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TITLE: High-Throughput Screening for Novel Drug Discovery Using Patient-Specific Induced Pluripotent Stem Cells for Familial Hypertrophic Cardiomyopathy

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CONTRACTING ORGANIZATION: Yale University, New Haven, CT

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14. ABSTRACT

The objective of this proposal is to discover novel drugs to treat hypertrophic cardiomyopathy (HCM) using cardiomyocytes (CMs) derived from human induced pluripotent stem cells (hiPSCs). HCM is one of the most prevalent heritable heart diseases in the world, affecting about 1 out of 500 people, including military families. It is characterized by a thickening of the heart tissue, reduced cavity size, impaired relaxation time, arrhythmias and ultimately sudden cardiac death (SCD). Here, we have validated two candidates of drugs that lead to inhibit calcineurin/NFAT or Mitigating enhanced actomyosin crossbridge formation in patient derived hiPSC-CMs. Additionally, we performed transcriptome analysis by RNA sequencing (RNAseq) with the control, isogenic MYH7-corrected, isogenic MLP-corrected and proband MLP-W4R;MYH7-R723C iPSC-CMs to elucidate the mechanism of pathological hypertrophy.

15. SUBJECT TERMS High-Throughput Screening, human induced pluripotent stem cells, cardiomyocytes					
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1. INTRODUCTION:

The primary objective of this proposal is to discover novel, small molecule therapeutics to treat hypertrophic cardiomyopathy (HCM) using cardiomyocytes (CMs) derived from human induced pluripotent stem cells (hiPSCs). HCM is one of the most prevalent heritable heart diseases in the world, affecting about 1 out of 500 people, including military families. It is characterized by a thickening of the heart tissue which leads to a reduced cavity size in the heart chambers, impaired relaxation time, arrhythmias and ultimately sudden cardiac death (SCD). Novel therapies targeting HCM can be discovered using a high-throughput screening approach based on patient iPSC-derived CMs of various HCM manifesting genotypes to evaluate candidate drugs.

2. KEYWORDS:

High-Throughput Screening, human induced pluripotent stem cells, cardiomyocytes

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of this proposal were to discover novel small molecules to treat HCM and evaluate the efficacy of the candidate drugs. Candidate drugs would be investigated with multiple hiPSC lines with varied HCM mutations, iPSC-based EHTs and transcriptome profiling through the RNA sequencing to determine their efficacy in treating HCM. This project aims to establish a new strategy which could provide a rapid path to the discovery of novel drugs targeting HCM and posit a new paradigm for drug discovery.

The first major goal is to elucidate the role of MYH7-R723C and MLP-W4R mutations in HCM pathogenesis and the effect of the drugs for alleviating HCM. To understand the mechanism of pathological hypertrophy is a very significant process to find novel drugs.

The second goal is to carry out the transcriptome profiling analysis with the isogenic control and MYH7-R723C/MLP-W4R mutant iPSC-CMs iPSC-CMs. Our group has a validated system for the molecular analysis and robust biomechanical analysis based on EHTs using iPSC-CMs, in which we could evaluate the effect of the drug precisely.

We expect that this creative approach posits a new paradigm for drug discovery and will help elucidate the fundamental mechanisms that underlie the development and pathogenesis of cardiac hypertrophy.

What was accomplished under these goals?

1. Major Activities

I have given talks at Yale Seminar Series in Biomedical Research, Department of Internal Medicine; at the Pathology Progress In Research in Progress Talk in January 11th, 2022. Our group holds regular joint meetings on engineered cardiovascular tissue with Dr. Stuart Campbell and cardiac physiology and Dr. Lawrence Young. I will give a presentation on February 4th, 2022. I also attend the weekly Yale Cardiovascular Biology Research In Progress meeting, the monthly Yale Stem Cell Center Research Forum, and the annual retreat of Yale VBT Program and Yale Stem Cell Center. Furthermore, I have mentored new postdocs, graduate students and visiting scholars in our group.

