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Viral expression of a SERCA2a-activating PLB mutant improves calcium cycling and synchronicity in dilated cardiomyopathic hiPSC-CMs

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Abstract

There is increasing momentum toward the development of gene therapy for heart failure (HF) that is defined by impaired calcium (Ca^{2+}) transport and reduced contractility. We have used FRET (fluorescence resonance energy transfer) between fluorescently-tagged SERCA2a (the cardiac Ca^{2+} pump) and PLB (phospholamban, ventricular peptide inhibitor of SERCA) to test directly the effectiveness of loss-of-inhibition/gain-of-binding (LOI/GOB) PLB mutants (PLB_M) that were engineered to compete with the binding of inhibitory wild-type PLB (PLB_{WT}). Our therapeutic strategy is to relieve PLB_{WT} inhibition of SERCA2a by using the reserve adrenergic capacity mediated by PLB to enhance cardiac contractility. Using a FRET assay, we determined that the combination of a LOI PLB mutation (L31A) and a GOB PLB mutation (I40A) results in a novel engineered LOI/GOB PLB_M (L31A/I40A) that effectively competes with PLB_{WT} binding to cardiac SERCA2a in HEK293–6E cells. We demonstrated that co-expression of PLB_M enhances SERCA Ca-ATPase activity by increasing enzyme Ca^{2+} affinity ($1/K_{Ca}$) in PLB_{WT} -inhibited HEK cell homogenates. For an initial assessment of PLB_M physiological effectiveness, we used human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) from a healthy individual. In this system, we observed that adeno-associated virus 2 (rAAV2)-driven expression of PLB_M enhances the amplitude of SR Ca^{2+} release and the rate of SR Ca^{2+} re-uptake. To assess therapeutic potential, we used a hiPSC-CM model of dilated cardiomyopathy (DCM) containing PLB mutation R14del, where we observed that rAAV2-driven expression of PLB_M rescues

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Disclosure Statement
None

arrhythmic Ca^{2+} transients and alleviates decreased Ca^{2+} transport. Thus, we propose that PLB_M transgene expression is a promising gene therapy strategy that directly targets the underlying pathophysiology of abnormal Ca^{2+} transport and thus contractility in underlying systolic heart failure.

Keywords

calcium transport; cardiomyocyte; dilated cardiomyopathy; gene therapy; phospholamban; SERCA

1. Introduction

Heart failure (HF) is characterized by alterations in myocardial contractility resulting in impaired ventricular function, constituting a leading cause of morbidity and mortality worldwide.¹ Over the past decades, researchers have made significant progress in identifying intracellular and molecular mechanisms that are altered during HF progression. Defective sarcoplasmic reticulum (SR) Ca^{2+} transport has been identified as a potential determinant of decreased contractility in the failing hearts.^{2–4} Ca^{2+} transport from the cytosol to the SR lumen in human cardiomyocytes (CM) is largely performed (~70% of Ca^{2+} ions transported) via the SR Ca-ATPase (SERCA2a).⁵ Despite recent advances in device and pharmacological therapies, the incidence of heart failure continues to rise due to an aging and increasingly obese population, so new therapeutic strategies targeting the underlying physiology are urgently needed. Increasing knowledge of the molecular mechanisms fundamental to cardiac function and disease – including HF – has expanded the therapeutic potential to target key players involved in Ca^{2+} handling, including SERCA2a.

