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Children's Hospital Corporation (DBA Boston Children's Hospital)

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14. ABSTRACT

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a inherited arrhythmia syndrome characterized by life-threatening arrhythmias during times of stress or exercise. Dominant mutations in the intracellular calcium (Ca^{2+}) release channel RYR2 are responsible for the majority of clinical cases. Despite maximal medical therapy, patients continue to have breakthrough events or therapy related complications. To response to this unmet clinical need, we have developed a targeted gene therapy to suppress arrhythmias by inhibiting the Ca^{2+} regulated kinase CaMKII. Using adeno-associated virus (AAV) vectors we demonstrated efficacy in cellular and animal models of CPVT by targeted expression of CaMKII peptide inhibitors. This grant proposal is focused on the further refinement and testing of a clinical CaMKII peptide inhibitory vector in preparation for a human clinical trial. During this granting period we have determined the cardiac-specific promoter for optimal transgene expression and laid the foundation for refinement of the peptide inhibitor. We have also expanded our clinical network of CPVT patients and performed analysis of current healthcare costs for CPVT management.

15. SUBJECT TERMS					
NONE LISTED					
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1. INTRODUCTION:

The purpose of this grant is to refine and optimize a novel cardiac gene therapy for the inherited arrhythmia disorder catecholaminergic polymorphic ventricular tachycardia (CPVT) in preparation for a first in human clinical trial. Mutations in the intracellular calcium (Ca^{2+}) release channel RYR2 are commonly associated with CPVT and lead to life-threatening ventricular arrhythmias during stress or exercise. Without any abnormality at baseline, activation of the Ca^{2+}/calmodulin dependent kinase II (CaMKII) in response to adrenergic stimulation is necessary to unmask the arrhythmogenic potential of RYR2 mutations. We have developed a gene therapy strategy to treat CPVT by targeted cardiac expression of CaMKII peptide-inhibitors using adeno-associated virus (AAV) delivery. Building on previous proof-of-concept research, this project encompasses the necessary steps to construct the optimal clinical vector, define the therapeutic dose, evaluate for possible toxicities, and identify eligible patients for potential treatment. In addition to being the first gene-specific treatment for an inherited cardiovascular syndrome, targeted inhibition of CaMKII may be more universally applicable because CaMKII dysregulation is implicated in several cardiac disorders.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Cardiac gene therapy
Catecholaminergic polymorphic ventricular tachycardia (CPVT)
Arrhythmia
Sudden cardiac death
Ventricular arrhythmias
Adeno-associated virus (AAV)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1 – To optimize the final product design.	**Timeline**	**Percent Completed**
Major Task 1	Months	
Subtask 1: Submit documents for ACURO approval (mouse studies)	1-4	100%
Milestone # 1 ACURO approval obtained	4	100%

Subtask 2: Optimize inhibitory peptide	1-8	50%
Subtask 3: Optimize promoter	1-8	90%
Subtask 4: Optimize capsid	1-8	Pending
Subtask 5: Compare self-complementary AAV to standard AAV	4-8	80%
Subtask 6: Develop an assay to detect transduced cells	4-8	Pending
Milestone # 2 Finalized design of the clinical candidate	8	Pending
Specific Aim 2 – To evaluate efficacy, dose-response, biodistribution, and safety of the clinical candidate in a murine CPVT model.		
Major Task 2.		
Subtask 1: Research grade vector production (AAV-nGFP then clinical candidate)	6-11	Pending
Milestone #3a Delivery of research grade AAV-nGFP	9	Pending
Milestone #3b Delivery of research grade clinical candidate	11	Pending
Subtask 2: Dose-finding and biodistribution of AAV-nGFP	9-12	Pending
Milestone #4 Define the % cardiomyocyte transduction for a range of viral doses and biodistribution of the AAV-nGFP test vector	12	Pending
Subtask 3: Efficacy and dose-response study	13-18	Pending

