Phospholamban as a Crucial Determinant of the Inotropic Response of Human Pluripotent Stem Cell–Derived Ventricular Cardiomyocytes and Engineered 3-Dimensional Tissue Constructs

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Background—Human (h) embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) serve as a potential unlimited ex vivo source of cardiomyocytes (CMs). However, a well-accepted roadblock has been their immature phenotype. hESC/iPSC-derived ventricular (v) CMs and their engineered cardiac microtissues (hvCMTs) similarly displayed positive chronotropic but null inotropic responses to β-adrenergic stimulation. Given that phospholamban (PLB) is robustly present in adult but poorly expressed in hESC/iPSC-vCMs and its defined biological role in β-adrenergic signaling, we investigated the functional consequences of PLB expression in hESC/iPSC-vCMs and hvCMTs.

Methods and Results—First, we confirmed that PLB protein was differentially expressed in hESC (HES2, H9)- and iPSC-derived and adult vCMs. We then transduced hES2-vCMs with the recombinant adenoviruses (Ad) Ad-PLB or Ad-S16E-PLB to overexpress wild-type PLB or the pseudophosphorylated point–mutated variant, respectively. As anticipated from the inhibitory effect of unphosphorylated PLB on sarco/endoplasmic reticulum Ca2+-ATPase, Ad-PLB transduction significantly attenuated electrically evoked Ca2+ transient amplitude and prolonged the 50% decay time. Importantly, Ad-PLB–transduced hES2-vCMs uniquely responded to isoproterenol. Ad-S16E-PLB–transduced hES2-vCMs displayed an intermediate phenotype. The same trends were observed with H9- and iPSC-vCMs. Directionally, similar results were also seen with Ad-PLB–transduced and Ad-S16E–transduced hvCMTs. However, Ad-PLB altered neither the global transcriptome nor I\(_{\text{Ca,L}}\), implicating a PLB-specific effect.

Conclusions—Engineered upregulation of PLB expression in hESC/iPSC-vCMs restores a positive inotropic response to β-adrenergic stimulation. These results not only provide a better mechanistic understanding of the immaturity of hESC/iPSC-vCMs but will also lead to improved disease models and transplantable prototypes with adult-like physiological responses.

Key Words: adrenergic effects • phospholamban • pluripotent stem cells • tissues

Human (h) embryonic stem cells (ESCs) can self-renew while maintaining their pluripotency to differentiate into all cell types, including cardiomyocytes (CMs).\(^1\) Alternatively, direct reprogramming of adult somatic cells to become hES-like induced pluripotent stem cells (iPSCs) has been achieved.\(^2\) Furthermore, directed cardiac differentiation protocols\(^3\) are available for deriving hESC/iPSC-CMs in large quantities with yields orders of magnitude higher than the traditional method of embryoid body formation.\(^4\) We have recently reported a method for efficient ventricular specification of hESC/iPSC.\(^5,6\) Therefore, hESC/iPSC-CMs serve well as a potential unlimited ex vivo source of human CMs for disease modeling, drug discovery, cardiotoxicity screening, and future cell-based heart therapies.

Despite these promises of hESC/iPSC-CMs, a major roadblock has been their general lack of mature adult–like characteristics. During an action potential of native adult ventricular (v) muscle CMs, Ca\(^{2+}\) entry into the cytosol...
Ca2+ exchanger to return to the resting Ca2+ level. Such a rise in the sarcoplasmic reticulum Ca2+-ATPase (SERCA) and extruded by the Na+-K+ ATPase escalates the cytosolic Ca2+ to activate the ryanodine receptors. This process, termed as Ca2+-induced Ca2+ release,7 escalates the cytosolic Ca2+ to activate the contractile apparatus for contraction. For relaxation, elevated Ca2+ gets pumped back into the SR by the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) and extruded by the Na+-K+ ATPase. PLB, whose expression is robust in adult but absent in hESC-CMs,8–10 here, we investigated the functional consequences of expressing this differentially expressed gene product in single hESC/iPSC-vCMs, as well as their 3-dimensional (3D) multicellular engineered cardiac microtissues (hvCMTs).

WHAT IS KNOWN
- A key physiological feature of the heart is its ability to chronotropically and inotropically respond to β-adrenergic stimulation.
- However, immature human pluripotent stem cell–derived cardiomyocytes display positive chronotropic but null inotropic responses.
- Phospholamban plays a defined biological role in β-adrenergic signaling.
- Phospholamban is robustly present in adult but poorly expressed in human pluripotent stem cell–derived ventricular cardiomyocytes.

