State of the Art

The Critical Role of Phospholamban in Cardiac Function

WEN ZHAO, QIUJING SONG, EVANGELIA G. KRANIAS

Department of Pharmacology and Cell Biophysics. University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

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Phospholamban (PLN) plays an important role in cardiac calcium cycling and contractility and is a crucial determinant of β-adrenergic stimulation in the heart. Generation of mouse models with genetically engineered alterations of PLN expression levels or PLN mutation allowed a thorough understanding of the physiological role of PLN *in vivo*. These studies also provided us insight for potential therapeutic approaches in human heart disease. However, cardiac calcium cycling and excitation-contraction coupling differ significantly between human and mouse and further studies are required to elucidate the physiological and pathological role of PLN in humans.

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Address:
Evangelia G. Kranias

Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, Ohio 45267-0575, USA
e-mail:
Litsa.Kranias@uc.edu

Cardiac contraction is induced by a small amount of calcium entering into myocytes through the L-type calcium channel, which then triggers a large calcium release from the sarcoplasmic reticulum (SR) through the ryanodine receptor (calcium-induced calcium release). The calcium is sequestered back into the SR by the SR Ca^{2+}-ATPase (SERCA2), and muscle relaxation occurs. Phospholamban (PLN), a low molecular weight phosphoprotein, is colocalized with SERCA2 in the SR, and reversibly regulates SERCA2 affinity for calcium (Figure 1).[1]

The cloning of PLN revealed that it is a 52-amino acid protein of 6.1kDa, which forms a homopentamer, accounting for the original observation that phosphorylated PLN has an apparent mass of ~25-28kDa.[2] Amino-acid sequence analysis suggested that PLN is organized in three domains. Cytosolic domain Ia (amino acids 1-20) contains Ser^{16}, the protein kinase A phosphorylation site, and Thr^{17}, the Ca^{2+}-calmodulin kinase phosphorylation site. Cytosolic domain Ib (amino acids 21-30) is rich in amidated amino acids, and domain II (amino acids 31-52) traverses the membrane.[3]

PLN is mainly expressed in cardiac SR, but low levels can also be detected in slow-twitch skeletal muscle,[4] smooth muscle,[5] and vascular endothelium.[6] *In vitro* studies, using purified cardiac SR membranes, have shown that PLN can be phosphorylated on Ser^{10} by protein kinase C (PKC), Ser^{16} by cAMP-dependent protein kinase (PKA), and Thr^{17} by Ca^{2+}-calmodulin-dependent protein kinase (CaMKII).[3,7,8] However, *in vivo* studies indicated that only Ser^{16} and Thr^{17} are phosphorylated in cardiac myocytes or perfused hearts,[9,10] while phosphorylation of PLN on Ser^{10} by PKC has not been detected in the intact heart. Dephosphorylated PLN binds to SERCA2a at resting Ca^{2+} concentrations and inhibits Ca^{2+} pump activity; phosphorylation of PLN relieves Ca^{2+}-pump inhibition by altering the PLN-SERCA interaction, and enhances relaxation rates (lusitropic effects) and contractility (inotropic effects).[11]