Milestone #5 Define the effective dose, efficacy, safety, and biodistribution of the clinical candidate.	18	Pending
Specific Aim 3 – To develop safety data on the final therapy vector in a large animal model		
Major Task 3		
Subtask 1: Submit documents for ACURO approval (swine dose-finding studies at BCH)	6-18	Pending
Milestone #6 ACURO approval obtained for swine	18	Pending
Subtask 1: Pre-IND meeting	19-21	Pending
Milestone #7 Finalize design of the large animal safety trial	21	Pending
Subtask 2: Production of clinical grade vector (AAV-GFP-P2A-IP1 then AAV-IP1)	13-24	Pending
Milestone #8a Delivery of Hyperstack scale AAV-GFP-P2A-IP1	18	Pending
Milestone #8b Delivery of Hyperstack scale clinical candidate (no GFP-P2A)	24	Pending
Subtask 3: Large animal dose-finding and biodistribution using GFP-containing vector and clinically applicable delivery route and equipment. [2 pigs x 5 groups = 10 pigs]	19-24	Pending
Milestone #9 Define target vector dose to match efficacious dose found in mouse model	24	Pending

Subtask 4: Large animal safety and biodistribution study [12 pigs x 2 doses = 24 pigs]	25-34	Pending
Milestone #10 Completion of large animal safety and biodistribution study	34	Pending
Specific Aim 4 – To lay the groundwork for a First-in-Human clinical trial		
Major Task 4		
Subtask 1: Establish a CPVT Network	1-12	75%
Milestone #11 Hold a meeting of CPVT Network participating members	12	Pending
Subtask 2: A retrospective chart review	8-24	25%
Milestone #12 Establish natural history and resource utilization of CPVT under the current standard of care.	24	50%
Subtask 3: Collect and test blood from patients with inherited arrhythmia for neutralizing antibodies	1-30	Pending
Milestone #13 Determine the frequency of neutralizing antibodies amongst the target population	30	Pending
Subtask 4. Finalize Phase I clinical protocol	25-32	Pending
Subtask 5. Prepare IND application	32-36	Pending
Milestone #14 Submission of FDA IND filing for a First-in-Human Phase I study	36	Pending

What was accomplished under these goals?

3.1 Major Activities

During the initial granting period we have focused our efforts on optimizing the components of the therapeutic viral vector and establishing a network of institutions to identify patients for potential future gene therapy. We have also performed an analysis of resource utilization for CPVT patients and established strategic partnerships for technology transfer.

3.2 Specific Objectives

Major Task 1

Optimization of the cardiac-specific promoter

In our initial proof of concept experiments, we used the cardiac troponin T promoter (cTnT) to drive the expression of the CaMKII peptide inhibitor. While this commonly used promoter restricts expression from non-cardiac organs it may not produce the highest level of transgene expression as compared to other promoters. To evaluate the relative strength of cardiac-specific promoters, we cloned three promoters along with the original cTnT promoter into AAV expression vectors containing specific barcodes (Fig. 1A). After the production of AAV isotype 9 (AAV9) virus-containing each barcoded-promoter, we performed subcutaneous injections at P3 and isolated hearts after 6-8 weeks of expression. After confirming the expression of each promoter by creating cardiac sections (Fig. 1B), we isolated DNA and RNA for amplicon sequencing. Quantification of the relative strength of each promoter demonstrated that cTnT with the inclusion of the CASQ2 enhancer and an intron derived from β-hemoglobin had the highest degree of expression (Fig. 1C). We will confirm that this promoter retains cardiac-specific expression to complete its optimization by measuring GFP expression in the heart, brain, liver, and muscle.

