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ORIGINAL ARTICLE

Highly efficient and specific modulation of cardiac calcium homeostasis by adenovector-derived short hairpin RNA targeting phospholamban

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Impaired function of the phospholamban (PLB)-regulated sarcoplasmic reticulum Ca^{2+} pump (SERCA2a) contributes to cardiac dysfunction in heart failure (HF). PLB down-regulation may increase SERCA2a activity and improve cardiac function. Small interfering (si)RNAs mediate efficient gene silencing by RNA interference (RNAi). However, their use for in vivo *gene therapy is limited by siRNA instability in plasma and tissues, and by low siRNA transfer rates into target cells. To address these problems, we developed an adenoviral vector (AdV) transcribing short hairpin (sh)RNAs against rat PLB and evaluated its potential to silence the PLB gene and to modulate SERCA2a-mediated Ca^{2+} sequestration in primary neonatal rat cardiomyocytes (PNCMs). Over a period of 13 days, vector transduction resulted in stable $>99.9\%$ ablation of PLB-mRNA at a multiplicity of infection of 100. PLB protein gradually decreased until day 7 ($7\pm2\%$ left), whereas SERCA, Na^+/Ca^{2+} exchanger (NCX1), calsequestrin and troponin I protein remained unchanged. PLB silencing was associated with a marked increase in ATP-dependent oxalate-supported Ca^{2+} uptake at 0.34 μM of free Ca^{2+}, and rapid loss of responsiveness to protein kinase A-dependent stimulation of Ca^{2+} uptake was maintained until day 7. In summary, these results indicate that AdV-derived PLB-shRNA mediates highly efficient, specific and stable PLB gene silencing and modulation of active Ca^{2+} sequestration in PNCMs. The availability of the new vector now enables employment of RNAi for the treatment of HF* in vivo.

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Keywords: *molecular therapy; RNA interference; phospholamban; heart failure*

Introduction

Heart failure (HF) remains a leading cause of mortality in the developed world. Deteriorated function of the failing heart has been partially attributed to dysfunction of the phospholamban (PLB)-controlled sarcoplasmic reticulum Ca^{2+} pump (SERCA2a).[1] Reduction of both SERCA2a expression and PLB phosphorylation[2,3] may contribute to this dysfunction. Non-phosphorylated PLB keeps the Ca^{2+} affinity of SERCA2a low, resulting in decreased sarcoplasmic reticulum (SR) Ca^{2+} uptake, slowed relaxation and decreased SR Ca^{2+} load, whereas PLB phosphorylation in response to β-adrenergic stimulation relieves this inhibition. Germline transgenic approaches for ablation of PLB expression and function in mice,[4] and somatic gene transfer for dominant-negative PLB mutants,[5,6] PLB-antisense-RNAs[7–10] or intracellular inhibitory PLB antibodies[11,12] were employed to increase cardiac SR Ca^{2+} transport activity and hence the contractile function of cardiomyocytes under physiological and diseased conditions. RNA interference (RNAi) mediated by chemically synthesized small interfering RNAs (siRNAs) was recently employed to silence PLB expression in cardiomyocytes.[13] RNAi relies on post-trancriptional, sequence-specific gene silencing via small homologous double-stranded RNAs.[14] Its silencing efficacy is higher than that of antisense RNAs.[15] Nevertheless, therapeutic use of synthetic siRNAs is significantly limited by their rapid degradation in target cells, resulting in only transient gene silencing,[13] and by the difficulties in achieving sufficient transfer rates into multiple cells of therapeutic interest including cardiomyocytes. These limitations are significantly aggravated *in vivo*, and despite intense efforts in recent years, systemic delivery of siRNAs remains a major hurdle for *in vivo* applications of RNAi.[16] As viral vector systems have been shown to be suitable to overcome these limitations *in vitro* and *in vivo*, we have developed a novel adenoviral vector (AdV) suitable for transcription of short hairpin RNA (shRNA) targeting PLB. Treatment with this vector resulted in

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highly efficient and specific PLB gene silencing in primary neonatal rat cardiac myocytes (PNCMs), which was stable over 2 weeks and associated with a marked increase in the SERCA2a-catalyzed SR Ca^{2+} sequestration.

Results

In order to select potential PLB-siRNA sequences for the generation of PLB-shRNAs, we initially co-transfected Cos-7 cells with a green fluorescent protein (GFP)-rat PLB fusion construct plus different siRNAs directed against rat PLB. Among five tested siRNAs, three were highly efficient in downregulating the fusion transcripts. The most efficient of these siRNAs was then cloned into an shRNA expression plasmid, as a DNA sequence encoding the PLB-shRNA. The co-transfection experiments with the GFP-rat PLB fusion constructs showed the very high efficacy of PLB-shRNA17 (Figure 1). Based on these results, we constructed an AdV designated $AdshPLB_r$ which generates PLB-shRNA17 (Figure 2).

