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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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14. ABSTRACT
The aim of this proposal is to discover a new medication for hypertrophic cardiomyopathy (HCM) using cardiomyocytes (CMs) generated from human induced pluripotent stem cells (hiPSCs). HCM is a prevalent hereditary heart disease affecting approximately 1 in 500 people worldwide, including military families. It is characterized by the thickening of heart tissue, which reduces the size of the heart chambers, impairs relaxation time, causes arrhythmias, and can ultimately result in sudden cardiac death (SCD). To identify potential treatments for HCM, a high-throughput screening approach can be used with patient-specific iPSC-derived CMs representing various HCM genotypes. In this study, we demonstrate the validation of hypertrophic defects using CMs derived from HCM patients with R723C/MLP-W4R mutations. We also show that declining MLP levels play a crucial role in stabilizing sarcomeres in cardiac cells, leading to remodeling sarcomeres and HCM disease progression. Additionally, we employed multiple HCM iPSC-CM lines to confirm our findings, including an enlarged cell area, elevated expression of BNP, prolonged twitch event with delayed relaxation, and increased peak force compared to the control. Subsequently, we have validated two drug candidates that can inhibit calcineurin/NFAT or mitigate enhanced actomyosin crossbridge formation in patient-derived hiPSC-CMs. Furthermore, we conducted transcriptome analysis using RNA sequencing (RNAseq) on control cells, isogenic MYH7-corrected cells, isogenic MLP-corrected cells, and proband MLP-W4R;MYH7-R723C iPSC-CMs to gain insight into the mechanisms underlying 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?

Our major goal was to discover novel drugs for the treatment of hypertrophic cardiomyopathy (HCM) by utilizing innovative high-throughput chemical screening with HCM patient-specific iPSC-CMs carrying the MYH7-R723C/MLP-W4R mutation. Our research group also has access to other HCM mutations, such as R442G and R663H. We studied the role of HCM signaling with these patient-specific iPSC-CMs using 2D-based molecular analysis and 3D-based EHT mechanical analysis. We anticipate that utilizing these diversified approaches will enable us to elucidate the mechanism of pathological hypertrophy, which is a significant process for identifying novel drugs for the treatment of HCM.

First was to discover novel drugs for the treatment of hypertrophic cardiomyopathy (HCM) by utilizing innovative high-throughput chemical screening with HCM patient-specific iPSC-CMs carrying the MYH7-R723C/MLP-W4R mutation. Our research group also has access to other HCM mutations, such as R442G and R663H. We studied the role of HCM signaling with these patient-specific iPSC-CMs using 2D-based molecular analysis and 3D-based EHT mechanical analysis. We anticipate that utilizing these diversified approaches will enable us to elucidate the mechanism of pathological hypertrophy, which is a significant process for identifying novel drugs for the treatment of HCM.

Second was to evaluate the efficacy of the candidate drugs through transcriptome profiling analysis and mechanical assessments using EHTs produced by isogenic control and MYH7-R723C/MLP-W4R mutant iPSC-CMs. 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.

Third was to clarify the role of MYH7-R723C and MLP-W4R mutations in the pathogenesis of HCM, and to determine the efficacy of drugs in alleviating HCM. Understanding the mechanism of pathological hypertrophy is a critical step in discovering novel drugs for HCM.

Fourth was to perform transcriptome profiling analysis using isogenic control and MYH7-R723C/MLP-W4R mutant iPSC-CMs, and to assess the effect of candidate drugs using a validated system for molecular and biomechanical analysis based on EHTs using iPSC-CMs. This approach allows us to evaluate the effectiveness of the drugs with high precision.

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 gave a talk 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 had joint meetings on engineered cardiovascular tissue with Dr. Stuart Campbell and cardiac physiology with Dr. Lawrence Young. I also attended 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.

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.

I also have three papers mentioning this grant support in the acknowledgements as follow.
1. https://doi.org/10.1085/jgp.202012640
2. https://doi.org/10.1016/j.yjmcc.2021.12.014
3. https://doi.org/10.1161/CIRCULATIONAHA.121.056265

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. Generation and characterization of MYH7 or MLP corrected iPSC line.

In the proposal, I showed 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)**.