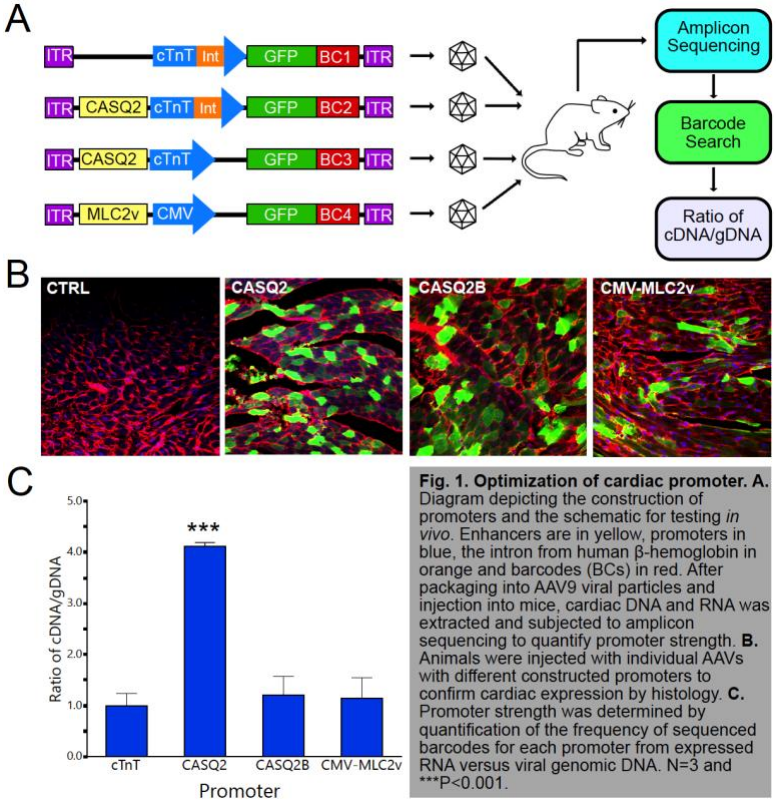

Fig. 1. Optimization of cardiac promoter. A. Diagram depicting the construction of promoters and the schematic for testing *in vivo*. Enhancers are in yellow, promoters in blue, the intron from human β-hemoglobin in orange and barcodes (BCs) in red. After packaging into AAV9 viral particles and injection into mice, cardiac DNA and RNA was extracted and subjected to amplicon sequencing to quantify promoter strength. **B.** Animals were injected with individual AAVs with different constructed promoters to confirm cardiac expression by histology. **C.** Promoter strength was determined by quantification of the frequency of sequenced barcodes for each promoter from expressed RNA versus viral genomic DNA. N=3 and ***P<0.001.

Selection of the optimal CaMKII inhibitory peptide

In our original proof-of-concept studies, we used the well-established CaMKII inhibitor AIP, to reverse the CPVT phenotype in both an animal model and induced pluripotent stem cell (iPSC) models of CPVT. However, more potent and specific CaMKII inhibitors have recently been developed. To determine the optimal CaMKII inhibitory peptide for inclusion in our therapeutic vector, we originally transfected candidate peptides using modified RNA (modRNA) into cardiomyocytes isolated from RYR2$^{R176Q/WT}$ mutant animals. Subsequent experiments demonstrated limited expression of putative peptide inhibitors by modRNA except at the highest concentrations, limiting comparative dose responses. To overcome this limitation, we generated AAV9 viruses containing putative CaMKII inhibitory peptides, the self-cleaving peptide sequence P2A, and mCherry as an expression marker (Fig. 2A). We included the original AIP and the novel optimized inhibitory peptide CN19o as either single peptides, multimerized or fused to the RYR2-binding protein FKBP12.6. After packaging into AAV9 viral particles, we performed subcutaneous injections in a novel CPVT mouse model with a human RYR2 mutation (RYR2-R4650I). In this model, administration of epinephrine and caffeine results in ventricular arrhythmias including bi-directional ventricular tachycardia (Bi-VT) which is characteristic of CPVT (Fig 2B, 2C). Adult cardiomyocytes were isolated and loaded with a fluorescent Ca^{2+} indicator (Fluo-4) and imaged using the high-throughput kinetic imaging cytometer (KIC) from Vala biosciences. Quantification of mCherry fluorescence will serve as a measure of CaMKII inhibitory peptide expression levels (Fig. 2D). Adult cardiomyocytes were electrically paced at 1Hz for 30s and then the frequency of post pacing Ca^{2+} events were quantified (Fig. 2E). A correlation of peptide expression levels as indicated by mCherry fluorescence and suppression of post pacing Ca^{2+} events will determine the potency of each expressed CaMKII inhibitory peptide.