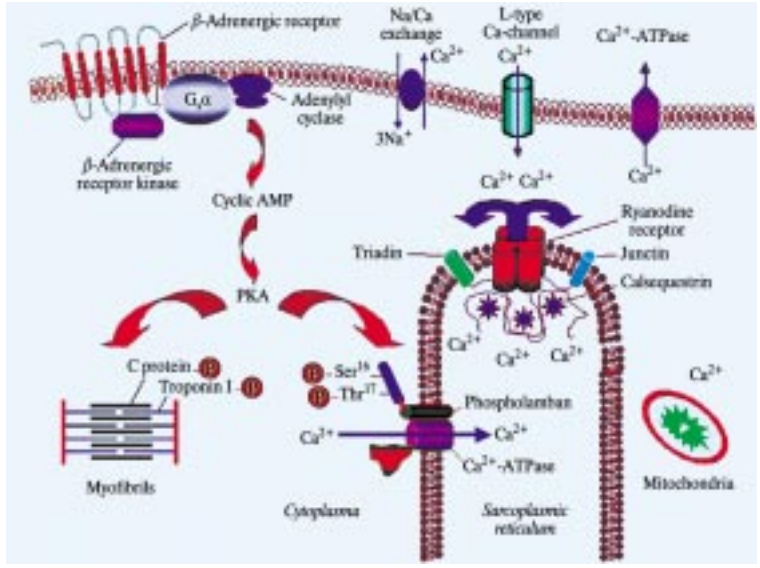

Figure 1. Cardiomyocyte signaling pathways and excitation-contraction coupling.

In this article, we discuss physiological studies of genetically engineered PLN, which revealed phospholamban's remarkable role in the regulation of the kinetics of cardiac contractility.

Role of phospholamban in basal cardiac function

PLN ablation

Much of our recent insight into the role of PLN in the regulation of cardiac function has been established through targeting the PLN gene in embryonic stem cells and generating heterozygous and homozygous PLN-deficient mice, in which PLN protein levels were reduced by 60% and 100%, respectively.[12,13] The decreases in PLN levels were associated with a linear increase in the affinity of SERCA2a for calcium,[13] and with a linear increase in contractile parameters of isolated cardiomyocytes, perfused hearts and intact mice.[13-16] The highly enhanced basal contractile parameters in PLN deficient hearts could be stimulated only minimally by β-agonists.[12,17] The attenuated responses were not associated with alterations in the β-agonist signal transduction pathway, such as β-adrenergic receptor density, adenylyl cyclase activity, cAMP levels or the phosphorylation levels of troponin I, C-protein or the 15-kDa sarcolemmal protein.[17] Furthermore, the hyperdynamic cardiac function in PLN deficient mice was not associated with any alterations in the levels of SERCA2a, calsequestrin, Na^+/Ca^{2+} exchanger, myosin, actin, troponin I or troponin T.[18] However, the protein levels of the ryanodine receptor were decreased by 25%, as a compensatory mechanism for the increased SR Ca^{2+}-load by the highly stimulated SERCA2a activity.[15,19] Another compensatory mechanism was the accelerated inactivation kinetics of the L-type Ca^{2+} channel, as a response to the increased SR Ca^{2+}-load.[20] In cardiac energenics, the levels of ATP, creatine kinase activity and the creatine kinase reaction velocity were not altered. However, ADP and AMP levels, as well as the active fraction of mitochondrial pyruvate dehydrogenase were increased.[18] These metabolic adaptations resulted in a new energetic steady state able to meet the increased ATP demands in the hyperdynamic PLN-null hearts. The hyperdynamic cardiac function of the PLN-null hearts persisted throughout the aging process, without any alterations in heart-to-body mass ratio, cardiac cell length, or sarcomere length.[21] PLN ablation did not compromise the animals' life span,[21] exercise performance,[22] or ability to compensate for sustained aortic stenosis.[23] These studies in PLN-targeted animals indicate that PLN is a crucial regulator of basal cardiac Ca^{2+} cycling and contractile parameters, and that PLN is an important determinant of β-agonist stimulatory response *in vivo*.

PLN overexpression

In contrast to PLN deficient mice, overexpression of PLN to twofold and fourfold higher levels in the heart, compared with their wild-type littermates, showed decreased fractional shortening and rates of contraction and relaxation in isolated, unloaded cardiomyocytes, which were reflected in a decreased amplitude of the Ca^{2+} signal and a prolonged time