To assess the efficacy of the vector $AdshPLB_r$ in downregulating endogenously expressed PLB in the actual target cells, we transduced PNCMs with $AdshPLB_r$ and investigated the dose- and time-dependency of its action. A control AdV ($AdshCAR4_m$) generating an shRNA irrelevant for PLB and cardiac Ca^{2+} metabolism was employed to test for target specificity of $AdshPLB_r$. The treatment of PNCMs with $AdshPLB_r$ resulted in strong dose-dependent downregulation of PLB-mRNA expression by 27% at a multiplicity of infection (MOI) of 10 and by 87% at an MOI of 100 two days after transduction (Figure 3a). Rapid, strong and enduring PLB silencing was observed after treatment of PNCMs with $AdshPLB_r$ (MOI = 100) over a prolonged 13-day investigation period. PLB-mRNA abundance was extremely low <0.1% of baseline as quantitated by real-time reverse transcription-polymerase chain reaction (RT-PCR) (not shown) as early as day 1 and remaining at this level until day 13 (end of experiment).

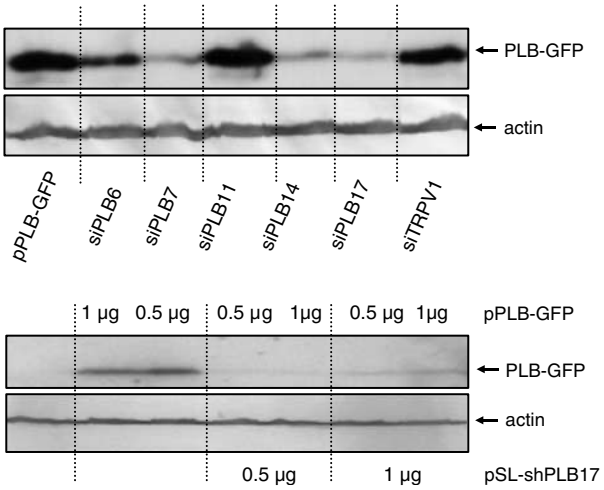

Figure 1 Evaluation of PLB siRNAs. Upper panel: Selection of PLB siRNAs. Cos-7 cells were co-transfected with the PLB-GFP fusions construct pPLB-GFP and synthetic PLB-siRNAs. Cells were harvested 20 h later and Western blot carried out. PLB-GFP fusion protein was detected by a rabbit antiserum against GFP. To confirm equal loading of the samples, membranes were stripped and reprobed with a monoclonal antibody against actin. Note: siTRPV1 is a synthetic siRNA directed against the vanilloid receptor 1 (TRPV1). Lower panel: Dose dependency of pSL-shPLB17 action. Cos-7 cells were co-transfected with the pSL-shPLB17 and pPLB-GFP as indicated. Immunoblots were carried out 24 h after transfection as described above.

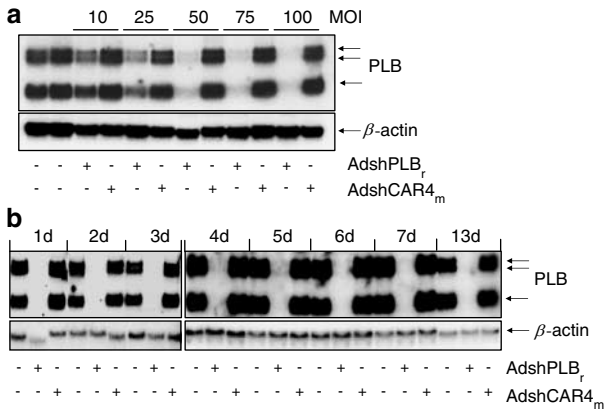

Figure 3 Silencing of PLB-mRNA expression by $AdshPLB_r$-generated PLB-shRNA. (**a**) Dose dependency of $AdshPLB_r$-mediated PLB-mRNA downregulation. PNCMs were transduced with $AdshPLB_r$ or a control vector as indicated. Cells were harvested 48 h later and Northern blot carried out using a rat PLB-specific probe. To confirm equal RNA loading blots were striped and rehybridized with a β-actin-specific probe. (**b**) Time dependency of $AdshPLB_r$-mediated PLB-mRNA downregulation. PNCMs were transduced with $AdshPLB_r$ or a control vector at an MOI of 100. Cells were harvested at indicated time points after transduction and Northern blot carried out as described above. Note: PLB-mRNA was undetectable during the complete 13-day investigation period in PNCMs transduced with $AdshPLB_r$, whereas its expression stayed unaffected in cells transduced with the control vector.

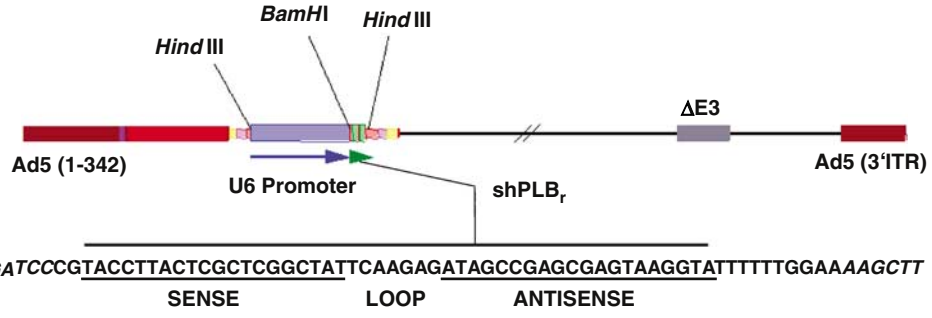

Figure 2 Map of $AdshPLB_r$ vector and PLB-shRNA sequence.