The activity of SERCA2a is regulated by phospholamban (PLB), a 52-residue single-pass transmembrane protein expressed in the SR of cardiac muscle. PLB binds to SERCA2a and reduces its apparent Ca^{2+} affinity, as measured from SERCA activity.⁶ PLB is in equilibrium between homopentamers and monomers, where the oligomeric state is proposed to function as a non-inhibitory reservoir.^{7–10} Previous studies have identified two functional regions on the transmembrane (TM) helix of PLB.^{9–11} Single-residue mutations on one side of the TM helix diminish inhibitory function without significantly affecting PLB oligomerization state(s), whereas mutations on the opposite face of the TM helix modulate PLB oligomerization and can lead to enhanced SERCA inhibition.^{10,12} The regulation of gene expression has been investigated in healthy individuals and HF patients, and decreased SERCA-to-PLB ratio is associated with deteriorated cardiac function, indicating that SERCA/PLB stoichiometry and SERCA2a activity are potential therapeutic targets.^{13–15} To this end, SERCA2a transgene expression via recombinant adeno-associated virus (rAAV) was achieved in HF animal models to increase the SERCA-to-PLB ratio in the heart. Exogenous SERCA2a expression significantly enhanced cardiac function in disease models by multiple metrics.^{16,17} Recently, gene therapy designed to increase SERCA2a expression in the human heart underwent clinical trials in patients diagnosed with end-stage HF.¹⁸ Despite positive preliminary results in Phase 1/2a,¹⁹ the trial did not meet its primary composite end point in phase 2b, and this has been ascribed to dosage constraints and the

heterogeneity of heart failure.²⁰ One likely factor is the large size of SERCA2a, which limits steady-state expression levels of the gene therapy construct. Other promising strategies to increase the SERCA-to-PLB ratio include expression of miRNAs that directly target PLB expression^{21–23} and oligonucleotide-based drugs,^{24,25} although these strategies have not yet produced an effective therapeutic agent.

In the present study, we have pursued an alternative approach to activate SERCA2a, by expressing a loss-of-inhibition (LOI) PLB mutant (PLB_M) that is engineered to compete with the inhibitory wild type PLB (PLB_{WT}) for SERCA2a binding (Fig.A) via viral delivery. In this approach, the ratio between endogenous SERCA and PLB is not targeted; instead, the exogenous PLB_M relieves SERCA2a inhibition and enhances Ca²⁺ transport activity. Thus, this strategy is designed to overcome limitations associated with SERCA2a overexpression-based gene therapy.

2. Materials and methods

2.1. Molecular biology

eGFP and tagRFP were fused to the N-terminus of human SERCA2a and human PLB, respectively. We have PLB demonstrated that attachment of the fluorescent proteins at these sites does not interfere with SERCA activity or inhibition.^{26,27} PLB cDNA mutations were introduced using the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA), and all expression plasmids were sequenced for confirmation.

2.2. Cell culture

Human embryonic kidney cells 293 (HEK293–6E, NRC, Canada) were cultured in FreeStyle F17 expression medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 2 mM L-glutamine (Invitrogen, Waltham, MA). Cell cultures were maintained in a circular shaker (125 rpm, 37°C, 5% CO₂ (Forma Series II Water Jacket CO₂ Incubator, Thermo Fisher Scientific, Waltham, MA). For displacement assays, HEK293–6E cells were transiently transfected using 293fectamine with GFP-SERCA2a, RFP-PLB_{WT}, and PLB_M or empty vector in a 1:7:7 molar ratio. Cells were then assayed 48 hours post-transfection.

The control hiPSC line (SKiPS-31.3) was cultured as previously described.²⁸ The R14del hiPSC line was derived from the SKiPS-31.3 line using homologous recombination via CRISPR/Cas9. Monolayer cardiac differentiation was performed as described,²⁸ yielding beating cardiomyocytes within 7–10 days.

2.3. Gene expression

RNA was extracted from uninfected and rAAV-infected hiPSC-CMs at day 37, and cDNA was synthesized as previously described.²⁸ Gene expression compared to the housekeeping gene β 2-microglobulin (B2M) was determined using qRT-PCR, as assessed by C_t analysis. See **Supplemental Table 1** for the list of primers used for qRT-PCR.

2.4. Immunoblot analysis

Samples were separated on a 4–20% polyacrylamide gradient gel (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) followed by overnight incubation at 4 °C with the primary target antibody: rabbit anti-GFP polyclonal antibody (pAb) (1:1000; ab290, Abcam, Cambridge, United Kingdom), mouse anti-SERCA2 monoclonal antibody (mAb) (1:1000; 2A7-A1, Abcam), rabbit anti-tagRFP pAb (1:1000; ab233, Evrogen), mouse anti-PLB mAb (1:1000, 2D12, Abcam), or rabbit anti- β -actin pAb (1:5000, ab8227, Abcam). Blots were incubated with anti-mouse or anti-rabbit secondary antibodies conjugated to IRDye 680RD or IRDye 800CW, respectively, for 1 h at 23 °C (1:20,000; LI-COR Biosciences). Blots were quantified on the Odyssey scanner (LI-COR Biosciences).

