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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 drug candidates that lead to remodeling sarcomere and HCM disease progression in patient derived hiPSC-CMs. Additionally, we employed multiple HCM iPSC-CM lines to validate our findings to find novel drugs for multiple variants of HCM its underlying mechanisms.

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 discover new drugs for alleviating HCM by innovative high-throughput chemical screening with a HCM patient-specific iPSC-CMs with MYH7-R723C/MLP-W4R mutations. In addition to the double mutations with MYH7-R723C/MLP-W4R, our group has the other HCM mutations, such as R442G and R663H, as well. We have studied the role of HCM signaling with these patient-specific iPSC-CMs using 2D based molecular analysis and 3D based engineered heart tissue (EHT) mechanical analysis. I expect that these diversified approaches will elucidate the mechanism of pathological hypertrophy. This is a very significant process to find novel drugs.

The second goal is to evaluate the effect of the candidate drugs with the transcriptome profiling analysis and mechanical assessments using EHTs produced by isogenic control and MYH7-R723C/MLP-W4R mutant iPSC-CMs iPSC-CMs. Our group has a validated system for 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 May 2020 and January 2021. Our group holds regular joint meetings on engineered cardiovascular tissue with Dr. Stuart Campbell and cardiac physiology and Dr. Lawrence Young. 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 am generating 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. Generation and characterization of MYH7 or MLP corrected iPSC line.

In the proposal, I demonstrateded that double heterozygote MYH7-R723C/MLP-W4R mutant iPSCs were larger than control iPSC-CMs, recapitulating one of the major HCM defects at the cellular level. To understand that how MYH7-R723C and MLP-W4R variants themselves cause HCM phenotype, we corrected R723C (MYH7-corrected) or W4R (MLP-corrected) in MYH7-R723C/MLP-W4R mutant iPSCs using gene editing techniques **(Figure 1A and 2A)**. After differentiating the corrected hiPSCs into CMs, cellular and mechanical defects were investigated in the corrected iPSC-CMs. The correction of MYH7-R723C or MLP-W4R mutation resulted in significant rescue of HCM phenotypes including cell size and expression of the BNP marker **(Figure 1B-D and 2B-D)**. In addition, 3D-EHTs from the correction of MYH7-R723C or MLP-W4R mutation revealed a significant rescue of the mechanical defects including the RT50, TTP and the peak force **(Figure 1E-G and 2E-G)**.