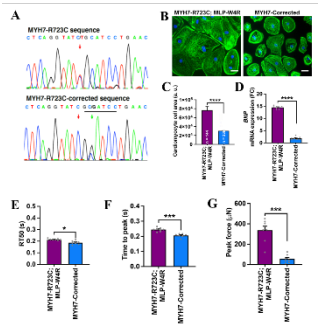


Figure 1. Generation and characterization of MYH7 corrected hiPSC from MYH7-R723C/MLP-W4R mutant iPSC. (A) Sequence showing successful genetic correction of MYH7-R723C mutation to wild-type sequence. Red arrow points to heterozygous mutant base, which is corrected to wild type base in the MYH7 corrected 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.

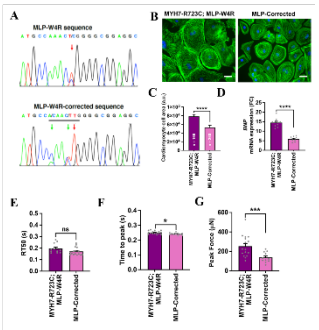


Figure 2. Generation and characterization of MLP corrected hiPSC from MYH7-R723C/MLP-W4R mutant iPSC. (A) Sequence showing successful genetic correction of MLP-W4R mutation to wild-type sequence. Red arrow points to heterozygous mutant base, which is corrected to wild-type base in the MLP corrected hiPSC 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 investigate 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.