2.5. Fluorescence data acquisition and analysis

Fluorescence lifetime (FLT) measurements were conducted using a top-read FLT plate reader (384-well plates) designed and built by Fluorescence Innovations, Inc. (St. Paul, MN). GFP donor fluorescence was excited with a 473 nm microchip laser from Concepts Research Corporation (Belgium, WI), and emission was acquired with 490 nm long-pass and 520/17 nm band-pass filters (Semrock, Rochester, NY). We previously validated the performance of this FLT plate reader with known fluorescence standards, as well as with a FRET-based high-throughput screening strategy that targeted 2-color SERCA.^{29,30} FLT waveforms from donor- and donor/acceptor-labeled samples were analyzed as described in our previous publications and the supplemental methods.^{29,30}

2.6. Ca-ATPase activity assay

An enzyme-coupled, NADH-linked ATPase assay was used to measure Ca^{2+} -activated ATPase activity of SERCA in 96-well microplates. Each well contained HEK293–6E homogenates, 50 mM MOPS (pH 7.0), 100 mM KCl, 1 mM EGTA, 0.2 mM NADH, 1 mM phosphoenol pyruvate, 10 IU/mL of pyruvate kinase, 10 IU/mL of lactate dehydrogenase, 3.5 $\mu\text{g/mL}$ of the Ca^{2+} ionophore A23187, and CaCl_2 added to set $[\text{Ca}^{2+}]_i$ to the desired values.¹² The assay was started by addition of Mg-ATP at a final concentration of 5 mM, and well absorbances were read in a SpectraMax 384 Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The Ca-ATPase assays were conducted over a range of $[\text{Ca}^{2+}]_i$, and the ATPase activities were fitted using the Hill function

$$V = \frac{V_{\max}}{1 + 10^{-n(pK_{\text{Ca}} - p\text{Ca})}} \quad \text{Eq. (1)}$$

where V is the initial rate of Ca^{2+} -dependent ATPase activity at a specified Ca^{2+} concentration ($p\text{Ca}$), $V_{\max}$ is the rate of Ca-ATPase activity at saturating $[\text{Ca}^{2+}]_i$, n is the Hill coefficient, pK_{Ca} is the fitted Ca^{2+} dissociation constant, and $p\text{Ca}$ is the concentration of ionized Ca^{2+} per specific well and V .

2.7. Live-cell Ca^{2+} transient measurements

hiPSC-CMs (day 35 of differentiation) were enzymatically dissociated using the Detach 2 kit (Promocell, Heidelberg, Germany) and plated on Matrigel-coated German glass coverslips. After 2 days, the plated hiPSC-CMs were loaded with a Ca^{2+} -sensitive fluorescent dye (Fura-2 AM, cell permeant, ThermoFisher, Rockville, MD, USA), and the ratio of fluorescence intensities (excitation ratio of 340/380 nm) were recorded using the IonOptix system (Ionoptix, Milton, MA). The electrically-induced Ca^{2+} transients were triggered by pulses from a MyoPacer (IonOptix, Milton, MA) at 40 V and 0.5 Hz, with cells at 23 °C. Ca^{2+} traces were analyzed using IonWizard software (IonOptix) to calculate the release amplitude (peak height relative to baseline) and tau (time of Ca^{2+} removal). The number of irregular Ca^{2+} transients was quantified using IonOptix software.