WHAT THE STUDY ADDS
- Upregulation of phospholamban expression in human embryonic stem cell-induced pluripotent stem cell–derived ventricular cardiomyocytes restores a positive inotropic response to β-adrenergic stimulation.
- Not only do these results provide a better mechanistic understanding of the immaturity of human embryonic stem cell-induced pluripotent stem cell–derived ventricular cardiomyocytes but will also lead to improved disease models and transplantable prototypes with adult-like physiological responses.

Materials and Methods

Isolation of Human Adult Left Ventricular CMs
Human adult left ventricular CMs were isolated at UC Davis according to the protocols approved by their International Union of Pure and Applied Chemistry and Institutional Review Board (protocol numbers 200614787-1 and 200614594-1). Hearts from healthy adults of 53 to 70 years of age were digested using the Langendorff system at 37°C as we previously reported.11

hESC/iPSC Culture and Ventricular Specification
Undifferentiated hES2 (Wicell, Madison, WI), H9, and iPSCs (re-programmed from CD34+ cells of the peripheral blood of a normal individual)6 were maintained on hESC-qualified Matrigel (BD Biosciences) in mTeSR1 medium (STEMCELL Technologies) as previously described elsewhere.12 For differentiation, we used a ventricular specification protocol that generates >90% hESC/iPSC-vCMs,6,14 as gauged by the positive expression of cTnT and MLC2v, as well as their ventricular-like action potential profile. In brief, hESC/iPSC colonies were dissociated by dispase into 50 to 100 cell clusters followed by culturing in differentiation media (StemPro34, 50 μg/mL ascorbic acid and 2 mmol/L GlutaMAX-I; Invitrogen, Carlsbad, CA) supplemented with cytokines and Wnt inhibitor as follows: day 1, BMP4 (1 ng/mL) and blebbistatin (5 μmol/L); days 2 to 4.5, BMP4 (10 ng/mL) and Activin-A (5 ng/mL); days 4.5 to 7, IWR-1 (1 μmol/L) in ultralow-attachment cell culture dishes (Corning, Lowell, MA). All recombinant proteins were purchased from R&D Systems (Minneapolis, MN).

Adenoviral Gene Transfer
The cDNAs of green fluorescence protein (GFP), wild-type, and S16E–PLB15 were subcloned into the shuttle vector pAd-RSV4. Replication deficient adenoviral (Ad) particles were packaged and amplified in HEK 293 cells by transfection with the corresponding vectors, followed by purification by the Vivapure Adenopack Kit (Vivascience) to yield titers on the order of 10^9 plaque forming units per milliliter as previously described.16 For transducing hESC/iPSC-vCMs, cardiospheres were digested with 0.05% trypsin at 37°C for 5 minutes. Cells were seeded on coverslips and cultured in a medium containing 90% DMEM, 10% defined FBS (HyClone), 1 mmol/L of L-glutamine, and 1% of NEAA. After 48 hours, plated hESC/iPSC-vCMs were transduced by adenoviruses with a multiplicity of infection of 10 (10 plaque forming units per cell) in DMEM with 2% FBS. Our previous work showed that a single transduction of Ad-GFP on hESC/iPSC-vCMs with 1 and 5 plaque forming units per cell yields >95% and almost 100% of positively transduced cells, respectively.6 No noticeable morphological changes were observed after transduction. Ca2+ measurements and other experiments were performed 24 to 48 hours post transduction.

Ica2,1 Measurements
Ica2,1 was recorded from single hES2-vCMs using the whole-cell patch-clamp technique with an EPC-10 amplifier and Pulse software
(Heka Elektronik) in a bath solution containing 160 mmol/L TEA-Cl, 5 mmol/L CaCl2, 1 mmol/L MgCl2, 10 mmol/L HEPES, 0.01 mmol/L TTX, 2 mmol/L 4-AP, and 10 mmol/L glucose (pH 7.4) at 37°C. Patch pipette solution contained 145 mmol/L CsCl2, 5 mmol/L NaCl, 2 mmol/L CaCl2, 5 mmol/L EGTA, 10 mmol/L HEPES, 5 mmol/L MgATP, and pH adjusted to 7.3 with KOH.