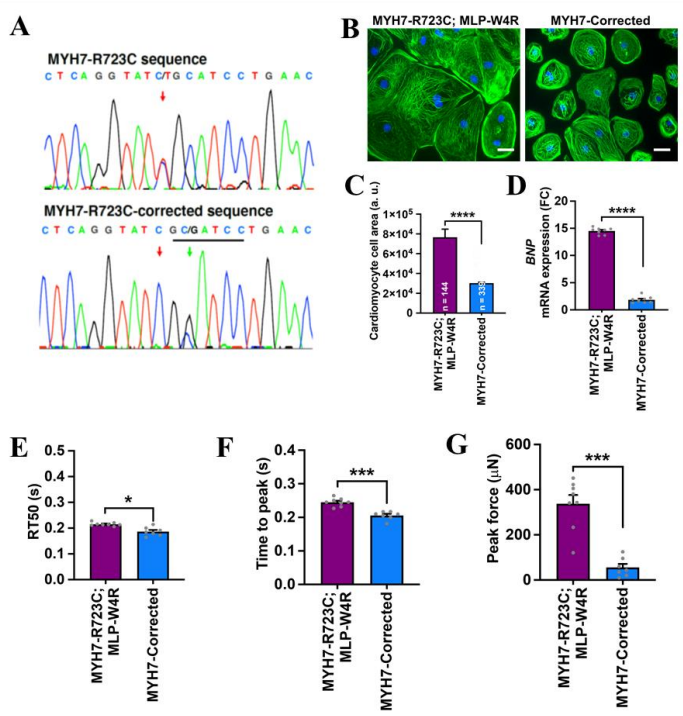

Figure 1. Generation and characterization of MYH7 corrected hiPSC from MYH7-R723C/MLP-W4R mutant iPSC. (A) Sequence showing successful genetic correction of MYH7-R723C mutation to wild-type sequence. Red arrow points to heterozygous mutant base, which is corrected to wild type base in the MYH7 corrected hiPSC. Green arrows point to silent base changes, which are introduced to generate *BamHI* restriction site for restriction digestion-based screening for the hiPSC clones with a successful correction event occurred at the target locus through homologous recombination mechanism. (**B)** Immunostaining of cTnT in 35 days old functional cardiomyocytes derived from MYH7-R723C/MLP-W4R mutant iPSC-CMs and MYH7 corrected hiPSC-CMs. **(C)** Cell size phenotype in MYH7-R723C/MLP-W4R mutant iPSC-CMs and MYH7 corrected hiPSC-CMs. Image J was used to measure hiPSC-CMs cell area. **(D)** qRT-PCR analyses of the expression levels of *BNP*. **(E-G)** RT50, TTP and peak force measurements are performed in EHTs from MYH7-R723C/MLP-W4R mutant iPSC-CMs and MYH7 corrected hiPSC-CMs. Statistical differences were evaluated using nonparametric Mann-Whitney test. . All data are presented as mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001; ns: not significant.

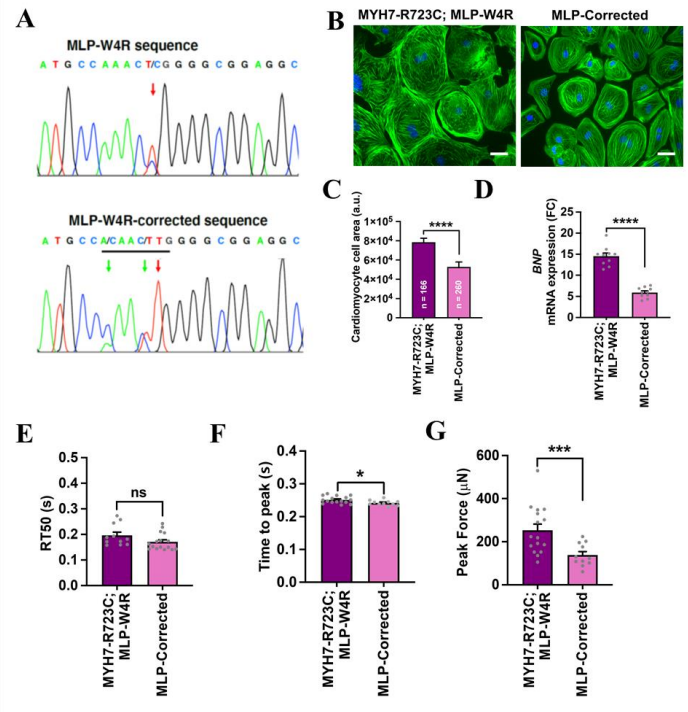

Figure 2. Generation and characterization of MLP corrected hiPSC from MYH7-R723C/MLP-W4R mutant iPSC. (A) Sequence showing successful genetic correction of MLP-W4R mutation to wild-type sequence. Red arrow points to heterozygous mutant base, which is corrected to wild-type base in the MLP corrected hiPSC clone. Green arrows point to the silent base changes, which are introduced to generate MfeI restriction site for digestion-based screening of the hiPSC clones harboring successful correction event at the target locus through homologous recombination mechanism. **(B)** Immunostaining of cTnT in 35 days old functional cardiomyocytes derived from MYH7-R723C/MLP-W4R mutant iPSCs and MLP corrected hiPSC-CMs. **(C)** Cell size phenotype in MYH7-R723C/MLP-W4R mutant iPSC-CMs and MLP corrected hiPSC-CMs. Image J was used to measure hiPSC-CMs cell area. **(D)** qRT-PCR analyses of the expression levels of *BNP*. **(E-G)** RT50, TTP and peak force

measurements are performed in EHTs from MYH7-R723C/MLP-W4R mutant iPSC-CMs and MLP corrected hiPSC-CMs. Statistical differences were evaluated using nonparametric Mann-Whitney test. . All data are presented as mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001; ns: not significant.