The transduction efficiency and transduction protocol using rAAV2 vectors to transduce hiPSC-CMs was previously documented.³¹ Briefly, rAAV2.L31A, rAAV2.I40A, and rAAV2.L31A/I40A PLB viruses were used to transduce hiPSC-CMs at day 30 of differentiation (1e4 MOI), enzymatically dissociated on day 35, and plated on Matrigel-coated German glass coverslips, as described above. Fura-2 fluorescence measurements were recorded in AAV-infected hiPSC-CMs on day 37 and compared to non-infected control hiPSC-CMs. AAV viruses were purified according to the two-plasmid method with iodixanol gradient as described.³²

2.8. Statistical analyses

All statistics were performed using Prism (GraphPad, La Jolla, CA), and analysis was done by one-way ANOVA followed by the Bonferroni post hoc test; analysis of two group comparisons was done by Student's t-test (* $P < 0.05$ and 'ns' is not significant). Data are presented as mean $\pm$ standard error of the mean (SEM), and all statistical values were calculated from a minimum of three separate experiments.

3. Results

3.1. FRET assay demonstrates SERCA-binding competition between PLB_{WT} and loss-of-function PLB_{M}

Previously, two classes of point mutations within the PLB TM domain had been identified via alanine-scanning mutagenesis, with disparate outcomes relative to SERCA: loss-of-inhibitory function (LOI) and gain-of-binding function (GOB).^{10,33} We hypothesized that combining LOI and GOB mutations in a single PLB would result in a LOI-PLB that can effectively compete with the inhibitory PLB_{WT} . To test this, we used a SERCA2a-PLB biosensor system that consists of (a) co-expressed GFP-tagged SERCA2a and RFP-tagged PLB_{WT} in HEK293–6E cells and (b) a fluorescence lifetime (FLT) readout of FRET. FLT-FRET is used to resolve changes in SERCA2a-PLB complex structure and binding (Fig. 1A).²⁹ We varied the ratio between the donor molecule (GFP-SERCA2a) and acceptor molecule (RFP- PLB_{WT}), and found that the maximal energy transfer efficiency E (fractional decrease of the fluorescence lifetime) in this live-cell based system saturates at 0.10, as previously reported.²⁹ We transfected cells expressing GFP-SERCA2a and RFP- PLB_{WT} with either untagged PLB_{WT} or PLB_{M} containing TM mutations (L31A, I40A, or L31A/I40A).

Displacement of the RFP-PLB_{WT} from its interaction with GFP-SERCA2a was observed as a decrease in the FRET efficiency relative to that measured in control cells expressing only the GFP-SERCA/RFP-PLB_{WT} donor-acceptor pair (Fig.1B). FRET between GFP-SERCA2a and RFP-PLB_{WT} decreased significantly upon co-expression of unlabeled PLB_{WT}, indicating that the RFP-PLB_{WT} and unlabeled PLB_{WT} are in equilibrium for hetero-dimeric binding to GFP-SERCA2a. An alanine substitution at L31A (LOI mutation) did not significantly alter the FRET value relative to the wild-type control. In contrast, an alanine substitution at I40A (associated with increased SERCA inhibition via decreased Ca²⁺ sensitivity) further decreased FRET, indicating that I40A-PLB_M competes effectively with RFP-PLB_{WT} with a potency (affinity) comparable to or greater than that of unlabeled PLB_{WT}. The combination of L31A and I40A mutations resulted in a PLB_M with binding similar to that of I40A-PLB_M, consistent with our hypothesis that PLB_M with both mutation types can retain high affinity toward SERCA2a.

Expression of the constructs was confirmed by SDS-PAGE and immunoblot (Fig.1C/D). GFP-tagged SERCA2a was resolved from endogenous SERCA2b, as verified by binding of a GFP-specific antibody. For GFP-SERCA, there were no bands of lower mobility (apparent proteolysis) and no bands of higher mobility (apparent aggregation) in intact cells. Expression of RFP-PLB produced a single band recognized by RFP- and PLB-specific antibodies in samples heated to 95°C prior to electrophoresis. The I40A mutation disrupts pentamer formation, and we observed the disappearance of the band corresponding to PLB pentamer when the I40A mutation is present (Fig.1D; lanes 11 and 12). PLB pentamer was detected for PLB_{WT} and L31A-PLB (Fig.1D; lanes 13 and 14). As the expression level of GFP-SERCA2a, RFP-PLB_{WT}, and PLB_M is not significantly different between respective samples, we conclude that the observed changes in FRET are due to displacement of RFP-tagged PLB_{WT} via competition (to GFP-SERCA2a) with untagged PLB_M. The Ca-ATPase activity of GFP-SERCA is similar to that of untagged SERCA, so the GFP tag does not perturb endogenous function.²⁶