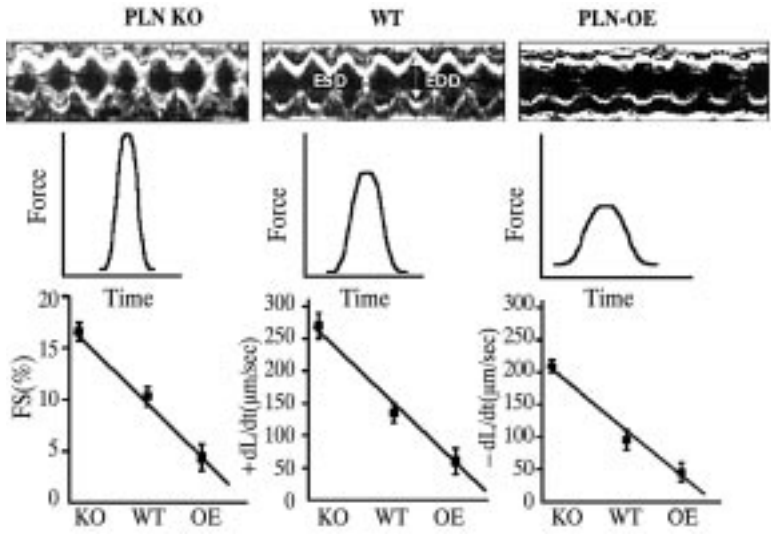

Figure 2. Effect of PLN levels on cardiac contractility in whole heart and in isolated cardiomyocytes.
FS: fractional shortening; +dL/dt: rate of contraction; -dL/dt: rate of relaxation.

for the decay of the Ca^{2+} transient.[24,25] Treatment with the β-agonist isoproterenol abolished these inhibitory effects by stimulating phosphorylation of PLN, thus relieving its inhibitory effects on SERCA2a affinity for Ca^{2+}. Mice with twofold PLN overexpression did not show any phenotypic alterations throughout the aging process,[25] while mice with fourfold PLN overexpression had elevated levels of adrenaline and noradrenaline, which could enhance the phosphorylation of PLN to relieve its inhibitory effects on cardiac function as a compensatory mechanism.[25] In the long term, this compensatory response became maladaptive, as adrenaline induced changes in transcription, which led to cardiac remodeling and progression to heart failure and early mortality. These findings, together with observations in rat cardiomyocytes, overexpressing PLN through adenoviral gene transfer,[26] suggested that a fraction of the SR Ca^{2+}-pumps is not regulated by PLN and the functional stoichiometric ratio of PLN/SERCA2a in the native SR is less than 1:1. Moreover, a close linear correlation was observed between the levels of PLN expression and the EC50 of SR Ca^{2+}-transport in PLN-overexpression, PLN wild-type, PLN-heterozygous and PLN-deficient hearts,[7] indicating that spare pumps in the SR are inhibited by the overexpressed PLN in transgenic hearts and the functional stoichiometry of PLN/SERCA2 is less than 0.5/1.0.

The phenotypes of mice that express null, normal and twofold PLN show that several physiological responses change in a linear fashion over a wide range of PLN expression levels, as outlined in Figure 2. This has provided strong experimental evidence that PLN is a principal regulator on the kinetics of cardiac contractility.

Phosphorylation of Phospholamban on Ser^{16} and Thr^{17} *In Vivo*

The availability of the PLN knockout mouse in combination with site-specific mutagenesis has provided us with an excellent opportunity to examine the structure-function relationship of PLN in a background free of the endogenous protein.[27-29] Cardiac hyperdynamic contractile parameters in PLN deficient mice were reversed by reintroduction of wild type PLN to the heart, demonstrating the feasibility of reinserting PLN phosphorylation site-specific mutants to examine their interdependence and functional relevance *in vivo*.[29] Mutations of Ser^{16} to Ala^{16} (S16A) or Thr^{17} to Ala^{17} (T17A) were introduced into the PLN cDNA by site-directed mutagenesis. Lines expressing similar levels of WT, S16A or T17A mutant PLN in the null background were selected for characterization studies. At basal condition, the fractional shortening, maximal rates of contraction and relaxation, Ca^{2+} amplitude and rate of Ca^{2+} transient decline were similar among myocytes expressing WT, S16A and T17A mutant PLN in the null background. After maximal isoproterenol stimulation, the myocyte mechanic and Ca^{2+} kinetic parameters were significantly lower in the S16A mutant PLN than WT or T17A mutant PLN cells. However, upon isoproterenol stimulation, rates of contraction and