3.2. Stability of MLP complex at the z-disc and its role in HCM signaling.

Next, we investigated the MLP stability. Cycloheximide was added to the CM culture to determine the MLP stability, and the results revealed that MLP had a markedly higher decay rate in the double mutant CMs than that in the control CMs **(Figure 3A and B)**. Importantly, genetic correction of MYH7-R723C or MLP-W4R resulted in an effective restoration of the MLP levels in MYH7-R723C/MLP-W4R mutant iPSC-CMs **(Figure 3 C-F)**. Together these results suggested that MLP expression is required for effective cardiac function.

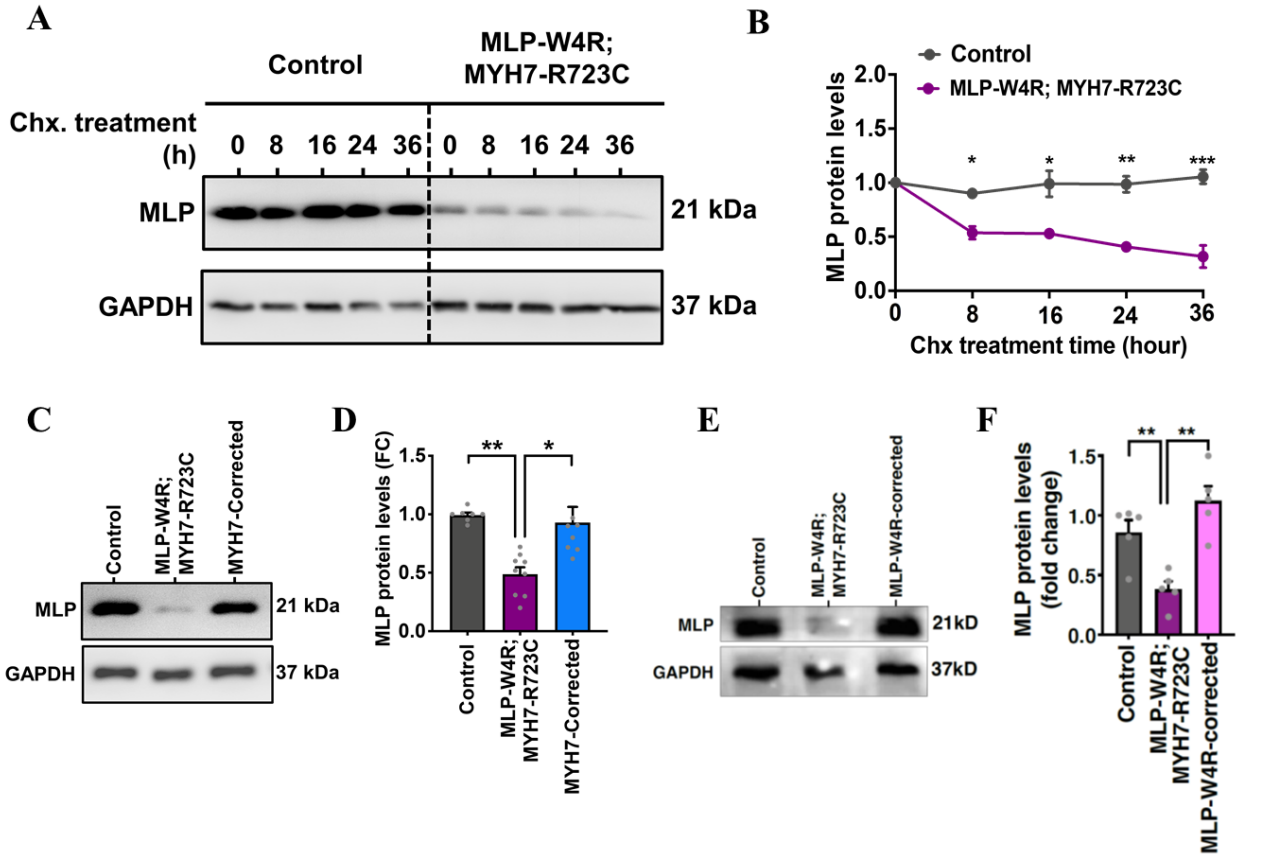

Figure 3. Stability of MLP complex at the z-disc and its role in HCM signaling. (A) Evaluation of MLP protein stability in control and MYH7-R723C/MLP-W4R mutant iPSC-CMs. Representative blot showing MLP protein in control and MYH7-R723C/MLP-W4R mutant iPSC-CMs at 0, 8, 16, 24, and 36 hours of treatment with 50 µg/ml cycloheximide. **(B)** Quantification of total MLP protein in control and MYH7-R723C/MLP-W4R mutant iPSC-CMs at different treatment time points of cycloheximide treatment. Statistical differences on each treatment time points are evaluated using Mann-Whitney nonparametric test; *p < 0.05; **p < 0.01; ***p < 0.001; ns: not significant. **(C and E)** Western blot analysis of MLP expression