2. Specific Objectives

In this funding period, I investigated hypertrophic defects using MYH7-R723C/MLP-W4R mutant iPSC-CMs iPSC-CMs, MYH7-R723C corrected iPSC-CMs or MLP-W4R corrected iPSC-CMs. In addition, I have optimized the system for RNA sequencing using multiple iPSC-CMs to elucidate the fundamental mechanisms that underlie the development and pathogenesis of cardiac hypertrophy. Furthermore, I generated an isogenic control line from MYH7-R723C corrected iPSC line or MLP-W4R corrected iPSC line with TALEN or CRISPR/Cas9-based genome editing methods. Our research group is confident these approaches will help to support this project.

3. Significant Results or Key Outcomes

3.1. Inhibiting Calcineurin/NFAT Mitigates Development of the HCM Phenotype.

In the last annual report, we have demonstrated that proband MYH7-R723C/MLP-W4R mutant iPSC-CMs were larger than control iPSC-CMs. It also showed an excessive force development, a significant reduction in the proband. I next investigated the potential mechanism by which reduced expression of MLP in the double mutant proband CMs leads to severe hypertrophic defects. A previous murine study reported the colocalization of MLP and calcineurin, a pro-hypertrophic phosphatase that dephosphorylates NFAT and promotes its nuclear translocation, at the Z-disc. To test this, we measured nuclear translocation of NFATc4 in control and proband MYH7-R723C/MLP-W4R iPSC-CMs. Nuclear translocation of NFATc4 in the proband CMs was markedly higher than that in the control CMs **(Figure 1A and 1B)**. Further, treatment of proband CMs with calcineurin inhibitor FK506 resulted in reduced nuclear localization of NFATc4 and a significant rescue of hypertrophic defects including normalizing cell area and expression of BNP **(Figure 1C-F)**.

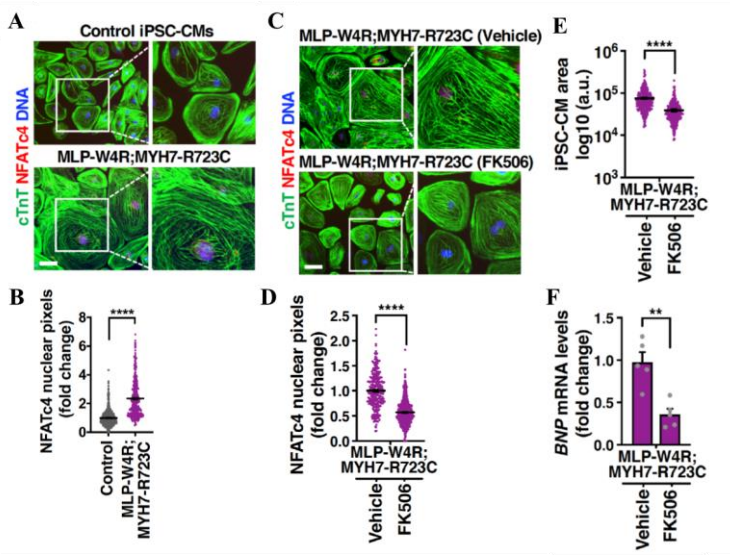

Figure 1. Inhibiting Calcineurin/NFAT Mitigates Development of the HCM Phenotype in Proband iPSC-Derived Cardiomyocytes.
(A) Immunostaining of NFATc4 (red) and cTnT (green) in day 35 control and MLP-W4R;MYH7-R723C iPSC-CMs. DNA was counterstained by DAPI. Scale bar, 100 μm. **(B)** Quantification of NFATc4 nuclear signals in panel D (two-tailed unpaired Student's t test). Nuclear NFATc4 pixels (gray value) were quantified by ImageJ from three independent cardiomyocyte differentiation batches (≥100 cells/batch). (**C)** Immunostaining of NFATc4 (red) and cTnT (green) in MLP-W4R;MYH7-R723C iPSC-CMs treated with DMSO (vehicle) or 0.5 μg/ml calcineurin inhibitor FK506. Scale bar, 100 μm. (**D-F)** Quantification of NFATc4 nuclear signals (**D**), cell area (**E**), and *BNP* gene expression (**F**) in DMSO- or FK506-treated MLP-W4R;MYH7-R723C iPSC-CMs. A two-tailed unpaired Student's t test was performed for nuclear NFATc4 and cell area analyses from three independent cardiomyocyte differentiation batches (≥100 cells/batch). A two-tailed unpaired Mann-Whitney U test was used for *BNP* gene analysis (n=5 independent cardiomyocyte differentiation batches per group; normalized to GAPDH).

