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Suppression-Replacement *KCNQ1* Gene Therapy for Type 1 Long QT Syndrome

Running Title: *Dotzler et al. KCNQ1 Gene Therapy for LQT1*

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Abstract

Background: Type 1 long QT syndrome (LQT1) is caused by loss-of-function variants in the *KCNQ1*-encoded $K_v7.1$ potassium channel α-subunit which is essential for cardiac repolarization, providing the slow delayed rectifier current (I_{Ks}). No current therapies target the molecular cause of LQT1.

Methods: A dual-component "suppression-and-replacement" (SupRep) *KCNQ1* gene therapy was created by cloning a *KCNQ1* shRNA and a "shRNA-immune" (shIMM) *KCNQ1* cDNA modified with silent variants in the shRNA target site, into a single construct. The ability of KCNQ1-SupRep gene therapy to suppress and replace LQT1-causative variants in *KCNQ1* was evaluated via heterologous expression in TSA201 cells. For a human *in vitro* cardiac model, induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) were generated from four patients with LQT1 (KCNQ1-Y171X, -V254M, -I567S, and -A344A/spl) and an unrelated healthy control. CRISPR-Cas9 corrected isogenic control iPSC-CMs were made for two LQT1 lines (correction of KCNQ1-V254M and KCNQ1-A344A/spl). FluoVolt voltage dye was used to measure the cardiac action potential duration (APD) in iPSC-CMs treated with KCNQ1-SupRep.

Results: In TSA201 cells, KCNQ1-SupRep achieved mutation-independent suppression of wild-type *KCNQ1* and three LQT1-causative variants (KCNQ1-Y171X, -V254M, and -I567S) with simultaneous replacement of KCNQ1-shIMM as measured by allele-specific qRT-PCR and western blot. Using FluoVolt voltage dye to measure the cardiac APD in the four LQT1 patient-derived iPSC-CMs, treatment with KCNQ1-SupRep resulted in shortening of the pathologically prolonged APD at both 90% (APD_{90}) and 50% (APD_{50}) repolarization resulting in APD values similar to those of the two isogenic controls.

Conclusions: This study provides the first proof-of-principle gene therapy for complete correction of LQTS. As a dual-component gene therapy vector, KCNQ1-SupRep successfully suppressed and replaced *KCNQ1* to normal wild-type levels. In TSA201 cells, co-transfection of LQT1-causative variants and KCNQ1-SupRep caused mutation-independent suppression-and-replacement of *KCNQ1*. In LQT1 iPSC-CMs, KCNQ1-SupRep gene therapy shortened the APD, thereby eliminating the pathognomonic feature of LQT1.

Key Words: Gene Therapy; KCNQ1; Long QT Syndrome; Induced Pluripotent Stem Cells; iPSC

Non-Standard Abbreviations and Acronyms

AAV9 – Adeno-Associated Virus Serotype 9
APD – Action Potential Duration
APD_{50} – Action Potential Duration at 50% Repolarization
APD_{90} – Action Potential Duration at 90% Repolarization
CFP – Cyan Fluorescent Protein
Ctrl – Control
ECG – Electrocardiogram
GFP – Green Fluorescent Protein
ICD – Implantable Cardioverter Defibrillator
I_{Kr} – Rapid Delayed Rectifier Current
I_{Ks} – Slow Delayed Rectifier Current
iPSCs – Induced Pluripotent Stem Cells

iPSC-CMs – Induced Pluripotent Stem Cell-Derived Cardiomyocytes
IRES – Internal Ribosome Entry Site
JLNS – Jervell and Lange-Nielsen Syndrome
KD – Knockdown
LCSD – Left Cardiac Sympathetic Denervation
LQTS – Long QT Syndrome
LQT1 – Type 1 Long QT Syndrome
LQT2 – Type 2 Long QT Syndrome
MOI – Multiplicity of Infection
PBMCs – Peripheral Blood Mononuclear Cells
PBS – Phosphate Buffered Saline
RPMI/B27-ins – RPMI 1640 GlutaMax with 25mM HEPES and B27 Minus Insulin Supplement
qRT-PCR – Quantitative Reverse Transcription Polymerase Chain Reaction
RNAi – RNA Interference
SCD – Sudden Cardiac Death
shCT – Non-Targeting Scramble Control shRNA
shIMM – shRNA-Immune
shRNA – Short Hairpin RNA
siRNA – Small Interfering RNA
SQTS – Short QT Syndrome
SupRep – Suppression and Replacement
TBS – Tris-Buffered Saline
WT – Wild Type

Clinical Perspective

What is new?

- This study establishes a novel, dual-component "suppression-and-replacement" *KCNQ1* (KCNQ1-SupRep) gene therapy approach for LQT1 that contains a *KCNQ1* shRNA to suppress endogenous expression and a codon-altered "shRNA-immune" copy of *KCNQ1* for gene replacement.
- KCNQ1-SupRep rescues the prolonged action potential duration in iPSC cardiomyocytes derived from four patients with unique LQT1-causative *KCNQ1* variants.

What are the clinical implications?

- This is the first proof-of-concept preclinical study for hybrid gene therapy in LQT1 (also first in cardiac disease), and is capable of providing complete rescue of *KCNQ1* function.
- KCNQ1-SupRep is applicable to *all* patients with LQT1 because it targets the whole *KCNQ1* gene rather than specific variants.

Introduction

Congenital long QT syndrome (LQTS) is an autosomal dominant disorder characterized by delayed repolarization of the myocardium associated with a prolonged QT interval on electrocardiogram (ECG). Patients with LQTS have increased risk for torsadogenic syncope/seizures, and sudden cardiac death (SCD). The prevalence of LQTS is about 1 in 2000, and when untreated, higher risk patients have an estimated 10-year mortality of 50%.[1, 2]

LQTS is caused by pathogenic variants in cardiac ion channels or their interacting regulatory proteins.[3] Type 1 LQTS (LQT1) is the most common form of LQTS accounting for approximately 35% of cases,[4] and is caused by loss-of-function variants in *KCNQ1*. *KCNQ1* encodes the α-subunit of the $K_v7.1$ voltage-gated potassium channel and is responsible for the slow delayed rectifier current (I_{Ks}) during repolarization of the cardiac action potential. Because the *KCNQ1*-encoded α-subunits tetramerize during $K_v7.1$ channel assembly, pathogenic missense variants commonly exhibit dominant-negative effect due to interference with the wild-type (WT) subunits translated from the non-affected allele.