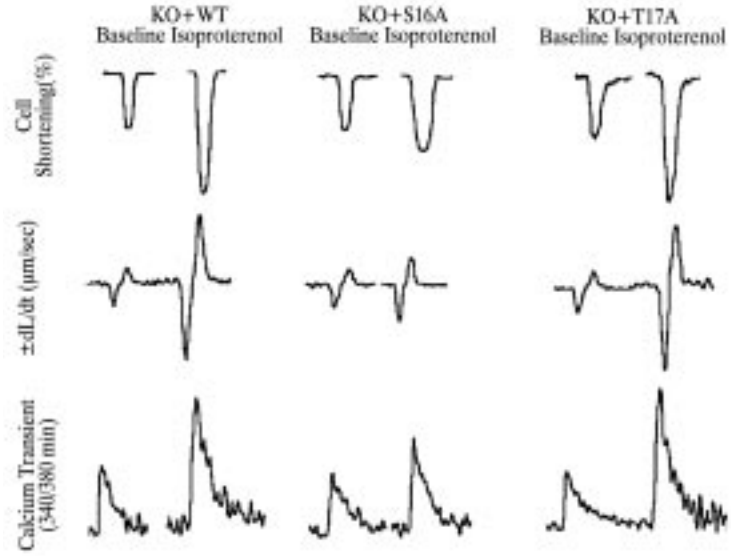

Figure 3. Phospholamban site-specific mutations and cardiac contractility at basal condition and upon isoproterenol stimulation.
+dL/dt: rate of contraction, -dL/dt: rate of relaxation.

relaxation as well as abbreviation of Ca^{2+} kinetics in T17A mutant PLN cardiomyocytes, were significantly increased. Also, the maximal stimulatory effects on mechanics and Ca^{2+} kinetics were similar to those observed in wild type cardiomyocytes. These findings suggested that mutation of S16A in PLN diminished the stimulatory effects of isoproterenol in cardiomyocytes, while mutation of T17A in PLN was not associated with any significant alteration in the stimulatory effects of PLN by isoproterenol (Figure 3).[27,29] Thus, phosphorylation of Ser^{16} in PLN may be sufficient in mediating the maximal cardiac responses to β-agonists, and the effects of dual site (Ser^{16} and Thr^{17}) PLN phosphorylation do not appear to be additive *in vivo*.

Recently, the role of the two PLN phosphorylation sites in the frequency-dependent contractile reserve was examined by several investigators. Using isolated adult rat myocytes, Hagemann et al[30] found that electrical pacing induced a selective enhancement in the CaMKII-mediated Thr^{17} phosphorylation of PLN in a frequency-dependent manner. They also indicated that frequency-dependent Thr^{17} phosphorylation of PLN by CaMKII occurs independently of PKA-mediated Ser^{16} phosphorylation. However, the force-frequency relationship was positive in wild type, but negative in PLN knockout mouse cardiac muscles, and PLN overexpression in rat cardiac myocytes showed an augmented positive force-frequency relationship.[31] More recent studies by Brixius et al[32] showed that the positive force-frequency relationship in human nonfailing myocardium was accompanied by a frequency-dependent increase in Ser^{16}-PLN, but not Thr^{17}-PLN phosphorylation. Interestingly, using isolated ventricular muscle or single myocytes from wild type and PLN knockout mice as well as rat ventricular myocytes, DeSantiage et al[33] found that frequency-dependent acceleration of relaxation in the heart, which indicates an intrinsic physiological mechanism and allows more rapid ventricular diastolic filling at higher heart rates, does not require PLN or PLN phosphorylation. Thus, the role of PLN in the cardiac force-frequency relationship is not well understood and further studies need to be performed.

