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ORIGINAL ARTICLE

Suppression and Replacement Gene Therapy for *KCNH2*-Mediated Arrhythmias

Sahej Bains, BS; Wei Zhou, MD; Steven M. Dotzler, BA; Katherine Martinez; CS; John Kim, PhD; David J. Tester, BS; Dan Ye, MD; Michael J. Ackerman, MD, PhD

BACKGROUND: *KCNH2*-mediated arrhythmia syndromes are caused by loss-of-function (type 2 long QT syndrome [LQT2]) or gain-of-function (type 1 short QT syndrome [SQT1]) pathogenic variants in the *KCNH2*-encoded K_v11.1 potassium channel, which is essential for the cardiac action potential.

METHODS: A dual-component "suppression-and-replacement" (SupRep) *KCNH2* gene therapy was created by cloning into a single construct a custom-designed *KCNH2* short hairpin RNA with ~80% knockdown (suppression) and a "short hairpin RNA-immune" *KCNH2* cDNA (replacement). Induced pluripotent stem cell-derived cardiomyocytes and their CRISPR-Cas9 variant-corrected isogenic control (IC) induced pluripotent stem cell-derived cardiomyocytes were made for 2 LQT2- (G604S, N633S) and 1 SQT1- (N588K) causative variants. All variant lines were treated with KCNH2-SupRep or non-targeting control short hairpin RNA (shCT). The APD at 90% repolarization (APD_{90}) was measured using FluoVolt voltage dye.

RESULTS: KCNH2-SupRep achieved mutation-independent rescue of both pathologic phenotypes. For LQT2-causative variants, treatment with KCNH2-SupRep resulted in shortening of the pathologically prolonged APD_{90} to near curative (IC-like) APD_{90} levels (G604S IC, 471±25 ms; N633S IC, 405±55 ms) compared with treatment with shCT (G604S: SupRep-treated, 452±76 ms versus shCT-treated, 550±41 ms; $P<0.0001$; N633S: SupRep-treated, 399±105 ms versus shCT-treated, 577±39 ms, $P<0.0001$). Conversely, for the SQT1-causative variant, N588K, treatment with KCNH2-SupRep resulted in therapeutic prolongation of the pathologically shortened APD_{90} (IC: 429±16 ms; SupRep-treated: 396±61 ms; shCT-treated: 274±12 ms).

CONCLUSIONS: We provide the first proof-of-principle gene therapy for correction of both LQT2 and SQT1. KCNH2-SupRep gene therapy successfully normalized the pathologic APD_{90}, thereby eliminating the pathognomonic feature of both LQT2 and SQT1.

Key Words: gene therapy ■ induced pluripotent stem cells ■ KCNH2 ■ long QT syndrome ■ variant

Long QT syndrome (LQTS) is a predominantly autosomal dominant, genetic heart disease characterized by a prolonged heart rate-corrected QT interval (QTc) on a 12-lead ECG.[1] LQTS confers an increased risk and predisposition toward syncope, seizures, and sudden cardiac death. The estimated prevalence for LQTS is 1:2000 individuals, and unfortunately, in 13% of LQTS cases, sudden cardiac death is the sentinel event.[2] Conversely, short QT syndrome is characterized by a shortened QTc on a 12-lead ECG, has been reported in ~250 cases worldwide, and is potentially lethal as well.[3]

About 30% of patients with LQTS host loss-of-function (LOF) mutations in the second most common LQTS-causative gene, *KCNH2*. While LOF mutations in the *KCNH2*-encoded I_{Kr} (K_v11.1) potassium channel cause type 2 LQTS (LQT2), gain-of-function mutations result in type 1 short QT syndrome (SQT1).[4,5] Importantly, no single pathogenic variant within *KCNH2* accounts for the

Correspondence to: Michael J. Ackerman, MD, PhD, Mayo Clinic Windland Smith Rice Sudden Death Genomics Laboratory, Guggenheim 501, Mayo Clinic, Rochester, MN 55905. Email ackerman.michael@mayo.edu
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Nonstandard Abbreviations and Acronyms

APD_{90}	action potential duration at 90% repolarization
I_{Kr}	rapid delayed rectifier current
iPSC-CM	induced pluripotent stem cell-derived cardiomyocyte
LOF	loss-of-function
LQTS	long QT syndrome
RNAi	interfering RNA
shCT	scramble control shRNA
shRNA	short hairpin RNA
SQT1	type 1 short QT syndrome
WT	wild type

majority of the disease, making targeted patient-specific therapy an ideal approach.

The current therapeutic options for patients with LQT2 include drug therapy (primarily β–blockers, BBs), denervation therapy (left cardiac sympathetic denervation), and/or device therapy (implantable cardioverter defibrillator). These efforts are usually efficacious, however, while most of the patients remain protected while on BB therapy, there is an underserved subset of patients suffering from breakthrough cardiac events including implantable cardioverter defibrillator shocks and sudden cardiac death.[6–8] Noncompliance is common with BBs due to intolerable side-effects, and failure of BB therapy may necessitate invasive strategies such as left cardiac sympathetic denervation or an implantable cardioverter defibrillator.[7,9,10] However, these invasive therapies are not without complications (eg, infection, lead fracture)[11,12] and more importantly, they do not treat the underlying pathology (i.e. dysfunction of ion channels). Therefore, there remains an unmet need for a precision medicine-based effective treatment for genetic heart diseases like LQT2 and SQT1.

Inadequacies in current treatments have prompted development of gene therapies that target the cause of the disease. One strategy uses interfering RNA (RNAi) including short hairpin RNA (shRNA), which utilizes the endogenous gene silencing mechanism to knockdown the target gene. Allele-specific RNAi that targets only mutant alleles have been developed and have proven effective in preliminary studies for cardiac diseases including LQT2, *MYH7*-mediated hypertrophic cardiomyopathy, and *RYR2*-mediated catecholaminergic polymorphic ventricular tachycardia.[13–15] However, this strategy is inadequate for LQTS where hundreds of unique pathogenic variants, often unique to individual families, have been described within the LQTS-susceptibility genes, which necessitate development of an individual RNAi for each and every variant. While initially thought to be a promising strategy, allele-specific, RNAi-based gene therapy for LQTS has insurmountable shortcomings. To overcome these limitations, we recently designed and developed the first suppression-and-replacement (SupRep) *KCNQ1* gene therapy vector for the potential treatment of patients with *KCNQ1*-mediated LQTS (LQT1).[16]

Herein, we describe the development of a novel and highly innovative SupRep gene therapy strategy for either *KCNH2*-mediated LQT2 or *KCNH2*-mediated SQT1 that is designed to treat patients regardless of which of the hundreds of pathogenic, nonsynonymous *KCNH2* variants that they may host. The development of SupRep-based gene therapy may be highly transformative not only to the therapeutic management of patients with LQTS/short QT syndrome but also to the entire field of cardiac channelopathies and genetic cardiology. Importantly, this innovative strategy has distinct advantages over other forms of RNAi: (1) applicability regardless of the patient's specific disease-causative variant because the shRNA is designed to knockdown the gene by targeting a conserved coding sequence rather than specific pathogenic variants and (2) replacement allows rescue of the disease phenotype beyond haploinsufficiency and could approach the level of "true normal" by adjusting the dose or expression of the replacement copy.[17]

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. Under the Mayo Clinic approved Institutional Review Board (IRB, 09-006465), human samples were obtained from 2 patients with LQT2 and 1 healthy control, following written informed consent. The detailed methods are available within the Supplement. The authors declare that all supporting data are available within the article and its Supplemental Material.