Over the past two decades, there have been substantial improvements in the management of LQTS, but current therapies have limitations.[5] Beta-blockers remain first line, but medication noncompliance is common, and breakthrough cardiac events (syncope, seizure, or SCD) still occur.[6, 7] More invasive therapies include left cardiac sympathetic denervation (LCSD) or an implantable cardioverter defibrillator (ICD). These strategies do not treat the underlying pathogenic substrate. Instead, LCSD decreases the risk of an LQTS-triggered breakthrough event[8, 9] while an ICD provides a rescue shock to restore normal rhythm if an event occurs.[10, 11] Though LCSD and ICDs are highly effective, they are not without complications including risk of infection or lead fracture.[12]

Gene therapy is an emerging area of interest for the treatment of LQTS. RNA interference (RNAi) including small interfering RNA (siRNA) or short hairpin RNA (shRNA), is one such strategy that utilizes the endogenous gene silencing mechanism to knockdown (KD) expression of the target gene. Early attempts to overcome dominant-negative *KCNH2* variants in LQT2 used allele-specific siRNAs to selectively KD only the mutant allele.[13, 14] Despite promising preliminary data, these studies were limited by resultant haploinsufficiency as the best possible outcome and the necessity of engineering and validating a separate siRNA for each unique LQT2-causative variant. In *KCNQ1* alone, there are hundreds of LQT1-causative variants, making it impractical to develop an individual RNAi for each one.[15]

To overcome these limitations, we designed and developed the first suppression-and-replacement (SupRep) *KCNQ1* gene therapy vector for the potential treatment of patients with LQT1 and demonstrate its potential therapeutic efficacy in two *in vitro* model systems. The SupRep strategy has two critical components that occur in tandem. First, suppression of *both* endogenous alleles, the WT allele and the LQT1 variant-containing allele, occurs via a *KCNQ1* shRNA. The second component involves replacement via expression of a shRNA-immune (shIMM) *KCNQ1* cDNA containing synonymous variants at the wobble base of each codon within the shRNA's binding sequence. These synonymous variants importantly do not alter the WT amino acid sequence, but do prevent KD by the shRNA thereby rendering it "immune" to the shRNA. KCNQ1-SupRep is mutation-independent, eliminating the need for design of multiple RNAi since the shRNA targets the gene itself rather than discrete mutations.

Herein, we describe the generation of a KCNQ1-SupRep gene therapy vector and validate its ability to suppress and replace *KCNQ1* via heterologous expression in TSA201 cells. Second, we show rescue of the LQT1 disease phenotype via shortening of the cardiac action

potential duration (APD) in an *in vitro* cardiac model using patient-specific, induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) generated from four patients with distinct LQT1-causative variants. Finally, we demonstrate how close to a "therapeutic cure" in terms of APD normalization that the KCNQ1-SupRep gene therapy achieves when compared to the gold standard of the patient's own corrected isogenic control cells.

Methods

The authors declare that all data are/will be available to other researchers for the purposes of reproducing the results or replicating the procedure. Human samples were obtained from patients with LQT1 and an unrelated healthy control (**Table I in the Supplement**), following written informed consent under Mayo Clinic Institutional Review Board (IRB, 09-006465) approved study. Procedures followed were in accordance with institutional guidelines. The comprehensive methods section is available as supplemental material.

Statistical Analysis

GraphPad Prism 8 was used for all statistical analysis. Individual data points are shown wherever practical along with the mean. Error bars represent standard deviation (S.D.) unless otherwise indicated in the figure legend. Specific statistical methods are indicated in each figure legend. Briefly, one-way ANOVA with post-hoc Tukey's or Dunnett's test for multiple comparisons was performed for comparisons among three or more groups as indicated. An unpaired two-tailed student's t-test was performed to determine statistical significance between two groups when indicated. A $p<0.05$ was considered to be significant.

Results

Generation of a KCNQ1-SupRep Gene Therapy Construct

To make KCNQ1-SupRep, four commercially available candidate *KCNQ1* shRNAs (sh#1-4) were purchased in the pGFP-C-shLenti lentiviral backbone from OriGene along with a non-targeting scramble control shRNA (shCT, **Table II in the Supplement**). The KD efficiency of each *KCNQ1* shRNA was determined by co-transfecting TSA201 cells with KCNQ1-WT and sh#1-4. Expression of *KCNQ1* was measured by quantitative reverse transcription PCR (qRT-PCR, **Figure IA in the Supplement**) and confirmed by western blot (**Figure IB-C in the Supplement**). Of the four shRNAs tested, sh#1, sh#2, and sh#4 all resulted in significant KD of *KCNQ1* (mRNA: 69-78% KD, protein: 50-77% KD) with no statistically significant differences between the three shRNAs. Any of these shRNAs could in theory have been used as part of the final KCNQ1-SupRep gene therapy vector. To select a final shRNA from the three potential candidates, by raw average KD, *KCNQ1* sh#4 provided the strongest KD of *KCNQ1* on both the mRNA (78%, p=0.004) and protein (77%, p=0.004) levels. Further, at the time of selection, the *KCNQ1* sh#4 target sequence (nucleotides c.1376-1404, exon 10-11 boundary) was assessed using the Genome Aggregation Database (gnomAD) and ClinVar and was devoid of both common genetic polymorphisms and all known pathogenic LQT1-causative variants that may interfere with KD efficiency (data not shown).

KCNQ1 sh#4 was selected for the final KCNQ1-SupRep and will be referred to as shKCNQ1 from here forward. To create the replacement shRNA-immune version of *KCNQ1*, called KCNQ1-shIMM, ten synonymous variants were introduced into the WT *KCNQ1* cDNA at the wobble base of each codon within the target site of shKCNQ1, nucleotides c.1376-1404 (**Figure 1A**). KCNQ1-shIMM was then cloned into the shKCNQ1-containing vector, pGFP-C-

shLenti, downstream of the CMV promoter. In this step, the original turboGFP reporter (which remained the reporter for shCT) was exchanged for an internal ribosome entry site (IRES) with CFP. The final KCNQ1-SupRep gene therapy vector used in this *in vitro* study is illustrated in **Figure 1B**.