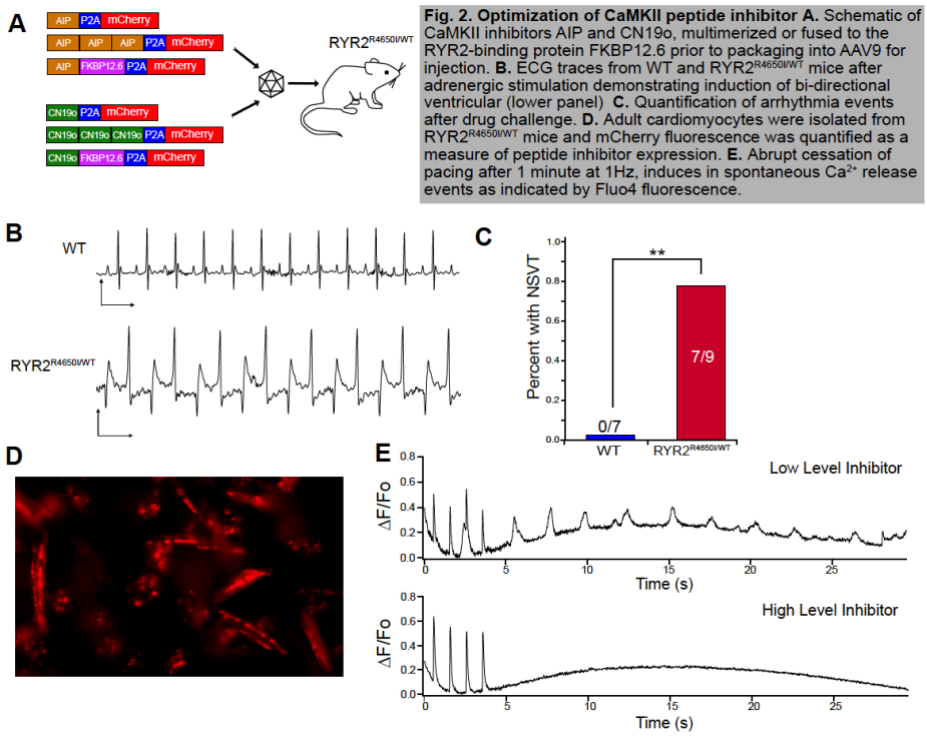

Fig. 2. Optimization of CaMKII peptide inhibitor A. Schematic of CaMKII inhibitors AIP and CN19o, multimerized or fused to the RYR2-binding protein FKBP12.6 prior to packaging into AAV9 for injection. **B.** ECG traces from WT and RYR2$^{R4650I/WT}$ mice after adrenergic stimulation demonstrating induction of bi-directional ventricular (lower panel) **C.** Quantification of arrhythmia events after drug challenge. **D.** Adult cardiomyocytes were isolated from RYR2$^{R4650I/WT}$ mice and mCherry fluorescence was quantified as a measure of peptide inhibitor expression. **E.** Abrupt cessation of pacing after 1 minute at 1Hz, induces in spontaneous Ca^{2+} release events as indicated by Fluo4 fluorescence.