RESULTS

Selection of the Lead shRNA Candidate for KCNH2-SupRep Gene Therapy

To generate the KCNH2-SupRep vector, 5 *KCNH2* shRNAs (sh#1-5) were custom designed. The target sequences of these shRNAs were assessed using ClinVar and the Genome Aggregation Database (gnomAD) and were devoid of common genetic polymorphisms and known pathogenic/likely pathogenic variants in *KCNH2* that could impact the knockdown efficiency (data not shown). After this initial quality control screen, the 5 *KCNH2* shRNAs along with a non-targeting scramble control shRNA (shCT) were purchased in the pGFP-C-shLenti lentiviral backbone from OriGene (Table S1). All 5 shRNAs were first screened for knockdown efficiency by co-transfecting each shRNA (#1-5) with KCNH2-wild-type (WT) in TSA201 cells. The expression of *KCNH2* was measured using quantitative reverse transcription

polymerase chain reaction (qRT-PCR, Figure 1A) and confirmed via western blot (Figure 1B). Of the 5 custom-designed shRNAs, only sh#4 achieved significant knockdown of *KCNH2* (mRNA: 75%–82%; *P*=0.013) and was selected for further studies.

KCNH2 sh#4, henceforth referred to as shKCNH2, was selected for the final KCNH2-SupRep gene therapy vector. To create the replacement "shKCNH2-immune" cDNA copy of *KCNH2*, called KCNH2-shRNA-immune (shIMM), ten synonymous nucleotide variants were introduced into the wobble base of each codon within the shKCNH2 target sequence (nucleotides c.2694-2722) as detailed within the Supplemental Material. KCNH2-shIMM was cloned downstream of the CMV promoter in the shKCNH2-containing pGFP-C-shLenti vector. In this step, the original turboGFP reporter was exchanged for a human influenza hemagglutinin (HA) tag.

KCNH2-SupRep Gene Therapy Both Suppresses and Replaces KCNH2-WT but not KCNH2-shIMM

To validate whether KCNH2-shIMM is indeed immune to knockdown by shKCNH2, TSA201 cells were co-transfected with KCNH2-WT or KCNH2-shIMM and shKCNH2 or shCT. The relative expression of *KCNH2* was quantified using quantitative reverse transcription polymerase chain reaction and confirmed via western blot. Each sample was run in four separate reactions using allele-specific primers (Table S3). These primers were used to quantify (1) total *KCNH2*, (2) endogenous *KCNH2* including WT and variant alleles but not KCNH2-shIMM, (3) KCNH2-shIMM, and (4) *GAPDH* as housekeeping. Total *KCNH2* was measured using commercially available primers. Endogenous *KCNH2* (both WT and variant alleles) and KCNH2-shIMM were measured exclusively using a common forward primer and 2 unique reverse primers. The reverse primers targeted the shKCNH2 site with one being complementary to the WT sequence and the other complementary to the modified KCNH2-shIMM sequence. A standard curve was used to correct for PCR amplification bias.

As expected, compared with shCT, shKCNH2 achieved suppression of KCNH2-WT by 80% (Figure 2A). Notably, both shCT and shKCNH2 were unable to reduce KCNH2-shIMM (expression of KCNH2-shIMM measured as 100% and 90%, respectively). This indicates the "immunity" of KCNH2-shIMM against knockdown by shKCNH2. Next, KCNH2-SupRep was co-transfected with KCNH2-WT which resulted in 70% suppression of KCNH2-WT with 93% replacement with KCNH2-shIMM. The dual component KCNH2-SupRep vector had similar knockdown of KCNH2-WT as compared with KCNH2-WT+shKCNH2 and similar replacement with KCNH2-shIMM as compared with KCNH2-shIMM+shCT. All results obtained via quantitative reverse transcription polymerase chain reaction were confirmed by western blot. Western blot results visibly indicated that shKCNH2 is able to knockdown KCNH2-WT but not KCNH2-shIMM (Figure 2B).

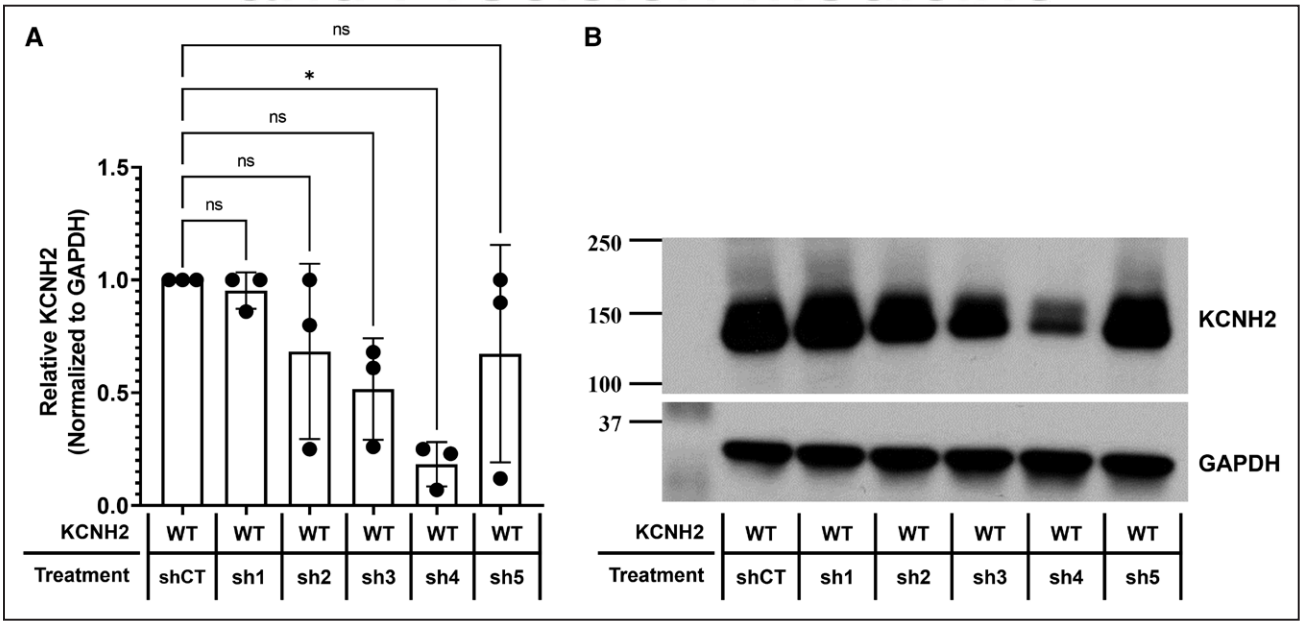

Figure 1. Selection of the lead short hairpin RNA (shRNA) candidate for KCNH2-SupRep.
TSA201 cells were co-transfected with KCNH2-WT and 5 custom-designed shRNAs (#1-5) or a non-targeting control shRNA (shCT). **A**, Relative expression of *KCNH2* normalized to *GAPDH* as measured by quantitative reverse transcription PCR (qRT-PCR). **B**, Confirms the results of qRT-PCR using Western blot. *KCNH2* sh4 (shKCNH2) provided the greatest knockdown and was selected for the final KCNH2-SupRep vector. Graphs show mean with SD error bars. One-way ANOVA (*P*=0.0321) with post-hoc Tukey test for multiple comparisons was used to analyze qRT-PCR results. ns indicates not significant; shRNA, short hairpin RNA; and WT, wild-type. **P*=0.0132.