KCNQ1-SupRep Gene Therapy Both Suppresses and Replaces KCNQ1-WT

To validate that KCNQ1-shIMM is immune to KD by shKCNQ1, TSA201 cells were co-transfected with KCNQ1-WT or KCNQ1-shIMM and shKCNQ1. The expression of KCNQ1-WT versus KCNQ1-shIMM was quantified using allele-specific qRT-PCR. Each sample was run in four separate reactions, using a unique set of allele-specific primers (**Table III in the Supplement**), to quantify: 1) total *KCNQ1*, 2) endogenous *KCNQ1* which includes WT or variant-containing alleles, but excludes KCNQ1-shIMM, 3) KCNQ1-shIMM, and 4) *GAPDH* as housekeeping. Commercial primers were used to amplify total *KCNQ1*. For exclusive amplification of endogenous *KCNQ1* or KCNQ1-shIMM, two forward primers were designed within the shKCNQ1 target site, one complementary to the WT sequence and the other complementary to the unique, modified sequence of KCNQ1-shIMM. A common reverse primer was used for both reactions, and a standard curve was used to correct for PCR amplification bias.

Compared to shCT, shKCNQ1 caused significant suppression of KCNQ1-WT by 87% ($p<0.0001$) as expected, but was unable to suppress KCNQ1-shIMM ($p=0.997$, **Figure 2A**). Notably, there was no difference in the expression of KCNQ1-WT compared to KCNQ1-shIMM ($p>0.999$), indicating that introduction of the synonymous variants in KCNQ1-shIMM did not disturb its expressivity. Next, KCNQ1-SupRep was co-transfected with KCNQ1-WT, which resulted in 52% suppression of KCNQ1-WT with 255% replacement of KCNQ1-shIMM (**Figure 2A**). The dual component KCNQ1-SupRep vector had less potent suppression compared to

shKCNQ1 alone, but exhibited stronger expression of KCNQ1-shIMM than KCNQ1-shIMM alone. While the reason for this is unclear, varying amounts of KCNQ1-SupRep were transfected and shown to cause dose-dependent suppression and replacement, suggesting that KCNQ1-SupRep expression can be adjusted as needed (**Figure II in the Supplement**). Results obtained by qRT-PCR were confirmed by western blot which demonstrated that shKCNQ1 was able to significantly KD KCNQ1-WT (p=0.037) but not KCNQ1-shIMM (p=0.61, **Figure 2B-C**). As an important safety metric for onset of the gene therapy, allele-specific qRT-PCR was used to measure the activation kinetics of KCNQ1-SupRep in a three day time course of TSA201 cells co-transfected with WT-KCNQ1 and shCT, shKCNQ1, KCNQ1-shIMM, or KCNQ1-SupRep. Compared to treatment with shCT, KCNQ1-SupRep caused reduction of KCNQ1-WT that was replaced with KCNQ1-shIMM, however the total *KCNQ1* was not altered at any time during the three day onset, avoiding over- or under-expression (**Figure III in the Supplement**).

Selection of Patients with LQT1-Causative Variants in *KCNQ1*

Four patients with LQT1 hosting unique variants, KCNQ1-Y171X, KCNQ1-V254M, KCNQ1-I567S, and KCNQ1-A344A/spl were selected for this study. All four KCNQ1 variants were classified as pathogenic (LQT1-causative) by current American College of Medical Genetics guidelines[16] and were chosen for this gene therapy pilot study to include a nonsense, premature truncation variant (KCNQ1-Y171X) producing haploinsufficiency in a patient with a mild phenotype, two dominant-negative missense variants (KCNQ1-V254M and KCNQ1-I567S) and a synonymous splice variant (KCNQ1-A344A/spl) that causes skipping of exon 7,[17] in three patients with a strong LQT1 phenotype including documented QTc greater than 500 ms, a positive history of LQTS-related symptoms (syncope, seizure, near drowning, sudden cardiac arrest), and a positive family history of LQTS-related symptoms (**Table I in the Supplement**).

All four variants have been reported previously in the literature, though only KCNQ1-V254M and KCNQ1-A344A/spl have been characterized functionally as dominant-negative variants.[17-20] Site-directed mutagenesis was used to introduce three of the four LQT1 patient variants, KCNQ1-Y171X, -V254M, and -I567S into KCNQ1-WT to evaluate the ability of KCNQ1-SupRep to suppress and replace *KCNQ1* variants in a mutation-independent manner. KCNQ1-A344A/spl was not included for heterologous expression studies in TSA201 cells since the KCNQ1-WT is a full length cDNA and does not contain the introns necessary to evaluate a splicing variant like KCNQ1-A344A/spl.

Validation of Function for KCNQ1-shIMM and KCNQ1 Pathogenic Variants

KCNQ1-WT and -shIMM, and LQT1-causative variants KCNQ1-Y171X, -V254M, and -I567S were co-transfected in TSA201 cells with the $K_V7.1$ channel β-subunit, *KCNE1*. The resulting I_{Ks} current was measured by standard whole cell patch clamp. Representative traces are shown in **Figure 3A**. Importantly, KCNQ1-shIMM produced robust I_{Ks} current with no significant difference from KCNQ1-WT (p=0.28, **Figure 3B-C**). All three LQT1 variants (KCNQ1-Y171X, -V254M, and -I567S) resulted in no functional I_{Ks} current beyond the minimal background ion channel activity of TSA201 cells, consistent with complete loss of function (**Figure 3A-C**). Null current was expected for a nonsense variant like KCNQ1-Y171X and additionally for KCNQ1-V254M whose null status was in concordance with previously published data.[19] Total lack of current from KCNQ1-I567S is a novel finding, but is not surprising given the patient's clinically definitive LQT1 and the fact that most LQT1-causative variants are missense variants.

To evaluate trafficking of KCNQ1 to the cell membrane, transfected TSA201 cells were assessed by immunofluorescence microscopy. Both KCNQ1-WT and KCNQ1-shIMM produced bright staining along the cell membrane, indicating that the synonymous variants in KCNQ1-

shIMM do not interfere with correct trafficking (**Figure IV in the Supplement**). Of the LQT1 variants, KCNQ1-Y171X produced no detectable protein as a result of premature truncation, while KCNQ1-V254M and KCNQ1-I567S exhibited normal cell membrane trafficking, though the overall expression of KCNQ1-I567S appeared to be decreased. Taken together, these results indicate that KCNQ1-shIMM has WT function and that KCNQ1-Y171X, -V254M, and -I567S are LQT1-causative variants with total loss of function.