Phosphorylation of PLN has been reported to decrease in human and animal heart failure[34,35] and this may be a contributing factor to the deteriorated function. In order to determine long-term effects of dephosphorylated PLN on SR Ca^{2+} cycling and cardiac function, a new mouse model was generated with a nonphosphorylatable double mutant form of PLN (PLN-DM, where both Ser^{16} and Thr^{17} are replaced by Ala) into the PLN-deficient background.[36] Transgenic lines expressing PLN-DM at levels similar to wild type were identified. Rates of basal twitch calcium decline were not different in PLN-DM versus wild type, while isoproterenol increased the rates of twitch calcium decline in wild type, but not PLN-DM myocytes, confirming the prominent role of PLN phosphorylation response. Increased L-type Ca^{2+} current density (ICa) was the major compensation in the DM myocytes. There were no changes in myofilament Ca^{2+} sensitivity, or the expression levels and Ca^{2+} removal activities of other Ca^{2+}-handling

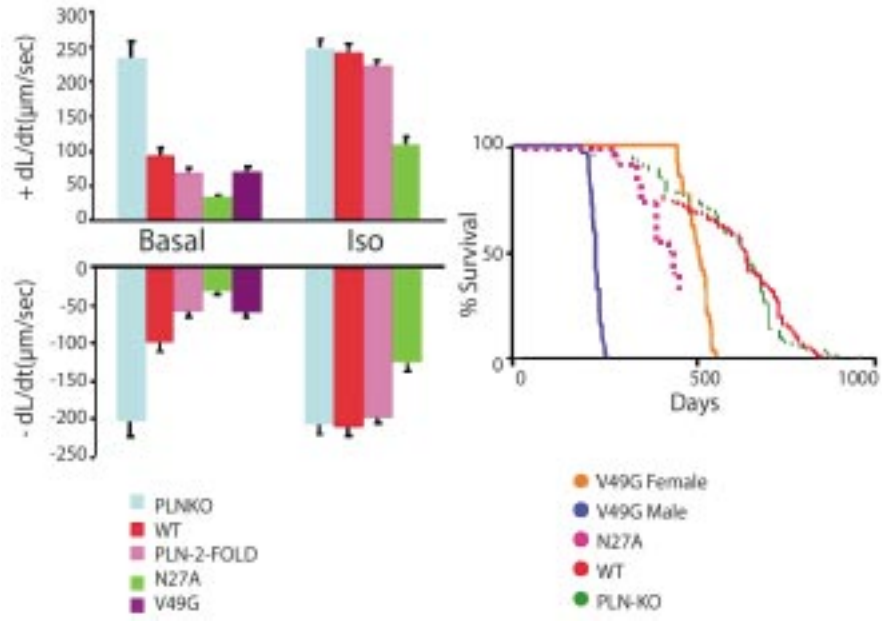

Figure 4. PLN site-specific mutation decreases basal cardiac contractility and life span in mice.
+dL/dt: rate of contraction, -dL/dt: rate of relaxation.

proteins. Nor was there evidence of cardiac remodeling up to 10 months of age. Thus, chronic inhibition of SERCA2a by ablation of PLN phosphorylation results in a unique cellular adaptation involving greater dynamic ICa modulation.[36]

Further studies on understanding the physiology and pathophysiology of the dual site PLN phosphorylation may provide novel insights into the regulatory mechanisms underlying cardiac function and the development of therapies targeting the PLN/SERCA2 interaction in heart disease.

Superinhibitory Effects of PLN Mutation

In order to determine whether superinhibitory mutations in PLN are associated with impaired cardiac function, a series of transgenic mice including Asn27Ala, Leu37Ala, Ile40Ala and Val49Gly were generated.[37-40] Each of these mice demonstrated increased inhibition of the affinity of cardiac SERCA2a for Ca^{2+}, which resulted in decreased Ca^{2+} uptake and a decrease in Ca^{2+} release from the SR.