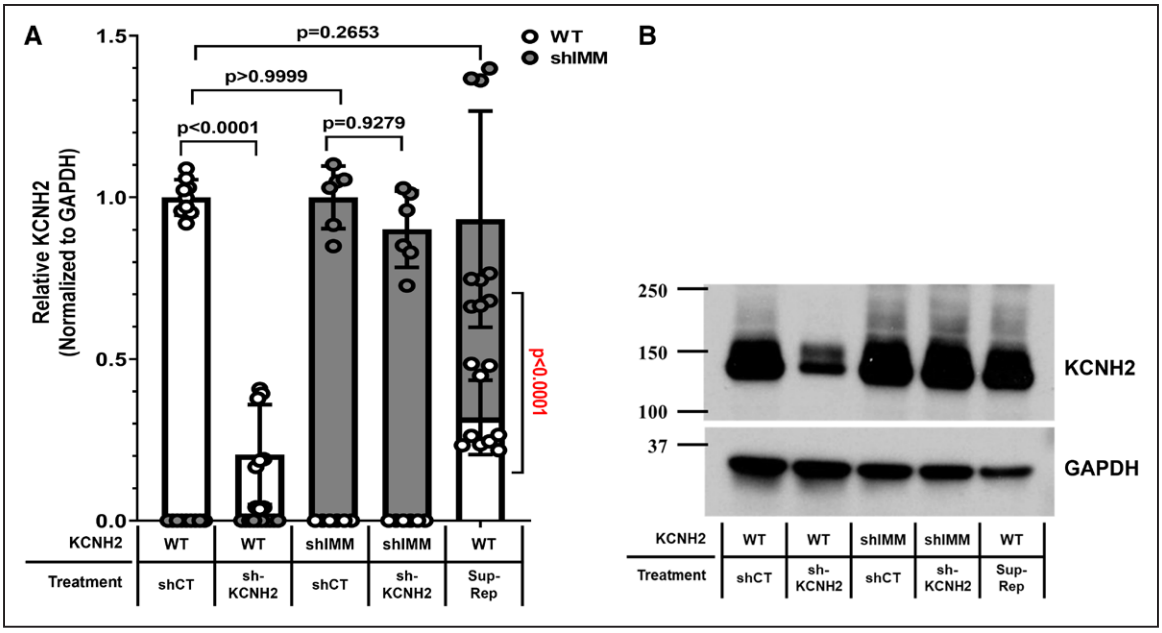

Figure 2. Selective knockdown of the wild-type (WT) but not shRNA-immune (shIMM) alleles.
shKCNH2 knocks down KCNH2-WT but not short hairpin RNA (shRNA)-immune KCNH2 (KCNH2-shIMM)in TSA201 cells co-transfected with KCNH2-WT or KCNH2-shIMM and non-targeting scramble shRNA (shCT), shKCNH2, or KCNH2-SupRep. **A**, Shows the relative *KCNH2* expression normalized to *GAPDH* as measured by allele-specific quantitative reverse transcription (qRT-PCR) quantifying KCNH2-WT (white) and KCNH2-shIMM (gray). **B**, Shows confirmation of the qRT-PCR results with Western blot for KCNH2 with GAPDH as housekeeping control. For relative KCNH2, 1-way ANOVA ($P<0.0001$) with post-hoc Tukey test for multiple comparisons (P shown in the panel in black) was performed to compare the total relative KCNH2. For the sample treated with KCNH2-SupRep, an unpaired 2-tailed Student t test (P shown in red) was done to compare the proportion of KCNH2-WT with KCNH2-shIMM.

Selection of Patients With LQT2-Causative Variants

From our biorepository comprising over 200 patients with LQT2, 2 patients hosting 2 unique LQT2-causative variants (KCNH2-G604S and KCNH2-N633S) were selected for this study based on their strong LQT2 phenotype including a QTc exceeding 500 ms, a positive history of LQTS-related symptoms (syncope, seizure, sudden cardiac arrest), and a positive family history of LQTS-related symptoms. These dominant-negative *KCNH2* missense variants have been characterized and described previously in the literature.[18]

Validation of Variant-Independent Suppression and Replacement Using KCNH2-SupRep

To evaluate whether KCNH2-SupRep can suppress and replace *KCNH2* in a variant-independent manner, KCNH2-WT, KCNH2-G604S, or KCNH2-N633S were co-transfected with either shCT, shKCNH2, or KCNH2-SupRep in TSA201 cells. As expected, compared with shCT, shKCNH2 achieved marked suppression of KCNH2-WT (80%), KCNH2-G604S (83%), KCNH2-N633S (78%) as measured by allele-specific quantitative reverse transcription polymerase chain reaction (Figure 3A). These results were confirmed with Western blot showing visible suppression of KCNH2-WT, KCNH2-G604S, and KCNH2-N633S by shKCNH2 (Figure 3B). Moreover, co-transfection of KCNH2-SupRep shows suppression of KCNH2-WT, KCNH2-G604S, and KCNH2-N633S and replacement with KCNH2-shIMM as seen via both quantitative reverse transcription polymerase chain reaction and Western blot. Overall, KCNH2-SupRep achieved suppression and replacement of 2 LQT2-causative *KCNH2* missense variants, validating its ability to function in a variant-independent manner. Furthermore, similar to a dose-dependent suppression and replacement shown in our previous studies with KCNQ1-SupRep,[16] we have provided a dose-dependent effect of KCNH2-SupRep in both HEK cells (Figure 4A) as well as in a patient-derived induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) model (Figure 4B). This shows that the expression level of KCNH2-SupRep can be adjusted by titrating the therapeutic dose.

Generation of LQT2 and SQT1 Patient-Derived iPSC-CMs

Patient-derived iPSC-CM models of both p.G604S and p.N633S were generated to test the ability of KCNH2-SupRep to shorten the pathologically prolonged APD, the hallmark pathognomonic feature of LQT2. CRISPR-Cas9 variant-corrected isogenic controls were created for both KCNH2-G604S and KCNH2-N633S to denote