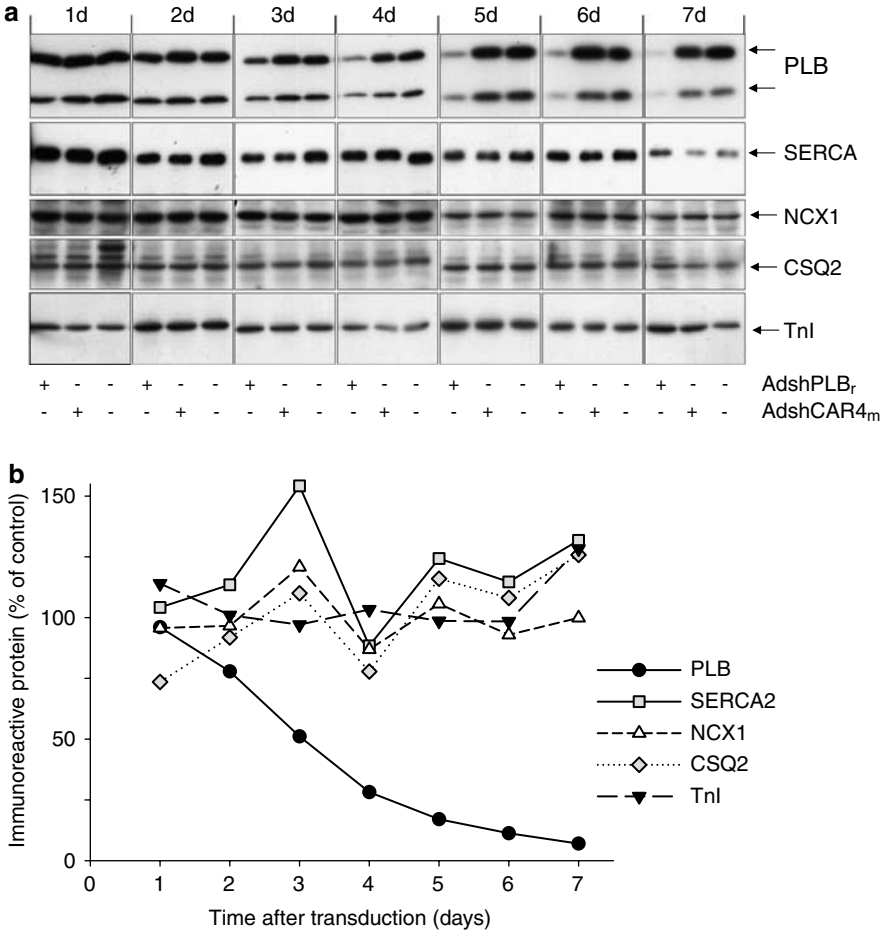

Figure 4 Time dependency of AdshPLB$_r$-mediated PLB protein downregulation. (**a**) Western blot analysis of PLB protein expression. PNCMs were transduced with AdshPLB$_r$ or a control vector at an MOI of 100. Cells were harvested at indicated time points after transduction and Western blots carried out. Significant PLB downregulation became visible 3 days post-transduction, whereas the expression levels of SERCA, NCX1, TnI and CSQ2 were unaffected during the 7 days investigation period. (**b**) Relative expression levels of PLB, SERCA, NCX1, TnI and CSQ2 protein normalized to the respective Western blot signals at the corresponding time points in control vector-transduced PNCMs.

By contrast, PLB-mRNA expression stayed at baseline level both in non-transduced controls and AdshCAR4$_m$-treated PNCMs (Figure 3b).

Similar but delayed changes occurred at the PLB protein level. A decrease in PLB protein became first visible on day 3 ($51 \pm 4\%$ remaining) as compared to controls. PLB protein then further decreased continuously until day 7 ($7 \pm 2\%$ remaining). By contrast, the protein levels of SERCA2, NCX1, troponin I (TnI) and calsequestrin (CSQ2) remained unchanged, indicating the absence of unspecific side effects on these cardiac proteins of both the vector itself and of the shRNA that it generates (Figure 4a and b).

To examine the functional consequences of the PLB-shRNA-induced decline in the PLB protein level, PNCMs were transduced with AdshPLB$_r$ or control vector AdshCAR4$_m$ (each at an MOI of 100). Cell homogenate SR Ca^{2+} uptake rates were determined at submicromolar (0.34 μM) and saturating (3.68 μM) free Ca^{2+} concentrations. At the latter, the SR Ca^{2+}-ATPase is known to be insensitive to non-phosphorylated PLB, whereas at submicromolar Ca^{2+} non-phosphorylated PLB has been shown to decrease the Ca^{2+} affinity of this enzyme.[17] The rate of Ca^{2+} uptake determined at 0.34 μM free Ca^{2+} and normalized to the maximum uptake rate (V_{max}) values at saturating Ca^{2+} (relative Ca^{2+} uptake rate) did not differ between non-transduced and AdshCAR4$_m$- or AdshPLB$_r$-treated PNCMs 1 day after transduction day 1 (approximately 45% of V_{max} each). The relative Ca^{2+} uptake rate remained at this level both in non-transduced and AdshCAR4$_m$-transduced PNCMs until day 7 after transduction. In contrast, it increased steadily in AdshPLB$_r$-transduced PNCMs reaching 100% of V_{max} on day 5 after transduction and remaining at this high level until the end of the experiment on day 7 (Figure 5a). This indicates an increase in the Ca^{2+} affinity of the SR Ca^{2+}-ATPase in the PLB-deficient PNCMs.

