Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via *in vivo* cardiac rAAV gene delivery

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The feasibility of gene therapy for cardiomyopathy, heart failure and other chronic cardiac muscle diseases is so far unproven. Here, we developed an *in vivo* recombinant adeno-associated virus (rAAV) transcoronary delivery system that allows stable, high efficiency and relatively cardiac-selective gene expression. We used rAAV to express a pseudophosphorylated mutant of human phospholamban (PLN), a key regulator of cardiac sarcoplasmic reticulum (SR) Ca^{2+} cycling in BIO14.6 cardiomyopathic hamsters. The rAAV/S16EPLN treatment enhanced myocardial SR Ca^{2+} uptake and suppressed progressive impairment of left ventricular (LV) systolic function and contractility for 28–30 weeks, thereby protecting cardiac myocytes from cytopathic plasma-membrane disruption. Low LV systolic pressure and deterioration in LV relaxation were also largely prevented by rAAV/S16EPLN treatment. Thus, transcoronary gene transfer of S16EPLN via rAAV vector is a potential therapy for progressive dilated cardiomyopathy and associated heart failure.

Heart failure remains a leading cause of mortality, and this reflects a lack of therapies targeted to the underlying biological processes within cardiomyocytes that lead to chronic dysfunction[1–3]. Currently, heart transplantation is the only curative procedure for end-stage heart failure, but is limited by donor heart availability.

Defects in sarcoplasmic reticulum (SR) Ca^{2+} cycling have a crucial role in heart-failure progression. The ablation of phospholamban (PLN), an endogenous inhibitor of SR Ca^{2+}-ATPase 2 (SERCA2), completely prevents heart-failure phenotypes in a mouse model of dilated cardiomyopathy[4] caused by deficiency of a cytoskeletal protein, the muscle-specific LIM domain protein (MLP)[5]. PLN ablation has a beneficial effect in other cardiomyopathy mouse models[6,7], and the short-term adenoviral-mediated gene transfer of SERCA2, or the overexpression of upstream regulators of SERCA2, such as a β-adrenergic receptor kinase inhibitory peptide, delays development of cardiac dysfunction[8–11]. However, the real therapeutic value of the enhancement of SR Ca^{2+} cycling in chronically progressive heart failure has not been directly tested.

Here we developed a transcoronary *in vivo* gene-delivery system for stable, highly efficient and relatively cardiac-specific gene expression using recombinant adeno-associated virus (rAAV) vectors. We tested rAAV in a well-characterized BIO14.6 cardiomyopathic (CM) hamster model of progressive heart failure[12], which models a human genetic disease of limb-girdle muscular dystrophy type F (ref. 13). Subsequently, we constructed a pseudophosphorylated mutant PLN peptide (S16EPLN) in a rAAV vector, designed to mimic the conformational change in PLN followed by protein kinase A phosphorylation, and delivered it into BIO14.6 CM hamsters showing a progressive stage of dilated cardiomyopathy and heart failure. Here we show that chronic enhancement of SR Ca^{2+} cycling promotes the improvement of progressive cardiac contractile dysfunction and assists in cardiac cytoprotection in a CM animal model.

Establishment of rAAV *in vivo* gene-delivery

We developed the rAAV-mediated *in vivo* cardiac gene-delivery system based on a variation of a transcoronary adenoviral system[14]. *In vivo* gene delivery was initiated in BIO14.6 CM hamsters at 5–6 weeks of age, when contractile dysfunction is already evident. Studies using a rAAV/*lacZ* vector revealed an efficiency of 79 ± 8% (5 wk after gene transfer, $n = 6$) by counting cells stained positively for LacZ in BIO14.6 CM hamsters in both the left and right ventricular walls (Fig. 1*a*). In our earlier unpublished studies in normal hamsters, the gene transfer efficiency at 5 weeks with rAAV/*lacZ* was 63 ± 10% ($n = 4$), suggesting that