levels in control, MYH7-R723C/MLP-W4R mutant iPSC-CMs and MYH7-corrected hiPSC-CMs **(C)** or MLP-corrected hiPSC-CMs **(E)**. GAPDH is used as the loading control. **(D and F)** Quantification of MLP protein levels using western blot in control, MYH7-R723C/MLP-W4R mutant iPSC-CMs and MYH7-corrected hiPSC-CMs **(D)** or MLP-corrected hiPSC-CMs **(F)**. Statistical differences were evaluated using one-way ANOVA with Tuckey multiple comparison test. All data are presented as mean ± SEM; *p < 0.05; **p < 0.01; ns: not significant.

3.3. MLP-W4R could induce severe HCM phenotype in multiple types of MHY7 mutant hiPSC lines (R723C, R663H and R442G).

To investigate whether MLP-W4R could induce severe HCM phenotype caused by the myosin mutation, HA-tagged MLP-W4R was ectopically expressed in the MYH7-R723C mutant and wild type control hiPSC-CMs **(Figure 4A)**. While MLP-W4R significantly exacerbated the HCM defects including the enlarged cell area and an elevated expression BNP in the MYH7-R723C mutant CMs, it did not affect the control CMs **(Figure 4 B-D)**. Additionally, MLP-W4R ectopic expression in cardiomyocytes differentiated from hiPSCs generated from two other HCM patient-derived iPSCs (MYH7-R663H and MYH7-R442G) resulted in more worsened HCM defects including the enlarged cell area and an elevated expression BNP **(Figure 5A-F)**.