KCNQ1-SupRep Gene Therapy Suppresses and Replaces KCNQ1 Variants in a Mutation-Independent Manner

To validate that treatment with KCNQ1-SupRep gene therapy is able to suppress and replace LQT1-causative variants in a mutation-independent manner, TSA201 cells were co-transfected with the three *KCNQ1* variants and shKCNQ1, KCNQ1-SupRep, or shCT control. All three LQT1-causative variants were suppressed by shKCNQ1 ranging from 87-93% KD relative to KCNQ1-WT as measured by allele-specific qRT-PCR (**Figure 4A**). While the suppression is visibly marked for each of the three variants, suppression by shKCNQ1 did not reach statistical significance for KCNQ1-Y171X and KCNQ1-I567S presumably due to lower baseline expression of these variants. It is however noteworthy that very few mRNA transcripts were detectable in any sample (WT or variant) that was treated with shKCNQ1. Notably, KCNQ1-Y171X had substantially decreased expression at baseline, likely due to its premature stop codon and predicted nonsense-mediated decay of mRNA transcripts.[21]

Results obtained by qRT-PCR were confirmed by western blot in which KCNQ1-Y171X produced no detectable protein as a result of premature truncation while KCNQ1-V254M was able to be suppressed by shKCNQ1, and KCNQ1-I567S had faint baseline expression that was also suppressed by shKCNQ1 (**Figure 4B**). Overall, KCNQ1-SupRep caused suppression and

replacement of three LQT1-causative *KCNQ1* variants, validating its ability to suppress and replace *KCNQ1* in a mutation-independent manner.

Generation of iPSC-CMs from Four Patients with LQT1

Among the 236 patients with LQT1 in our iPSC biorepository, we selected four patients with distinct LQT1-causative variants in *KCNQ1* to generate iPSC-CMs and test the APD-shortening potential of KCNQ1-SupRep gene therapy for this proof-of-principle, therapy-in-the-dish study. A healthy unrelated individual was included as a control, and two isogenic controls were created by CRISPR-Cas9 correction of KCNQ1-V254M and KCNQ1-I567S, respectively. These isogenic controls served as the gold standard for a "therapeutic cure" thereby providing a marker for the ideal normalization of the prolonged APD and determine how close to this ideal did treatment with KCNQ1-SupRep gene therapy reach.

Dermal fibroblasts or peripheral blood mononuclear cells (PBMCs) were collected from each patient with informed consent under Mayo Clinic IRB-approved protocol (09-006465) and were used to generate iPSCs. Standard quality control assays were performed on each iPSC line including Sanger sequencing of the LQT1-causative variant (**Figure VA in the Supplement)**, bright field morphology (**Figure VB in the Supplement**), karyotype (**Figure VC in the Supplement**), and immunofluorescence microscopy for pluripotent markers including Tra-1-60, Nanog, SSEA-4, and Oct4 (**Figure VI in the Supplement**). Additionally, for the two isogenic controls the top ten predicted off-target CRISPR-Cas9 edit sites were analyzed *in silico* for deleterious effect on the cardiac action potential by searching the gene spanning or nearest the edit site within a large protein-protein interactome of 1629 genes that may influence cardiac repolarization (i.e. the LQTS interactome).[22] Only three genes matched within the LQTS interactome (*PTPRU*, *DNM3*, and *PHACTR3*). However, none of these edit sites fell in protein

coding sequences and as such were not predicted to have any confounding effect on the action potential (**Table IV in the Supplement**). Differentiation of iPSCs was induced by previously published methods to generate spontaneously beating iPSC-CMs.[23, 24] Since the cardiac APD is known to shorten as iPSC-CMs mature over time, all experiments were conducted at least 30 days after the induction of differentiation.[25]

KCNQ1-SupRep Gene Therapy Increases KCNQ1 in LQT1 iPSC-CMs

To assess the ability of lentiviral KCNQ1-SupRep to transduce iPSC-CMs and increase WT KCNQ1 expression, unrelated control and LQT1 iPSC-CMs were transduced with lentiviral KCNQ1-SupRep or shCT and evaluated using immunofluorescence microscopy. Cardiac troponin T (cTnT) was used as a marker of cardiomyocytes. Antibodies targeting the lentiviral reporters (turboGFP for shCT or CFP for KCNQ1-SupRep) were used to identify transduced cells, and lastly KCNQ1 was stained to visualize the effects of KCNQ1-SupRep on overall expression of KCNQ1. Results for KCNQ1-V254M iPSC-CMs (**Figure 5**) and remaining unrelated control and LQT1 iPSC-CMs (**Figure VII in the Supplement**) showed high purity cardiomyocytes within the iPSC-CM cultures that had been evenly transduced with lentiviral KCNQ1-SupRep or shCT. At baseline in iPSC-CMs treated with shCT, KCNQ1 is only faintly detectable by confocal microscopy whereas iPSC-CMs treated with KCNQ1-SupRep display robust staining for KCNQ1 (**Figure 5**, **Figure VII in the Supplement**). This suggests that in iPSC-CMs, treatment with KCNQ1-SupRep gene therapy drives substantial overexpression of KCNQ1-shIMM.

KCNQ1-SupRep Gene Therapy Shortens the Cardiac APD in LQT1 iPSC-CMs as Measured by FluoVolt Voltage Dye

To test whether treatment with KCNQ1-SupRep gene therapy is able to rescue the pathognomonic feature of LQT1 by shortening the pathologically prolonged APD, FluoVolt voltage dye was used to measure optical action potentials in iPSC-CMs derived from four patients with LQT1 stemming from either KCNQ1-Y171X, -V254M, -I567S, or -A344A/spl treated with either the lentiviral shCT control or KCNQ1-SupRep gene therapy. The unrelated control was measured without any treatment as a measure for a healthy APD. All iPSC-CMs were paced at 1Hz during recording to eliminate beat rate-dependent changes to the APD. Representative optical action potentials are shown in **Figure 6A**. When treated with shCT, all LQT1 iPSC-CMs had significantly longer APD at 90% repolarization (APD_{90}) and three of the four also had significantly longer APD at 50% repolarization (APD_{50}) compared to untreated unrelated healthy control iPSC-CMs, validating the LQT1 iPSC-CMs as an *in vitro* model of LQT1 for this therapeutic intervention trial.

A full summary of APD_{90} and APD_{50} values and APD shortening due to KCNQ1-SupRep are shown in **Table 1**. Specifically, the APD_{90} of the unrelated control was 332 ± 53 ms (n=50) as compared to LQT1 iPSC-CMs with APD_{90} of 585 ± 77 ms (KCNQ1-Y171X, n=52, $p<0.0001$), 580 ± 57 ms (KCNQ1-V254M, n=42, $p<0.0001$), 452 ± 72 ms (KCNQ1-I567S, n=45, $p<0.0001$), and 553 ± 98 ms (KCNQ1-A344A/spl, n=61, $p<0.0001$, **Figure 6B**). When treated with KCNQ1-SupRep, the APD_{90} and APD_{50} of both LQT1 lines shortened significantly. The APD_{90} shortened by 117 ms in KCNQ1-Y171X to 468 ± 43 ms (n=63, $p<0.0001$), by 111 ms in KCNQ1-V254M to 469 ± 89 ms (n=55, $p<0.0001$), by 85 ms in KCNQ1-I567S to 367 ± 60 ms

(n=45, p<0.0001), and by 210 ms in KCNQ1-A344A/spl to 343 ± 133 ms (n=63, p<0.0001, **Figure 6B**).