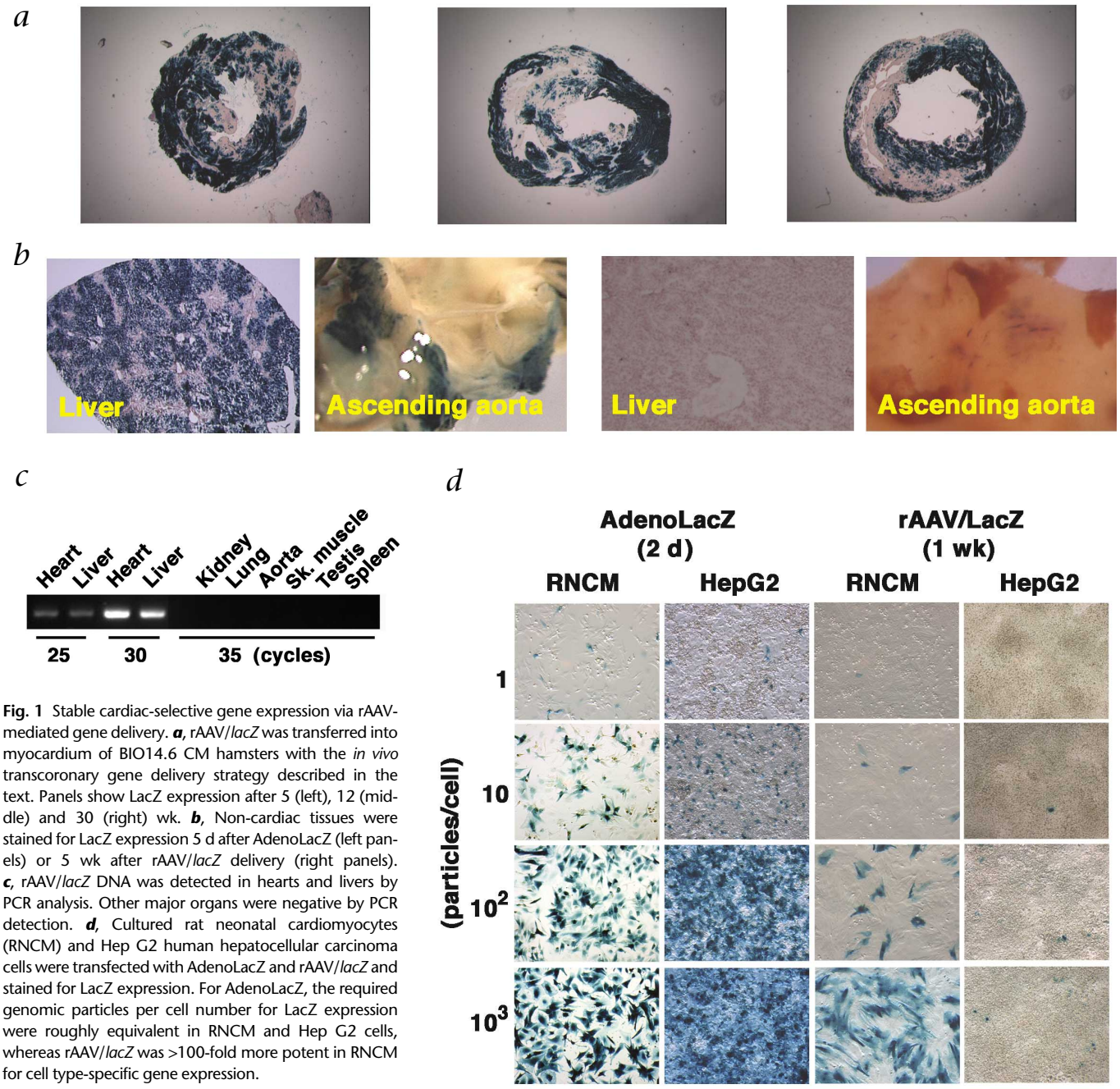

Fig. 1 Stable cardiac-selective gene expression via rAAV-mediated gene delivery. ***a***, rAAV/*lacZ* was transferred into myocardium of BIO14.6 CM hamsters with the *in vivo* transcoronary gene delivery strategy described in the text. Panels show LacZ expression after 5 (left), 12 (middle) and 30 (right) wk. ***b***, Non-cardiac tissues were stained for LacZ expression 5 d after AdenoLacZ (left panels) or 5 wk after rAAV/*lacZ* delivery (right panels). ***c***, rAAV/*lacZ* DNA was detected in hearts and livers by PCR analysis. Other major organs were negative by PCR detection. ***d***, Cultured rat neonatal cardiomyocytes (RNCM) and Hep G2 human hepatocellular carcinoma cells were transfected with AdenoLacZ and rAAV/*lacZ* and stained for LacZ expression. For AdenoLacZ, the required genomic particles per cell number for LacZ expression were roughly equivalent in RNCM and Hep G2 cells, whereas rAAV/*lacZ* was >100-fold more potent in RNCM for cell type-specific gene expression.

this markedly enhanced efficacy of gene delivery is not related to the cardiomyopathic changes in the CM hamsters. Long-term studies in CM hamsters revealed that LacZ expression was stable for 28–30 weeks with a slight decline in the efficiency (61 ± 5% at 28–30 weeks after gene transfer, $n = 6$, P = n.s., versus 5 wk) (Fig. 1*a*). The quantitative analysis of *lacZ* mRNA by northern blotting revealed identical expression of reporter gene at 5 weeks and 12–13 weeks, whereas at 28–30 weeks the *lacZ* mRNA levels were approximately 40% less (data not shown), probably due to advanced cardiomyopathic myocyte loss and replacement fibrosis in CM hamsters.

Surprisingly, the rAAV-mediated *lacZ* gene expression was largely restricted to the myocardium, with only trace expression of *lacZ* in the liver and ascending aorta (Fig. 1*b*). We could detect a few LacZ hepatocytes across the entire transverse hepatic sections. Skeletal muscle, lung, spleen, kidney, testis, descending aorta and brain were negative for staining (data not shown). Aside from expression in the heart, PCR analysis revealed a lower level of rAAV genomic DNA only in liver and none in other tissues, which were negative for LacZ staining (Fig. 1*c*). In contrast, the transfection of an adenoviral vector carrying *lacZ* (AdenoLacZ) by the identical method resulted in the highest gene expression in liver, as well as in several other tissues at lower levels (Fig. 1*b* and data not shown). The postmortem macroscopic examination of major organs and the microscopic examination of hearts and livers revealed no sign of oncogenicity in animals transferred with rAAV/*lacZ* for 28–30 weeks. Results from *in vitro* infection of isolated rat neonatal cardiomy-

ocytes and HepG2 cells with the rAAV/*lacZ* vector indicate the inherent cardiac-selective nature of the cardiac rAAV delivery method (Fig. 1*d*).

S16EPLN activates inotropic and lusitropic effects

PLN is a highly conserved 52-amino-acid peptide with two distinct phosphorylation sites: Ser16 (protein kinase A) and Thr17 (Ca^{2+}-calmodulin dependent kinase)[15,16]. The *in vivo* inotropic and lusitropic effects of β-adrenergic agonists are primarily regulated by the phosphorylation of PLN at Ser16 by protein kinase A and the dephosphorylation mediated primarily by the SR-bound phosphatase type 1. Therefore, we generated a pseudophosphorylated PLN mutant by replacing Ser16 with an acidic amino acid, glutamate, thereby introducing a negative charge at position 16 (S16EPLN) (Fig. 2*a*). Subsequently, we directly tested the ability of S16EPLN to have an inotropic effect in single cardiac cells utilizing an *in vivo* neonatal adenoviral vector delivery[4]. Expression of the S16EPLN led to the constitutive activation of contractility and relaxation in wild-type mouse ventricular cells in the absence of catecholamines (Fig. 2*b*). This effect of S16EPLN to constitutively enhance myocardial contractility and relaxation was further confirmed by generating transgenic mice, which harbor an S16EPLN gene driven by cardiac specific α-myosin heavy chain promoter (data not shown).