As shown in Figure 5b and a linear relationship between the relative rates of Ca^{2+} uptake and the respective relative PLB protein levels was observed in AdshPLB$_r$-transduced PNCMs. In addition, we investigated the degree of stimulation of Ca^{2+} uptake by protein kinase A (PKA)-dependent *in vitro* phosphorylation following transduction of PNCMs with AdshPLB$_r$ at an MOI of 100. On day 1 after transduction, the PKA-induced increase in the rate of Ca^{2+} uptake was 82% both in non-transduced and in AdshPLB$_r$-transduced PNCMs. In homogenates of AdshPLB$_r$-transduced PNCMs, this

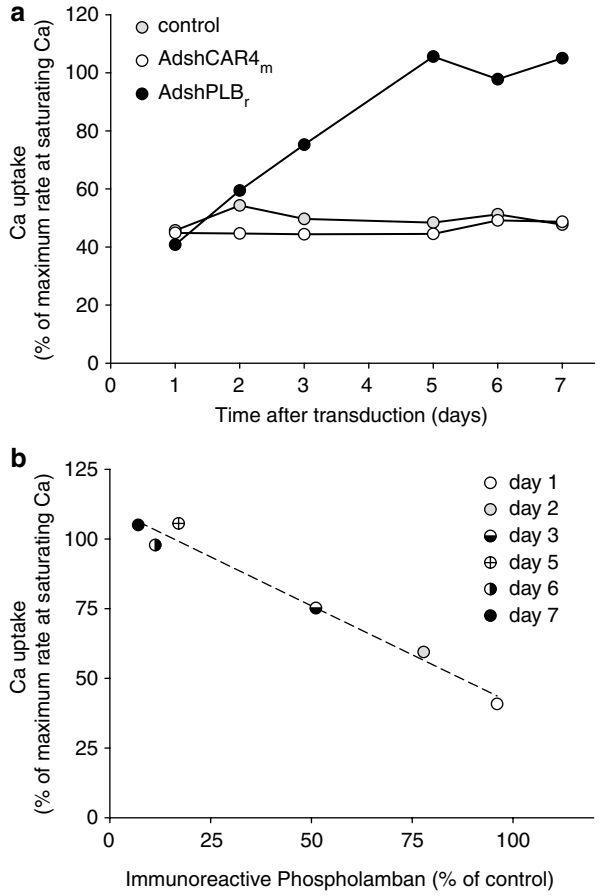

Figure 5 SR Ca^{2+} uptake in PNCM homogenates after $AdshPLB_r$-mediated PLB silencing. (**a**) Relative Ca^{2+} uptake at 0.34 μM free Ca^{2+} in homogenates of PNCMs. PNCMs were transduced with $AdshPLB_r$ or a control vector at an MOI of 100. Cells were harvested at indicated time points and SR Ca^{2+} uptake rates were determined at submicromolar (0.34 μM) and saturating (3.68 μM) free Ca^{2+} concentrations. Values after 3 min of uptake were normalized to the maximum uptake at a saturating Ca^{2+} of 3.68 μM. Control: non-transduced cells. (**b**) Relative Ca^{2+} uptake vs percent change in PLB protein in PNCMs homogenates on days 1–7 post-transduction. Data were calculated from the results obtained from Ca^{2+} uptake experiments (**a**) and PLB protein expression (Figure 4b).

PKA stimulation became completely lost around day 4/5 after transduction, whereas approximately 1.5-fold stimulation of Ca^{2+} uptake was observed on days 4–7 in non-transduced PNCMs (Figure 6).

Discussion

We demonstrated highly efficient and specific ablation of endogenous PLB expression in PNCMs by PLB-shRNA expressed from an AdV. shRNA transcription resulted in the downregulation of endogenous PLB-mRNA below 0.1% of baseline, persisting for 13 days. No changes of the expression of other cardiac proteins including Ca^{2+} handling proteins occurred, indicating high target specificity of the PLB-shRNA vector. A control vector had no effect on PLB, indicating the absence of unspecific effects of shRNA *per se* on PNCMs. At the functional level, the SERCA2a Ca^{2+} affinity was markedly increased after PLB silencing accompanied by a loss of responsiveness to PKA-dependent stimulation of Ca^{2+} uptake.

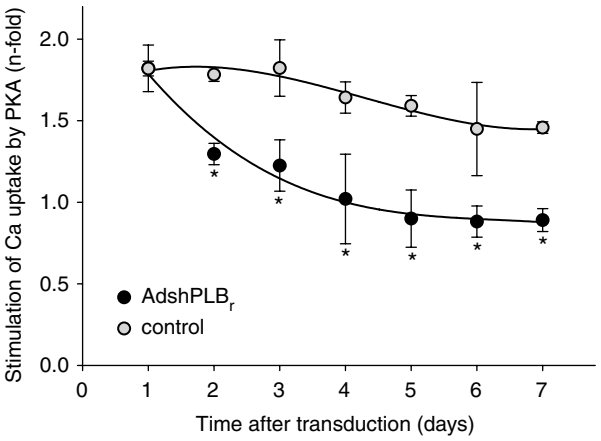

Figure 6 Stimulation of Ca^{2+} uptake in PNCMs homogenates by the catalytic subunit of PKA. Values in the presence of added PKA normalized to those measured in the presence of 2 μM synthetic PKA peptide inhibitor; shown are means $\pm$ s.d. for four separate transductions. Control: non-transduced cells.