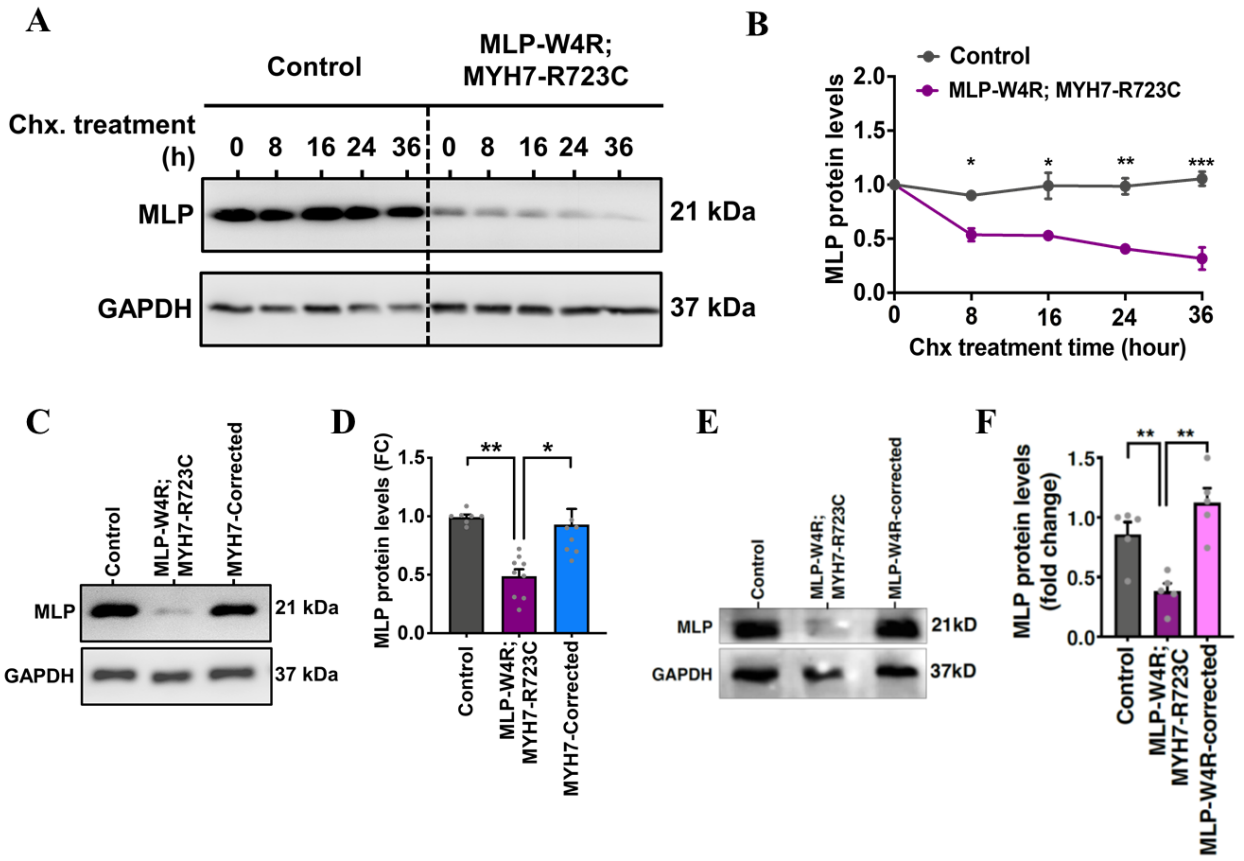


Figure 3. Stability of MLP complex at the z-disc and its role in HCM signaling. (A) Evaluation of MLP protein stability in control and MYH7-R723C/MLP-W4R mutant iPSC-CMs. Representative blot showing MLP protein in control and MYH7-R723C/MLP-W4R mutant iPSC-CMs at 0, 8, 16, 24, and 36 hours of treatment 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)**.

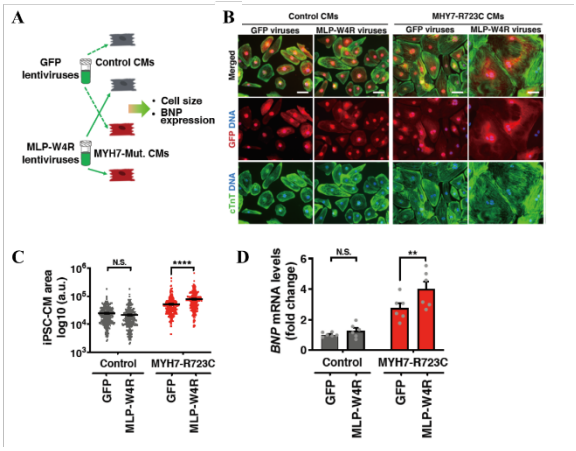


Figure 4. MLP-W4R induces severe HCM phenotype in MHY7-R723C mutant hiPSC. (A) Schematic illustration showing MLP-W4R mediated exacerbation of HCM phenotype strategy. **(B)** Immunostaining of cTnT in 35 days old cardiomyocytes derived from the control and MHY7-R723C mutant hiPSC-CMs treated with GFP 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.