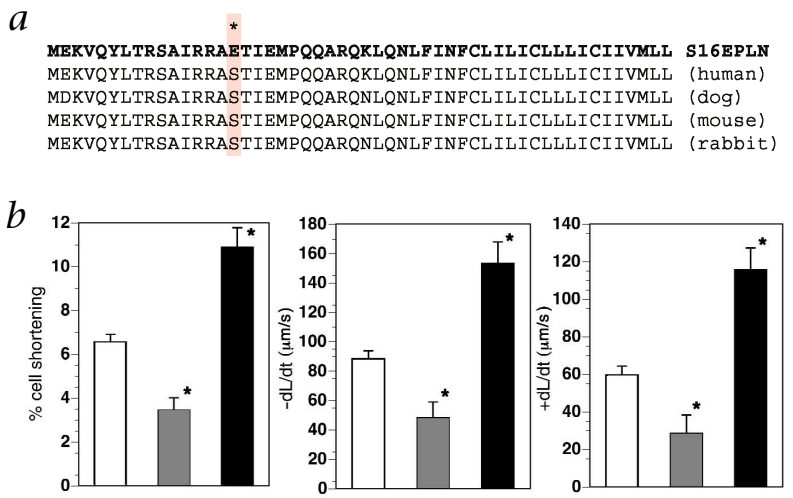

Fig. 2 The positive inotropic and lusitropic effects of a pseudophosphorylated mutant of phospholamban (S16EPLN). ***a***, PLN, a 52-amino-acid peptide, is highly conserved in mammals. The phosphorylation site at Ser(S)16 catalyzed by protein kinase A was mutated to Glu(E)16 to generate S16EPLN. ***b***, AdenoPLN and AdenoS16EPLN with AdenoEGFP, an expression marker vector, were injected in day 0–3 neonatal mouse hearts *in vivo* and the isolated cells were analyzed as described[4,38]. AdenoS16EPLN (■, $n = 19$) enhanced the contraction (% cell shortening and –dL/dt) and relaxation (+dL/dt) of single isolated cells at 3–4 wk post-gene-transfer analysis, whereas the over-expression of a wild type PLN peptide suppressed (■, $n = 17$). *, $P < 0.05$ versus control non-transfected cells (□, $n = 85$). Error bars are means ± s.e.m.

Suppression of heart-failure progression by rAAV/S16EPLN

We tested the physiological effects of *in vivo* rAAV mediated expression of S16EPLN on the progression of heart failure in BIO14.6 CM hamsters. At 5–6 weeks of age, the CM hamsters had clear evidence of cardiac dysfunction, with a significant decrease in percent fractional shortening (%FS) of the left ventricle (LV), as assessed by echocardiography (Fig. 3*a*). Subsequently, the CM hamsters developed progressive heart failure characterized by echocardiography with a marked decrease in %FS (at 12 wk: normal hamster, 44.0 ± 1.3, $n = 14$ versus CM hamster, 24.1 ± 1.4, $n = 10$; $P < 0.001$) and chamber dilation indicated by an increase in end-diastolic LV chamber dimension (LVEDD) (at 12 wk: normal hamster, 4.46 ± 0.10 mm, $n = 14$ versus CM hamster, 5.28 ± 0.13 mm, $n = 10$; $P < 0.001$). Here, we evaluated a total of 59 CM hamsters at the age of 5–6 weeks by echocardiography and randomly separated them into three groups (22 rAAV/S16EPLN treatment, 19 rAAV/*lacZ* treatment, 18 animals without virus treatment), immediately followed by the gene delivery. These three groups of CM animals did not show any statistical variation in cardiac function before gene transfer. Subsequently, we examined animals by echocardiography at 5, 12–13 weeks and 28–30 weeks after gene transfer (Fig. 3). To monitor for any independent effects of the rAAV vectors or the gene-delivery protocol itself, five non-operated animals that did not receive any treatment and another group of five saline-injected animals that were exposed to the *in vivo* delivery protocol with a saline injection were compared at five weeks after the procedure. Statistical comparison of these two groups for echocar-