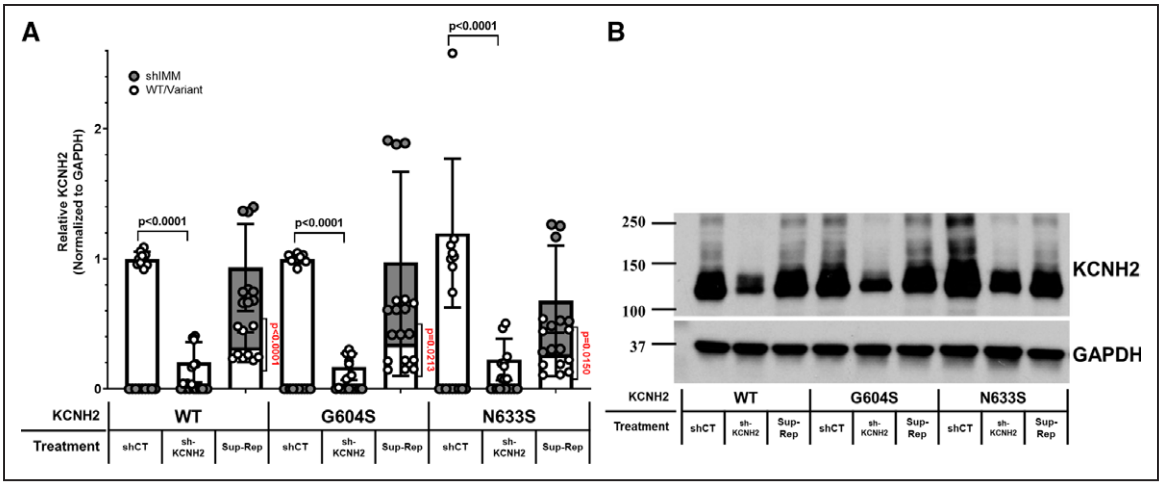

Figure 3. KCNH2-SupRep achieves variant-independent knockdown.
KCNH2-SupRep knocks down type 2 long QT syndrome (LQT2) disease-causing *KCNH2* missense variants and replaces with KCNH2-short hairpin RNA (shRNA)-immune (shIMM). TSA201 cells were co-transfected with KCNH2-wild-type (WT) or KCNH2-variants and non-targeting scramble shRNA (shCT), shKCNH2, or KCNH2-SupRep. shKCNH2 knocks down *KCNH2* in a variant-independent manner. KCNH2-SupRep knocks down *KCNH2* variants via shKCNH2 and expresses shRNA-immune KCNH2 (KCNH2-shIMM), which is knockdown immune. **A**, Shows proportional expression of KCNH2-WT/variants and KCNH2-shIMM detected using allele-specific quantitative reverse transcription PCR (qRT-PCR) measuring KCNH2-WT/variant (white) and KCNH2-shIMM (gray). **B**, Shows overall KCNH2 expression (not allele-specific) validated in Western blot with GAPDH as housekeeping control. For relative KCNH2 expression between the shCT-(control) and shKCNH2-treated samples (WT or variant), 1-way ANOVA (P<0.0001) with post-hoc Tukey test for multiple comparisons (P shown in black in the panel) was performed to compare total relative KCNH2. For the samples treated with KCNH2-SupRep, an unpaired 2-tailed Student t test (P shown in red) was done to compare the proportion of endogenous KCNH2 (WT or variant) with KCNH2-shIMM.

the curative level for the APD (Table S2). Considered to be the gold standard for a therapeutic cure, isogenic controls for each variant were used to determine how close to ideal APD the treatment with KCNH2-SupRep could achieve.

The LQT2 iPSC lines used in this study were generated and assessed for standard quality control including sanger sequencing, karyotyping, and immunofluorescence microscopy for pluripotent markers by our group previously.[19] The previously reported SQT1-causative p.N588K variant[20] was created by variant insertion into a healthy control iPSC line using CRISPR-Cas9 technology and was assessed subsequently for quality control (Figure S1). Differentiation of iPSCs into spontaneously beating iPSC-CMs was induced as described previously.[21,22] The cardiac APD is known to shorten as iPSC-CMs mature over time. Thus, all experiments were conducted at least 30 days after the induction of differentiation.[23]

KCNH2-SupRep Shortens the Pathologically Prolonged Cardiac APD in LQT2 iPSC-CMs as Measured by FluoVolt Voltage Dye

To determine if KCNH2-SupRep can shorten the pathologically prolonged APD to normal levels, FluoVolt voltage dye was used to measure the optical action potentials in KCNH2-G604S and KCNH2-N633S iPSC-CMs with either lentiviral shCT control or KCNH2-SupRep. Isogenic control iPSC-CMs were treated with lentiviral shCT to eliminate any differences in APD arising from the viral vector itself. All iPSC-CMs were paced at 1 Hz during recording to avoid beat-rate dependent changes to the APD. Representative optical action potential tracings for various treatment configurations for KCNH2-G604S and KCNH2-N633S are shown in Figure 5A and 5C, respectively.

When treated with shCT, both KCNH2-G604S (550±41 ms) and KCNH2-N633S (577±39 ms) iPSC-CMs had significantly prolonged APD at 90% repolarization (APD_{90}) compared with their respective isogenic controls (471±25 ms, P<0.0001; and 405±55 ms, P<0.0001, respectively). However, treatment with KCNH2-SupRep significantly shortened the APD_{90} of KCNH2-G604S to 452±76 ms and KCNH2-N633S to 399±105 ms as compared with shCT (Figure 5B and 5D, respectively). In KCNH2-G604S, treatment with KCNH2-SupRep decreased the pathologically prolonged APD_{90} by 98 ms whereas in KCNH2-N633S, the APD_{90} was reduced by 178 ms. A full summary of APD_{90} values along with the APD shortening associated with KCNH2-SupRep is shown in the Table 1.

Notably, after treatment with KCNH2-SupRep, there was no significant difference in the APD_{90} of KCNH2-G604S (452±76 ms) and its isogenic control (471±25 ms; P=0.1153) or in APD_{90} of KCNH2-N633S (399±105 ms) and its isogenic control (405±55 ms; P=0.5104). Additionally, treatment of an isogenic control iPSC-CM line with KCNH2-SupRep did not have a

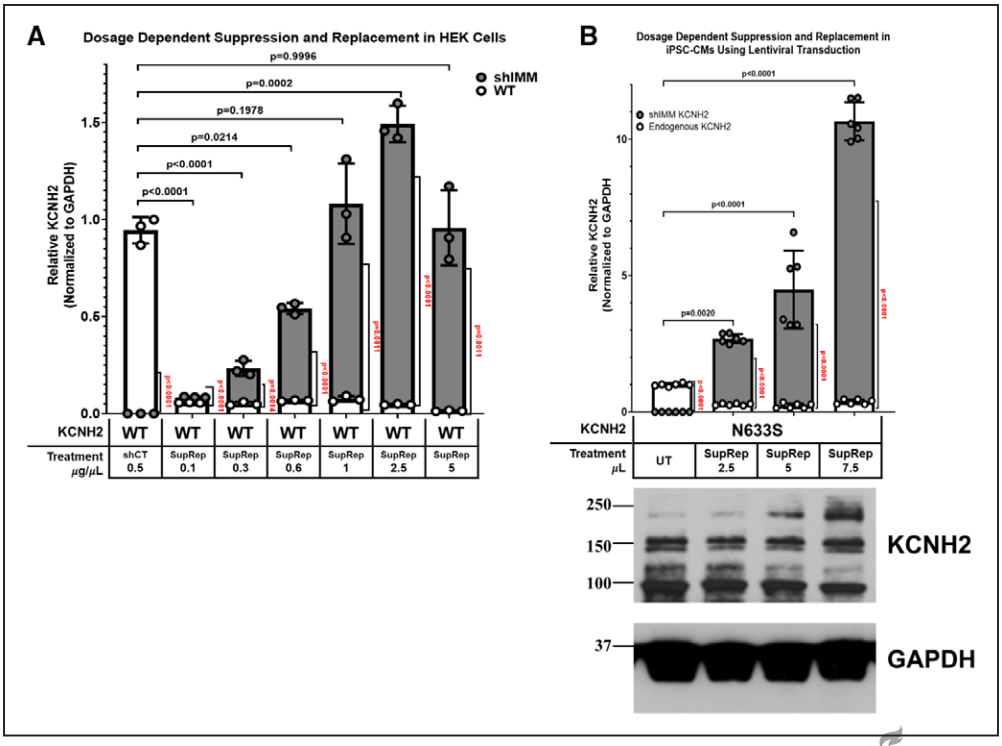

Figure 4. KCNH2-SupRep achieves dose-dependent replacement of KCNH2-shRNA-immune (shIMM). **A**, TSA201 (HEK) cells were co-transfected with KCNH2-wild-type (WT; 0.5 μg/μL) and either control short hairpin RNA (shCT; 0.5 μg/μL) or varying doses of KCNH2-SupRep (range: 0.1–5 μg/μL). **B**, KCNH2-N633S-induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs) were transduced with varying doses of KCNH2-SupRep. For both experiments, KCNH2 expression was measured using allele-specific qRT-PCR and normalized to GAPDH. The allele-specific proportions of WT/endogenous KCNH2 are represented with white bars and KCNH2-shIMM with gray bars. **B**, The corresponding Western blot is shown. A 1-way ANOVA (P<0.0001 for both panels) with post-hoc test for multiple comparisons (P shown in black) was performed to compare the expression level of total WT-KCNH2/endogenous KCNH2 and KCNH2-shIMM at different doses of SupRep. An unpaired 2-tailed Students t test (P shown in red) was performed to compare the allele-specific proportions of KCNH2-WT and KCNH2-shIMM in samples treated with suppression-and-replacement (SupRep). UT indicates untreated.

statistically significant effect on APD_{90} when compared with shCT-treated cells (Figure S2). Overall, treatment with KCNH2-SupRep gene therapy achieved correction and rescue of the pathologically prolonged action potential in LQT2 iPSC-CMs without overcorrection of the APD_{90}.