To validate whether the observed APD shortening due to KCNQ1-SupRep represents complete rescue to WT or if these shorter APD values were incomplete or overcorrection, two CRISPR-Cas9 corrected isogenic controls were created from the KCNQ1-V254M and KCNQ1-A344A/spl parent LQT1 iPSC cell lines. When measured by FluoVolt, and plotted against the shCT and KCNQ1-SupRep treatment data from **Figure 6B**, both isogenic controls had significantly shorter APD_{90} and APD_{50} compared to their shCT-treated counterparts (**Figure 7A-B**).

Isogenic correction of KCNQ1-V254M shortened the APD_{90} by 200 ms to 380 ± 112 ms (n=58, p<0.0001), and isogenic correction of KCNQ1-A344A/spl shortened the APD_{90} by 176 ms (n=57, p<0.001). A full summary of the APD_{90} and APD_{50} values for KCNQ1-V254M and KCNQ1-A344A/spl with isogenic controls is shown in **Table V in the Supplement**. Comparing the shortened APD values of the KCNQ1-V254M and KCNQ1-A344A/spl iPSC-CMs treated with KCNQ1-SupRep gene therapy to their isogenic controls, there was variability in the degree of rescue. In KCNQ1-V254M, there was incomplete shortening of the APD_{90} and concomitant overcorrection of the APD_{50} while in KCNQ1-A344A/spl the APD_{90} had complete rescue with no significant difference, but did show overcorrection of the APD_{50}. Despite this variability, treatment with KCNQ1-SupRep gene therapy demonstrated ability to completely rescue the prolonged action potential in LQT1 iPSC-CMs.

KCNQ1-SupRep Gene Therapy Shortens the Cardiac APD in 3D-Organoid Culture of LQT1 iPSC-CMs

As proof of concept to see if the APD-shortening ability of KCNQ1-SupRep is translatable from 2D syncytial monolayer iPSC-CM culture to a three-dimensional environment, LQT1 iPSC-CM 3D-organoids were generated from one of the four LQT1 variants using the KCNQ1-Y171X iPSC-CMs. The KCNQ1-Y171X iPSC-CMs were dissociated and embedded in a Matrigel spheroid mold and allowed to reorganize naturally on the collagenous extracellular architecture to create a 3D-cardiac organoid (**Figure VIIIA in the Supplement**). The organoids were treated with shCT or KCNQ1-SupRep, were cryosectioned and stained for immunofluorescence using cardiac troponin T (cTnT) to mark cardiomyocytes and the lentiviral reporters (turboGFP for shCT and CFP for KCNQ1-SupRep) to mark infected cells. Immunofluorescence revealed networks of cardiomyocytes and prominent staining of turboGFP and CFP indicating even transduction by shCT and KCNQ1-SupRep (**Figure VIIIB in the Supplement**). The APD of untreated and KCNQ1-SupRep treated organoids were assessed by FluoVolt where KCNQ1-SupRep resulted in shortening of the APD_{90} and APD_{50} (**Figure VIIIC-D in the Supplement**), suggesting that KCNQ1-SupRep retains APD-shortening ability in a simple 3D organoid environment.

Discussion

In 1996, *KCNQ1* was discovered as the gene responsible for LQT1.[26] For more than two decades, the molecular mechanism for LQT1 has been known, yet no current therapy directly targets the defective *KCNQ1*-encoded $K_V7.1$ potassium channel. Instead, beta-blockers and LCSD reduce the risk of breakthrough cardiac events while ICDs are an implanted antidote to restore normal

sinus rhythm should an LQT1-triggered ventricular arrhythmia fail to self-terminate. On the other hand, gene therapy represents the only therapeutic option with the potential to directly correct the dysfunctional ion channel activity at the source of the disease.

By design, replacement is the most straightforward gene therapy strategy in which expression of a defective gene is restored. There are currently two gene therapies that have been approved by the United States' Food and Drug Administration– voretigene neparvovec-rzyl (Luxturna) and onasemnogene abeparvovec-xioi (Zolgensma). Both of these gene therapies use a replacement strategy, expressing *RPE65* in cases of retinal dystrophy and *SMN1* in cases of spinal muscular atrophy, respectively.[27, 28] In LQT1, however, where *KCNQ1* variants often exhibit dominant-negative effect, the replacement strategy alone is thought to be insufficient because it would require abnormally high expression to overcome this interference.

As an alternative to replacement, allele-specific RNAi was developed with targeted mutant KD to remove dominant-negative interference under the assumption that the remaining WT allele would provide enough function to ameliorate the disease phenotype, despite haploinsufficiency. In two previous studies of LQT2, allele-specific siRNAs rescued the rapid delayed rectifier current (I_{Kr}) produced by the *KCNH2*-encoded $K_v11.1$ channel via specific KD of the dominant-negative missense variants KCNH2-E637K and KCNH2-G1681A.[13, 14] This allele-specific RNAi strategy has several limitations. First, haploinsufficiency is the best theoretical outcome since only one WT allele remains after KD of the mutant allele. Second, allele-specific KD can be difficult to achieve, especially for single nucleotide variants. And third, the allele-specific nature necessitates design of a separate RNAi for each discrete disease-causing variant, which would be impractical when there are hundreds of known variants in each of the common LQTS genes.

To overcome the limitations of allele-specific RNAi, Millington-Ward et al. developed a new strategy involving suppression-and-replacement (SupRep).[29] Previously, the SupRep strategy yielded promising preliminary results in studies of *RHO*-mediated retinitis pigmentosa, a genetic disorder that causes progressive loss of vision, and *AAT*-mediated α-1-antitrypsin deficiency, a genetic disorder that predisposes to emphysema and liver cirrhosis.[30-32]

As described in the current study, SupRep gene therapy offers several advantages for the treatment of LQTS in general and LQT1 in particular over other gene therapy strategies. First, in KCNQ1-SupRep gene therapy, the *KCNQ1* shRNA targets the gene rather than individual mutations. As a result, KCNQ1-SupRep provides mutation-independent suppression, allowing generalizability to essentially all known LQT1-causative (and also type 2 short QT syndrome-causative) pathogenic variants in *KCNQ1*, including those with dominant-negative effect. Universal suppression also eliminates the need for design of multiple RNAi. Second, expression of the uniquely engineered "knockdown-immune" KCNQ1-shIMM cDNA replaces the lost *KCNQ1* which allows for disease rescue beyond haploinsufficiency dependent upon the level of expression. Finally, it is noteworthy that due to KD of *both* endogenous alleles, KCNQ1-SupRep is the first attempted gene therapy that is theoretically applicable to patients with autosomal recessive LQT1 and Jervell and Lange-Nielsen syndrome (JLNS) where there are variants involving both *KCNQ1* alleles. Current therapies for these malignant versions of *KCNQ1*-mediated disease remain suboptimal.