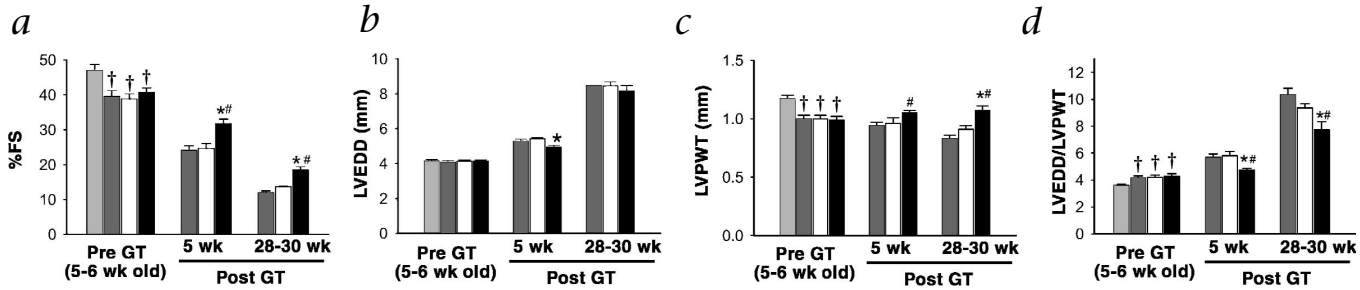

Fig. 3 Chronic suppression of heart-failure development by rAAV/S16EPLN gene delivery. Normal hamsters (■, $n = 14$), BIO14.6 CM hamsters without virus treatment (■, $n = 10$ for 5 wk; $n = 11$ for 28–30 wk), rAAV/*lacZ*-treated CM hamsters (□, $n = 11$ for 5 wk; $n = 7$ for 28–30 wk), and rAAV/S16EPLN-treated CM hamsters (■, $n = 11$ for 5 wk; $n = 6$ for 28–30 wk) were analyzed by echocardiography. ***a*** and ***b***, The rAAV/S16EPLB treatment chronically suppressed the decline in fractional shortening (%FS) (*a*) with a mild effect on LV end-diastolic chamber size (LVEDD) (*b*) in BIO14.6 CM hamsters. ***c*** and ***d***, LV posterior-wall thickness (LVPWT) was higher in rAAV/S16EPLN-treated animals, resulting in a lower value of an estimate of diastolic wall stress (LVEDD/LVPWT) (*d*). †, $P < 0.05$ versus normal hamster; *, $P < 0.05$ versus rAAV/*lacZ*; #, $P < 0.05$ versus no virus treatment. Error bars are means ± s.e.m.

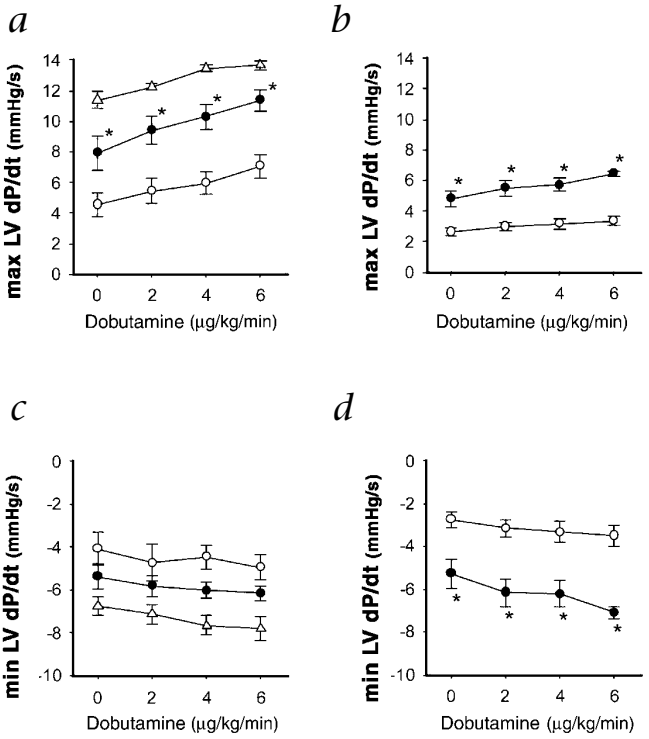

Fig. 4 Sustained improvement of the cardiac contractility and relaxation. ***a*–*d***, LV contractility (max LV dP/dt) (*a* and *b*) and relaxation (min LV dP/dt) (*c* and *d*) were assessed at baseline and in response to the increasing doses of dobutamine at 5 wk (*a* and *c*) and 28–30 wk (*b* and *d*) after rAAV/S16EPLN delivery (●, $n = 6$ for 5 wk and $n = 4$ for 28–30 wk) and rAAV/*lacZ* delivery (○, $n = 7$ for 5 wk and $n = 4$ for 28–30 wk) in BIO14.6 CM hamsters. In parallel, age-matched normal control F1B hamsters (△, $n = 7$) were analyzed. *, $P < 0.05$ versus rAAV/*lacZ*. Error bars are means ± s.e.m.

diographic and hemodynamic analysis did not yield any significant differences (data not shown). Therefore, we employed non-operated animals for the later stages of analysis of the post gene transfer (post-GT) groups as 'no-virus' animals. In addition, because of the limited sample number of this late-stage animal analysis, the echocardiographic measurements of 7 age-matched BIO14.6 CM hamsters were added to that of the four original 28–30-week post-GT no virus animals (total $n = 11$). At each time point, we sampled paired animals for high-fidelity LV pressure measurements and subjected them to further histological and biological analysis. One rAAV/S16EPLN animal and a no-virus animal were lost during the post-GT observation.

rAAV/S16EPLN treatment strongly suppressed the progressive impairment of cardiac contractility in the rAAV/*lacZ*-treated animals at five weeks post-GT, with a mild effect on chamber dilation (Fig. 3*a* and *b*). An inotropic effect of rAAV/S16EPLN treatment was evident for 28–30 weeks (Fig. 3*a*). The maximum first derivative of LV pressure (max LV dP/dt) was greatly improved toward normal levels by rAAV/S16EPLN treatment at baseline, as well as in response to the increasing doses of the β-adrenergic agonist, dobutamine, at 5 weeks after gene delivery (Fig. 4*a*), and remained increased by 100% at baseline over 28–30 weeks in the rAAV/S16EPLN-transferred animals compared with rAAV/*lacZ*-transferred animals (Fig. 4*b*). Two independent parameters of cardiac relaxation, the minimum first derivative of LV pressure (min LV dP/dt) (Fig. 4*d*) and exponential Tau (the time constant of LV pressure decay) (Fig. 5*c*) were markedly improved at 28–30 weeks after the rAAV/S16EPLN delivery. In fact, diastolic wall stress estimated by LVEDD/LVPWT was significantly lower (Fig. 3*d*), and wall thickness (LVPWT) was well preserved over 28–30 weeks in rAAV/S16EPLN-treated animals in contrast to the other two animal groups (Fig. 3*c*). These findings suggest that rAAV/S16EPLN treatment enhanced diastolic cardiac function and protected myocardial cell loss in CM hamsters. The cross-sectional analysis of individual cardiomyocytes from three groups (no-virus, rAAV/*lacZ* and rAAV/S16EPLN-treated animals) did not indicate significant hypertrophic changes in rAAV/S16EPLN-treated cardiomyocytes (data not shown), suggesting the effects of S16EPLN treatment are on SR Ca^{2+} cycling versus calcium signaling for hypertrophy.