Measurements of Cytosolic Ca2+ Transients
Intracellular Ca2+ transients were analyzed by loading hESC/iPSC-vCMs with 2.5 μmol/L X-Rhod-1 AM (Invitrogen) for 45 minutes at 37°C in DMEM/F12, followed by imaging with a complementary metal-oxide semiconductor–based camera (MiCAM ULTIMA, SciMedia USA Ltd, CA) in Tyrode’s solution containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 10 mmol/L glucose, 10 mmol/L HEPES, and pH adjusted to 7.4 with NaOH. Electric stimulation or 50 mmol/L caffeine was applied to evoke Ca2+ transients. Isoproterenol (1 μmol/L) was incubated with hESC/iPSC-vCMs for 3 to 5 minutes at 37°C before measurement; 10 μmol/L ryanodine or 1 μmol/L thapsigargin was incubated with hES2-vCMs, as indicated, for 30 minutes at 37°C before measurements.

Microtissue Fabrication and Force Quantification
As for engineering hvCMTs,15 polydimethylsiloxane (Sylgard 184, Dow-Corning)-microfabricated tissue gauge substrates were molded from the SU-8 masters, with embedded fluorescent microbeads (Fluoresbrite 17147; Polysciences, Inc.) on the cantilever ends. A cooled suspension of 1010 trypsin-digested hESC-vCMs within a re-constitution mixture, consisting of 1.5 mg/mL liquid neutralized collagen I (BD Biosciences) and 0.5 mg/mL fibrinogen (Sigma-Aldrich), was added to the substrate, and the entire assembly was centrifuged to drive the cells into the micropatterned wells. For quantifying microtissue forces, brightfield and fluorescence images were taken at 30 Hz each within template by Photometrics Evolve EMCCD camera (Photometrics). Only tissues that were uniformly anchored to the tips of the cantilevers were included in the analysis. The displacement of fluorescent microbeads at the top of the cantilevers was then tracked using the SpotTracker plug-in in ImageJ (National Institutes of Health). Four days after manufacture and tissue compaction, the microtissues were transduced by Ad-GFP, Ad-PLB, or Ad-S16E-PLB, respectively. Measurements were performed 2 days post transduction.

Western Blot
Proteins (20 μg) were electrophoresed in SDS–polyacrylamide (12%) gels. After transfer to nitrocellulose membranes, they were probed with antibodies against SERCA2a (ab2861, Abcam), PLB (ab2865, Abcam), or β-actin (ab8226, Abcam). Detection was performed with an ECL Plus Western blotting detection system.

Real-Time Polymerase Chain Reaction
Total RNA was extracted with the RNeasy Mini kit (Qiagen). cDNAs were prepared using the SuperScript® CellsDirect cDNA Synthesis Kit (Life Technologies) following the manufacturer’s protocol. Gene expressions were quantified using the StepOnePlus® Real-Time Polymerase Chain Reaction system (Applied Biosystems). Polymerase chain reaction amplifications were carried out in 96-well optical plates with SYBR® Green Polymerase Chain Reaction Master Mix (Applied Biosystems). Transcriptomic Analysis
Total RNAs of nontransduced, Ad-GFP–transduced, or Ad-PLB–transduced hES2-vCMs were harvested by the RNeasy Mini kit (Qiagen). The quality was confirmed by using the Affymetrix GeneChip Human Exon 1.0 ST Array. Data analysis was based on cell intensity files generated from the arrays using Affymetrix’s GeneSpring GX software.

Immunostaining
Ad-PLB–transduced hES2-CMs were immunostained with an anti-PLB antibody (ab2865, Abcam). Primary antibodies were diluted in PBS with 1% BSA and incubated at room temperature for 1 hour. Alexa Fluor 488–conjugated goat antimouse IgGs (Invitrogen) were used as secondary antibodies and stained for 1 hour at room temperature. The cells were mounted in Prolong Gold mounting medium with DAPI (Invitrogen), and samples were imaged on LSM Carl Zeiss 510 Meta (Carl Zeiss).

Statistical Analysis
Unless stated otherwise, all data were expressed as means±SD. Unpaired t test was performed to evaluate for differences between
the mean values within the same study. A difference of $P<0.05$ was considered significant. For transcriptomic analysis, data were filtered with a 20% cutoff of flag value or signal intensity. Significance analysis was carried out by 1-way ANOVA with $P<0.05$ cutoff.