KCNH2-SupRep Prolongs the Pathologically Shortened Cardiac APD in SQT1 iPSC-CMs as Measured by FluoVolt Voltage Dye

Conversely, to determine whether KCNH2-SupRep can prolong the pathologically shortened APD in SQT1 iPSC-CMS, thereby eliminating the disease phenotype, p.N588K iPSC-CMs were treated with KCNH2-SupRep. Treatment with KCNH2-SupRep prolonged the APD_{90} as measured by FluoVolt to 396±61 ms as compared with treatment with shCT (274±12 ms, P<0.0001). Additionally, SupRep resulted in prolongation of the APD_{90} to near isogenic control levels (429±16 ms), thus demonstrating the efficacy of this therapeutic in SQT1 (Figure 6). A full summary of APD_{90} values is detailed in the Table 1.

DISCUSSION

Although current treatment strategies and outcomes reflect the tremendous progress that has been made in the management of patients with these genetic heart diseases,[8] one cannot overlook the need for continuous improvement wherein the clinician does not become complacent with the status quo therapeutic options. In diseases like LQT2 and SQT1 where the underlying mechanism of disease (dysfunction of ion channels) is clearly outlined, the quest for mechanism-based novel therapeutics is warranted. Although current therapies work via suppression of the arrhythmic activity by preventing sympathetic action of the ventricular myocardium (BBs and left cardiac sympathetic denervation) or by delivering a rescue shock to restore sinus rhythm if an event occurs (implantable cardioverter defibrillator), they do not address LQT2 or SQT1 at the substrate level. Herein, we describe a single suppression-replacement (SupRep) gene therapy for both LQT2 and SQT1.

Although replacement-only or suppression-only gene therapies have been approved in Europe or in the United States, there are currently no approved hybrid

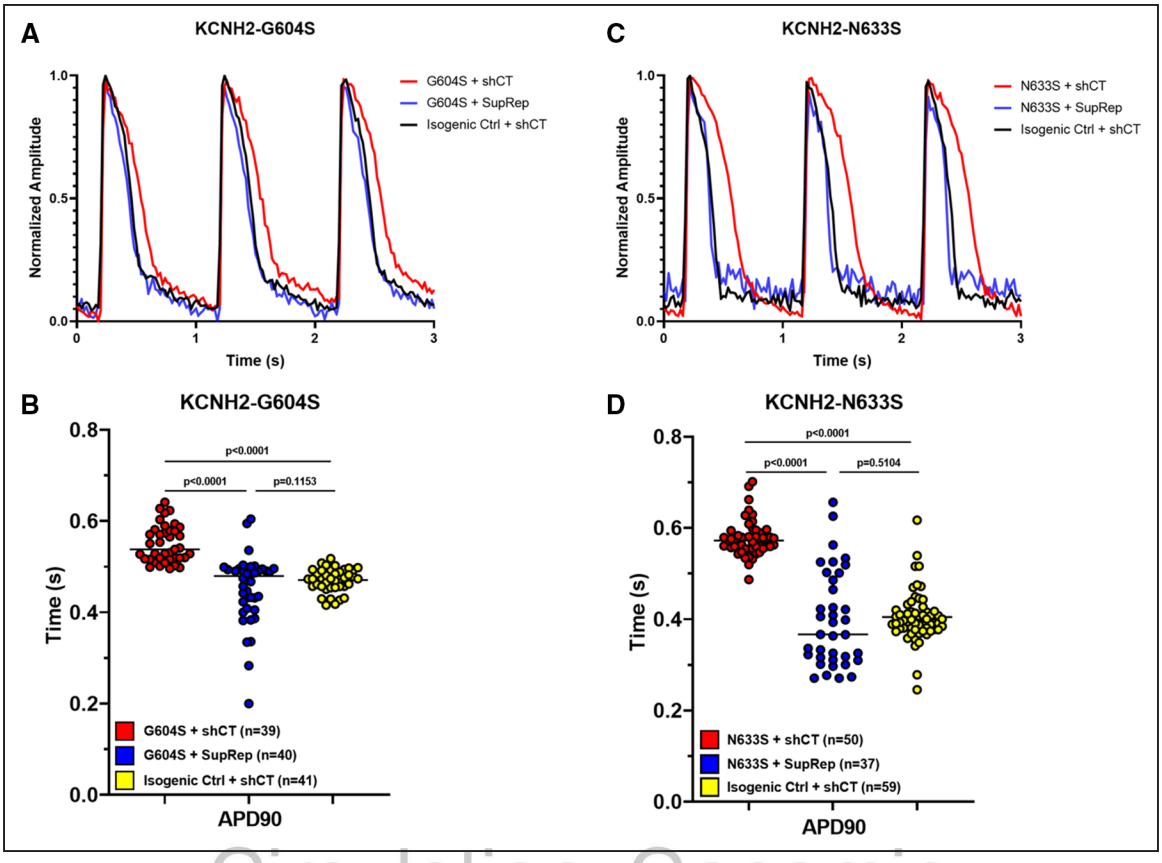

Figure 5. KCNH2-suppression-and-replacement (SupRep) shortens the pathologically prolonged action potential duration (APD) in type 2 long QT syndrome (LQT2) patient lines as compared with their respective isogenic controls.
FluoVolt voltage dye measurement of the cardiac APD in p.G604S and p.N633S iPSC-CMs and their respective isogenic control induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) are shown. APD_{90} values for isogenic controls treated with scramble control shRNA (shCT; yellow) and KCNH2-variant treated with shCT (red) or KCNH2-SupRep (blue) are shown. The isogenic control iPSC-CMs (yellow) had significantly shorter APD_{90} than the LQT2 iPSC-CMs treated with shCT, which indicates that correction of the single pathogenic LQT2 variant in *KCNH2* is able to rescue the disease phenotype in vitro. Treatment of LQT2 iPSC-CMs with KCNH2-SupRep results in APD_{90} shortening. For both variants, KCNH2-SupRep achieved shortening of the prolonged APD_{90} as compared with isogenic control treated with shCT. Action potential trace videos were obtained for 20-second duration at 50 fps with 1 Hz 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} values for all action potentials within a 20-second 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 1-way ANOVA with post-hoc Tukeys test comparing all pairs for APD_{90} was used. Following ANOVA, $P<0.0001$ for both **B** and **D**. Pairwise *P* following log transformation are shown in the panels. Ctrl indicates control.

suppression and replacement gene therapies.[24–33] The hybrid SupRep gene therapy involves 2 critical components that occur in tandem. First, suppression of both mutant and wild-type (WT) endogenous alleles occurs via a shRNA. Simultaneously, replacement occurs using a shRNA-immune or shIMM *KCNH2* cDNA copy which contains synonymous variants at the wobble base of each codon within the shRNA sequence. Due to the

Table 1. Summary of FluoVolt Optical Action Potential Data for LQT2 and SQT1 iPSC-CMs Treated With KCNH2-SupRep or shCT

iPSC-CMs	shCT APD_{90} (ms)	SupRep APD_{90} (ms)	Δ APD_{90} (ms)	*P* value (SupRepv. shCT)
KCNH2-G604S	550±41 (n=39)	452±76 (n=40)	−98	*P*<0.0001
Isogenic Control (for G604S)	471±25 (n=41)	…	…	…
KCNH2-N633S	577±39 (n=50)	399±105 (n=37)	−178	*P*<0.0001
Isogenic Control (for N633S)	405±55 (n=59)	…	…	…
KCNH2-N588K	274±12 (n=31)	396±61 (n=49)	+121	*P*<0.0001
Isogenic Control (for N588K)	429±16 (n=53)	…	…	…

APD indicates action potential duration; iPSC-CM, induced pluripotent stem cell-derived cardiomyocyte; shCT, non-targeting control shRNA; and SupRep, suppression and replacement gene therapy.