The rAAV/S16EPLN-treated hamsters had significantly higher peak LV systolic pressure than rAAV/*lacZ*-treated CM hamsters over 28–30 weeks without a significant difference in heart rate (Fig. 5*a* and *b*). This high systolic LV pressure in rAAV/S16EPLN-treated animals, along with a sustained improvement in β-adrenergic responsiveness (Fig. 4*b* and *d*), which should improve exercise ability, suggests the significant improvement of the clinically relevant endpoints in late-stage heart failure.

The studies of rAAV/S16EPLN delivery were then expanded to include a more advanced stage of BIO14.6 CM hamsters. 26 animals (11–13 weeks old: %FS, 24.2 ± 0.4, $n = 26$ versus normal hamsters, 44.0 ± 1.3, $n = 14$; $P < 0.001$) were randomly separated into four groups (8 rAAV/S16EPLN treatment, 9 rAAV/*lacZ* treatment, 4 saline-injection and 5 non-operated), immediately followed by the gene-transfer procedure. There were three early deaths related to surgical procedures out of 21 animals (2 rAAV/*lacZ*-treated animals and 1 rAAV/S16EPLN-treated animal), and we did not observe any increase in delayed mortality or subsequent major physical complications. The *lacZ* gene expression in this late-stage cardiomyopathy group was almost identical to that of our gene-transfer studies in 5-week-old animals (data not shown). These four groups of CM animals did not show any statistical variation in cardiac function before gene transfer, and the data from 4 saline-injected and 5 non-operated animals are combined as a no-virus group. An inotropic effect of rAAV/S16EPLN was evident over 5 weeks, with a substantial improvement in %FS (rAAV/S16EPLN: 24.3 ± 0.8, $n = 7$, versus rAAV/*lacZ*, 21.1 ± 0.3, $n = 7$, $P < 0.05$; versus no-virus, 21.4 ± 0.6, $n = 9$; $P < 0.05$). The diastolic wall stress estimated by LVEDD/LVPWT was significantly lower (rAAV/S16EPLN: 6.5 ± 0.1, $n = 7$, versus rAAV/*lacZ*,

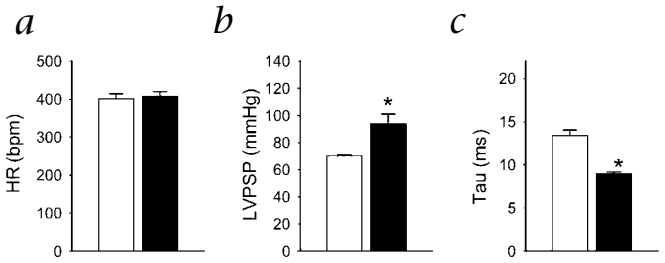

Fig. 5 Sustained hemodynamic effects of rAAV/S16EPLN gene delivery. ***a*–*c***, Heart rate (HR) remained stable (*a*) and the peak LV systolic pressure (LVPSP) was maintained high (*b*) and Tau was largely suppressed (*c*) at 28–30 wk after rAAV/S16EPLN gene delivery (■, $n = 4$) in BIO 14.6 CM hamsters compared with rAAV/*lacZ* (□, $n = 4$). *, $P < 0.05$ versus rAAV/*lacZ*. Error bars are means ± s.e.m.