Based on alanine-scanning mutagenesis of PLN, followed by co-expression of the mutant forms with SERCA2a, findings indicated that some monomeric mutants such as I40A (100% monomer) or L37A (94% monomer) were far more effective in decreasing the affinity of SERCA2a for Ca^{2+} than wild type PLN.[41,42] Thus, it was proposed that monomeric PLN is the active inhibitory form of the protein[41,42] and that gain of inhibitory function for monomeric mutants is due to mass action effects of the increased content of monomeric PLN.[41] The depression of myocyte Ca^{2+} kinetics and mechanics, observed with the monomeric L37A and I40A PLN mutant transgenic mice, was reversed by isoproterenol. However, *in vitro* expression studies have shown that mutants N27A, Q29A, and N30A in PLN domain Ib also gain inhibitory function with only about 10% monomer.[43] The N27A is the strongest superinhibitory mutant noted in a heterologous cell preparation.[43] Even though neither N27A nor V49G alter the pentameric:monomer ratio of PLN *in vivo*, the pentameric PLN mutations can also act as superinhibitors of the affinity of SERCA2a for Ca^{2+} and of cardiac contractility.[37,39] Furthermore, the impaired function could not be reversed fully with the experimental application of isoproterenol. Therefore, PLN becomes a chronic inhibitor and SERCA2a no longer has the potential to be fully functional. Mice that overexpress the N27A mutation on the PLN-null background survive for less than one year and die of dilated cardiomyopathy,[40] whereas transgenic males that overexpress V49G die of dilated cardiomyopathy when they are six months old (Figure 4).[39]

The PLN site-specific mutation studies described above provided us with a more detailed understanding of interaction between SERCA2a and PLN, which may be beneficial for the treatment of heart failure.

Modulation of Phospholamban Activity and Heart Failure in Animal Models

A hallmark of hypertrophied and failing myocardium is depressed SR calcium cycling, caused by down-regulation of the SR calcium pump, or by relative overabundance of the inhibitor of the pump, PLN, or both.[44] Therefore, the development of a PLN inhibitor may represent a promising therapeutic approach for heart failure. In this regard, the genetically engineered mouse, lacking PLN protein, was a powerful research tool in addressing the effect of enhanced SR calcium cycling on progression of heart failure. As described earlier, the PLN knockout mouse featured permanent supernormal cardiac contraction[12,21] without any compromise to SR energetic efficiency,[45] exercise capacity[22] or longevity.[21] Furthermore, PLN ablation has been demonstrated to improve SR calcium cycling and/or contractile function in transgenic mice overexpressing slow-skeletal muscle troponin I,[46] a non-phosphorylatable troponin I mutant,[47] and SERCA1a.[48] More importantly, to gain insight into the role of PLN ablation in cardiac disease, PLN knockout mice have been bred with a variety of experimental animal models recapitulating heart failure and its common antecedent, cardiac hypertrophy.[49-54] Experimental data obtained from cardiac hypertrophic responses induced by overexpression of the SR luminal calcium regulatory protein calsequestrin have demonstrated that ablation of PLN not only restored depressed calcium transients and contractile function, but also alleviated the re-introduction of fetal gene program and myocyte hypertrophy.[49] Strikingly, PLN gene deletion in the MLP mouse model of dilated cardiomyopathy prevented the progressive defects in cardiac SR, and rescued the depressed ventricular function and remodeling.[50] A recent study provided further evidence that PLN ablation rescued functional, morphological, and molecular characteristics of heart failure in β_1-adrenergic receptor-overexpression transgenic mice.[51] Collectively, these investigations suggest: 1) a nodal role of SR dysfunction in the complicated etiology of heart failure; and 2) modulation of SR function through inhibition of PLN as a promising therapeutic strategy for heart failure. These predominant successes of rescue of heart failure with genetic ablation of PLN have generated great enthusiasm concerning the value of PLN as a potential drug target. However, the improved contractile function associated with disruption of the PLN gene exhibited no beneficial effects on the heart of a dilated cardiomyopathy transgenic model that overexpressed tropomodulin.[54] Targeting of the PLN gene also failed to rescue the secondary hypertrophic response observed in a mouse model overexpressing a mutant myosin heavy chain, although systolic dysfunction was significantly improved.[52] More recently, the effect of PLN ablation on two well-characterized genetic models of cardiac hypertrophy and heart failure, Gαq overexpression and human familial hypertrophic cardiomyopathy mutant myosin binding protein C (MyBP-CMUT) expression[53] was examined. The hypertrophy and global cardiac dysfunction were not altered following PLN ablation, although the impaired contractility and depressed calcium kinetics were restored to supernormal levels in single cardiomyocytes isolated from these two cross-models.