**Results**

**Positive Chronotropic but Null Inotropic Responses of hvCMTs and Single hESC/iPSC-vCMs to β-Adrenergic Stimulation**

Shortening of single hESC-CMs or their clusters has been measured as an index for contractile forces.\(^\text{10,18}\) A multicellular cardiac microtissue system has been developed, where tension generated in real time can be measured.\(^\text{17}\) Figure 1 shows that hCMTs engineered from $\approx 1000$ hES2-vCMs each of $\approx 0.5$ mm in length allowed continuous measurements of their spontaneous beating rate, as well as developed twitch tension under baseline control conditions. On addition of isoproterenol (10 $\mu$mol/L), a β-adrenergic agonist, the spontaneous twitch frequency increased significantly (75.6±44.6%; $P<0.01$; $n=5$). However, the developed twitch tension was not altered by isoproterenol ($P=0.729$). These results were in complete accordance with our recent report that human ventricular cardiac tissue strips that are $\approx 10$ mm consisting of $\approx 10^6$ cells per tissue elicit a positive chronotropic but negligible inotropic response as gauged by their twitch forces on β-adrenergic stimulation by isoproterenol.\(^\text{19}\) Consistent with these measurements at the tissue level, isoproterenol increased the spontaneous action potential ($P=0.008$; $n=4$) and Ca\(^{2+}\) transient firings ($P=0.006$; $n=6$) of single hES2-vCMs but not the transient amplitude ($P=0.182$; $n=6$; Figure 2). Considering the effects of frequency on the force and Ca\(^{2+}\) transients, where appropriate, the experiments that followed were electrically paced as indicated for comparison.

**Effects of Ad-GFP, Ad-PLB, and Ad-S16E-PLB Transduction on the Expression Levels of PLB and SERCA of hES2-vCMs**

Figure 3A shows that PLB protein was poorly expressed in hES2-vCMs compared with human adult vCMs. Adenoviral transduction of hES2-vCMs with Ad-PLB upregulated the PLB transcript and protein to levels not statistically different from those of the adult counterparts; similarly, Ad-S16E-PLB for overexpressing the pseudophosphorylated point–mutated S16E variant\(^\text{15}\) also upregulated the PLB mRNA (Figure 3C) and protein (Figure 3B) levels in hES2-vCMs compared with those of untransduced and Ad-GFP–transduced controls. The same PLB

![Figure 3](http://circep.ahajournals.org/)

**Figure 3.** Effects of adenovirus (Ad)-green fluorescence protein (GFP), Ad-phospholamban (PLB), and Ad-S16E-PLB transduction on the expression levels of PLB, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). **A**, Western blot showing Ad-PLB transduction increased PLB protein expression in hES2-ventricular cardiomyocytes (vCMs; left), H9-vCMs (middle), and induced pluripotent stem cell (iPSC)-vCMs (right) but did not reach the adult level ($n=2$; $P=0.226, 0.644$, and 0.001 for hES2-vCMs, H9-vCMs, and iPSC-vCMs). **B**, Western blot analysis shows that the PLB protein levels of the Ad-PLB and Ad-S16E-PLB groups were significantly increased, but those for SERCA were not different from the untransduced and Ad-GFP–transduced groups ($n=3$). **C**, PLB transcript levels as gauged by quantitative polymerase chain reaction ($n=3$). **D**, Immunostaining images show that Ad-PLB–transduced hES2-vCMs were 100% positive: cell nucleus indicated by DAPI (left), PLB staining (middle), and merged image (right).
expression trends were observed with H9-vCMs and iPSC-vCMs before and after transduction. Immunostaining of hES2-vCMs showed results consistent with this pattern (Figure 3D). By contrast, SERCA levels in all groups examined were not different (Figure 3B), suggesting a PLB-specific effect.