3.2. Determination of mavacamten effective dose, the effects of mavacamten and calcineurin/NFAT activity

We examined the effect of mavacamten in rescuing the HCM defects, including the elevated expression of *BNP* and enlarged cell area, in proband iPSC-CMs at different doses to evaluate possible differences in sensitivity. A dose range of 0.1, 0.3, 0.5, and 1.0 μM of mavacamten was employed to treat proband iPSC-CMs on day 25 of cardiac differentiation for one day. DMSO was used as the vehicle control. Results showed that mavacamten treatment resulted in a dose-dependent rescue of elevated expression of *BNP*, with a concentration at 0.5 μM reaching statistical significance. Additionally, there appeared to be no significant differences in rescuing *BNP* expression between the treatments of mavacamten at 0.5 and 1.0 μM **(Figure 2A-C)**.

We previously reported a rescue of HCM defects in proband iPSC-CMs by mavacamten, including the enlarged cell area and elevated expression of *BNP*. To further confirm the effects of mavacamten on inhibiting cellular hypertrophy in the proband CMs, the expression levels of three additional cardiac hypertrophic genes, *ANF*, *ACTA1*, and *ANKRD1* were investigated after the administration of mavacamten. Results revealed that mavacamten treatment led to a significant reduction in the expression of these three hypertrophic markers **(Figure 3A-C)**. To obtain a more direct assessment of NFAT transcriptional activity in the proband CMs after mavacamten treatment, cells were transfected with p9xNFAT-GL, a luciferase reporter plasmid driven by nine NFAT-binding sites, in the presence and absence of mavacamten, and luciferase activity was analyzed. Results showed that administration of mavacamten resulted in a reproducible downregulation of NFAT-luciferase activity in the proband CMs **(Figure 3D-E)**. Additionally, consistent with enhanced nuclear localization of NFATc4 in proband CMs, a higher NFAT transcriptional activity was observed in proband CMs compared with controls based on NFAT-luciferase activity **(Figure 3F)**.