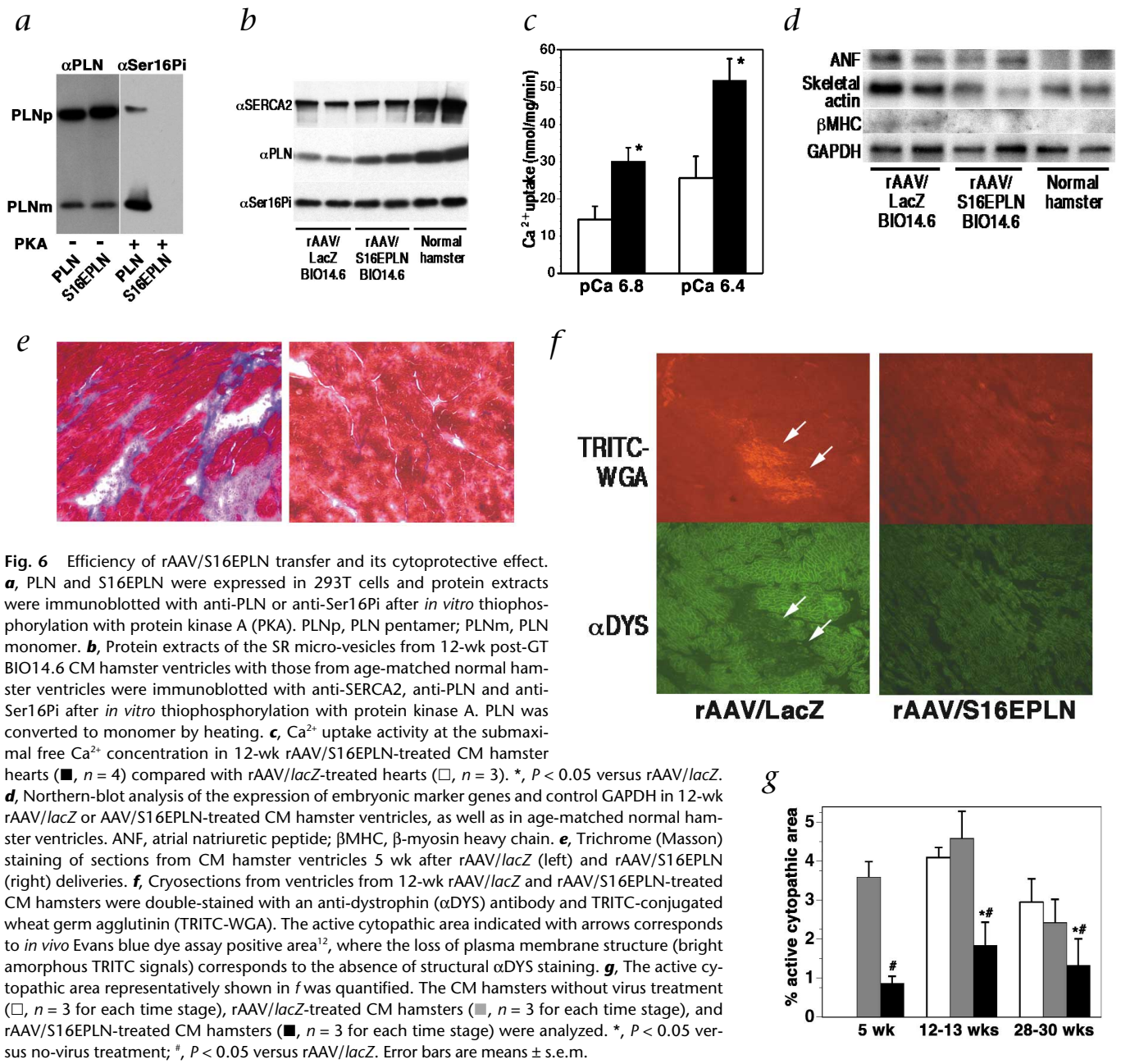

Fig. 6 Efficiency of rAAV/S16EPLN transfer and its cytoprotective effect. ***a***, PLN and S16EPLN were expressed in 293T cells and protein extracts were immunoblotted with anti-PLN or anti-Ser16Pi after *in vitro* thiophosphorylation with protein kinase A (PKA). PLNp, PLN pentamer; PLNm, PLN monomer. ***b***, Protein extracts of the SR micro-vesicles from 12-wk post-GT BIO14.6 CM hamster ventricles with those from age-matched normal hamster ventricles were immunoblotted with anti-SERCA2, anti-PLN and anti-Ser16Pi after *in vitro* thiophosphorylation with protein kinase A. PLN was converted to monomer by heating. ***c***, Ca^{2+} uptake activity at the submaximal free Ca^{2+} concentration in 12-wk rAAV/S16EPLN-treated CM hamster hearts (■, $n = 4$) compared with rAAV/*lacZ*-treated hearts (□, $n = 3$). *, $P < 0.05$ versus rAAV/*lacZ*. ***d***, Northern-blot analysis of the expression of embryonic marker genes and control GAPDH in 12-wk rAAV/*lacZ* or AAV/S16EPLN-treated CM hamster ventricles, as well as in age-matched normal hamster ventricles. ANF, atrial natriuretic peptide; βMHC, β-myosin heavy chain. ***e***, Trichrome (Masson) staining of sections from CM hamster ventricles 5 wk after rAAV/*lacZ* (left) and rAAV/S16EPLN (right) deliveries. ***f***, Cryosections from ventricles from 12-wk rAAV/*lacZ* and rAAV/S16EPLN-treated CM hamsters were double-stained with an anti-dystrophin (αDYS) antibody and TRITC-conjugated wheat germ agglutinin (TRITC-WGA). The active cytopathic area indicated with arrows corresponds to *in vivo* Evans blue dye assay positive area[12], where the loss of plasma membrane structure (bright amorphous TRITC signals) corresponds to the absence of structural αDYS staining. ***g***, The active cytopathic area representatively shown in *f* was quantified. The CM hamsters without virus treatment (□, $n = 3$ for each time stage), rAAV/*lacZ*-treated CM hamsters (■, $n = 3$ for each time stage), and rAAV/S16EPLN-treated CM hamsters (■, $n = 3$ for each time stage) were analyzed. *, $P < 0.05$ versus no-virus treatment; #, $P < 0.05$ versus rAAV/*lacZ*. Error bars are means ± s.e.m.

7.3 ± 0.3, $n = 7$; $P < 0.05$; versus no-virus, 7.6 ± 0.2, $n = 9$; $P < 0.05$), with a higher value of LVPWT (rAAV/S16EPLN: 1.02 ± 0.01 mm, $n = 7$, versus rAAV/*lacZ*, 0.94 ± 0.03 mm, $n = 7$; $P < 0.05$; versus no-virus, 0.91 ± 0.02 mm, $n = 9$; $P < 0.05$) in animals treated with rAAV/S16EPLN.

We also confirmed that the effect of S16EPLN was not specific for BIO14.6 CM hamsters. We transferred AdenoS16EPLN into the myocardium of MLP-deficient CM mouse neonates and documented a significant improvement in the basal contractility and relaxation of transgene-positive cardiomyocytes isolated at 4–6 weeks after neonatal gene transfer (data not shown).

We analyzed the persistent expression of the S16EPLN peptide by immunoblotting with an antibody against PLN and a Ser16 phosphorylation-specific antibody to PLN (anti-Ser16Pi) in SR micro-vesicles isolated from the CM hamster ventricles after rAAV/S16EPLN gene delivery. The anti-PLN recognized both the wild-type PLN and S16EPLN, whereas anti-Ser16Pi did not detect S16EPLN after the treatment with the catalytic subunit of protein kinase A in the presence of adenosine-5′-[γ-thio] triphosphate, a condition where wild-type PLN was fully thiophosphorylated (Fig. 6*a*). Accordingly, the densitometric measurement of anti-PLN and anti-Ser16Pi immunoblot signals was used to estimate the relative molecular ratio of S16EPLN peptide versus the endogenous PLN, which was 3–5:1 (Fig. 6*b*).