3.2. Effects of loss-of-function and gain-of-binding PLB mutants on SERCA2a regulation

The effects of PLB_M overexpression on the Ca²⁺-dependence of SERCA2a activity (measured by pK_{Ca}) were determined in HEK293–6E homogenate samples, enabling co-expression of PLB_{WT} and PLB_M in a cell-based system similar to the FRET assays. Ca-ATPase activity in HEK293–6E cells expressing SERCA2a alone (Fig.2A, black) increases with Ca²⁺ at physiological Ca²⁺ concentrations (*e.g.*, between pCa 7 and pCa 6) and saturates at higher Ca²⁺ concentrations (pCa 5), consistent with previous measurements.²⁹ Concomitant expression of PLB_{WT} (Fig.2A/B, blue) decreases Ca²⁺ affinity (increases pK_{Ca}) of SERCA2a, and the additional expression of PLB_{WT} (purple) or L31A-PLB_M (cyan dashes) does not significantly decrease the apparent Ca²⁺ affinity; indicating that SERCA2a is fully inhibited and that L31A-PLB_M is not sufficient to relieve PLB_{WT} inhibition under these experimental conditions. We observed a further decrease in the apparent Ca²⁺ affinity upon co-expression of I40A-PLB_M (red) with SERCA2a and PLB_{WT}, suggesting that I40A-PLB_M shows competitive binding to SERCA2a in the presence of PLB_{WT} (consistent with FRET measurements) and acts as a super-shifter/inhibitor.^{10,12,34} Co-expression with L31A/I40A-PLB_M (green) increased Ca²⁺ affinity to values similar to the SERCA-only sample,

indicating that the PLB inhibition was relieved. Taken together, the structural (FRET) and functional (Ca-ATPase) assay results demonstrate that L31A/I40A-PLB_M is non-inhibitory and binds to SERCA2a in HEK293–6E cells.

3.3. rAAV-driven expression of L31A/I40A-PLB_M enhances Ca²⁺ transients in hiPSC-CMs

We differentiated hiPSCs derived from a healthy individual (SKiPS-31.3 line) into cardiomyocytes using established protocols that yields a predominant ventricular-like population.²⁸ To assess physiological relevance in this native SERCA-PLB cardiac system, we tested the effects of rAAV-delivered PLB_M by infecting hiPSC-CMs with empty virus, rAAV2.L31A-, rAAV2.I40A-, or rAAV2.L31A/I40A-PLB_M at day 30 of differentiation and recording Ca²⁺ transients via fura-2AM upon electrical stimulation at day 37 (Fig.3A). Ca²⁺ transients were regular in appearance (Fig.3B). hiPSC-CMs expressing rAAV2.L31A-PLB_M or rAAV2.I40A-PLB_M did not show significant changes in peak amplitude or the rate of Ca²⁺ removal relative to control cells expressing empty vector (Fig.3C). However, there were striking improvements in Ca²⁺ transient parameters (increased peak amplitude, increased Ca²⁺ removal rate) in hiPSC-CMs infected with rAAV2.L31A/I40A-PLB_M (Fig.3B/C). Expression of the exogenous PLB_M was confirmed by qRT-PCR where virally-infected hiPSC-CMs had approximately two PLB_M per one PLB_{WT} (Fig.3D). We observed no differences in SERCA2a levels indicating that enhanced Ca²⁺ transport is due to altered PLB regulation and not a compensatory change in SERCA2a expression (Fig.3D). Cardiac troponin T expression levels were also unchanged, suggesting that PLB_M does not significantly alter hiPSC-CM differentiation.