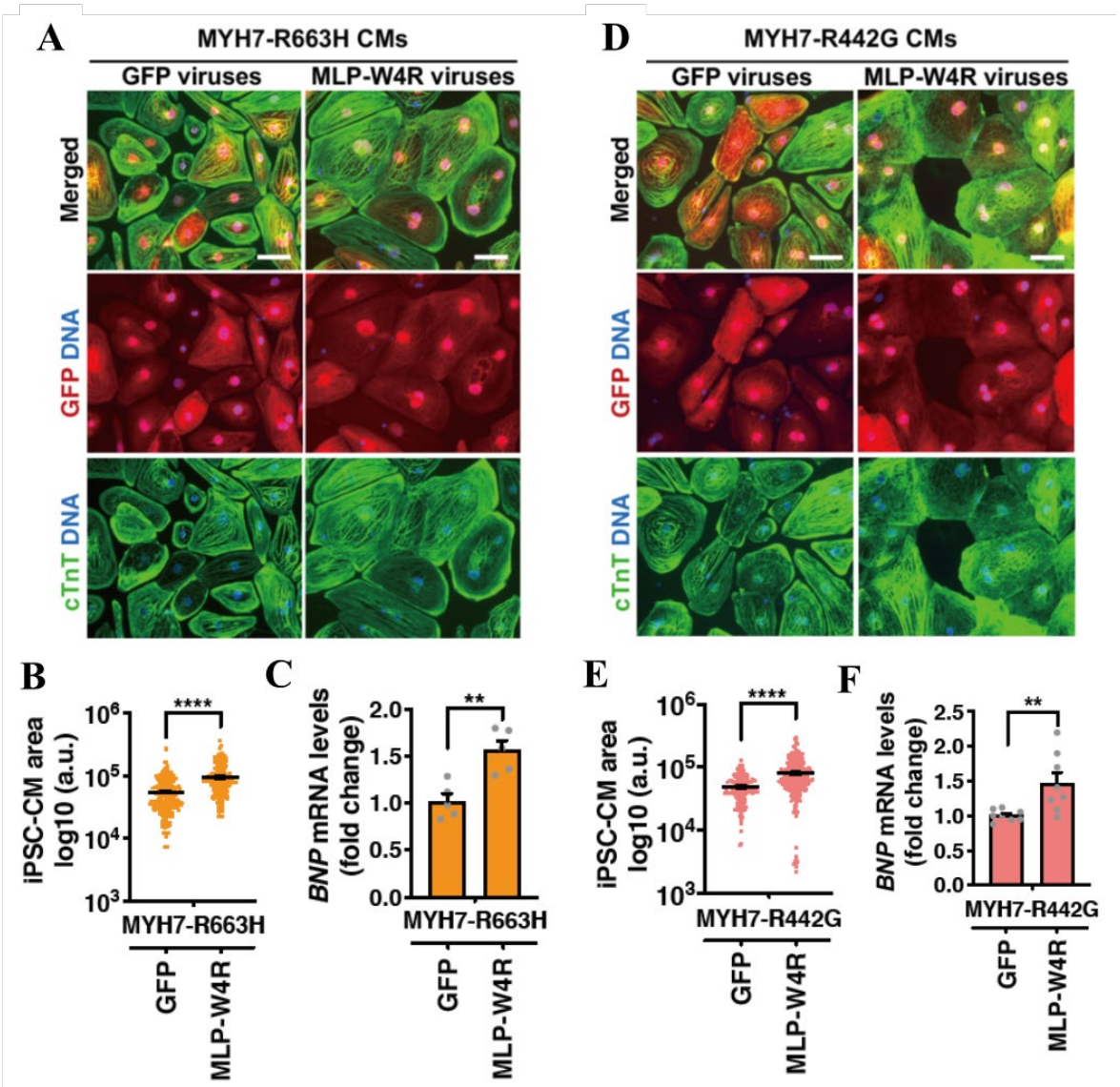


Figure 5. MLP-W4R induces severe HCM phenotype in the other HCM mutations (MYH7-R663H and MYH7-R442G). (A and D) Immunostaining of cTnT in 35 days old cardiomyocytes derived from the control and MYH7-R663H mutant hiPSC-CMs **(A)** or MYH7-R442G mutant hiPSC-CMs **(D)** treated with GFP control and MLP-W4R 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 the 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-bridge 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.