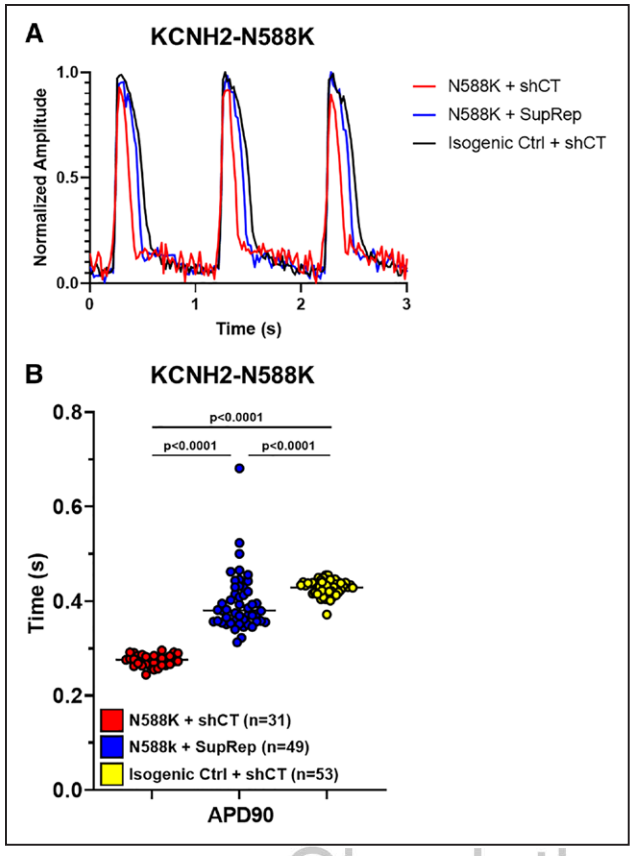

Figure 6. KCNH2-SupRep prolongs the pathologically shortened action potential duration (APD) in type 1 short QT syndrome (SQT1) induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs).
FluoVolt voltage dye measurement of the cardiac APD in p.N588K iPSC-CMs and their respective isogenic control iPSC-CMs are shown. APD_{90} values for isogenic controls treated with scramble control shRNA (shCT; yellow) and KCNH2-N588K treated with shCT (red) or KCNH2-SupRep (blue) are shown. Treatment with SupRep prolongs the APD to near isogenic control levels. Action potential trace videos were obtained for 20-second duration at 50 fps with 1 Hz 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} values for all action potentials within a 20-second 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 1-way ANOVA with post-hoc Tukey test comparing all pairs for APD_{90} was used. Following ANOVA, $P<0.0001$ for **B**. Pairwise P following log transformation are shown in the panel. Ctrl indicates control.

synonymous variants, the shRNA is unable to bind to this new copy, which importantly does not alter the WT amino acid sequence, thus rendering it immune to knockdown.

SupRep gene therapy has several advantages over suppression-only or replacement-only therapies. First, by suppressing both endogenous alleles and by targeting the gene rather than individual variants, a single, gene-specific SupRep therapeutic is theoretically applicable to all patients with either LQT2 or SQT1 without necessitating the development of multiple patient-specific RNAi. To date, nearly 500 LQT2-associated pathogenic variants have been identified with 60% representing missense variants which cause $K_v11.1$ channel LOF by either disrupting channel trafficking to the membrane, disruption of channel gating, and/or negatively affecting channel conductance and the remaining 40% representing nonsense, frameshift, or splice-site variants that inhibit $K_v11.1$ protein synthesis and lead to haploinsufficiency when present in a heterozygote state.[34,35]

Moreover, unlike suppression therapies, which could exacerbate LOF diseases, SupRep can be used to treat both gain-of-function (SQT1) or LOF (LQT2) *KCNH2*-mediated diseases. Alternatively, replacement therapies in which a missing/null protein is replaced by a healthy copy can help in diseases where a protein is deficient such as alipogene tiparvovec (Glybera) for the treatment of lipoprotein lipase deficiency caused by LOF variants in LPL-encoded lipoprotein lipase.[25] However, in the case of LQTS, augmented expression or replacement with a WT copy is not sufficient. The majority of LQTS is caused by dominant negative missense variants, which would interfere with the replacement WT *KCNH2*, rendering replacement-only gene therapy insufficient in addressing the disease pathobiology.[36]

In theory, SupRep gene therapy is generalizable to all monogenetic disease patients with any given pathogenic variant in the gene of interest. Previously, SupRep gene therapy has been attempted for only *RHO*-mediated retinitis pigmentosa,[37] *AAT*-mediated α-1-antitrypsin deficiency,[38] and *PABPN1*-mediated oculopharyngeal muscular dystrophy.[39] Recently, we developed a SupRep gene therapy strategy for *KCNQ1*-mediated LQTS type 1 (LQT1), the most common form of LQTS.[16] In our previous study, we were able to shorten the pathologically prolonged APD at 90% (APD_{90}) repolarization in 4 LQT1 patient-derived iPSC-CMs models (KCNQ1-Y171X, -V254M, -I567S, and -A344A/splice error) resulting in APD values similar to isogenic controls.[16]

Herein, we provide the first proof-of-principle, therapy-in-the-dish study using SupRep to successfully shorten and normalize the too long disease phenotype for *KCNH2*-mediated LQT2 and also to successfully lengthen and normalize the too short disease phenotype for *KCNH2*-mediated SQT1 using a custom designed potent shRNA selected in the region of *KCNH2* devoid of >99% of all known pathogenic variants and common variants listed in gnomAD. For LQT2, our work consistently showed that KCNH2-SupRep shortened the APD in a variant-independent manner in patient-derived iPSC-CMs. Moreover, the APD_{90} of both variant lines (p.G604S and p.N633S) treated with KCNH2-SupRep was not significantly different from the APD_{90} of the respective isogenic controls, showing that SupRep achieved "close to isogenic/WT" level of correction in patient lines. Conversely for SQT1, KCNH2-SupRep prolonged the APD to near isogenic control levels.

Though further investigation in animal models of LQT1, LQT2, and SQT1 will be needed for the advancement of these therapies toward the clinical stage in humans, we have demonstrated a promising glimpse of the potential of KCNQ1-SupRep[16] and now KCNH2-SupRep therapy in addressing both LQT1 and LQT2/SQT1 at the underlying substrate level. Given the successes of these preclinical studies, the development of additional SupRep gene therapies for other potentially lethal monogenic cardiac channelopathies and potentially cardiomyopathies are warranted and in progress.

LIMITATIONS

Though KCNH2-SupRep effectively corrected the APD in LQT2/SQT1 patient iPSC-CMs, there are several areas in which this current therapy can be improved. First, throughout this study, lentivirus-mediated delivery of KCNH2-SupRep was used for iPSC-CM experiments. Lentivirus was chosen due to its high transduction efficiency in vitro as compared with AAV9 which has strong cardiac tropism in vivo.[40–42] For future animal studies, AAV9 will be pursued over lentivirus due to its superior safety profile. Moreover, unlike lentiviruses, which integrate into the host genome and are at risk of insertional mutagenesis, AAVs do not integrate. Further, AAVs generate minimal immune response, and may require single dosing due to their indefinite presence in nondividing cells likes cardiomyocytes.[43] Second, in this study, FluoVolt voltage dye was used to assess the changed in APD upon treatment. Although FluoVolt is a reasonable method for action potential measurement, patch-clamp remains the gold standard. Future studies could be conducted using patch-clamp assays to assess the effect of KCNH2-SupRep on both the APD and the I_{Kr} channel in patient-derived iPSC-CMs. Third, whether KCNH2-SupRep will be efficacious in treating an LQT2 and/or SQT1 animal model remains unknown. Using this study as the foundation, KCNH2-SupRep gene therapy for either LQT2 or SQT1 rabbit models, which closely mimics the human LQT2 and SQT1 phenotype, respectively,[44,45] represent the critical next step.