In the current study, a KCNQ1-SupRep gene therapy vector was developed in a lentiviral backbone. Lentivirus was used due to high transduction efficiency compared to adeno-associated virus serotype 9 (AAV9) which has strong cardiac tropism *in vivo*, but has poor efficiency *in vitro*.[33-35] For future animal model studies, AAVs are preferred over lentivirus in studies

involving gene therapy due to superior safety profile. Lentiviruses integrate into the host genome and have risk for insertional mutagenesis whereas AAVs are non-integrating, generate minimal immune response, and may persist indefinitely in non-dividing cells such as cardiomyocytes, raising the possibility of single dosing.[36]

In creating KCNQ1-SupRep, a potent *KCNQ1* shRNA was selected. Importantly, the shKCNQ1 target sequence was free of common polymorphisms and known pathogenic variants, ensuring that future treatment in patients would not be confounded by somatic variation that could interfere with shRNA binding affinity. KCNQ1-shIMM was then created by introducing synonymous variants at the degenerate wobble base of each codon within the shKCNQ1 target sequence, rendering it resistant or immune to KD while maintaining the identical WT amino acid sequence. This KCNQ1-shIMM cDNA construct was validated to have WT-like i) expression (qRT-PCR and western blot), ii) trafficking (immunofluorescence microscopy), and iii) I_{Ks} current (patch clamp). The balance of shKCNQ1 and KCNQ1-shIMM is critical in determining the degree of APD rescue, and ultimately the efficacy of KCNQ1-SupRep as a clinical therapeutic for LQT1, JLNS1, or even SQT2.

In principle, the ideal suppression-and-replacement of *KCNQ1* for patients with either LQT1, JLNS1, or SQT2 treated with KCNQ1-SupRep gene therapy would produce I_{Ks} current density matching exactly that of a healthy individual. Like traditional pharmaceuticals, KCNQ1-SupRep should theoretically have a therapeutic window, providing enough I_{Ks} to ameliorate the LQTS phenotype without overcompensating and causing SQTS. In LQT1 and JLNS, disease severity correlates with the degree of lost I_{Ks}.[37] Heterozygous nonsense or frameshift variants cause haploinsufficiency and typically result in mild LQT1 with ~50% I_{Ks}. Dominant-negative missense variants reduce I_{Ks} beyond 50% and are more strongly associated with breakthrough

cardiac events. In the most severe cases, patients with JLNS inherit two mutant alleles that result in either minimal (<10%) or no I_{Ks}.[38] Conversely, *KCNQ1* variants with *substantial* gain of function cause SQT2, though the exact degree of increased I_{Ks} is not well established.[39-42] Taken together, the therapeutic window for KCNQ1-SupRep in humans may actually be relatively wide, potentially allowing some flexibility for achieving optimal efficacy. Future investigations of KCNQ1-SupRep dosing in animal model studies will be of paramount importance, but could be modified at several points including the promoters driving expression, or most basically, the amount of viral particles delivered.

Limitations

Several studies have revealed heterogeneity regarding the role of *KCNQ1*-encoded $K_v7.1$ (I_{Ks}) and the I_{Ks} current across model systems. In humans and canines, I_{Ks} plays a prominent role at baseline due to basal sympathetic tone, but upon *ex vivo* dissociation, cardiomyocytes may require adrenergic stimulation to observe a substantive I_{Ks} effect.[43, 44] This phenomenon is likely a result of the slow-activation kinetics of I_{Ks} which result in minimal passage of current during the normal action potential duration. However, upon adrenergic stimulation, I_{Ks} is greatly increased and provides a major source of cardiac repolarization. Lack of basal I_{Ks} has also been observed in some iPSC-CMs in a direct comparison to human left ventricular cardiomyocytes.[45] However, multiple studies have used iPSC-CMs to successfully model LQT1 and JLNS, demonstrating irrefutable evidence of I_{Ks} at both baseline and in response to adrenergic stimulation.[46-48] The role of I_{Ks} and its variability across different model systems warrants additional exploration. Though KCNQ1-SupRep caused consistent APD shortening in this study, direct measurement of I_{Ks} by patch clamp in future studies would help to clarify the role of I_{Ks} in iPSC-CMs while simultaneously solidifying the direct mechanism by which KCNQ1-SupRep

shortens the cardiac action potential. Regardless, LQT1 animal model studies are a crucial next step for a novel therapeutic like KCNQ1-SupRep to ensure both a physiologic I_{Ks} effect and generalizability across models.

For KCNQ1-SupRep to be translated beyond APD rescue in 2D or 3D iPSC-CMs to normalizing the QTc of a patient with LQT1, advances in viral transduction of gene therapy to the heart must occur. Efficiency of cardiac viral transduction is one of the major challenges in the field of cardiac gene therapy primarily due to limitations in delivery methods and cardiac tropism of viral vectors.[49] Even with efficient transduction, some cardiomyocytes are virtually guaranteed to remain untransduced. Rescue of the APD in some, but not all cardiomyocytes may create heterogeneity in repolarization across the myocardium which could predispose to reentrant arrhythmias.[50] Alternatively, if the KCNQ1-SupRep gene therapy is efficiently delivered to the majority of cardiomyocytes, it is possible that the gene therapy-mediated restoration of repolarization reserve may distribute via gap junctions to compensate for neighboring untransduced cardiomyocytes. Future studies are needed to answer this question, though in this study it is noteworthy that during measurement of optical action potentials, no arrhythmic activity was observed in electrically coupled iPSC-CMs transduced with KCNQ1-SupRep.