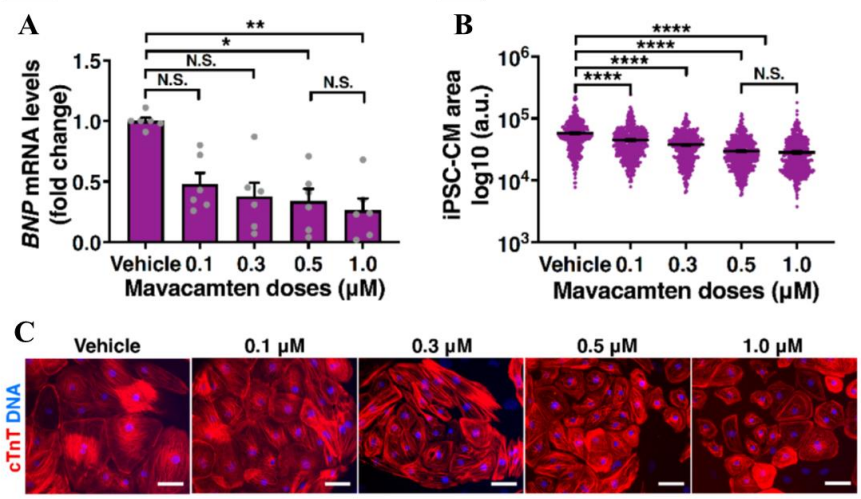

Figure 2. Determination of mavacamten effective dose in proband MLP-W4R;MYH7-R723C iPSC-CMs. (A) *BNP* mRNA expression levels in MLP-W4R;MYH7-R723C iPSC-CMs treated with either vehicle control (DMSO) or different doses (0.1, 0.3, 0.5, and 1.0 μM) of mavacamten. Treatment was started on day 25 of cardiac differentiation. *BNP* expression levels were analyzed 24 hours after drug treatment using qRT-PCR and normalized to the housing keeping gene *GAPDH*. Kruskal–Wallis with Dunn's multiple comparisons test was performed for *BNP* expression from six independent cardiac differentiation batches (H(4)=16.060, p=0.0029). **(B)** Immunostaining of cTnT (red) in MLP-W4R;MYH7-R723C iPSC-CMs treated with either vehicle control (DMSO) or mavacamten at different doses. Treatment was started on day 25 of cardiac differentiation for 4 days. Scale bar, 100 μm. **C**, Cell areas were quantified in vehicle (DMSO)- or mavacamten-treated MLP-W4R;MYH7-R723C iPSC-CMs. One-Way ANOVA with Tukey's multiple comparison test was performed for cell area differences (F(4,2380)=124.700, p<0.0001). Image J was used to quantify of iPSC-CM area in panel C from five independent cardiac differentiation batches (≥50 cells per experiment for each dose). All data are presented as mean ± S.E.M; *p<0.05; **p<0.01; ****p<0.0001; N.S.: not significant.

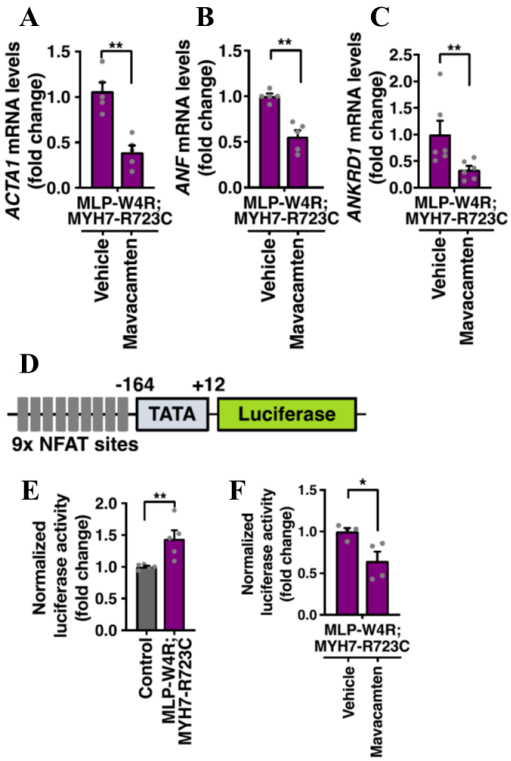

Figure 3. Quantification of the effects of mavacamten on the expressions of hypertrophic genes and the calcineurin/NFAT activity using a NFAT-luciferase reporter in proband MLP-W4R;MYH7-R723C iPSC-CMs. mRNA expression levels of three known HCM marker genes ACTA1 **(A)**, ANF **(B)**, and ANKRD1 **(C)** were measured in vehicle control (DMSO)- or 0.5 μM mavacamten-treated proband iPSC-CMs. Cardiomyocytes were treated at day 25 of cardiac differentiation for 24 hours. mRNA expression levels were normalized to the GAPDH gene. **(D)** Schematic of the NFAT-luciferase reporter construct. Nine copies of an NFAT binding site from the IL-4 promoter were placed 5' to a minimal promoter of the α-myosin heavy chain gene (-164 to +16) and introduced upstream of the luciferase reporter in pGL-3 Basic plasmid (Promega) to generate the NFAT-luciferase reporter. **(E)** Measurement of normalized NFAT-luciferase activity in control and proband MLP- W4R;MYH7-R723C iPSC-CMs. Cardiomyocytes were transiently transfected at day 25 of cardiac differentiation with the NFAT-luciferase reporter (9xNFAT-luciferase) and Renilla-luciferase (pLX313 from Addgene) that is driven by the constitutively active EF-1α promoter and works as an internal transfection control. Luciferase activities were measured two days after transfection using the Dual-Glow kit (Promega). NFAT-