3.4. Relief of SERCA2a inhibition by PLB_M rescues irregular Ca²⁺ transients in cardiomyopathic R14del-PLB hiPSC-CMs

Autosomal dominant mutations in PLB have been linked to DCM and these mutations include R9C³⁵, R14del^{36–38}, R25C³⁹, and L39stop⁴⁰. Recent work using hiPSC-CMs derived from patients heterozygous for the R14del-PLB mutation show mislocalization of R14del-PLB into aggregates leading to higher levels of autophagy and accompanying irregular Ca²⁺ transients.^{41,42} Mice heterozygous for the R14del-PLB mutation have severe defects in SERCA2a activity and Ca²⁺ transport. We sought to characterize the effects of viral expression of PLB_M in this dilated cardiomyopathic model to evaluate therapeutic potential. To accomplish this, we generated a R14del-PLB knock-in hiPSC line using homologous recombination via CRISPR/Cas9 to insert the R14del-PLB mutation into the control SKiPS-31.3 line (Fig.3), producing an isogenic control to test our therapies.²⁸ While the R14del-PLB hiPSC-CMs display no differences in Ca²⁺ transients parameters measured at day 21 (compared to wild type cells), we observed an arrhythmic (irregular) Ca²⁺ transient profile in R14del-PLB hiPSC-CMs after day 37 of differentiation. This irregularity occurred at a frequency of 58 ± 19% of Ca²⁺ transient recorded under paced conditions. (n = 85 cells) (Fig.4A/D). This phenotype switch was also observed in patient-derived R14del hiPSC-CMs.⁴¹ Upon infection with rAAV2.L31A/I40A-PLB, we observed an improvement in Ca²⁺ handling properties of the R14del-PLB hiPSC-CMs. Representative Ca²⁺ tracings show a significant reduction in irregular Ca²⁺ transients and improvements in Ca²⁺ amplitude and tau upon rAAV2.L31A/I40A-PLB infection (Fig.4B/C). Finally, no irregular Ca²⁺ transients (n = 20 cells) were observed after infection with rAAV2.L31A/I40A-PLB

(Fig.4D). Altogether, these data indicate an impairment in Ca^{2+} cycling in R14del-PLB hiPSC-CMs that is corrected by exogenous expression of the SERCA activating L31A/I40A-PLB_M.

4. Discussion

Hallmarks of heart failure include decreased contractile velocity, decreased relaxation rates, and pathological remodeling.¹ Although the critical events that lead to impaired cardiac performance are still being determined, it is clear that pathways controlling intracellular Ca^{2+} homeostasis significantly contribute to decreased cardiomyocyte and contractile function.²⁻⁴ SERCA2a and PLB are major determinants of SR Ca^{2+} transport in the heart, and alterations to their function has profound effects on intracellular Ca^{2+} cycling.⁵ We demonstrated previously that the interaction between the cardiac Ca^{2+} pump, SERCA2a, and its principal inhibitor, PLB, can be measured via FRET in HEK293 cells.²⁹ In the present study, we applied this FRET assay to measure the competition of non-inhibitory PLB_M and RFP-labeled PLB_{WT} for binding to GFP-SERCA2a. We used this assay to measure the relative affinity of non-inhibitory PLB_M and identified a double mutant (L31A/I40A) that binds with affinity greater than PLB_{WT}. Expression of L31A/I40A-PLB_M increased Ca-ATPase activity in PLB_{WT}-expressing HEK293 cells. Mechanistically, these results strongly suggest that L31A/I40A-PLB_M enhances SERCA activity by competitively displacing the inhibitory form of PLB. These results demonstrate that it is possible to separate the inhibitory potency of PLB from its binding affinity. Specifically, the properties of the PLB double mutant (L31A = loss-of-inhibition = LOI; I40A = gain-of-binding = GOB) show the dominant effect of the LOI mutation (without inhibition by the GOB mutation), while preserving binding affinity for SERCA2a. Ryanodine receptor (RyR), the SR Ca^{2+} -release channel, is hyperphosphorylated by PKA in chronic cardiac diseases, causing dissociation of FKBP12.6 from RyR2 and increased RyR2 open probability (SR Ca^{2+} leak), resulting in reduced SR Ca^{2+} content.⁴³ We observed that the basal (unstimulated) cytosolic Ca^{2+} concentration was similar in the control (empty vector) transduced hiPSC-CMs, as compared to PLB or PLB_M transduced cells, indicating that there is not a significant change in RyR function or RyR leak (Fig.3B; Fig.4A).