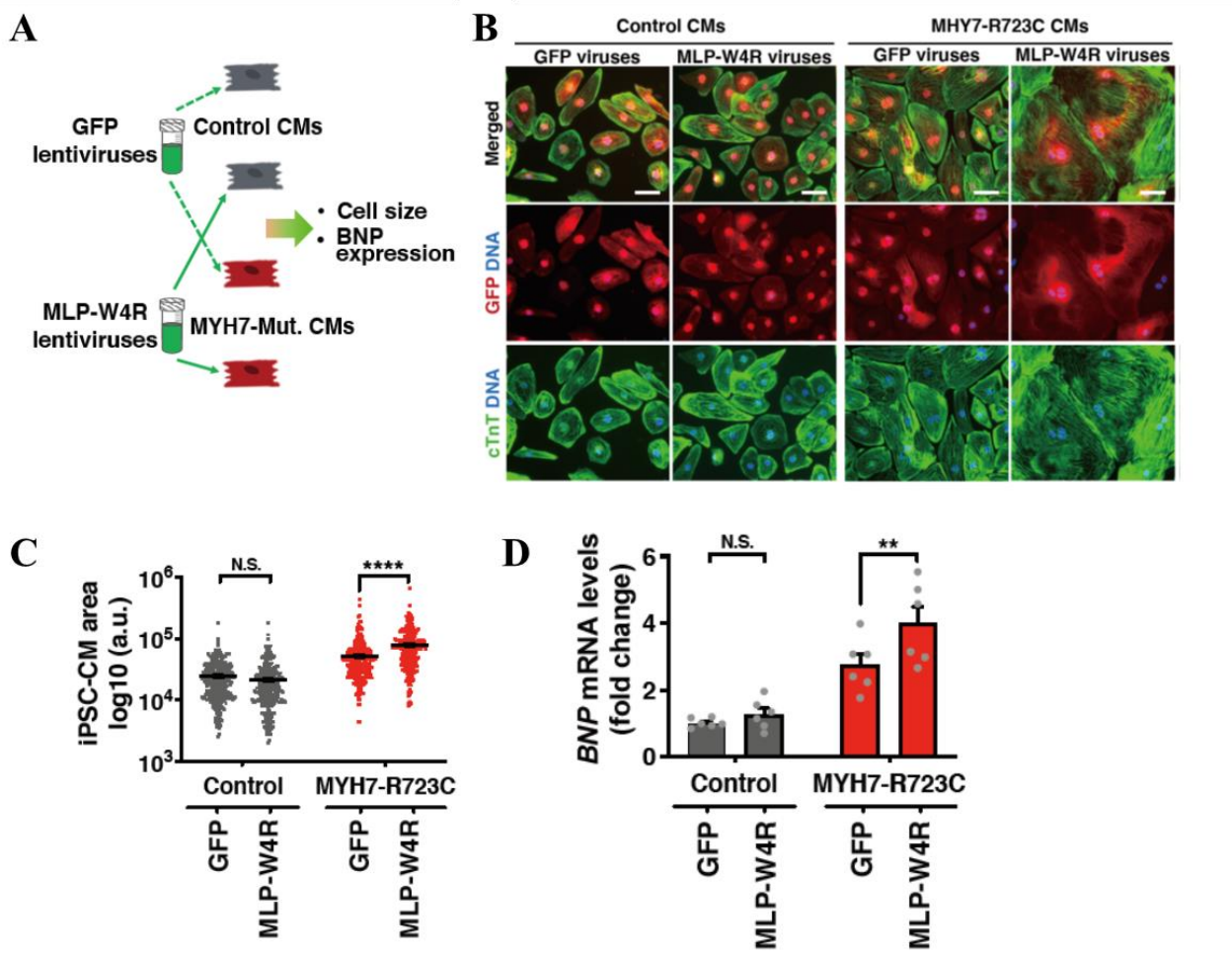

Figure 4. MLP-W4R induces severe HCM phenotype in MHY7-R723C mutant hiPSC. (A) Schematic illustration showing MLP-W4R mediated exacerbation of HCM phenotype strategy. **(B)** Immunostaining of cTnT in 35 days old cardiomyocytes derived from the control and MHY7-R723C mutant hiPSC-CMs treated with GFP control and MLP-W4R lentiviruses. **(C)** Quantification of cell area in the control and MHY7-R723C mutant hiPSC-CMs. ImageJ was used to measure cell area. **(D)** RT-qPCR expression analysis of BNP mRNA in 35-days cardiomyocytes derived from the control and MHY7-R723C mutant hiPSC-CMs treated with GFP control and MLP-W4R lentiviruses. Statistical evaluation is performed using two-way ANOVA test with Tuckey multiple comparison test. The individual groups were compared using one-way ANOVA. The data are presented as mean ± SEM; **p < 0.01; ****p < 0.0001; ns: not significant.