SERCA2a and PLB form a functional complex regulating Ca^{2+} uptake. Altered levels of either protein may therefore have profound effects by changing the PLB/SERCA2a ratio and thereby altering intracardiac Ca^{2+} homeostasis. This was demonstrated in normal and diseased hearts by use of transgenic animal models or gene transfer approches targeting PLB or SERCA2a.[5,18–21] Reduction of the PLB/SERCA2a ratio was achieved by classical overexpression of cDNAs encoding SERCA2a, a dominant-negative PL mutant, or PLB-targeted antibodies. We and others have employed antisense RNAs directed against PLB-mRNA.[7,9]

In contrast, RNAi-based therapeutic strategies are not yet widely employed in the cardiovascular field. Synthetic siRNAs were recently employed to downregulate PLB in cardiomyocytes.[13] As siRNAs enable only transient gene silencing, we have developed an AdV generating shRNAs mediating efficient, specific and stable PLB silencing. With respect to the duration of silencing, a striking difference between previous work using synthetic siRNAs[13] and the current AdV-based approach was observed. siRNAs resulted in the downregulation of PLB-mRNA to 5% of baseline within 12 h, but this effect was almost completely lost after 4 days. In contrast, PLB-mRNA levels remained below 0.1% of baseline over a 2-week-period after PLB-shRNA-AdV treatment. Vector-based shRNA generation was clearly superior to synthetic siRNAs with respect to silencing stability. With respect to the efficacy of silencing, the PLB-shRNA-AdV resulted in $>99.9\%$ ablation of PLB-mRNA, whereas a former study using a PLB-antisense-RNA-AdV achieved maximal PLB-mRNA ablation $\approx 75\%$.[7]

In line with previous reports, we found PLB ablation to improve SERCA2a-dependent intracellular Ca^{2+} handling. Significant upregulation of SERCA2a Ca^{2+} affinity was first observed when PLB protein was down to 50% of baseline. Interestingly, PLB downregulation to 20% was sufficient to mediate maximal increase in the Ca^{2+} affinity of SERCA2a; further reduction had no additional effect. There was a steady loss of responsiveness to PKA-dependent Ca^{2+} uptake stimulation by PLB-shRNA, and complete loss of responsiveness was observed on days

4–7. There was no loss of responsiveness to PKA-dependent Ca^{2+} uptake stimulation in the control groups. This indicates that the RNAi-mediated PLB silencing was linked to the anticipated loss of responsiveness of the SR Ca^{2+} transport system to PKA-dependent phosphorylation normally mediated through PLB.

Although RNAi is a powerful method for gene silencing, its implementation for therapeutic purposes in humans requires that two technical problems are solved. First, instability of chemically synthesized siRNAs in plasma and cells requires their repetitive administration *in vivo*. The loss of initially efficient target gene silencing in cardiomyocytes within 3 days[13] allows an estimate of the required siRNA application frequency for this specific target tissue. The problem may be overcome by viral vector systems producing shRNAs over long time periods *in vivo*. For intermediate stability, AdV-shRNA systems as used here may suffice, and even provide advantages over long-term stable AAV vectors[22–25] if the RNAi effect is needed only temporarily in an acute and potentially reversible condition (e.g. HF due to viral or autoimmune myocarditis). Second, cardiac targeting of synthetic siRNAs is currently only possible by experimental methods unsuitable for possible transfer to any clinical setting.[16] In contrast, recent developments in vector technology (e.g. pseudotyped AAVs with cardiotropic properties) are likely to allow cardiac targeting of transgene and shRNA expression cassettes by simple intravenous injection.[23] Importantly, the small expression cassette and specific PLB-shRNA sequence used in this study can easily be incorporated in different AAV and other vectors selected for this purpose. Within the framework of our proof-of-concept study that introduces a novel PL-shRNA tool, we have not performed *in vivo* work, as the efficacy of PLB ablation for HF therapy in animal models has already been demonstrated.[5,18–21] In future cardiac gene therapy studies, we will use pseudotyped AAV8 and AAV9 vectors, as two recent studies[26,27] have demonstrated important advantages of these pseudotypes over lentiviruses, adenoviruses and previously used AAV vectors. One remaining challenge is the modification of the cassette in such a way as to allow exogenously regulatable shRNA expression and adjustment of the degree of PLB modulation to changing physiological conditions. This appears to be particularly important in humans, as chronic PLB deficiency owing to genomic mutations was associated with cardiomyopathies.[28–30] Whereas in mice complete knockout of PLB (as may also be achieved by RNAi) was able to rescue the severe cardiomyopathic phenotype of MLP knockout mice,[31] suggesting that unregulated PLB silencing is appropriate in this species, application in humans most probably requires regulatable RNAi.