The rate and amplitude of Ca^{2+} transients in cardiac cells provides two key parameters that correlate with effects on the excitation-contraction coupling in the heart.⁴⁴ We found that rAAV2-driven expression of L31A/I40A-PLB_M significantly improved Ca^{2+} release amplitude and Ca^{2+} removal in both healthy and pathogenic hiPSC-CM lines. Using CRISPR/Cas9 we created a knock-in hiPSC cell line that is heterozygous for the DCM-causing mutation R14del-PLB. We differentiated this cell line into cardiomyocytes to study potential defects in Ca transport and homeostasis. Similar to hiPSC-CMs derived from R14del-PLB human patients,⁴¹ our cell line developed an arrhythmia-like Ca^{2+} transient phenotype by day 37 of differentiation. We demonstrated that expression of L31A/I40A-PLB_M reversed Ca transport dysfunction and irregular Ca^{2+} transients in R14del-PLB hiPSC-CMs.

In this study, we have combined FRET-based SERCA and PLB biosensors in HEK293 cells with the use of control and cardiomyopathic hiPSC-CMs to correlate effects on SERCA2a

structure-function. We acknowledge the limitations of the HEK293 and hiPSC cell models. HEK293 cells expressing human SERCA and PLB have been a standard in biophysical approaches.^{10,26,29,30,45,46} However, these cells do not express other components that compose the cardiomyocyte proteosome, including key Ca^{2+} handling components and contractile elements. hiPSC-CM is a renewable human cardiac cell line that retains the disease mutation and can be gene-edited, avoids invasive approaches to obtain human cardiac tissue, and can be utilized for drug discovery. However, hiPSC-CMs are immature compared to adult myocytes and are a heterogeneous population.⁴⁷ These results may justify future studies to test potential therapeutic effects *in vivo* and in human heart tissues. Altogether, this work paves the way for potential novel therapies for heart diseases associated with impaired Ca^{2+} transport and decreased contractility.

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Abbreviations:

Ca^{2+}	calcium
B2M	β 2-microglobulin
DCM	dilated cardiomyopathy
ER	endoplasmic reticulum
FLT	fluorescence lifetime
FRET	fluorescence resonance energy transfer
GOB	gain of binding function
GFP	green fluorescent protein
hiPSC-CM	human induced pluripotent stem cell-derived cardiomyocyte
HEK	human embryonic kidney
HF	heart failure
LOI	loss of inhibitory function
mAb	monoclonal antibody
miRNA	microRNA
pAb	polyclonal antibody
PLB	phospholamban

PLB_M	phospholamban mutant
PLB_{WT}	wild type phospholamban
rAAV	recombinant adeno-associated virus
RFP	red fluorescent protein
SERCA2a	sarcoendoplasmic reticulum calcium ATPase 2a
SR	sarcoplasmic reticulum

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Highlights

- SERCA activation improves contractility in cardiac diseases with impaired Ca^{2+} transport.
- Engineered PLB mutant (PLB_M) binds SERCA to relieve inhibition by PLB_{WT}.
- PLB_M increased the Ca^{2+} affinity and Ca^{2+} -activated ATPase activity of SERCA.
- Viral expression of PLB_M in hiPSC-CMs enhanced the Ca^{2+} uptake rate and release.
- PLB_M rescued irregular Ca^{2+} transients in dilated cardiomyopathic hiPSC-CMs.

Fig. 1. Co-expression of SERCA, PLB, and PLB_M.