CONCLUSIONS

The work detailed in this study has demonstrated the genesis of KCNH2-SupRep for the treatment of both LQT2 and SQT1 using an in vitro cardiac cellular model. KCNH2-SupRep effectively corrected the APD and rescued the LQT2 and SQT1 disease phenotypes in patient derived iPSC-CMs. These results provide a compelling justification to further test SupRep gene therapy in established rabbit models of these genetic heart diseases. Our suppression-replacement-based gene therapy, if successful, will be highly transformative not only to the therapeutic management of patients with LQTS and short QT syndromes but also to the entire field of cardiac channelopathies and genetic cardiology.

ARTICLE INFORMATION

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Affiliations

Medical Scientist Training Program (S.B., S.M.D.), Department of Molecular Pharmacology and Experimental Therapeutics (Windland Smith Rice Sudden Death Genomics Laboratory) (S.B., W.Z., S.M.D., K.M., C.S.J.K., D.J.T., D.Y., M.J.A.), Department of Pediatric and Adolescent Medicine, Division of Pediatric Cardiology (D.J.T., M.J.A.), and Division of Heart Rhythm Services, Department of Cardiovascular Medicine, Windland Smith Rice Genetic Heart Rhythm Clinic (D.J.T., M.J.A.), Mayo Clinic, Rochester, MN.

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Disclosures

Dr Ackerman is a consultant for Abbott, ARMGO Pharma, Boston Scientific, Bristol Myers Squibb, Daichii Sankyo, Invitae, LQT Therapeutics, Medtronic, and UpToDate. MJA and Mayo Clinic are involved in an equity/royalty relationship with AliveCor, Anumana, and Pfizer. However, none of these entities were involved in this study. Other authors declare no conflicts.

Supplemental Material

Supplemental Methods
Tables S1–S3
Figures S1 and S2
References[16,19,21,22]

REFERENCES

1. aMoss AJ, Kass RS. Long QT syndrome: from channels to cardiac arrhythmias. *J Clin Invest*. 2005;115:2018–2024. doi: 10.1172/JCI25537
2. Priori SG, Schwartz PJ, Napolitano C, Bloise R, Ronchetti E, Grillo M, Vicentini A, Spazzolini C, Nastoli J, Bottelli G, et al. Risk stratification in the long-QT syndrome. *N Engl J Med*. 2003;348:1866–1874. doi: 10.1056/NEJMoa022147
3. Campuzano O, Sarquella-Brugada G, Cesar S, Arbelo E, Brugada J, Brugada R. Recent advances in short QT syndrome. *Front Cardiovasc Med*. 2018;5:149. doi: 10.3389/fcvm.2018.00149
4. Tester DJ, Will ML, Haglund CM, Ackerman MJ. Compendium of cardiac channel mutations in 541 consecutive unrelated patients referred for long QT syndrome genetic testing. *Heart Rhythm*. 2005;2:507–517. doi: 10.1016/j.hrthm.2005.01.020
5. Napolitano C, Priori SG, Schwartz PJ, Bloise R, Ronchetti E, Nastoli J, Bottelli G, Cerrone M, Leonardi S. Genetic testing in the long QT syndrome: development and validation of an efficient approach to genotyping in clinical practice.[see comment]. *JAMA*. 2005;294:2975–2980. doi: 10.1001/jama.294.23.2975
6. Schwartz PJ, Periti M, Malliani A. The long Q-T syndrome. *Am Heart J*. 1975;89:378–390. doi: 10.1016/0002-8703(75)90089-7
7. Vincent GM, Schwartz PJ, Denjoy I, Swan H, Bithell C, Spazzolini C, Crotti L, Piippo K, Lupoglazoff JM, Villain E, et al. High efficacy of beta-blockers in long-QT syndrome type 1: contribution of noncompliance and QT-prolonging drugs to the occurrence of beta-blocker treatment "failures". *Circulation*. 2009;119:215–221. doi: 10.1161/CIRCULATIONAHA.108.772533
8. Rohatgi RK, Sugrue A, Bos JM, Cannon BC, Asirvatham SJ, Moir C, Owen HJ, Bos KM, Kruisselbrink T, Ackerman MJ. Contemporary outcomes in patients with long QT syndrome. *J Am Coll Cardiol*. 2017;70:453–462. doi: 10.1016/j.jacc.2017.05.046