Effects of PLB Overexpression on the SR Load and Ca²⁺ Transient of hES2-vCMs

We next functionally characterized un transduced, Ad-GFP–transduced, Ad-PLB–transduced, and Ad-S16E-PLB–transduced hES2-vCMs. Ad-PLB–transduced hES2-vCMs displayed a significantly reduced SR Ca²⁺ load as assessed by the addition of caffeine (50 mmol/L) compared with untransduced and Ad-GFP–transduced controls (Figure 4A and 4B). As anticipated from the inhibitory effect of unphosphorylated PLB on SERCA, Ad-PLB transduction significantly attenuated the electrically evoked Ca²⁺ transient amplitude compared with untransduced and Ad-GFP–transduced controls (Figure 4C and 4D). As for the transient kinetics, both the upstroke and decay of Ad-PLB–transduced cells became significantly slow compared with controls (Figure 4E and 4F). Because Ca²⁺ influx via L-type Ca²⁺ channels modulates Ca²⁺ transients, we also examined I_{Ca,L} of Ad-PLB–transduced hES2-vCMs. As shown in Figure 4G and 4H, Ad-PLB transduction did not alter I_{Ca,L}. Ad-S16E-PLB transduction similarly reduced the SR load and decreased the transient amplitude but to a significantly lesser extent than those of wild-type PLB (Figure 4).

Ad-PLB Transduction Restored the Positive Inotropic Response of hES2/iPSC-vCMs to β-Adrenergic Stimulation

We next investigated the effect of isoproterenol on Ca²⁺ transient in hESC/iPSC-vCMs. Isoproterenol application did
not affect the Ca\textsuperscript{2+} transient amplitude of untransduced and Ad-GFP–transduced controls but uniquely augmented that of Ad-PLB–transduced hES2-vCMs by 69.6±47.4% (n=15; P=0.0001; Figure 5). Isoproterenol did not increase the transient amplitude of Ad-PLB-S16E–transduced cells (P=0.245).

As for the 50% decay time, the Ad-PLB group was significantly reduced by isoproterenol (P=0.0001), whereas those of untransduced, Ad-GFP–transduced, and Ad-PLB-S16E–transduced cells were not changed (n=15, 15, and 25; P=0.621, 0.083, and 0.122).

Ad-PLB transduction attenuated the amplitude of Ca\textsuperscript{2+} transients in H9-vCMs (F/F\textsubscript{0}=2.1±0.6, n=7 versus F/F\textsubscript{0}=10.5±7.9, n=8 of untransduced; P=0.015) and hiPSC-vCMs (F/F\textsubscript{0}=2.4±1.0, n=6 versus F/F\textsubscript{0}=8.4±5.6, n=7 of untransduced; P=0.042) and prolonged their 50% decay time (H9-vCMs: 281.1±101.5 ms, n=7 versus 168.2±63.9 ms, n=8 of untransduced; P=0.0214 and iPSC-CM: 272.3±62.6 ms, n=6 versus 190.0±65.6 ms, n=7 of untransduced; P=0.042; Figure 6).

Interestingly, isoproterenol increased the amplitude (H9-vCMs: F/F\textsubscript{0}=3.3±0.8 versus F/F\textsubscript{0}=2.4±1.0, n=7
it remained unclear whether this gain-of-function would likewise be observed at the level of force generation by multicellular 3D tissues. We, therefore, tested whether the twitch tension of hvCMTs (made out of hES2-vCMs) also responded to β-adrenergic stimulation (Figure 8A). Human vCMTs could be thoroughly transduced by Ad-GFP as demonstrated by the pattern of epifluorescence (Figure II in the Data Supplement), consistent with what we recently reported for larger engineered human cardiac tissue strips (10⁶ versus 500–1000 cells). Similar to the Ca²⁺ transient amplitude of single hESC/iPSC-vCMs, the twitch tension of hvCMTs was decreased after Ad-PLB transduction (2.2±1.0 μN, n=9 versus 5.8±1.2 μN, n=4 for control; P=0.004). In contrast, these were not changed by either Ad-GFP or Ad-S16E-PLB (6.4±2.7 μN and 8.4±2.5 μN; n=8 and 5; P=0.634 and 0.186; Figure 8B). Isoproterenol application uniquely increased the twitch tension of Ad-PLB–transduced hvCMTs by 119.0±86.6% (4.5±1.9 μN; n=9; P=0.004) but not those of untransduced, Ad-GFP–transduced, and Ad-S16E-PLB–transduced hvCMTs (P=0.521; 0.164 and 0.906). However, the 50% diastolic time was unaffected in all our recordings (Figure 8C).