Materials and methods

PNCM cultures

Our *in vitro* study was designed to investigate the biological effects of PL-shRNA transfer over as long a period of time as possible. Adult cardiomyocyte cultures were less suitable than PNCM cultures for this purpose because of their known fragility and dedifferentiation after only few days. PNCM culture is a well-proven model to study hormonal, stretch and gene transfer effects and was therefore chosen as test system. PNCM cultures were prepared from ventricular tissue of 1- to 3-day-old Wistar rat pups (Charles River, Berlin, Germany) as described previously.[32] Briefly, the removed ventricles of 30–50 animals were placed into ice-cold calcium ion-free phosphate-buffered cell isolation medium (CIM) containing 120 mM NaCl, 4.56 mM KCl, 0.44 mM KH_2PO_4, 0.42 mM Na_2HPO_4, 25 mM $NaHCO_3$ and 5.55 mM glucose, pH 7.5, as well as 0.5 mg/ml streptomycin and 5000 IE/ml penicillin G (Biochrom KG, Berlin, Germany). Ventricular tissue was transferred to a Petri dish and minced into pieces of approximately 1 mm^3 in size using two sterile scalpels. Stepwise disaggregation for 15 min each of tissue pieces into single cells was performed at 37°C under continuous mixing with a magnetic stirrer at 150 r.p.m. in a 50-ml Erlenmeyer flask containing 15 ml CIM supplemented with 0.12% porcine trypsin 1:250 (Belger Biochemie, Kleinmachnow, Germany). The first tissue digest was discarded. The following 3–6 supernatants obtained after each 15 min digestion period were poured into single sterile 50-ml cell culture tubes each containing 4 ml of ice-cold, heat-inactivated fetal calf serum (FCS) (Biochrom KG). Cells were gently sedimented at 300 *g* for 8 min using a non-refrigerated centrifuge. The sedimented cells were resuspended in cell growth medium CMRL 1415-ATM, which was supplemented with 10% FCS, 10% horse serum (HS) and 0.02 mg/ml gentamicin (all constituents from Biochrom KG) and adjusted to pH 7.4 using 1 N NaOH. For enrichment of cardiomyocytes, 20 ml of this suspension containing approx. 2×10^8 cardiac cells were incubated in 175-cm^2 plastic Corning culture flask in a water-saturated atmosphere for 90 min at 37°C. The cardiomyocyte-enriched supernatant was carefully removed and pooled in an Erlenmeyer flask. The number of cells was counted using a light microscope and a hemocytometer. The cell density was adjusted with additional growth medium to 1.2×10^6 cells/ml and the cells were seeded to six-well Nunc culture plates and incubated in a water-saturated atmosphere at 37°C. After 24 h of incubation, the FCS/HS-supplemented growth medium was replaced by 10% FCS-supplemented CMRL 1415-ATM medium containing 2 μM fluorodeoxyuridine (Sigma Aldrich Chemie, Steinheim, Germany) for inhibition of proliferation of contaminating non-muscle cells.

siRNAs

siRNAs against the following target sites on the rat PLB mRNA with two-nucleotide overhangs were used in this study:

siPLB6 5′-GUCCAAUACCUUACUCGCU-3′;
siPLB7 5′-CCUCCAGAACCUCUUUAUC-3′;
siPLB11 5′-GGCCUCCUAAAAGGAGACA-3′;
siPLB14 5′-AAGUCCAAUACCUUACUCG-3′;
siPLB17 5′-UACCUUACUCGCUCGGCUA-3′.

As a control, an siRNA against the human vanilloid receptor 1 (TRVP1) which is not expressed in the cell lines used in this study was used:

siTRPV1 5′-GCGCAUCUUCUACUUCAAC-3′

The siRNAs were purchased from Dharmacon (Lafayette, CO, USA) or from IBA GmbH (Göttingen, Germany). siPLB17 was obtained as a kind gift from Noxxon Pharma (Berlin, Germany).