Whether AAV9-mediated delivery of this KCNQ1-SupRep gene therapy construct will normalize the APD in a more physiologically relevant animal model of LQT1 remains to be determined. There are significant challenges to overcome when translating from a lentiviral-based delivery of the therapeutic vector in iPSC-CMs to an AAV9-based delivery of the gene therapy to the whole animal or to the patient in the future. Application of KCNQ1-SupRep gene therapy to a model that more closely recapitulates the complex intricacies of the human heart will be of utmost importance in determining if the APD-attenuating efficacy observed in this

study will translate to the global cardiac environment. Further, experiments must be undertaken to ensure that deliberate gene therapeutic intervention in the electrical system of the heart is safe and does not instigate the very arrhythmias that KCNQ1-SupRep gene therapy was designed to prevent. Nevertheless, because of the *in vitro* success of this KCNQ1-SupRep gene therapy in the patient-specific LQT1 iPSC-CM model system, KCNQ1-SupRep– the first hybrid gene therapy for any form of genetic heart disease– can now advance to animal model studies of LQT1.

Conclusion

Using two *in vitro* model systems, we have engineered and validated the desired APD-attenuating effect of the first hybrid suppression-and-replacement gene therapy construct for genetic heart disease in general, LQTS specifically, and LQT1 in particular. If the "therapeutic efficacy" of this disease-in-the-dish gene therapy trial with KCNQ1-SupRep can be replicated in a nonhuman, animal model of LQT1, then suppression-replacement gene therapy may be a promising strategy for directly targeting the pathogenic substrate and ameliorating the resultant disease for not only LQT1, but also LQTS in general, and in theory almost any sudden death-predisposing autosomal dominant genetic heart disease.

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Disclosures

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Supplemental Materials

Expanded Methods

Online-only Supplemental Tables I-V

Online-only Supplemental Figures I-VIII

References 51-56

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Table 1. Summary of Figure 6 FluoVolt optical action potential data showing APD_{90} and APD_{50} values (mean ± S.D.) obtained from untreated, "healthy" unrelated control iPSC-CMs and LQT1 iPSC-CMs transduced with shCT or KCNQ1-SupRep lentivirus.

iPSC-CMs	shCT APD_{90} (ms)	SupRep APD_{90} (ms)	Δ APD_{90} (ms)	p-value (SupRep v. shCT)	shCT APD_{50} (ms)	SupRep APD_{50} (ms)	Δ APD_{50} (ms)	p-value (SupRep v. shCT)
Unrelated Control	[Untreated] 332 ± 53 (n=50)	—	—	—	[Untreated] 184 ± 23 (n=50)	—	—	—
KCNQ1-Y171X	585 ± 77 (n=52) p<0.0001 ****	468 ± 43 (n=63) p<0.0001 ****	–117	p<0.0001 ****	230 ± 26 (n=52) p=0.0015 **	181 ± 23 (n=63) p=0.9997 n.s.	–49	p<0.0001 ****
KCNQ1-V254M	580 ± 57 (n=42) p<0.0001 ****	469 ± 89 (n=55) p<0.0001 ****	–111	p<0.0001 ****	353 ± 112 (n=42) p<0.0001 ****	224 ± 96 (n=55) p=0.0073 **	–129	p<0.0001 ****
KCNQ1-I567S	452 ± 72 (n=45) p<0.0001 ****	367 ± 60 (n=45) p=0.1945 n.s.	–85	p<0.0001 ****	184 ± 24 (n=45) p>0.9999 n.s.	149 ± 24 (n=45) p<0.0424 *	–35	p<0.0001 ****
KCNQ1-A344A/spl	553 ± 98 (n=61) p<0.0001 ****	343 ± 133 (n=63) p<0.9757 n.s.	–210	p<0.0001 ****	350 ± 94 (n=61) p<0.0001 ****	142 ± 47 (n=63) p<0.0033 **	–208	p<0.0001 ****

APD_{90} and APD_{50} values were assessed by one-way ANOVA with post-hoc Dunnett's test comparing each *KCNQ1* variant treated with shCT or KCNQ1-SupRep to the untreated, unrelated control (all p-values except those listed in the SupRep v. shCT columns). All four LQT1 iPSC-CMs treated with shCT had significantly longer APD_{90} than the unrelated control, and three of the four had significantly longer APD_{50} as well, validating that these LQT1 lines display prolonged APD, the hallmark feature of LQT1. APD shortening due to KCNQ1-SupRep compared to treatment with shCT was then assessed by unpaired two-tailed student's t-tests at both the APD_{90} and APD_{50} levels separately for each variant. KCNQ1-SupRep resulted in statistically significant attenuation of both APD_{90} and APD_{50} in all four LQT1 iPSC-CMs. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, (n.s.) not significant.

Figure Legends

Figure 1. *KCNQ1* suppression-replacement (KCNQ1-SupRep) vector design. A, Sequence alignment of shKCNQ1 to KCNQ1-WT cDNA (top) and "shRNA-immune" *KCNQ1* (KCNQ1-shIMM, bottom) which includes 10 wobble base synonymous variants (red). Sequence shown is KCNQ1 p.V458-P469 (c.1372-1407, NM_000218.2). **B**, KCNQ1-SupRep vector map. (U6) U6 promoter; (CMV) CMV promoter; (IRES) internal ribosome entry site; (CFP) cyan fluorescent protein.

Figure 2. shKCNQ1 knocks down KCNQ1-WT but not KCNQ1-shIMM in TSA201 cells co-transfected with KCNQ1-WT or KCNQ1-shIMM and shCT, shKCNQ1, or KCNQ1-SupRep. A, Relative *KCNQ1* expression normalized to *GAPDH* measured by allele-specific qRT-PCR quantifying KCNQ1-WT (white) and KCNQ1-shIMM (grey). **B**, Results confirmed with western blot for KCNQ1 with cofilin as housekeeping control. **C**, ImageJ quantification of western blot pixel density. Results and representative images were obtained from three independent experiments (defined as three identical repeats of each experiment conducted from start to finish on separate weeks with one biological replicate per treatment group per run). Graphs show mean with S.D. error bars. For relative *KCNQ1*, one-way ANOVA with post-hoc Tukey's test for multiple comparisons was used in both (**A**) and (**C**). For the sample treated with KCNQ1-SupRep in (**A**), an unpaired 2-tailed student's t-test was used to compare the proportion of KCNQ1-WT compared to KCNQ1-shIMM (red bracket). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, (n.s.) not significant.

Figure 3. Patch clamp analysis of I_{Ks} in TSA201 cells co-transfected with KCNQ1-WT, KCNQ1-shIMM, or KCNQ1-variants and the K_v7.1 beta-subunit, *KCNE1*. KCNQ1-shIMM produces WT I_{Ks} current. Previously uncharacterized functionally, KCNQ1-Y171X and KCNQ1-I567S produced no I_{Ks} current. KCNQ1-V254M similarly produced no I_{Ks} current consistent with previous characterization in the literature. **A**, Voltage clamp I_{Ks} representative traces. **B**, Peak current density. **C**, Peak current density at +80mV depolarization step. Error bars represent (**B**) S.E. for clarity and (**C**) S.D. One-way ANOVA with post-hoc Tukey's test for multiple comparisons was used in (**C**). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, (n.s.) not significant.