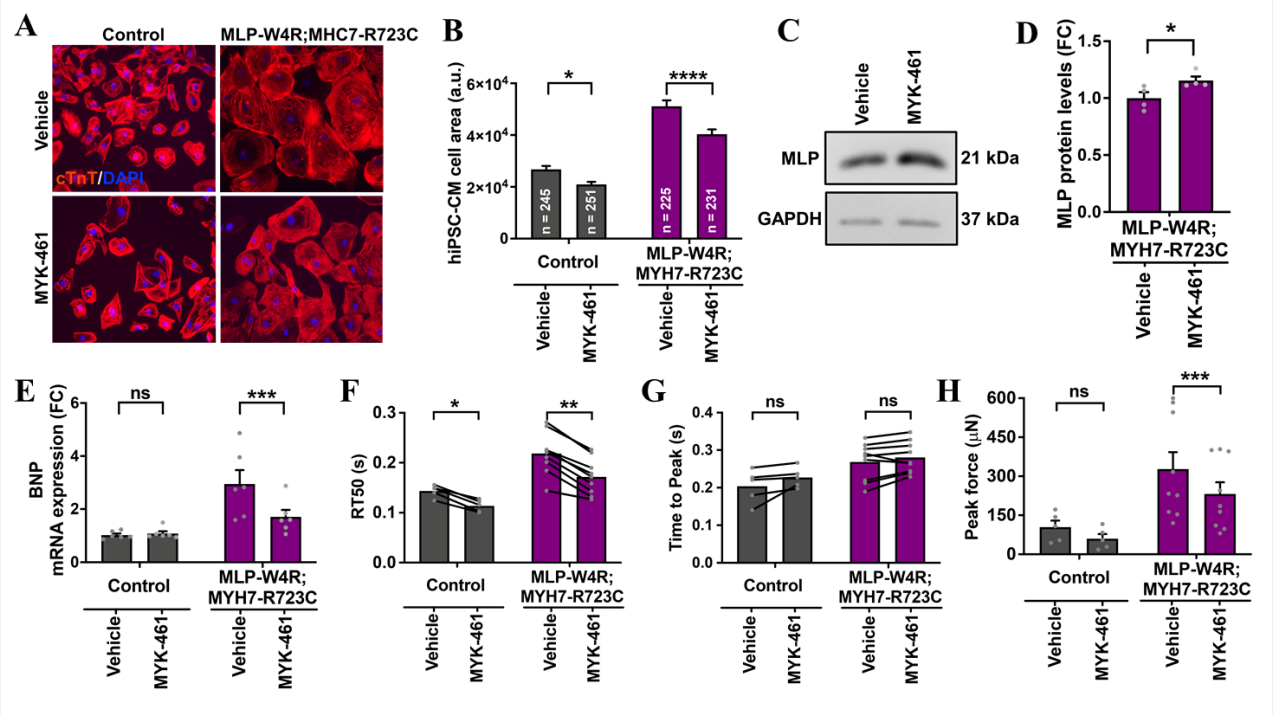


Figure 6. Pharmacological interventions to rescue the HCM phenotype. (A) Representative images for 30-days control and MYH7-R723C/MLP-W4R mutant iPSC-CMs treated with 0.5uM MYK461 or with only DMSO as a vehicle. **(B)** Cell area of the vehicle and MYK461 treated cardiomyocytes from control and MYH7-R723C/MLP-W4R 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. 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 7A and 7B)**. 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 7C-F)**.

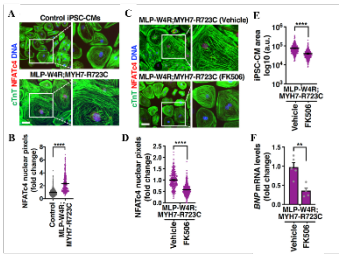


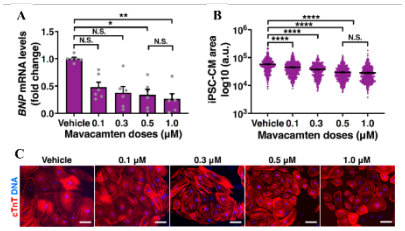
Figure 7. Inhibiting Calcineurin/NFAT Mitigates Development of the HCM Phenotype in Proband iPSC-Derived Cardiomyocytes.
(A) Immunostaining of NFATc4 (red) and cTnT (green) in day 35 control and MLP-W4R;MYH7-R723C iPSC-CMs. DNA was counterstained by DAPI. Scale bar, 100 μm. **(B)** Quantification of 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).

3.6. 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 8A-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 9A-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 10A-D)**. 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 10F)**.

Figure 9. 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.

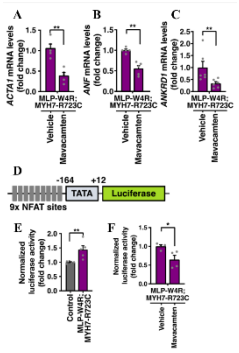


Figure 10. Quantification of the effects of mavacamten on the expressions of hypertrophic genes and the calcineurin/NFAT activity using a NFAT-luciferase reporter in proband MLP-W4R;MYH7-R723C iPSC-CMs. mRNA expression levels of three known HCM marker genes ACTA1 **(A)**, ANF **(B)**, and ANKRD1 **(C)** 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.

3.7. 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 11A, 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 11B)**. 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 12A)**. Additionally, the signaling pathways associated with the cardiac hyperthrophy were obtained by the ingenuity pathway analysis **(Figure 12B)**.

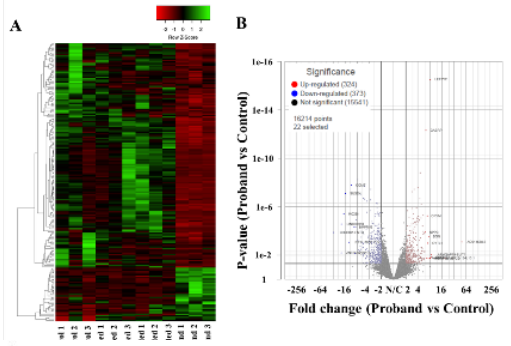


Figure 11. Transcriptomic profiling of proband MLP-W4R;MYH7-R723C iPSC-CMs. (A) Heatmap of differentially expressed genes among the control, isogenic MYH7-corrected proband, isogenic MLP-corrected proband and proband MLP-W4R;MYH7-R723C iPSC-CMs (P<0.05; fold change >2). **(B)** Volcano plot obtained from DESeq2 analysis of the control and the proband MLP-W4R;MYH7-R723C iPSC-CMs (P<0.05; fold change >2).