Construction of plasmids

The plasmid pPLB-GFP was generated by insertion of the PLB-cDNA (deleted in the TGA stop codon), which was amplified by RT-PCR from rat heart mRNA using the primer pair 5′-CGC AGC TGA GCT CCC AGA C-3′/5′-CCA GAA GCA TCA CAA TGA TGC-3′. The PCR fragment was inserted into the plasmid pcDNA3.1/CT-GFP (Invitrogen, Karlsruhe, Germany) by TOPO cloning according to the manufacturer's protocol. Based on siPLB17, an shRNA expression vector was generated by cloning the corresponding DNA oligonucleotides into the plasmid pSilencer 1.0-U6 from Ambion (Austin, TX, USA). For construction of the plasmid pSL-PLB17, the following oligonucleotides from TIB Molbiol (Berlin, Germany) were used: 5′-GATCCCGTACCTTACTCGCT CGGCTATTCAAGAGATAGC-CGAGCGAGTAAGGTA TTTTTTGGAAA-3′/5′-AGCTTTTCCAAAAAATACCTT ACTCGCTCGGCTAT-CTCTTGAATAGCCGAGCGAGT AAGGTACGG-3′. The oligonucleotides cover the sense and antisense sequence of rat PLB (Accession no.: ×71068.1) from nucleotide position 189 to 209 (underlined, corresponding to siPLB17), and a 7 bp loop. Annealing of the oligonucleotides resulted in a duplex containing a *Bam*HI site at its 5′ end and a *Hin*dIII site at the 3′ end. This duplex was ligated into *Bam*HI/*Hin*dIII-digested pSilencer 1.0-U6 generating pSL-PLB17. The expression cassette consisting of the U6 promoter and the PLB shRNA17 was then amplified from pSL-PLB17 using the primer pair 5′-TAGGGTTTAAACCCAGGGTTTTCC CAGTCA-3′/5′-ATGGACTAGTCCAGAGAATTACCCT CAC-TAA-3′. The PCR fragment was digested with *Spe*I and filled on with T4 polymerase and then cloned into pCR4Blunt-TOPO (Invitrogen). The fragment was then cut out with *Spe*I/*Not*I and inserted into the plasmid pAd.ME[33] via *Spe*I/*Not*I. The resulting plasmid was digested with *Sac*I/*Nhe*I, overhanging ends were filled and the plasmid was religated. The resulting adenoviral shuttle plasmid was termed pAdshPLB$_r$. For generation of the control adenoviral shuttle plasmid pAdshCAR4$_m$, the plasmid pAdshPLB$_r$ was digested with *Pme*I and religated resulting in elimination of an *Eco*RI site. Then, the plasmid was digested with *Eco*RI and religated resulting in elimination of a *Spe*I site, which was essential to generate the AdV. Thereafter, we generated an shRNA for mouse coxsackievirus-adenovirus-receptor (CAR) and inserted it via *Bam*HI/*Hin*dIII into the plasmid intermediate, resulting in generation of pAdshCAR4$_m$. For generation of the mouse CAR-shRNA, four oligonucleotides (Oligoservice, Berlin, Germany) were used:

5′-GATCCCCGGATCGGAAGAGATTGGAAATTTC-3′
5′-AAGAGAATTTCCAATCTCTTCCGATCCTTTTTA-3′
5′-AGCTTAAAAAGGATCGGAAGAGATTGGAAATT-3′
5′-CTCTTGAAATTTCCAATCTCTTCCGATCCGGG-3′

The oligonucleotides cover the sense and antisense sequence of mouse (Accession no.: Y10320.1) from nucleotide position 448 to 468 (underlined). Initially, each oligonucleotide was phosphorylated with T4 polynucleotide kinase (New England Biolabs, Frankfurt, Germany). Then the oligo's were mixed, annealed and cloned via open *Bam*HI/*Hin*dIII sites in the fragment directly into *Hin*dIII/*Bam*HI-digested pAdshPLB$_r$. Plasmid sequences were verified by sequencing using the ABI Prism Genetic Analyser 310 (Perkin-Elmer, Wellesley, USA).

Construction of AdVs

The adenoviral shuttle plasmids pAdshPLB$_r$ and pAdshCAR4$_m$ were linearized with *Spe*I, ligated to the 5′ long arm of *Xba*I-digested E1$^-$E3$^-$ adenovirus mutant RR5, transfected into HEK293 cells and propagated as described,[34] generating the AdVs termed AdshPLB$_r$ and AdshCAR4$_m$. All adenoviral constructs were tested against contamination with replication-competent adenovirus by PCR as described,[35] and the viral titers were determined using standard plaque assays on HEK293 cell.

Transfection of siRNAs and shRNA-generating plasmids

For transfection, Cos-7 cells were plated in 24-well plates at a density of 0.6×10^5 cells/well in a volume of 500 ml without antibiotics. The next day, cells were transfected with 10 nM siRNA and 1 μg of the pPLB-GFP fusion construct in 2.5 ml of Lipofectamine 2000 (Invitrogen) per well, following the manufacturer's instructions. The cells were harvested 20 h after transfection. For plasmid co-transfection, the Cos-7 cells were transfected with 0.5 or 1 μg pSL-shPLB17 and 0.5 or 1 μg of pPLB-GFP, respectively, using 2.5 ml of Lipofectamine 2000 (Invitrogen) per well, following the manufacturer's instructions. Cells were harvested 24 h after transfection.

Adenovector-mediated gene transfer

PNCMs were seeded at a density of 1.2×10^6 cells per well on six-well plates. Forty-eight hours later, cell growth medium was removed and PNCMs were incubated for 1 h with 0.5 ml serum-free CMRL 1415-ATM medium containing AdVs. Thereafter, 2.5 ml 10% FCS-supplemented cell growth medium was added and replaced every 24 h with 3 ml/well of fresh FCS-supplemented cell CMRL 1415-ATM growth medium. Transduced PNCMs were cultured in this medium in a water-saturated atmosphere at 37°C up to 13 days.

Northern blot analyses

Ten micrograms of total RNA were separated on a 1% formaldehyde agarose gel and transferred to a Hybond N nylon membrane (Amersham/GE Healthcare, Freiburg, Germany). After prehybridization, the membranes were hybridized with a [^{32}P]dCTP labeled ss-antisense PLB probe in ExpressHyb Solution (Clontech, Germany) following the manufacturer's instructions. Rehybridization was carried out with heart muscle cell-specific [^{32}P]dCTP-labeled ss-antisense SERCA (generated from rat SERCA cDNA) and CSQ2 probes (generated from mouse CSQ2 cDNA) and for standardization of PLB-mRNA expression levels with [^{32}P]dCTP-labeled ss-antisense β-actin-DNA probe generated from human cDNA for detection of housekeeping gene expression. Labeling of probes was performed by PCR-like reactions as described previously.[36] Hybridized filters were exposed to Kodak Biomax MS film (Integra Biosciences,

Fernwald, Germany). The hybridization signal intensity was determined by phosphoimaging in a Fuji Film BAS-1500 (Fuji Photo Film, Frankfurt, Germany) imager.