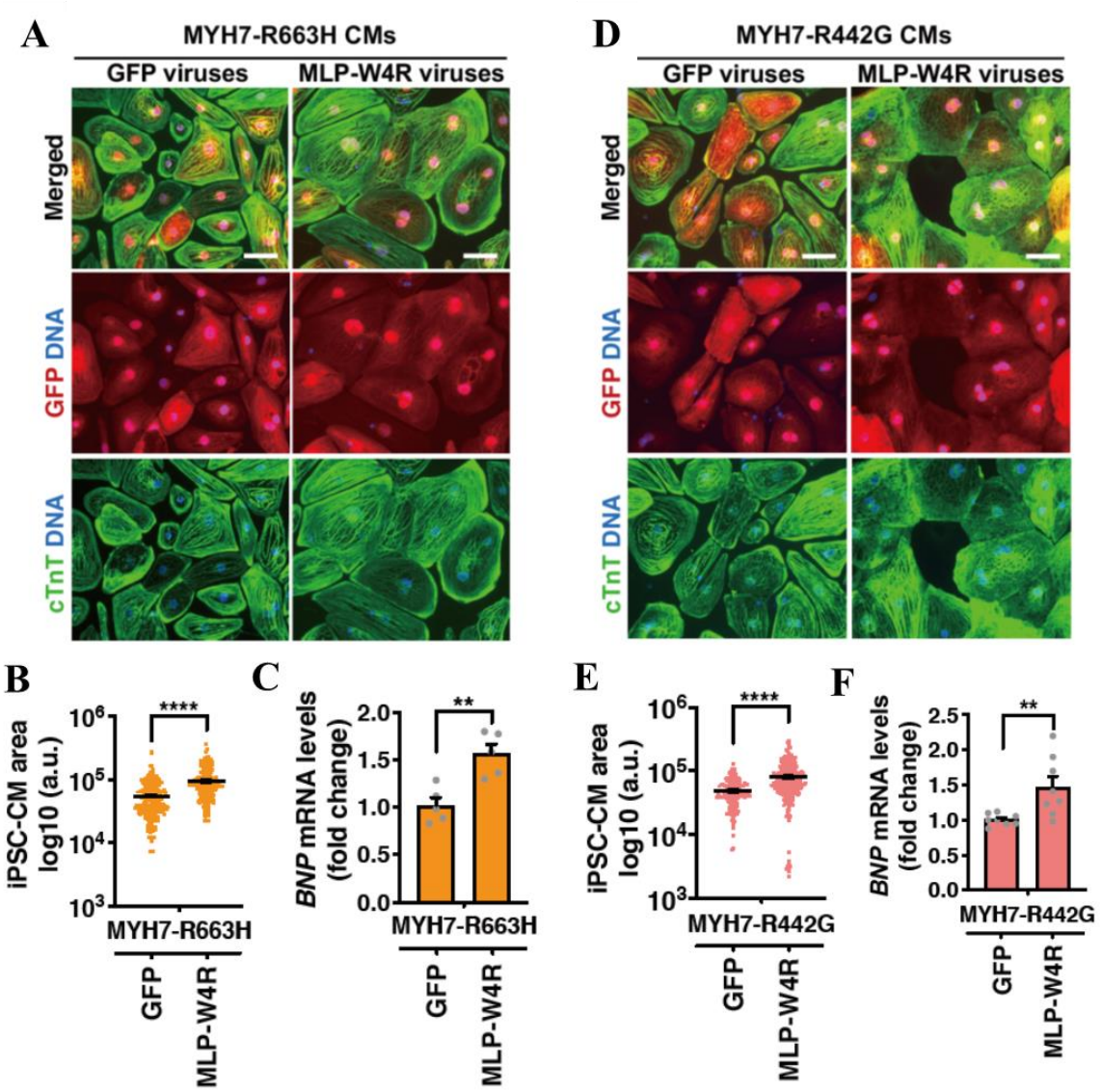

Figure 5. MLP-W4R induces severe HCM phenotype in the other HCM mutations (MYH7-R663H and MYH7-R442G). (A and D) Immunostaining of cTnT in 35 day old cardiomyocytes derived from the control and MYH7-R663H mutant hiPSC-CMs **(A)** or MYH7-R442G mutant hiPSC-CMs **(D)** treated with GFP control and MLP-W4R lentiviruses. **(B and E)** Quantification of cell area in the control and MYH7-R663H mutant hiPSC-CMs **(B)** or MYH7-R442G mutant hiPSC-CMs **(E)** treated with GFP control and MLP-W4R lentiviruses. ImageJ was used to measure cell area. **(C and F)** RT-qPCR expression analysis of BNP mRNA in 35-days cardiomyocytes derived from the control and MYH7-R663H mutant hiPSC-CMs **(C)** or MYH7-R442G mutant hiPSC-CMs **(F)** treated with GFP control and MLP-W4R lentiviruses. Statistical evaluation was performed using nonparametric Mann-Whitney test. All data are presented as mean ± SEM; **$p < 0.01$; ****$p < 0.0001$.