luciferase activities were normalized by Renilla-luciferase, and fold change was calculated in relation to control CMs. N= 5 independent cardiac differentiation batches. A two-tailed unpaired Mann-Whitney U test was used to evaluate statistical difference. **(F)** Measurement of normalized NFAT-luciferase activity in vehicle (DMSO) and mavacamten-treated (0.5 μM) MLP-W4R;MYH7-R723C iPSC-CMs. Cardiomyocytes were treated with DMSO or mavacamten on day 25 of cardiac differentiation and transiently transfected with NFAT-luciferase reporter (9xNFAT-luciferase) and Renilla-luciferase (pLX313 from Addgene) that is driven by the constitutively active EF-1α promoter and functioned as an internal transfection control on day 27. Cells were then further treated with DMSO or mavacamten for two more days, and luciferase activities were measured using the Dual-Glow assay kit (Promega). NFAT-luciferase activity normalized by Renilla-luciferase was determined, and fold change was calculated in relation to DMSO treatment. A two-tailed unpaired Mann-Whitney U test was used to evaluate statistical difference. All data are presented as mean ± S.E.M; **p<0.01.

3.3. Transcriptome profiles and signaling pathways of proband MLP-W4R;MYH7-R723C iPSC-CMs

Differentially expressed genes (DEGs) were identified using DESeq2. Then, heatmap was generated by differentially expressed genes among the control, isogenic MYH7-corrected proband, isogenic MLP-corrected proband and proband MLP-W4R;MYH7-R723C iPSC-CMs. Consistent with isogenic correction of the proband revealed a significant attenuation of the defects, the differentially expressed genes of the isogenic correction of proband showed the similar gene expression pattern with the control **(Figure 4A, P<0.05; fold change >2)**. Next, we investigated the up-regulated and down-regulated genes between the control and the proband MLP-W4R;MYH7-R723C iPSC-CMs, as shown in the volcano plot derived from DESeq2 analysis **(Figure 4B)**. It showed 324 up-regulated genes and 373 down-regulated genes in the proband. I confirmed many pathways associated with the pathological hypertrophy, such as TGF-β signaling, protein kinase A signaling, role of NFAT in cardiac hypertrophy and cardiac hypertrophy signaling, from the ingenuity pathway analysis using the differentially expressed genes in the proband MLP-W4R;MYH7-R723C iPSC-CMs and the control **(Figure 5A)**. Additionally, the signaling pathways associated with the cardiac hyperthrophy were obtained by the ingenuity pathway analysis **(Figure 5B)**.