Comparing self-complementary AAV to standard AAV

The rate-limiting step in AAV target tissue transduction is the conversion of its single DNA genome to double-stranded DNA, prior to transcription. To overcome this limitation, modification of one of the internal terminal repeat (ITR) sequences can establish a self-complementary DNA molecule that greatly increases the efficiency of conversion to dsDNA. To directly compare the resulting transduction efficiency of self-complementary AAV (scAAV) to standard or single-stranded AAV (sAAV), we injected $2x10^{10}$ viral genomes/gram of mouse weight (vg/g) at P1. Cardiac sections prepared within one week after viral injection demonstrated increased expression with scAAV9 as compared to sAAV at one day after injection but with largely similar levels of transduction afterwards (Fig. 3). Therefore it is unclear whether incorporation of self-complementary AAV in the final therapeutic vector design will be necessary, although we will perform detailed bio-distribution studies in the next granting periods to determine any other significant differences in cardiac transgene expression.

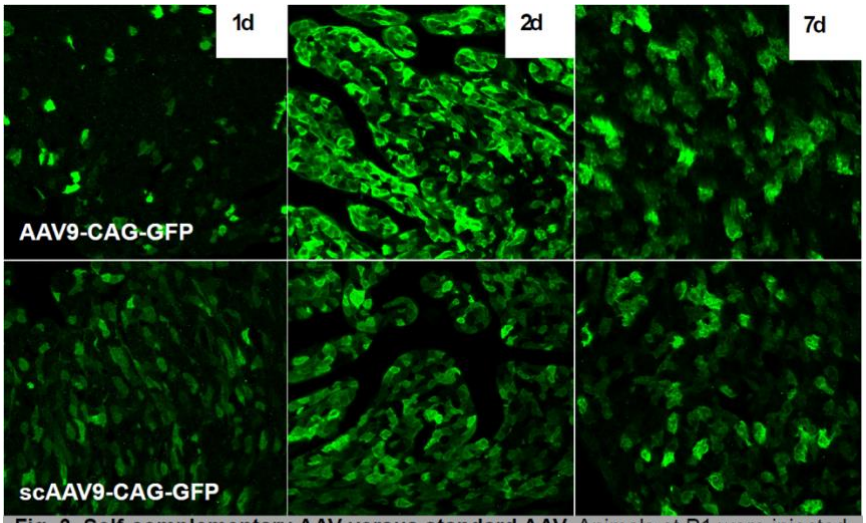

Fig. 3. Self-complementary AAV versus standard AAV. Animals at P1 were injected with either standard AAV9 or self-complementary AAV9 (scAAV9) expressing GFP at a dose of 2x1010 viral genomes/gram (vg/g). Animals were sacrificed and cardiac sections were prepared at one, two and seven days after virus administration.

Major Task 4

Establish a CPVT Network

To facilitate the identification of additional CPVT patients eligible for a potential clinical trial thereby increasing the clinical and molecular diversity of patients and better define the natural history of CPVT, we partnered with several institutions as part of the International CPVT network. Dr. Abrams is in the final stages of IRB approval that facilitates the participation of Boston Children's Hospital in this multi-institutional international registry, which includes over 1000 patients from 26 number of programs in Europe, North America, Asia and Australasia. Participation in this expansive registry will dramatically increase the available data for clinical analysis and to identify potential candidates for our proposed gene therapy.

Determine resource utilization for current CPVT treatment

To further develop strategic partnerships, we sought to define the current cost of care for CPVT patients. Partnering with the Health-lab at MIT Sloan, an analysis of health-care costs for CPVT patients based on BCH charges was performed. Cost modeling demonstrated that the first-annual treatment cost was approximately $64,293 with continued annual costs. Additionally, an analysis of large commercial health insurance providers demonstrated that while the current cost of gene therapies is high compared to current treatment, benefits to patients are likely to significantly drive demand and bridge the cost gap.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

During the next granting period, we will finalize the design of the clinical vector including the viral capsid and CaMKII inhibitory peptide cargo. We have also initiated a comprehensive screen for additional modifiers of restrictive expression based on microRNA target sequences and will consider incorporation of one or more of these sequences into the final vector. In parallel with final selection of the CaMKII inhibitory peptide, we will design RNAScope probes to further validate bio-distribution of our optimized therapeutic transgene. We will finalize the approval of the large animal protocol for testing of our clinical vector. To facilitate the efficient progress of our dose finding experiments and testing of the optimized therapeutic vector, we will recruit a master's level research assistant/investigator to perform the electrophysiology testing.