We also studied S16EPLN expression in gene-transferred CM hamster myocardium by Ca^{2+} uptake analysis of isolated SR micro-vesicles (Fig. 6*c*). At pCa ($-\log_{10}[Ca^{2+}]$) of 6.8 and 6.4, SERCA2 activity was enhanced by 109 and 103%, respectively, in 12-week rAAV/S16E-treated versus rAAV/*lacZ*-treated animals. Thus, the S16EPLN treatment markedly increases SR Ca^{2+} cycling in the heart. There was no significant difference in the protein level of SERCA2 expression in the ventricles of rAAV/S16EPLN-

treated animals compared with rAAV/*lacZ*-treated animals; however, SERCA2 expression as well as PLN expression in CM hamsters are generally down-regulated compared with age-matched normal hamsters (Fig. 6*b*).

The mRNA expression levels of several embryonic gene markers were analyzed for the efficacy of rAAV/S16EPLN treatment. Three representative markers, atrial natriuretic peptide, skeletal actin and β-myosin heavy chain, were upregulated in CM hamsters compared with control normal hamsters, as in other heart failure/cardiomyopathy models. rAAV/S16EPLN treatment largely suppressed the increase in these marker-gene expressions (Fig. 6*d*). Thus, the slight increase in wall thickness recorded in the rAAV/S16EPLN-treated animals most likely reflects a reduction in the progressive thinning of the ventricular wall accompanying the cardiomyopathic process.

rAAV/S16EPLN limits progressive myocardial injury

Catecholamine toxicity is known to cause cardiac myocyte injury and cell death, and a recent study has documented the development of dilated cardiomyopathy in the mice over-expressing a constitutively active protein kinase A in the heart[17]. Because S16EPLN constitutively activates cardiac contractility in the absence of increases in cAMP stimulation *per se*, we questioned if chronic stimulation of cardiac performance by targeting the downstream point in excitation-contraction coupling would lead to an increase in cardiomyocyte injury.

Paradoxically, the histological analysis at five weeks after gene transfer revealed that cardiac interstitial fibrosis, which is characteristic of the hamster and some types of human dilated cardiomyopathies[18], was markedly decreased by rAAV/S16EPLN treatment (Fig. 6*e*). Moreover, the degree of cytopathic myocardial cell injury, as assessed by the stability of the dystrophin complex and the loss of plasma membrane structure, was significantly diminished in the rAAV/S16EPLN-treated animals over 28–30 weeks (Fig. 6*f* and *g*). This cell-injury area estimation only indicates the active cytopathic process within a given myocardial region, and does not include the tissue that is already replaced by fibrosis. Our previous report[12] and unpublished observations indicate that normal hamsters (F1B strain) exhibit no detectable area of cardiac injury, as assessed by essentially the same methods. The intensity of cytopathic effects observed in rAAV/*lacZ*-treated animals was the same as in the animals without virus treatment (Fig. 6*g*), suggesting that these histological differences are not due to a pathological effect of the rAAV vectors or the *lacZ* gene but rather due to the base-line progressive disease process in CM hamsters. Nevertheless, these studies support the concept that one of the mechanisms by which chronic expression of S16EPLN suppresses heart-failure progression is via reductions in wall stress and associated effects on myocardial cell injury.

Discussion

Gene therapy for dilated cardiomyopathy, heart failure and other chronic cardiac muscle diseases has been an elusive goal for many years. The therapeutic utility has been mitigated by the relatively short-term expression, low efficiency and frank myocarditis that accompanies the use of adenoviral vectors. Clearly, for gene therapy to become a feasible therapeutic strategy for chronic cardiac muscle diseases, it will be necessary to express the target gene for many months at high levels to drive a change in the global cardiac chamber phenotype. In addition, strategies for transcoronary *in vivo* gene delivery would be essential to allow optimal clinical implementation.

In this regard, there has been widespread interest in developing new protocols to achieve high-efficiency transcoronary cardiac-gene delivery. Some attempts have used vascular permeabilizing agents, including histamine *in vivo*[19] or *in vitro*[20]. Other studies have used transcoronary infusion of the virus vector in allografts protected with cardioplegic solutions[21,22]. The *in vivo* use of rAAV, which is expected to achieve long-term gene expression, has been limited in direct cardiac muscle injection procedures in the myocardial wall which results in relatively low efficiency[23,24]. Our study describes a new *in vivo* protocol for the transcoronary delivery of genes with rAAV vectors that holds considerable promise for the long-term molecular remodeling of the failing myocardium. Using this transcoronary *in vivo* rAAV vector delivery system, we have achieved high efficiency (over 60% of the ventricular muscle) and long-term expression (over 7 months of continuous expression after the initial gene delivery). In addition, the gene was expressed in a relatively cardiac-selective manner without evidence of local inflammation. Several groups have reported sustained transgene expression via rAAV vectors in skeletal muscle[25–27]. Notably, widely used AAV type 2 vectors interact with membrane-associated heparan sulfate proteoglycans (HSPGs)[28], and in skeletal muscle, rAAV type 2 predominantly transduces slow myofibers[29], which share many characteristics with cardiomyocytes. In this regard, the high level expression of glypican[30], glycosylphosphatidylinositol-anchored HSPG, in adult cardiomyoctyes might serve as a high-affinity binding site for rAAV. As opposed to the high-efficiency cardiac gene-expression profile in this study, direct intraportal vein injection of a higher amount of rAAV is reported to transduce only 2–5% of hepatocytes in mice[31]. Furthermore, Nakai *et al*. reported that CMV promoter activity was rapidly downregulated in hepatocytes after rAAV-mediated gene delivery[32]. Thus, we speculate that relatively cardiac-selective gene expression via rAAV vectors reflects an inherent muscle avidity for the rAAV vector itself, as well as the relative tissue selectivity of CMV enhancer/promoter and greater first-pass uptake through the coronary circulation.