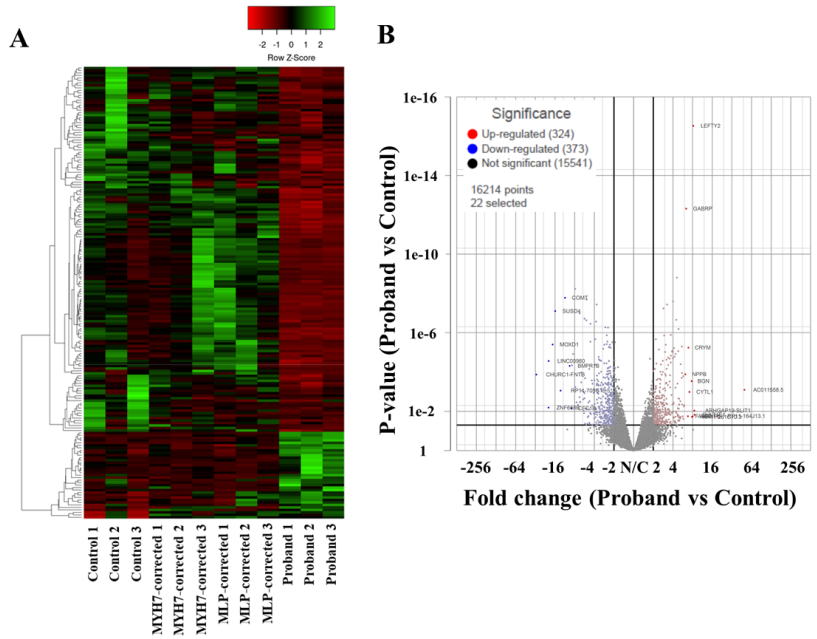

Figure 4. Transcriptomic profiling of proband MLP-W4R;MYH7-R723C iPSC-CMs. (A) Heatmap of differentially expressed genes among the control, isogenic MYH7-corrected proband, isogenic MLP-corrected proband and proband MLP-W4R;MYH7-R723C iPSC-CMs (P<0.05; fold change >2). **(B)** Volcano plot obtained from DESeq2 analysis of the control and the proband MLP-W4R;MYH7-R723C iPSC-CMs (P<0.05; fold change >2).