3.4. Pharmacological interventions to rescue the HCM phenotype.

In this proposal, I demonstrated that EHTs generated from double heterozygote (MYH7-R723C;MLP-W4R) iPSC-CMs show a prolonged twitch event with delayed relaxation and increased peak force than those of control with the enlarged cell area and an elevated expression BNP at the cellular level. In addition, I showed that the correction of MYH7-R723C or MLP-W4R

mutation revealed a significant rescue of the mechanical defects as well as cellular defects in the current result **(Figure 1-3)**. Therefore, I hypothesize that severe HCM phenotype could be rescued by reducing HCM defects. We next investigated whether MYK-461 (a.k.a. mavacamten), a novel small-molecule that modulates actin-myosin cross-bridges by reducing ATPase activity in sarcomere could attenuate cardiac remodeling and hypertrophic signaling. To confirm whether mavacamten normalizes HCM defects, MYH7-R723C/MLP-W4R mutant iPSC-CMs were treated with mavacamten. We observed that significant rescue of HCM phenotypes including cell size, MLP expression and expression of the BNP marker **(Figure 6A-E)**. In addition, 3D-EHTs from MYH7-R723C/MLP-W4R mutant iPSC-CMs revealed a significant rescue of the mechanical defects including the RT50, TTP and the peak force **(Figure 6F-H)**. Notably, mavacamten differentially reduced RT50 in double mutant EHTs compared to healthy control EHTs (2-way anova, interaction p value <0.05), suggesting a targeted effect of the drug on the mutant myosin. These data suggest that reducing cardiomyocyte contractility can attenuate the severe pathology in MYH7-R723C/MLP-W4R mutant iPSC-CMs.