Detection of PLB-GFP fusion protein by immunoblotting

Expression and knockdown of the PLB-GFP fusion protein was analyzed by immunoblotting. Twenty-four hours after transfection, cells were lysed in 24-well plates in a buffer consisting of 125 mM Tris-HCl (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 1.4 mM β-mercaptoethanol, 25% (v/v) glycerol and 0.05% (w/v) bromophenol blue. Lysates were boiled at 95°C for 5 min, and proteins were subsequently separated on a 12.5% polyacrylamide gel. Transfer of proteins to PVDF membranes (Amersham/GE Healthcare) was performed by semi-dry blotting (Peqlab, Erlangen, Germany). Membranes were incubated with rabbit antiserum against GFP at 1:5000 dilution (Invitrogen). Secondary antibodies were conjugated with alkaline phosphatase at 1:5000 dilution (Chemicon, Hampshire, UK) and chemiluminescence was detected with CDP-Star (Roche, Mannheim, Germany) according to the manufacturer's protocol. To confirm equal loading of the samples, membranes were stripped and reprobed with a monoclonal antibody against actin at 1:5000 dilution (Chemicon).

Western blot analyses

Cells were washed with phosphate-buffered saline and scraped in 200 μl of Laemmli buffer. The suspensions were sonicated on ice water in a Bioruptor (Diagenode, Liege, Belgium) at setting 'high' and 30 s on/off intervals for 15 min to disrupt DNA. Samples were heated for 5 min at 37°C and centrifuged for 1 min at 9700 g_{av}. An aliquot of the clear supernatant was removed for protein determination using the RCDC protein assay (Bio-Rad Laboratories, Munich, Germany). Samples were stored at −80°C. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5–15% gradient gels. Samples were reheated for 5 min at 37°C before use and 5 μg of protein/lane was applied onto the gels. After electrophoresis proteins were blotted overnight at 40 V onto PVDF membranes (immunblots) (Bio-Rad Laboratories). Blots were stained reversibly with Ponceau Red to check protein loading and transfer. Blots were pre-incubated in tris-buffered saline Tween 20 (TTBS) (10 mmol/l Tris-HCl, pH 7.6, 150 mmol/l NaCl, 0.1% Tween-20) supplemented with 0.5% non-fat milk powder for 1 h at room temperature and incubated overnight at 4°C with diluted primary antibodies. Anti-PLB (1:20 000, mouse monoclonal) was from Affinity Bioreagents and anti-SERCA2 (1:20 000, rabbit polyclonal) was from Abcam, anti-TnI was from RDI (1:45000, rabbit polyclonal) and anti-CSQ2 (1:1000, rabbit polyclonal) and anti-NCX1 (1:8000, goat polyclonal) were both from Santa Cruz Biotechnology (Heidelberg, Germany). Blots were probed for 3 h at room temperature with either diluted horseradish peroxidase-conjugated goat anti-mouse or goat antirabbit or rabbit antigoat secondary antibody (Pierce Biotechnology, Rockford, IL, USA). In between the incubations, the blots were washed extensively with TTBS. A rat cardiac sarcoplasmic reticulum membrane preparation was used as positive control. Signals were visualized using Supersignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and Hyperfilm ECL (Amersham/GE Healthcare). Signal densities were quantified using a Bio-Rad Calibrated GS-800 Scanner and Quantity One quantitation software (Bio-Rad Laboratories).

SR Ca^{2+} uptake measurements

SERCA2a-catalyzed transport of Ca^{2+} into SR vesicles was determined in cell homogenates as oxalate-supported Ca^{2+} uptake as essentially described earlier.[37] The reaction medium of 0.2 ml contained 40 mM imidazole (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, 6 mM phosphocreatine, 10 mM K-oxalate, 0.2 mM ethyleneglycol tetraacetate, 10 mM NaN_3, 3–4 μg of homogenate protein and 0.034 or 3.68 μM free $^{45}CaCl_2$ (2×10^{12} Bq/mol). For determination of the degree of stimulation of Ca^{2+} uptake by PKA-dependent phosphorylation at 0.34 μM free $^{45}CaCl_2$, either 2 μM synthetic PKA inhibitor peptide (PKI(6–22)amide) or 2 μM catalytic subunit of PKA was added to the uptake medium. After pre-incubation of the reaction mixture for 2 min at 37°C, the Ca^{2+} uptake was started by addition of homogenate. After 3 min, a 150 μl sample was filtered through 0.45-μm cellulose ester ME 25 membranes (Schleicher and Schuell BioSience, Dassel, Germany) using a vacuum pump. Washing of filters and determination of radioactivity were carried out as described.[35]

Abbreviations

AdV, adenovector; AdV, adenoviral vector; PLB, phospholamban; PNCM, primary neonatal rat cardiomyocytes; RNAi, RNA interference; SERCA2a, sarcoplasmic reticulum Ca^{2+} pump; shRNA, short hairpin RNA; siRNA, small interfering RNA

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