9. Waddell-Smith KE, Li J, Smith W, Crawford J, Skinner JR. β-blocker adherence in familial long QT syndrome. *Circ Arrhythm Electrophysiol*. 2016;9: doi: 10.1161/circep.115.003591
10. O'Donovan CE, Waddell-Smith KE, Skinner JR, Broadbent E. Predictors of β-blocker adherence in cardiac inherited disease. *Open Heart*. 2018;5:e000877. doi: 10.1136/openhrt-2018-000877
11. Schwartz PJ. Efficacy of left cardiac sympathetic denervation has an unforeseen side effect: medicolegal complications. *Heart Rhythm*. 2010;7:1330–1332. doi: 10.1016/j.hrthm.2010.04.038
12. Kleemann T, Becker T, Doenges K, Vater M, Senges J, Schneider S, Saggau W, Weisse U, Seidl K. Annual rate of transvenous defibrillation lead defects in implantable cardioverter-defibrillators over a period of 10 years. *Circulation*. 2007;115:2474–2480. doi: 10.1161/CIRCULATIONAHA.106.663807
13. Matsa E, Dixon JE, Medway C, Georgiou O, Patel MJ, Morgan K, Kemp PJ, Staniforth A, Mellor I, Denning C. Allele-specific RNA interference rescues the long-QT syndrome phenotype in human-induced pluripotency stem cell cardiomyocytes. *Eur Heart J*. 2014;35:1078–1087. doi: 10.1093/eurheartj/eht067
14. Jiang J, Wakimoto H, Seidman JG, Seidman CE. Allele-specific silencing of mutant Myh6 transcripts in mice suppresses hypertrophic cardiomyopathy. *Science*. 2013;342:111–114. doi: 10.1126/science.1236921
15. Bongianino R, Priori SG. Gene therapy to treat cardiac arrhythmias. *Nat Rev Cardiol*. 2015;12:531–546. doi: 10.1038/nrcardio.2015.61
16. Dotzler SM, Kim CSJ, Gendron WAC, Zhou W, Ye D, Bos JM, Tester DJ, Barry MA, Ackerman MJ. Suppression-replacement KCNQ1 gene therapy for type 1 long QT syndrome. *Circulation*. 2021;143:1411–1425. doi: 10.1161/CIRCULATIONAHA.120.051836
17. Millington-Ward S, O'Neill B, Tuohy G, Al-Jandal N, Kiang AS, Kenna PF, Palfi A, Hayden P, Mansergh F, Kennan A, et al. Strategems in vitro for gene therapies directed to dominant mutations. *Hum Mol Genet*. 1997;6:1415–1426. doi: 10.1093/hmg/6.9.1415
18. Anderson CL, Kuzmicki CE, Childs RR, Hintz CJ, Delisle BP, January CT. Large-scale mutational analysis of Kv11.1 reveals molecular insights into type 2 long QT syndrome. *Nat Commun*. 2014;5:5535. doi: 10.1038/ncomms6535
19. 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. doi: 10.1161/CIRCGEN.120.002950
20. Cordeiro JM, Brugada R, Wu YS, Hong K, Dumaine R. Modulation of I(Kr) inactivation by mutation N588K in KCNH2: a link to arrhythmogenesis in short QT syndrome. *Cardiovasc Res*. 2005;67:498–509. doi: 10.1016/j.cardiores.2005.02.018
21. Burridge PW, Matsa E, Shukla P, Lin ZC, Churko JM, Ebert AD, Lan F, Diecke S, Huber B, Mordwinkin NM, et al. Chemically defined generation of human cardiomyocytes. *Nat Methods*. 2014;11:855–860. doi: 10.1038/nmeth.2999
22. Mummery CL, Zhang J, Ng ES, Elliott DA, Elefanty AG, Kamp TJ. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. *Circ Res*. 2012;111:344–358. doi: 10.1161/CIRCRESAHA.110.227512
23. Shaheen N, Shiti A, Huber I, Shinnawi R, Arbel G, Gepstein A, Setter N, Goldfracht I, Gruber A, Chorna SV, et al. Human induced pluripotent stem cell-derived cardiac cell sheets expressing genetically encoded voltage indicator for pharmacological and arrhythmia studies. *Stem Cell Rep*. 2018;10:1879–1894. doi: 10.1016/j.stemcr.2018.04.006
24. He X, Urip BA, Zhang Z, Ngan CC, Feng B. Evolving AAV-delivered therapeutics towards ultimate cures. *J Mol Med (Berl)*. 2021;99:593–617. doi: 10.1007/s00109-020-02034-2
25. Carvalho M, Sepodes B, Martins AP. Regulatory and scientific advancements in gene therapy: state-of-the-art of clinical applications and of the supporting European regulatory framework. *Front Med (Lausanne)*. 2017;4:182. doi: 10.3389/fmed.2017.00182
26. Scott LJ. Alipogene tiparvovec: a review of its use in adults with familial lipoprotein lipase deficiency. *Drugs*. 2015;75:175–182. doi: 10.1007/s40265-014-0339-9
27. Maguire AM, Russell S, Wellman JA, Chung DC, Yu ZF, Tillman A, Wittes J, Pappas J, Elci O, Marshall KA, et al. Efficacy, safety, and durability of voretigene neparvovec-rzyl in RPE65 mutation-associated inherited retinal dystrophy: results of phase 1 and 3 trials. *Ophthalmology*. 2019;126:1273–1285. doi: 10.1016/j.ophtha.2019.06.017
28. Kolb SJ, Coffey CS, Yankey JW, Krosschell K, Arnold WD, Rutkove SB, Swoboda KJ, Reyna SP, Sakonju A, Darras BT, et al. Natural history of infantile-onset spinal muscular atrophy. *Ann Neurol*. 2017;82:883–891. doi: 10.1002/ana.25101
29. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, Lowes L, Alfano L, Berry K, Church K, et al. Single-dose gene-replacement therapy for spinal muscular atrophy. *N Engl J Med*. 2017;377:1713–1722. doi: 10.1056/NEJMoa1706198
30. Al-Zaidy S, Pickard AS, Kotha K, Alfano LN, Lowes L, Paul G, Church K, Lehman K, Sproule DM, Dabbous O, et al. Health outcomes in spinal muscular atrophy type 1 following AVXS-101 gene replacement therapy. *Pediatr Pulmonol*. 2019;54:179–185. doi: 10.1002/ppul.24203
31. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, Tournev I, Schmidt HH, Coelho T, Berk JL, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med*. 2018;379:11–21. doi: 10.1056/NEJMoa1716153
32. Heras-Palou C. Patisiran's path to approval as an RNA therapy. *Nature*. 2019;574:S7. doi: 10.1038/d41586-019-03070-w
33. Scott LJ. Givosiran: first approval. *Drugs*. 2020;80:335–339. doi: 10.1007/s40265-020-01269-0
34. Anderson CL, Kuzmicki CE, Childs RR, Hintz CJ, Delisle BP, January CT. Large-scale mutational analysis of Kv11.1 reveals molecular insights into type 2 long QT syndrome. *Nat Commun*. 2014;5:5535–5535. doi: 10.1038/ncomms6535
35. Mehta A, Ramachandra CJA, Singh P, Chitre A, Lua CH, Mura M, Crotti L, Wong P, Schwartz PJ, Gnecchi M, et al. Identification of a targeted and testable antiarrhythmic therapy for long-QT syndrome type 2 using a patient-specific cellular model. *Eur Heart J*. 2017;39:1446–1455. doi: 10.1093/eurheartj/ehx394
36. Bongianino R, Priori SG. Gene therapy to treat cardiac arrhythmias. *Nat Rev Cardiol*. 2015;12:531–546. doi: 10.1038/nrcardio.2015.61
37. Kiang AS, Palfi A, Ader M, Kenna PF, Millington-Ward S, Clark G, Kennan A, O'Reilly M, Tam LC, Aherne A, et al. Toward a gene therapy for dominant disease: validation of an RNA interference-based mutation-independent approach. *Mol Ther*. 2005;12:555–561. doi: 10.1016/j.ymthe.2005.03.028
38. Mueller C, Tang Q, Gruntman A, Blomenkamp K, Teckman J, Song L, Zamore PD, Flotte TR. Sustained miRNA-mediated knockdown of mutant AAT with simultaneous augmentation of wild-type AAT has minimal effect on global liver miRNA profiles. *Mol Ther*. 2012;20:590–600. doi: 10.1038/mt.2011.292
39. Greenwald DL, Cashman SM, Kumar-Singh R. Mutation-independent rescue of a novel mouse model of retinitis pigmentosa. *Gene Ther*. 2013;20:425–434. doi: 10.1038/gt.2012.53
40. Zhao J, Pettigrew GJ, Thomas J, Vandenberg JI, Delriviere L, Bolton EM, Carmichael A, Martin JL, Marber MS, Lever AM. Lentiviral vectors for delivery of genes into neonatal and adult ventricular cardiac myocytes in vitro and in vivo. *Basic Res Cardiol*. 2002;97:348–358. doi: 10.1007/s00395-002-0360-0
41. Ellis BL, Hirsch ML, Barker JC, Connelly JP, Steininger RJ 3rd, Porteus MH. A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with Nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype. *Virol J*. 2013;10:74. doi: 10.1186/1743-422X-10-74
42. Ambrosi CM, Sadananda G, Han JL, Entcheva E. Adeno-associated virus mediated gene delivery: implications for scalable in vitro and in vivo cardiac optogenetic models. *Front Physiol*. 2019;10:168. doi: 10.3389/fphys.2019.00168
43. Penaud-Budloo M, Le Guiner C, Nowrouzi A, Toromanoff A, Chérel Y, Chenuaud P, Schmidt M, von Kalle C, Rolling F, Moullier P, et al. Adeno-associated virus vector genomes persist as episomal chromatin in primate muscle. *J Virol*. 2008;82:7875–7885. doi: 10.1128/JVI.00649-08
44. Brunner M, Peng X, Liu GX, Ren XQ, Ziv O, Choi BR, Mathur R, Hajjiri M, Odening KE, Steinberg E, et al. Mechanisms of cardiac arrhythmias and sudden death in transgenic rabbits with long QT syndrome. *J Clin Invest*. 2008;118:2246–2259. doi: 10.1172/JCI33578
45. Odening KE, Choi BR, Liu GX, Hartmann K, Ziv O, Chaves L, Schofield L, Centracchio J, Zehender M, Peng X, et al. Estradiol promotes sudden cardiac death in transgenic long QT type 2 rabbits while progesterone is protective. *Heart Rhythm*. 2012;9:823–832. doi: 10.1016/j.hrthm.2012.01.009