Table 1 summarizes the mouse models with different cardiac phenotype and the cross models, which were generated by breeding genetically different mice with PLN deficiency mice. Nevertheless, caution should prevail in interpreting the results of such studies investigating the rescue of murine cardiomyopathy by genetic ablation of PLN. PLN deficiency is associated with a number of cellular adaptations to accommodate the enhanced Ca^{2+} cycling and energetic demand of the hyperdynamic cardiac function,[28,55] and these may contribute to or even mask the effects of PLN deletion in the various cardiomyopathy models.

Targeting PLN by Adenoviral-Mediated Gene Transfer

Targeting PLN by adenoviral-mediated gene transfer, an alternative experimental paradigm, has been demonstrated to slow the progression of heart failure. In cultured rat neonatal cardiomyocytes, infection of anti-sense RNA of PLN under the control of different promoters resulted in significantly decreased endogenous PLN level, which was associated with an increased calcium affinity of SR calcium pump and enhanced calcium uptake.[67,68] Importantly, in human failing cardiomyocytes, targeting PLN by adenovirus-based transfer of anti-sense PLN improved the contractile function concomitant with increased SR calcium kinetics.[69] Furthermore, the force-frequency relationship in failing human cardiomyocytes was restored to normal.[69] These *in vitro* studies have revealed unequivocal beneficial effects of targeting PLN by gene transfer in cardiomyocyte contractile function. Consistent with this notion, in-

Table 1. Effects of Phospholamban Ablation on Genetically Altered Mouse Models

Genotype	Cardiac Phenotype	Influences of Phospholamban Ablation
B1-receptor overexpression[56]	Cardiac hypertrophy and progressive heart failure	Prevented the heart from detrimental functional and morphological consequences of chronic β-adrenergic stimulation[51]
Muscle Lim Protein deletion[57]	Disrupted myocyte cytoarchitecture, DCM with hypertrophy	Rescued the whole spectrum of the dilated cardiomyopathic phenotype[50]
Calsequestrin overexpression[58]	Depressed cardiac function and concentric cardiac hypertrophy	Improved the cardiac function and alleviated the cardiac hypertrophy[49]
α-MHC Arg403Gln mutant[59]	Cardiac hypertrophy progresses into cardiomyopathy with fibrosis	Increased the systolic cardiac function but exhibited no effects on cardiac hypertrophy[52]
Tropomodulin overexpression[60]	DCM in juvenile mice with severe myofibril disorganization	No beneficiary effects on cardiac function and remodeling[54]
Gαq overexpression[61]	Cardiac hypertrophy and heart failure	Rescued the dysfunction in cardiomyocyte but failed to prevent ventricular failure[53]
Homozygous MyBP-C mutant[62]	Neonatal onset progressive DCM with myocyte hypertrophy	Improved cardiomyocyte contractility and calcium kinetics without influences on hypertrophy and DCM[53]
SERCA1a overexpression[63]	Significant increases in cardiac contractile parameters	Further enhancement of cardiac function[48]
Slow skeletal TnI overexpression[64]	Increased relaxation under β-adrenergic stimulation	Further enhancement of relaxation and no changes during β-adrenergic stimulation[46]
Ψ RACK overexpression[65]	Sustained cardioprotection from ischemia injury	Enhanced cardiac recovery of PLN-KOs from ischemia-reperfusion injury and decreased CK release[66]