Figure 4. KCNQ1-SupRep knocks down LQT1 disease-causing *KCNQ1* variants including both nonsense and missense variants and replaces with KCNQ1-shIMM. TSA201 cells were co-transfected with KCNQ1-WT or KCNQ1-variants and shCT, shKCNQ1, or KCNQ1-SupRep. shKCNQ1 knocks down *KCNQ1* in a variant-independent manner. KCNQ1-SupRep knocks down *KCNQ1* variants via shKCNQ1 and expresses KCNQ1-shIMM which is knockdown immune. **A**, Proportional expression of KCNQ1-WT/variants and KCNQ1-shIMM was detected using allele-specific qRT-PCR measuring KCNQ1-WT/variant (white) and KCNQ1-shIMM (grey). **B**, Overall KCNQ1 expression (not allele-specific) was validated in western blot with cofilin as housekeeping control. Results and representative images were obtained from three independent experiments (defined as three identical repeats of each experiment conducted from start to finish on separate weeks with one biological replicate per treatment group per run). Graph shows mean with S.D. error bars. For relative *KCNQ1*, a separate one-way ANOVA with post-hoc Tukey's test for multiple comparisons was conducted for each *KCNQ1* variant to

compare the three treatments and avoid extraneous comparisons between variants. In samples treated with KCNQ1-SupRep, an unpaired two-tailed student's t-test was used to compare the proportion of KCNQ1-WT compared to KCNQ1-shIMM (red brackets). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, (n.s.) not significant.

Figure 5. Immunofluorescence of iPSC-CMs derived from a patient with KCNQ1-V254M mediated LQT1 one week after transduction with lentiviral shCT or KCNQ1-SupRep. The patient-derived iPSC-CMs were stained with three separate antibodies to demonstrate 1) presence of cardiomyocytes [cardiac troponin T (cTnT, pink)], 2) transduction by lentivirus as indicated by the turboGFP reporter in shCT (GFP, top panel, red) or by the CFP reporter in KCNQ1-SupRep (CFP, bottom panel, red), and 3) the presence of KCNQ1 (green) either endogenous or as the result of treatment with KCNQ1-SupRep. The results show high purity populations of cardiomyocytes that are evenly transduced with lentiviral shCT or KCNQ1-SupRep. In shCT, there is weak staining for KCNQ1, but in treatment with KCNQ1-SupRep, KCNQ1 staining is bright and indicates robust expression. Cells were counterstained with DAPI for nuclear stain (blue). Representative images of iPSC-CMs from one LQT1 variant (KCNQ1-V254M). Immunofluorescence results for iPSC-CMs derived from the unrelated control and other three LQT1 variants (KCNQ1-Y171X, -I567S, and -A344A/spl) can be found in **Figure VII in the Supplement**. Scale bars 50μm.

Figure 6. FluoVolt voltage dye optical action potentials show action potential duration (APD) shortening in LQT1 iPSC-CMs treated with lentivirus containing KCNQ1-SupRep compared to shCT. A, Representative traces showing three consecutive action potentials paced

at 1Hz. **B**, APD_{90} and APD_{50} values for untreated, unrelated healthy control and KCNQ1-Y171X, KCNQ1-V254M, KCNQ1-I567S, and KCNQ1-A344A/spl iPSC-CMs treated with shCT or KCNQ1-SupRep. Action potential trace videos were obtained for 20s duration at 50fps with 1Hz pacing. Regions of interest containing flashing cells were identified, and the changes in fluorescence intensity over time were measured to produce optical action potentials from which APD_{90} and APD_{50} values were determined. APD_{90} and APD_{50} values for all action potentials within a 20s trace were averaged to produce a single data point. The total number of measurements (n) is shown. Dot plots show median (horizontal black line). APD shortening due to KCNQ1-SupRep compared to treatment with shCT was assessed by unpaired two-tailed student's t-tests at both the APD_{90} and APD_{50} levels separately for each variant. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, (n.s.) not significant.

Figure 7. CRISPR-Cas9 corrected isogenic controls serve as a marker for "ideal" therapeutic correction of the cardiac APD. FluoVolt voltage dye measurement of the cardiac APD in isogenic control iPSC-CMs generated from two of the four LQT1 iPSCs (KCNQ1-V254M and KCNQ1-A344A/spl). Data for treatment with shCT (red) or KCNQ1-SupRep (blue) was shown here unchanged from **Figure 6**. Both isogenic control iPSC-CMs (yellow) had significantly shorter APD_{90} and APD_{50} than the LQT1 iPSC-CMs treated with shCT, which indicates that correction of the single pathogenic LQT1 variant in KCNQ1 is able to rescue the disease phenotype *in vitro*. As with the unrelated control, the isogenic controls were measured untreated as to provide the purest signal for a normal APD. Treatment of LQT1 iPSC-CMs with KCNQ1-SupRep results in APD shortening, however the degree of shortening is variable. For KCNQ1-V254M, KCNQ1-SupRep undercorrected the prolonged APD_{90} and overcorrected the

APD_{50}. In KCNQ1-A344A/spl, ideal correction for the APD_{90} was achieved and matched the isogenic control APD_{90}, however overcorrection of the APD_{50} also occurred. **A**, Representative traces showing three consecutive action potentials paced at 1Hz. **B**, APD_{90} and APD_{50} values for untreated, isogenic controls (yellow) and KCNQ1-V254M and KCNQ1-A344A/spl iPSC-CMs treated with shCT (red) or KCNQ1-SupRep (blue). APD_{90} and APD_{50} values are summarized in **Table V in the Supplement**. Action potential trace videos were obtained for 20s duration at 50fps with 1Hz pacing. Regions of interest containing flashing cells were identified, and the changes in fluorescence intensity over time were measured to produce optical action potentials from which APD_{90} and APD_{50} values were determined. APD_{90} and APD_{50} values for all action potentials within a 20s trace were averaged to produce a single data point. The total number of measurements (n) is shown. Dot plots show median (horizontal black line). A one-way ANOVA with post-hoc Tukey's test comparing all pairs for APD_{90} and all pairs for APD_{50} was used for each *KCNQ1* variant tested. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, (n.s.) not significant. (Ctrl) control.