(A) Schematic of gene therapy strategy: a tight-binding, non-inhibitory PLB mutant (PLB_M, dark green) displaces inhibitory RFP-PLB (PLB_{WT}, dark red), resulting in a FRET decrease. (B) FRET assay of PLB_M competition in HEK293-6E. GFP-SERCA-RFP-PLB_{WT} FRET (binding, white bar) is decreased (arrow) by PLB variants. LOI = Loss-of-inhibitory function mutant. GOB = Gain-of-inhibition mutant. (C/D) Immunoblots of HEK homogenate samples incubated at 95°C (disrupting PLB pentamer, lanes 1–7) or at 70°C (lanes 8–14) from untransfected HEK293-6E cells (lane 1&8), cells expressing GFP-SERCA2a (lanes 2&9), or cells expressing GFP-SERCA2a+RFP-PLB (lanes 3–7&11–14) +L31A/I40A-PLB (lanes 4&11), +I40A-PLB (lanes 5&12), +L31A-PLB (lanes 6&13), and +WT-PLB (lanes 7&14). Primary antibodies used (from top to bottom of 1D panels) are anti-GFP, anti-SERCA2, anti-RFP, anti-PLB, and anti-β-actin. **Expression levels were quantitated by densitometry in lanes 1–7.** Error bars indicate SEM (n=3). *P < 0.05.

Fig. 2. SERCA activation by PLB_M competition.

(A) Normalized Ca-ATPase activity of HEK293–6E cell homogenates was measured 48 hours after transfection using +empty vector (negative control, not shown), +WT-SERCA2a (black), +WT-SERCA2a/WT-PLB (blue), +WT-SERCA2a/WT-PLB +WT-PLB (purple), +WT-SERCA2a/WT-PLB +L31A-PLB (cyan, dashed line), WT-SERCA2a/WT-PLB +I40A-PLB (red), or WT-SERCA2a/WT-PLB +L31A/I40A-PLB (green). (B) Quantification of apparent Ca²⁺ affinity (1/pK_{Ca}) and PLB_M-induced change () from (A). Error bars indicate SEM (n = 3).

Fig. 3. In normal, healthy hiPSC-CMs, rAAV2-driven expression of L31A/I40A-PLB enhances Ca²⁺ release amplitude and Ca²⁺ removal rate.

(A) Schematic of experimental design for hiPSC differentiation, transduction, and cytosolic Ca²⁺ assay. (B) Representative Ca²⁺ transients in control hiPSC-CMs (*left*) compared to rAAV2-L31A/I40A PLB transduced hiPSC-CMs (*right*). (C) Quantification of Ca²⁺ release amplitude (peak height) normalized to uninfected control (CTRL) and (D) Ca²⁺ removal time constant (Tau) of hiPSC-CMs normalized to uninfected control (CTRL). Error bars indicate SEM (n = 3). *P < 0.05. (E) qRT-PCR analysis of gene expression of Ca²⁺ transport and contractility proteins: SERCA2a (*left*), cardiac troponin T (*center*), and PLB (*right*; black = endogenous PLB_{WT}, gray = exogenous PLB_M) of uninfected (CTRL) or infected (rAAV2-L31A/I40A PLB) hiPSC-CMs. Gene expression values are normalized to uninfected control.

Fig. 4. In cardiomyopathic R14del hiPSC-CMs, viral delivery of L31A/I40A-PLB rescues arrhythmogenic Ca^{2+} transients and enhances cellular Ca^{2+} cycling.

(A) Representative Ca^{2+} transients in R14del PLB hiPSC-CMs (left) and R14del hiPSC-CMs infected with rAAV2.L31A/I40A-PLB (right). Arrhythmogenic Ca^{2+} transients are identified by red arrow. (B) Ca^{2+} release peak amplitude (fluorescence intensity) normalized to uninfected R14del hiPSC-CMs. (C) Ca^{2+} re-uptake time constant (Tau) normalized to uninfected R14del hiPSC-CMs. (D) Irregular Ca^{2+} transients determined by quantifying the number of individual cells exhibiting irregular Ca^{2+} transients. Error bars indicate SEM (n = 3). *P < 0.05.