In collaboration with Dr. Abrams, we will arrange a meeting with the collaborating centers to facilitate the completion of the clinical research study. We have recently recruited a cardiovascular genetics fellow who along with a clinical research assistant will establish an arrhythmia severity score for patients in the CPVT registry. These data will be combined with on-going quality of life and neurocognitive assessments to create a comprehensive database for a possible clinical trial.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our development of a simple method to test the relative promoter strength of transgenes delivered by AAV will greatly facilitate the optimization of new cardiac gene therapies. This method leavages NGS technology and is easily adaptable to testing other aspects of AAV expression including capsid isotype or other transgene expression modifiers.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

We have initiated a partnership with a gene therapy company based on our proof of concept data for CaMKII inhibition to treat CPVT.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

5.1 Changes in approach

In our initial studies to optimize the promoter, we prepared cardiac sections and performed quantitative PCR (qPCR) to determine the frequency and expression levels of GFP after treatment with different AAV-containing promoter constructs as a measure of promoter strength. It became evident that variation in effective viral titer and heterogeneity in cardiomyocyte transduction (Fig. 1B) would greatly impair an accurate comparison of promoter strength. To overcome this limitation, we quantified the levels of expressed mRNA transcripts and corresponding viral genomes by next generation sequencing (NGS) by measuring the frequency of recovered promoter-specific barcodes. This technique overcomes variations in viral titer, transduction efficiency or regional heterogeneity and can be applied to multiple promoter constructs at a time in the same heart to accurately determine the relative promoter strength. We will use a similar approach to compare transduction efficiencies of different AAV capsids in the next granting period.

Our preliminary data used modRNA to express CaMKII inhibitory peptides in adult cardiomyocytes isolated from the CPVT mouse model $RYR2^{R176Q/WT}$. Subsequent experiments demonstrated that expression was highly variable and was only appreciably detectable at the highest concentrations of transfected modRNA. Therefore, to readily compare different CaMKII inhibitory peptides, we generated AAV constructs for each of the putative inhibitors and administered them to P3 pups carrying the RYR2-R4650I mutation. This CPVT line was recently generated by genomic engineering at BCH and has a robust CPVT phenotype without the need for cardiac catheterization and ventricular pacing. This experimental model more accurately recapitulates the arrhythmias seen in CPVT patients including bi-directional ventricular tachycardia and also demonstrates abnormal Ca^{+2} release events in response to adrenergic stimulation. Using a high-throughput kinetic imaging cytometer we are able to record from hundreds of cells per sample correlating mCherry fluorescence as a marker for CaMKII inhibitory peptide expression to the suppression of abnormal Ca^{+2} release events.

Actual or anticipated problems or delays and actions or plans to resolve them

5.2 Actual or anticipated problems or delays

The global coronavirus pandemic caused significant delays in our experimental progress because of the necessary shutdowns at Boston Children's Hospital. Our research enterprise was extremely limited from March 15th to June 1st with significant reduction in animal numbers, restricted access to research laboratories and only the maintenance of biologic lines. During the reopening process, personnel were only allowed back into the laboratories at a limited capacity until early July. This protracted slowdown in research dramatically impacted our expected timelines to meet the goals of finalizing the selection of the optimal gene therapeutic vector, dose-finding in a small animal model and preparing for large-scale AAV productions. To overcome these significant setbacks, we have dramatically increased the breeding of our CPVT mouse lines, developed new methods for high-throughput acquisition of adult cardiomyocyte Ca^{+2} imaging data and refined methods for testing multiple promoter or capsid constructs at once. We plan to employ these methods to rapidly finalize the construction of our optimized therapeutic vector and prepare for viral production. We will also use our new CPVT mouse line $RYR2^{R4650I/WT}$ to perform dose finding experiments because arrhythmia assessment is technically more straightforward, increasing experimental throughput. The final therapeutic dose will be confirmed in both $RYR2^{R4650I/WT}$ and $RYR2^{R176Q/WT}$ mutant lines prior to large-scale viral production for the proposed large animal experiments.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