MLP-PLN double-knockout mice displayed a complete, chronic prevention of the onset of heart failure[4]. However, it remained unclear whether PLN inhibition would prove therapeutically efficacious after the onset of cardiomyopathy, and whether this effect would carry a long-term benefit over several months of inhibition. Using an *in vivo* rAAV delivery system, we show that the sustained expression of the pseudophosphorylated PLN mutant leads to the activation of SR Ca^{2+} cycling, as assessed by directly measuring the Ca^{2+} uptake activity of SR micro-vesicles from CM hamsters. Chronic S16EPLN expression constitutively activates LV contractility and relaxation in the absence of adrenergic stimuli, in a manner analogous to the PLN-null mutant mice, although LV dilation was only partially prevented in the progressive disease process of CM hamsters. The *in vivo* therapeutic effects of the transcoronary AAV/S16EPLN delivery are not directly related to dystrophin-glycoprotein complex defects, which are responsible for the myopathic process in CM hamsters[12,13,33], suggesting that this therapeutic strategy may not be limited to genetic causes of cardiomyopathy. In fact, our preliminary studies support the efficacy of *in vivo* rAAV/S16EPLN treatment in a rat post-myocardial infarction heart-failure model in triggering the chronic improvement of cardiac dysfunction, suggesting the efficacy of rAAV/S16EPLN gene transfer across species in multiple acquired and genetic forms of heart failure.

In conclusion, chronic improvement of excitation-contraction coupling in a cAMP-independent manner with rAAV/S16EPLN gene-delivery system represents a novel therapeutic strategy for slowing heart-failure progression. The effects of chronic S16EPLN expression to largely maintain peak LV systemic pressure and to reduce an index of diastolic wall stress, suggest that S16EPLN gene therapy may allow for the chronic improvement of critical features of human end-stage heart failure, particularly in settings where the processes of cell loss and fibrosis are chronic and slower and less severe than in the CM hamster. Given that there is currently no therapy for end-stage heart failure aside from heart transplantation, future studies are warranted to translate these findings into larger animal models and the clinical setting.

Methods

Virus vectors. rAAV type-2 vectors were made by three plasmids cotransfection method as described[34]. To generate S16EPLN, PCR-based site-directed mutagenesis was carried out following the manufacturer's protocols (Stratagene, La Jolla, California). Human PLN cDNA subcloned in pBluescript SKII was used as the PCR template. Subsequently, S16EPLN was inserted into a *cis*-vector with CMV enhancer/promoter and SV40 late polyadenylation signal fragments, which are from pCI (Promega, Madison, Wisconsin). rAAV vectors were recovered from three sequential CsCl gradients, or by a single-step heparin-column method[35]. The vector genome titer was determined by real-time PCR, using TaqMan (Perkin Elmer Biosystems, Foster City, California). AdenoLacZ, AdenoPLN, AdenoS16EPLN and AdenoEGFP, which are recombinant adenovirus type-5 vectors with a CMV enhancer/promoter, were prepared as described[4,14].

Animal procedures. rAAVs were delivered into the coronary arteries of BIO14.6 CM hamsters following the protocol modified from the adenovirus vector transfer procedure we established recently[14]. Briefly, hamsters were anesthetized with sodium pentobarbital (75 mg/kg i.p.), ventilated, and the right carotid artery was cannulated with a catheter placed at the aortic root. The animals were subjected to general hypothermia by external cooling until the core temperature reached below 26 °C. The ascending aorta and pulmonary arteries were then occluded and histamine pretreatment was administrated into the aorta (20 mmol/L, volume; 2.5 μL/gram, body weight) for 3 min. The virus solution was then bolus-injected and the occlusions were released 0.5–1 min later, and animals were resuscitated. 1×10^9 virus particles per gram body weight of rAAV vectors were used. All of the animal experimental protocols received formal Institutional Review Board approval.

Post-gene transfer analysis. The methods for cardiac functional analysis of CM hamsters by echocardiography and cardiac catheterization were described[18]. For determination of gene-transfer efficiency, the heart was transversely sliced and 10-mm sections were obtained from basal and mid-LV slices. The percentage of β-gal-positive myocyte nuclei was estimated using a stereological point-counting method[14]. For cytopathic area analyses, 10-mm cryosections (6 transmural sections from each animal) were double stained with an antibody against the C-terminal domain of dystrophin (Novocastra Laboratory, Newcastle, UK) followed by FITC-conjugated anti-mouse IgG antibody incubation with TRITC-conjugated wheat germ agglutinin (Sigma, St. Louis, Missouri)[12]. The composite image was used to define the injured cell area, which corresponds to the area positive for the *in vivo* Evans blue dye assay, with loss of plasma membrane structure and absence of dystrophin staining[12]. The identified cytopathic area was quantified by the use of NIH image software (http://rsb.info.nih.gov/nih-image). SR microvesicles were purified by a sequential centrifugation as described[36]. Protein was determined, thiophosphorylated with the catalytic subunit of protein kinase A (Sigma)[37] and then subjected to immunoblotting analysis with anti-PLN and anti-Ser16Pi antibodies (Upstate, Lake Placid, New York) and an anti-SERCA2 antibody (Affinity Bioreagents, Golden, Colorado), or calcium-45 uptake analysis[36].

Statistics. Comparison between 2 groups was performed by Student's unpaired or paired *t*-tests. Comparisons between repeated values were done by repeated measure of ANOVA, followed by Student–Newman–Keuls post hoc test. All values are mean ± s.e.m.

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Competing interests statement

The authors declare competing financial interests: see the website (http://medicine.nature.com) for details.

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