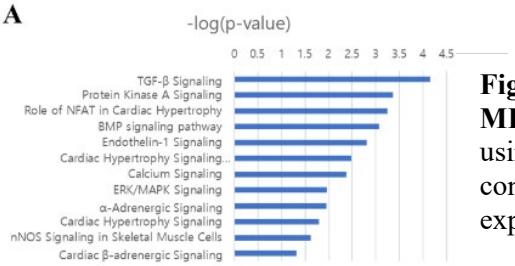
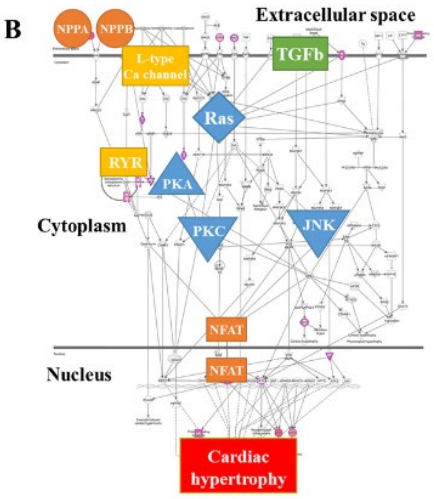


Figure 12. Transcriptomic profiling of signaling pathways in proband MLP-W4R;MYH7-R723C iPSC-CMs. (A) Ingenuity pathway analysis using the differentially expressed genes in proband compared with the control (P<0.05; fold change >2). **(B)** Signaling pathways of differentially expressed genes in proband compared with the control.



3.5. Conclusion.

Our research has demonstrated that the sarcomeric contraction/MLP/calcineurin mechanotransduction pathway described here is relevant to sarcomeric HCM mutations. Furthermore, the decrease in MLP levels plays a critical role in stabilizing the sarcomere and transducing sarcomere stress in cardiac cells, leading to rapid remodeling of cardiac muscles and progression of HCM disease. Additionally, we have confirmed the proband's transcriptome profiling through RNA sequencing and identified signaling pathways associated with cardiac hypertrophy in the proband. We anticipate that these findings will strongly support the discovery of a novel drug for HCM patients and provide a better understanding of the underlying mechanisms of HCM.

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

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 a system using patient-derived iPSCs to model heart disease. To ensure accurate interpretation, we validated HCM disease phenotypes in our model using isogenic MYH7 or MLP-corrected iPSC-CMs and tested the efficacy of drugs like mavacamten and FK506. In the next reporting period, we plan to conduct high-throughput screening. I will participate in various scientific meetings, including the Yale Cardiovascular Biology Research In Progress meeting, the monthly Yale Stem Cell Center Research Forum, and the annual retreat of the Yale VBT Program and Yale Stem Cell Center. I will also present our research project at these meetings to get feedback from experts. Finally, I plan to submit a 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 established a strong system for both drug screening and studying HCM disease using patient-derived iPSCs in 2D culture and 3D EHTs, as well as isogenic lines generated through gene editing. Significantly, we have validated the pathological hypertrophic phenotype using RNAseq analysis. Additionally, we have identified a crucial role for MLP as a mechanosensing protein in the z-disk, regulating HCM disease progression through sarcomere remodeling. These findings have the potential for significant impact in the field of heart disease research.

What was the impact on other disciplines?

The combination of transcriptome profiling and the development of a screening system using 2D culture and 3D EHT from patient-derived iPSCs and isogenic lines via gene editing has the potential to revolutionize drug discovery for heart disease patients.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
We have made a slight modification to our proposal by adding a reporter system. One of the reviewers had raised a concern about whether cell size is the most suitable phenotype for screening. Therefore, we have developed an additional system that employs a reporter to address this concern. To identify a suitable marker in the differentially expressed genes for screening, we compared the transcriptome of the control with that of the MLP-W4R;MYH7-R723C proband iPSC-CMs. We plan to use this reporter system in our high-throughput screening. We also added a luciferase assay to close the vulnerability

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.*