hibition of PLN activity using adenoviral-mediated gene transfer in the intact heart also exhibited remarkable salutary effects on heart failure. As discussed previously, phosphorylation of PLN relieves its inhibition of the SR calcium pump and leads to increased SR calcium cycling. Chien and colleagues have reported that chronic inhibition of PLN by delivering a pseudophosphorylated form of PLN mutant (S16E-PLN) into the heart successfully prevented progressive heart failure in inherited cardiomyopathic hamsters, and also rescued the cardiac dysfunction and remodeling induced by myocardial infarction, in a model of acquired heart failure.[70,71] These *in vivo* studies validate the premise that inhibition of PLN by gene transfer may represent a new therapeutic modality in heart failure. Nevertheless, we should remain cautiously optimistic about the clinical value of gene-transfer mediated inhibition of PLN in heart failure, as further basic and clinical research is required.

PLN Inhibition: Differences between Mouse and Human

There is no doubt that animal models have offered us a great deal of information as we unravel complex human diseases and identify therapeutic targets. However, since there are significant difference in calcium handling between mouse and human myocardium, caution should prevail in extrapolation of find-

ings in mouse "heart failure" models to the human condition, as illustrated by recent studies on PLN gene mutations in human heart failure patients.[72,73] In one study, a mutation at residue 9 of PLN (R9C) in one patient out of 20 unrelated patients with inherited cardiomyopathy was identified.[73] Sequencing of the PLN gene of this individual's extended family revealed that only the members with this PLN mutation (R9C) developed cardiomyopathy. Cellular and biochemical studies indicated that this mutant PLN escaped phosphorylation by protein kinase A and dominantly suppressed the phosphorylation of wild-type PLN, which constitutively inhibited the activity of SERCA2a. Transgenic mice overexpressing the mutant PLN developed heart failure in early life and died prematurely, consistent with their human counterparts. These findings suggested that defects in genes encoding SR calcium regulatory proteins, such as PLN, can cause heart failure in humans.

Surprisingly, parallel studies of human inherited cardiomyopathy identified a terminal codon for lysine 39 (L39stop) in the human PLN gene, which is presumed to produce a truncated protein unable to associate with SERCA2a.[72] Although the function of this mutation as a true null allele has not been conclusively demonstrated, there were no detectable PLN protein levels in heart tissue of patients who had inherited the mutation from both parents and developed heart failure before the age of 30. Thus, PLN ablation in human appears to be detrimental, unlike findings in the mouse. The explanation for this discrepancy between the cardiac phenotypes of PLN ablation in mice and humans may exist in the inherent differences in cardiac physiology and Ca^{2+}-cycling mechanisms between the two species. The Ca^{2+} removal from the cytosol during cardiac relaxation in mice relies almost exclusively on SERCA2a (92% of total), while in humans Na^+/Ca^{2+} exchanger activity accounts for approximately one third of Ca^{2+} removal, with SERCA2a function largely responsible for the remainder. Mice with heart rates ranging up to 800 beats per min have relatively little cardiac reserve compared to humans.[74,75] A major part of human cardiac reserve is the ability to enhance SR Ca^{2+} uptake and load. If PLN inhibits SERCA2a function, then the cardiac reserve is compromised, as observed in human heart failure.[76] Therefore, the role of PLN may be more important in humans. The characterization of other PLN mutants in the human population will further elucidate the functional role of PLN in cardiac physiology and its effects on predisposition to heart disease.

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