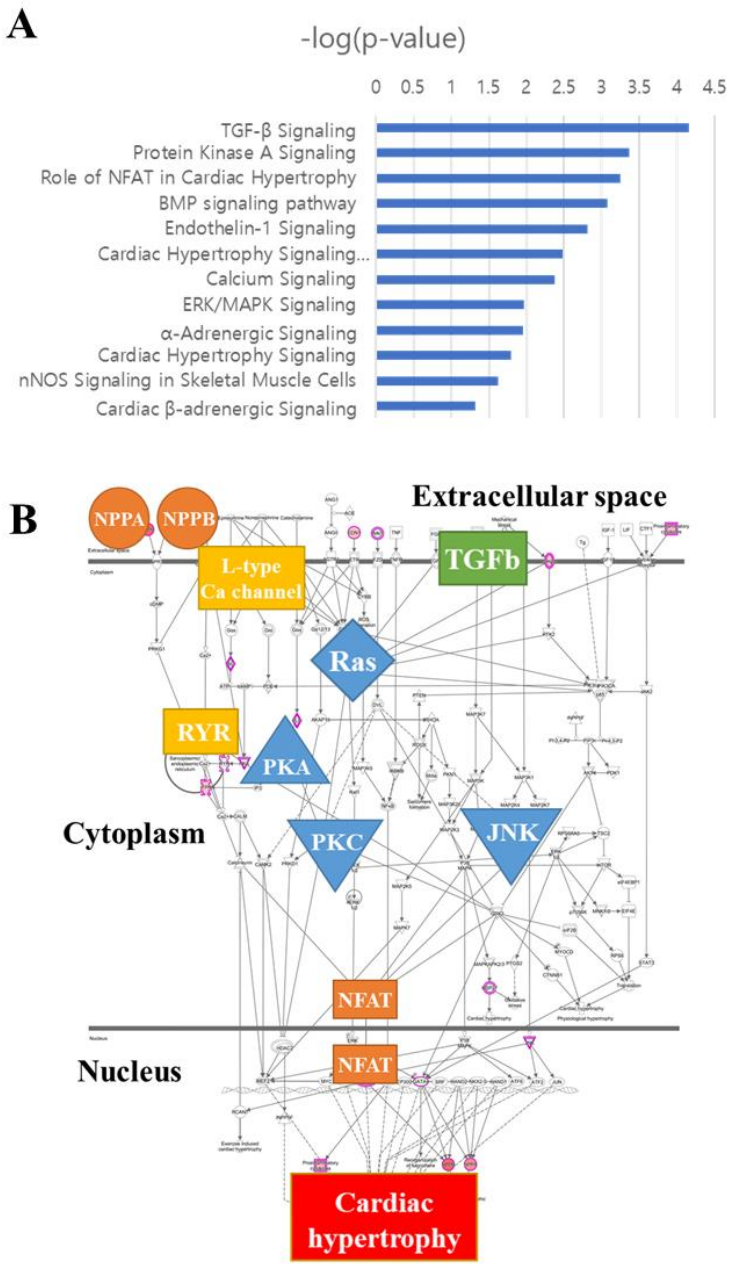

Figure 5. Transcriptomic profiling of signaling pathways in proband MLP-W4R;MYH7-R723C iPSC-CMs. (A) Ingenuity pathway analysis using the differentially expressed genes in proband compared with the control ($P<0.05$; fold change >2). **(B)** Signaling pathways of differentially expressed genes in proband compared with the control.

3.5. Conclusion.

Through the research supported by this seed grant, we showed that the sarcomeric contraction/MLP/calcineurin mechanotransduction pathway described here would have relevance to the sarcomeric HCM mutations. Moreover, declining MLP levels play a crucial role in stabilization of sarcomere and transducing the sarcomere stress in cardiac cells, leading to rapid cardiac muscles remodeling and HCM disease progression. In addition, we confirmed the transcriptome profiling in the proband through the RNA sequencing. We found the signaling pathways associated with the cardiac hyperthrophy in the proband. I expect the current findings could support strongly to find a novel drug for HCM patient as well as understand the mechanism underlying HCM.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

I could expand my career in heart disease modeling with the goal of understanding the mechanisms of HCM and finding novel drugs using patient-derived iPSCs. As using the corrected iPSC-CMs, we could figure out the defect in the proband MLP-W4R;MYH7-R723C iPSC-CMs triggered by each mutation as well as the effect of the drugs.

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?

We have established the system for the disease modeling to study on heart disease using patient-derived iPSCs. For the accurate interpretation, we needed to validate HCM disease phenotypes in our model using isogenic MYH7 or MLP corrected iPSC-CMs each and the efficacy of the drug, such as mavacamten and FK506. During the next reporting period, we will do the high-throughput screening. I plan to join scientific meetings including Yale Cardiovascular Biology Research In Progress meeting, the monthly Yale Stem Cell Center Research Forum, and the annual retreat of Yale VBT Program and Yale Stem Cell Center. In addition, I will give a presentation at the meeting to get the feedback from the experts. Finally, I have a plan to submit one manuscript based on this research project supported by DOD.

4. IMPACT:

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

We have developed a robust system for a drug screening and understanding HCM disease using 2D culture and 3D EHT from patient derived iPSCs and isogenic line via gene editing. Importantly, we confirmed the pathological hypertrophic phenotype using RNAseq analysis. In addition, we found that declining MLP levels is a clear indication of severe HCM and MLP plays a crucial role as a mechanosencing in z-disk to remodel the cardiac sarcomere and regulate HCM disease progression. These finding could be a significant impact in the field of heart disease.

What was the impact on other disciplines?

The transcriptome profiling, developing screening system using 2D culture and 3D EHT from patient derived iPSCs and isogenic line via gene editing could be a new paradigm in a new drug discovery for heart patients.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

We have made a modest change in adding a reporter system. Because one reviewer raised the question in my proposal about whether cell size is the most ideal phenotype to screen. It is also one of what we concern. So, we made an additional system to screen. It is to employ reporter system. For that, we compared the transcriptome of the control with the proband MLP-W4R;MYH7-R723C iPSC-CMs to find the good marker in differentially expressed genes for screening. Therefore, we will employ a report system for a high-throughput screening.

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

Nothing to Report.

Changes that had a significant impact on expenditures

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

Nothing to Report.

Significant changes in use or care of vertebrate animals

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**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Nothing to Report.

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?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Nothing to Report.