces. The traces were corrected for photobleaching and the amplitude was normalized as change in fluorescence divided by the baseline minimum fluorescence ($\Delta F/F_{min}$) using an in-house custom Excel program. Common action potential parameters including APD_{90}, APD_{50}, amplitude, rise time, upstroke velocity, etc. were detected for each individual optical action potential and averaged across all beats within a 20s trace. The average of all beats within a 20s trace represents a single data point. For representative traces, the maximum amplitude was further normalized to 1.0 to allow for accurate visualization of APD differences.

Multi-Electrode Array (MEA) Measurement

iPSC-CMs were dissociated and seeded at 50,000 cells per well on 48-well Biocircuit MEA plate (Axion BioSystems, Inc. M768-BIO-48) pre-coated with Matrigel. Cells were cultured in a humidified incubator at 37°C and 5% CO_2 for 7-10 days after dissociation, and media was changed every 2 days. Media was changed 1-4 hours before starting experiment and the MEA plate was placed in Maestro MEA device (Axion BioSystems, Inc.) with automatically adjusted

and controlled environment (37°C and 5%CO_2), and equilibrated for at least 20 minutes before baseline recordings.

All testing compounds were prepared fresh on each day of the experiment in warm RPMI+ins culture media. Medroxyprogesterone Acetate (Selleck Chemicals Catalog No. S2567), aka Depo, was diluted to 20 µM (2X) and added to the testing well at equal volume creating a final concentration of 10 µM. Depo-treated wells were allowed to rest in the MEA climate-controlled machine for 30 minutes before recording. Isoproterenol was diluted to 2 µM (2X) and added to the testing wells at equal volume creating a final concentration of 1 µM. Wells with Depo + ISO were diluted at 4X for the same final concentrations based on the concentrations and volumes already present in the wells. Nadolol [Sigma-Aldrich Solutions Catalog No. 1449700] was added to Depo+ISO treated wells at time of Iso treatment for a final concentration of 10 µM. MEA measurements were taken on the field potential configuration and recorded for 20 seconds from 10-12 minutes from the time ISO was added. The Cardiac Analysis Tool (Axion BioSystems, Inc. Version 3.1.4) was used for data analysis.

Statistical Analysis

GraphPad Prism 9 was used for all statistical analyses. Individual data points shown where appropriate as well as the mean ± standard error (SEM). Statistical analysis methods are specified in each figure legend, but 1-way ANOVA with multiple comparisons was compared when comparing >2 groups and an unpaired Student's t-test was used when comparing 2 groups. A $P<0.05$ was considered statistically significant.

Supplemental Tables:

Supplemental Table 1 | List of sequences used for genome editing and *KCNH2* sequencing.

List of Sequences used for genome editing and sequencing (5'-3')	
gRNA #1	ACAGTCGGGCCGCCAGTACC
gRNA #2	GAGGGAGCTCCTGGTACTGG
ssODN	CTGCAGGCGCCTTCTCAGGAGTGTCCAACATTTTCAGCTT CTGGGGGGACAGTCGGgGcCGCCAaTAtCAGGAGCTCCCT CGATGCCCCGCCCCCACCCCCAGCCTCCTCAACATCCCCC TCTCCAGCCCGG

***KCNH2* Exon 13 Primers (5'-3')**	
Forward	CTCACCCAGCTCTGCTCTCTG
Reverse	CACCAGGACCTGGACCAGACT

Supplemental Table 2 | List of antibodies used for immunofluorescence.

List of Antibodies used for Immunofluorescence				
Primary Antibodies	**Manufacture**	**Host**	**Type**	**Dilution**
Nanog	ThermoFisher Scientific	Rabbit	Polyclonal	1:200
Tra-160	Santa Cruz	Mouse	Monoclonal	1:200
SSEA4	ThermoFisher Scientific	Mouse	Monoclonal	1:200
Oct4	ThermoFisher Scientific	Rabbit	Polyclonal	1:200
cTNT	abcam	Rabbit	Polyclonal	1:200
α-Actinin	Sigma	Mouse	Monoclonal	1:200

Supplementary Table 3 | Expanded Data for LQT2 iPSC-CM Fluovolt Data

	APD90		APD50		APD30	
iPSC-CM Line	**Baseline**	**Depo**	**Baseline**	**Depo**	**Baseline**	**Depo**
KCNH2-G1006Afs*49	394±10 n=35	303±10 n=42	318±8 n=35	208±4 n=42	275±7 n=35	175±3 n=
	P<0.0001		p<0.0001		p<0.0001	

Values listed in milliseconds demonstrate averages ± SEM. N represents single cell measurements. Cells were measured at baseline and subsequently treated with 10 µM Depo. Measurements recorded 10-20 minutes after Depo-treatment.

Supplementary Table 4 | Expanded Data for LQT2 iPSC-CMs with Multi-Electrode Array (MEA).

Erratic Beating Frequency

iPSC-CM Line	Baseline	Depo + ISO	Depo	ISO
Isogenic Control	0±0 n=15	10±3 n=13	1±1 n=10	14±5 n=16
KCNH2-G1006Afs*49 combined clones	18±5 n=25	54±5 n=38	35±8 n=15	39±8 n=21

Values listed demonstrate averages ± SEM. N represents number technical replicates of independent wells. Each well contains 16 total recording electrodes, if electrode was not beating, it was excluded from denominator in frequency calculation. Erratic beating frequency was defined as dropped beats, alternans, or early after depolarization-like phenomena. Depo-treated wells were pretreated with 10 μM Depo for 30 minutes before treatment and incubation with 1 μM ISO for 10-12 minutes at time of measurement. Number of independent experiments are as follows: Isogenic Control= 3, p.G1006Afs*49-KCNH2 clone #1= 3, p.G1006Afs*49-KCNH2 clone #2= 1.

Supplemental Citations

1. Dotzler SM, Kim CSJ, Gendron WAC, et al.: Suppression-Replacement KCNQ1 Gene Therapy for Type 1 Long QT Syndrome. Circulation 20210128th Edition. 2021; 143:1411–1425.