Discussion

Using a combination of 2D and 3D models, we reported that hESC/iPSC-vCMs and their engineered hvCMTs and cardiac tissue strips similarly displayed positive chronotropic but
null inotropic responses to β-adrenergic stimulation. Forced expression of PLB, which was otherwise lowly expressed in hESC/iPSC-vCMs, sufficed to restore a positive inotropic response to isoproterenol. Despite this gain-of-function, the amplitude and kinetics of immature Ca2+ transients, as well as the SR load of hESC/iPSC-vCMs, were further reduced by PLB overexpression. Consistently, these cellular changes were reflected at the level of multicellular tissue constructs in terms of the twitch tension of hvCMTs.

Mechanistically, Ca2+-induced Ca2+ release ceases to operate when the SR Ca2+ load gets sufficiently low.1 Despite the significant reduction of the SR content by Ad-PLB transduction, such was not observed. This reduction in the SR load likely underlies that reduced transient amplitude observed and could be attributed to the inhibitory effect of SERCA reuptake by PLB as demonstrated by the slowed decay. A decreased SR Ca2+ concentration in turn would negatively affect ryanodine receptor opening and therefore slow the upstroke.20

Adult CMs can undergo compensatory changes as a result of alterations in the environment, altering the expression or activity of certain specific gene products. Indeed, SERCA2 gene silencing has been shown to lead to remodeling of the Ca2+ signaling mechanisms in CMs.21 These responses of adult CMs are generally considered adaptations for maintenance or homeostasis because, for instance, protein synthesis is largely determined by protein turnover rather than active synthesis.21–23 By contrast, hESC/iPSC-vCMs are immature cells that robustly undergo developmental changes with active protein synthesis and regulation. Our microarray profiling showed that the global transcriptome was not altered after PLB overexpression. Therefore, the functional consequences observed after Ad-PLB transduction could be attributed to the change in PLB protein expression in hPSC-vCM per se. This notion is consistent with the known biological role in β-adrenergic signaling and Ca2+ handling, as supported by the intermediate effect observed with the pseudophosphorylated S16E-PLB, and further implicates that PLB plays a compartmentalized role in specific functionality, namely inotropic responsiveness to β-adrenergic stimulation and SERCA modulation in hESC-vCMs. As such, other gene products are likely responsible for global Ca2+-handling maturation.

Pharmacologically (Figure I in the Data Supplement), thapsigargin, a specific SERCA inhibitor, significantly decreased the transient amplitude and slowed the decay of control and Ad-GFP–transduced hES2-vCMs as we previously reported8,9; likewise, ryanodine significantly reduced the electrically evoked Ca2+ transient amplitude and slowed the upstroke. Interestingly, although similar inhibitory effects on the peak amplitude, decay, and upstroke by thapsigargin or ryanodine were seen with Ad-PLB–transduced hES2-vCMs, the changes were significantly less compared with the controls, indicating that Ad-PLB transduction rendered cells are less sensitive to these pharmacological agents. As anticipated from pseudophosphorylation, the effects of Ad-PLB-S16E on the responses to thapsigargin and ryanodine resembled those of controls, consistent with the notion that PLB modulates the pharmacological profile of hESC/iPSC-vCMs.

Of note, the human PLB has an amino acid difference from the rabbit isoform that was used in this study at residue K27N.24 Experimentally, this difference has been shown to confer on human PLB a more potent ability to inhibit SERCA activity in a mouse model.25 However, extensive studies have reported that this species-specific difference at position 27 does not affect the basal inhibitory effect on SERCA, which itself is modulated and can be unleashed by adrenergic stimulation.25–27 Indeed, cardiac pumps and ion channels of different species are commonly studied in CMs of different species for functional and mechanistic insights.28–30 Considering that rabbit PLB is functional, the low endogeneous expression level in hESC-CMs and the extent of restoration of chronotropic effect, it is, therefore, felt that our observations and interpretation would qualitatively remain the same.

To date, we have reported that calsequestrin 2, PLB, SERCA, and Na+-Ca2+ exchanger are differentially expressed in hESC-CMs, fetal and adult human CMs, with defined specific roles in Ca2+ handling.8,9,31,32 Partial maturation of Ca2+ transient properties (with an augmented amplitude, accelerated kinetics, and increased SR load) in hESC-CMs can be accomplished by overexpressing calsequestrin.9 As demonstrated in this study, forced expression of PLB restores inotropic responsiveness, albeit other transient properties remain immature. Such incomplete maturation by engineering a single Ca2+-handling gene product is perhaps not surprising and could result from the insufficient expression of specific regulatory or accessory proteins. For
instance, junctin and triadin that interact with ryanodine receptor remain absent after calsequestrin transduction.9 Similarly, Ca2+ handling and key ion channel transcripts are essentially unchanged after PLB overexpression. Our collective observations raise the intriguing possibility that a combinatorial approach can lead to a more mature Ca2+-handling phenotype. Positive inotropic responses to adrenergic stimulation are important for adaptation33,34; a lack of such responses in hPSC-derived grafts could present a major obstacle to accurately model the native heart and to functional integration after transplantation. Taken together, our results not only provide a better mechanistic understanding of the immaturity of hPSC-vCMs but will also lead to better disease models and transplantable prototypes with adult-like physiological responses.