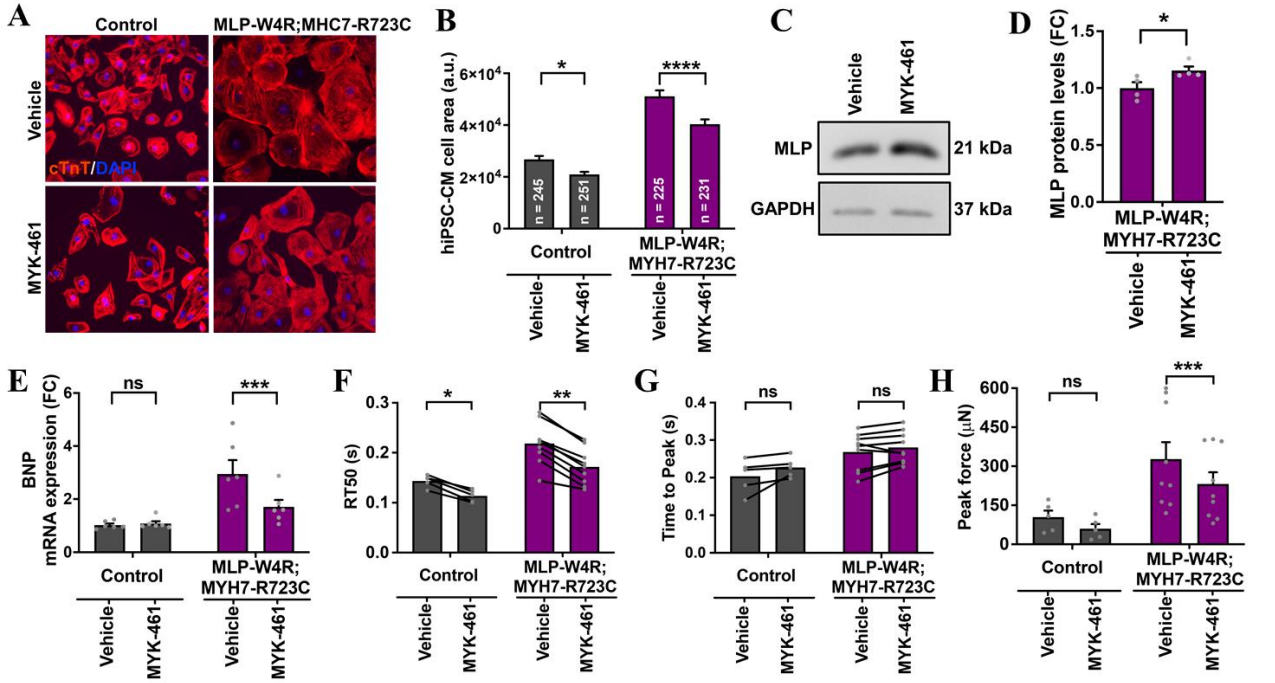

Figure 6. Pharmacological interventions to rescue the HCM phenotype. (A) Representative images for 30-days control and MYH7-R723C/MLP-W4R mutant iPSC-CMs treated with 0.5uM MYK461 or with only DMSO as a vehicle. **(B)** Cell area of the vehicle and MYK461 treated cardiomyocytes from control and MYH7-R723C/MLP-W4R mutant iPSC-CMs was measured using ImageJ. **(C)** Western blot analysis of MLP expression levels in MYH7-R723C/MLP-W4R mutant iPSC-CMs treated with 0.5uM MYK461 or with only DMSO as a vehicle. **(D)** Quantification of MLP protein levels using western blot in MYH7-R723C/MLP-W4R mutant iPSC-CMs treated with 0.5uM MYK461 or with only DMSO as a vehicle. **(E)** RT-qPCR expression analysis of BNP mRNA in 30-days old control and MYH7-R723C/MLP-W4R mutant iPSC-CMs treated with 0.5uM MYK461 or with only DMSO as a vehicle. **(F-H)** RT50, TTP and peak force measurements are performed in EHTs from control and MYH7-R723C/MLP-W4R mutant iPSC-CMs treated with 0.5uM MYK461 or with only DMSO as a vehicle. Statistical evaluation is performed using two-way ANOVA test with Tuckey multiple comparison test. The individual groups were compared using one-way ANOVA. The data are presented as mean ± SEM; *p < 0.05; **p < 0.01; ***p < 0.001; ns: not significant.

3.5. Conclusion.

Through the research supported by this seed grant, we showed that the validation of hypertrophic defects using HCM patient derived R723C/MLP-W4R mutant iPSC-CMs. 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 employed multiple HCM iPSC-CM lines to validate our findings including the enlarged cell area, an elevated expression BNP, a prolonged twitch event with delayed relaxation and increased peak force than those of control. 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?

I could expand my career in heart disease modeling with the goal of understanding the mechanisms of HCM and finding a novel drugs using patient-derived iPSCs. Specially, I have learned to generate genome editing via TALEN or CRISPR/Cas9 method. As using the corrected iPSC-CMs, we could figure out the defect in R723C/MLP-W4R mutant iPSC-CMs triggered by each mutation. These finding could support to elucidate the effect of the novel drug.

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, mavacamten, reducing myosin hypercontractility. During the next reporting period, we will establish an isogenic control iPSC line by correcting both of MYH7 and MLP. We will employ this isogenic control as a real control for transcriptome profiling and 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 talk 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 found that to introduce MLP-W4R in three different HCM patient-derived iPSC-CMs (MYH7-R723C, MYH7-R663H and MYH7-R442G) could induce a severe HCM phenotype. In addition, 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 success of 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. We will compare the transcriptome of the isogenic control with those of R723C/MLP-W4R mutant iPSC-CMs to find the marker in differentially expressed genes for screening. Therefore, we will employ two systems, such as using cell size difference and 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?

Name:	Jinkyu Park, Ph.D.
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	1234567
Nearest person month worked:	12
Contribution to Project:	Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support:	The Ford Foundation (Complete only if the funding support is provided from other than this award.)

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.