There are no significant changes in the care or use of vertebrate animals, biohazards and/or select agents. The IRB to participate in the international CPVT registry is currently under review. No further clinical research concerning subjects outside of BCH will be performed until the new IRB is approved.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

We submitted a provisional US patent application on 7/6/2017 for our proposed gene therapy. A formal application was submitted on 1/2/2020 and currently is pending under number 16/628,162. We have partnered with a gene therapy company as part of a sponsored research agreement with an option for license.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	William Pu, MD
Project Role:	PI
Researcher Identifier:	0000-0002-4551-8079
Nearest person month worked:	1
Contribution to Project:	Overall co-direction of the project along with Dr. Bezzerides

Funding Support:	Committed effort fully supported by this award

Name:	Vassilios Bezzerides MD, PhD
Project Role:	Co-investigator
Researcher Identifier:	0000-0003-0825-6580
Nearest person month worked:	1
Contribution to Project:	Overall co-direction of the project along with Dr. Pu
Funding Support:	Committed effort fully supported by this award

Name:	Dominic Abrams MBBS
Project Role:	Institutional Collaborator
Researcher Identifier:	0000-0003-0825-6580
Nearest person month worked:	1
Contribution to Project:	Assistance with establishment of CPVT network
Funding Support:	Departmental

Name:	Bayush Dinegde
Project Role:	Laboratory Assistant
Researcher Identifier:	NA
Nearest person month worked:	3
Contribution to Project:	Assist research assistants and fellows with preparation of experiments and project needs.
Funding Support:	Committed effort fully supported by this award

Name:	Sofi de la Serna Buzon
Project Role:	Postdoctoral Fellow
Researcher Identifier:	NA
Nearest person month worked:	8
Contribution to Project:	Dr. de la Serna Buzon completed the promoter optimization and is performing the experiments to select the CaMKII inhibitory peptide. She will continue with the production of additional AAVs for dose-finding.
Funding Support:	Committed effort fully supported by this award

Name:	Suya Wang
Project Role:	Postdoctoral Fellow
Researcher Identifier:	NA
Nearest person month worked:	6
Contribution to Project:	Dr. Wang performed the comparison of self-complementary versus standard AAV. She also assisted with the design of testing the CaMKII peptides
Funding Support:	Committed effort fully supported by this award

Name:	Danielle Heims Waldron
Project Role:	Research assistant
Researcher Identifier:	NA
Nearest person month worked:	3
Contribution to Project:	Ms. Waldron assisted with construction of the AAVs for peptide and promoter testing.
Funding Support:	Committed effort fully supported by this award

Name:	Thoms Samenuk
Project Role:	Research assistant
Researcher Identifier:	NA
Nearest person month worked:	2
Contribution to Project:	Mr. Samenuk determined the lack of effect of microRNA targeting sequence for miR124. Currently improving on non-promoter expression specification.
Funding Support:	Committed effort fully supported by this award

Name:	Daisuke Yoshinaga
Project Role:	Postdoctoral Fellow
Researcher Identifier:	NA
Nearest person month worked:	1
Contribution to Project:	Dr. Yoshinaga assisted with imaging of cardiomyocytes along with other research members.
Funding Support:	Committed effort fully supported by this award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Organization Name: University of British Columbia
Location of Organization: (if foreign location list country) British Columbia, Canada
Partner's contribution to the project:
The University of British Columbia is part of international registry of CPVT patients providing clinical information for analysis.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*