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Disclosures
None.

References


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SUPPLEMENTAL MATERIAL

Supplemental Results

We investigated the pharmacological responses of the various groups of hES2-βCMs to thapsigargin and ryanodine, specific SERCA and RyR inhibitors, respectively. Application of 1µmol/L thapsigargin to control hES2-βCMs (un- and Ad-GFP-transduced) significantly decreased the transient amplitude and slowed the decay (P<0.05; Supplemental Figure 1). Likewise, ryanodine application (10µmol/L) significantly reduced the electrically evoked Ca\(^{2+}\) transient amplitude and slowed the upstroke (P<0.01; Supplemental Figure 1). Interestingly, while similar inhibitory effects on the peak amplitude, decay and upstroke by thapsigargin or ryanodine were seen with Ad-PLB-transduced hES2-βCMs, the changes were significantly less compared to the controls, indicating that Ad-PLB transduction rendered cells less sensitive to these pharmacological agents (Supplemental Figure 1). As anticipated from pseudo-phosphorylation, the effects of Ad-PLB-S16E on the responses to thapsigargin and ryanodine resembled those of controls (Supplemental Figure 1).

Supplemental Figure and Legends
Supplemental Figure 1

Effects of thapsigargin or ryanodine on electrically-evoked Ca\textsuperscript{2+} transient of un-, Ad-GFP-, Ad-PLB-, Ad-S16E-PLB-transduced hES2-\textit{v}CMs.

A) Representative raw tracings of electrically-induced Ca\textsuperscript{2+} transients of un-, Ad-GFP-, Ad-PLB-, Ad-S16E-PLB-transduced hES2-\textit{v}CMs recorded in the absence
and presence of 1µM thapsigargin as indicated. Peaks recorded under control drug-free condition of all groups have been normalized for comparison. B) Bar graphs summarizing the transient parameters of the same groups from A. At 1µmol/L, thapsigargin significantly decreased the amplitude of control, Ad-GFP and Ad-S16E-PLB- but not Ad-PLB-transduced hES2-vCMs. 50% decay time was affected in all groups except Ad-PLB-transduced hES2-vCMs. Upstroke time was not affected in any of the groups. (N=13, 16, 10 and 12 for control, Ad-GFP, Ad-PLB and Ad-S16E-PLB respectively.) C) Representative raw tracings of electrically-induced Ca2+ transients of un-, Ad-GFP-, Ad-PLB-, Ad-S16E-PLB-transduced hES2-vCMs recorded in the absence and presence of 10µmol/L ryanodine as indicated. Peaks recorded under drug-free condition of all groups have been normalized. D) Bar graphs summarizing the transient parameters of the same groups from A. At 10µmol/L, ryanodine significantly decreased the amplitude of control, Ad-GFP and Ad-S16E-PLB- but not Ad-PLB-transduced hES2-vCMs. 50% decay time was unaffected in all groups. Upstroke time was affected in control, Ad-GFP and Ad-S16E-PLB- but not Ad-PLB-transduced hES2-vCMs (N=11, 11, 10 and 11 for control, Ad-GFP, Ad-PLB and Ad-S16E-PLB respectively). *P<0.05, **P<0.01, compared to parameters under drug-free condition.
Supplemental Figure 2

Bright and fluorescence images of hvCMT after Ad-GFP transduction demonstrated that the hvCMT could be fully transduced by Ad.
Supplemental Figure 3

*Role of PLB in Ca^{2+} handling of hPSC-CM.*

In adult CM, stimulation of β-adrenergic receptors leads to phosphorylation of PLB by PKA, which is a fundamental mechanism of the positive inotropic effect of β-adrenergic stimulation. However, PLB is missing in hPSC-CM. Overexpression of PLB in hPSC-CMs restored the positive inotropic response of hPSC-CM.