Long-Term Cardiac-Targeted RNA Interference for the Treatment of Heart Failure Restores Cardiac Function and Reduces Pathological Hypertrophy

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**Background**—RNA interference (RNAi) has the potential to be a novel therapeutic strategy in diverse areas of medicine. Here, we report on targeted RNAi for the treatment of heart failure, an important disorder in humans that results from multiple causes. Successful treatment of heart failure is demonstrated in a rat model of transaortic banding by RNAi targeting of phospholamban, a key regulator of cardiac Ca\(^{2+}\) homeostasis. Whereas gene therapy rests on recombinant protein expression as its basic principle, RNAi therapy uses regulatory RNAs to achieve its effect.

**Methods and Results**—We describe structural requirements to obtain high RNAi activity from adenoviral and adeno-associated virus (AAV9) vectors and show that an adeno-viral short hairpin RNA vector (AdV-shRNA) silenced phospholamban in cardiomyocytes (primary neonatal rat cardiomyocytes) and improved hemodynamics in heart-failure rats 1 month after aortic root injection. For simplified long-term therapy, we developed a dimeric cardiotropic adeno-associated virus vector (rAAV9-shPLB) to deliver RNAi activity to the heart via intravenous injection. Cardiac phospholamban protein was reduced to 25%, and suppression of sarcoplasmic reticulum Ca\(^{2+}\) ATPase in the HF groups was rescued. In contrast to traditional vectors, rAAV9 showed high affinity for myocardium but low affinity for liver and other organs. rAAV9-shPLB therapy restored diastolic (left ventricular end-diastolic pressure, dp/dt\(_{\text{min}}\)), systolic (fractional shortening) functional parameters to normal ranges. The massive cardiac dilation was normalized, and cardiac hypertrophy, cardiomyocyte diameter, and cardiac fibrosis were reduced significantly. Importantly, no evidence was found of microRNA deregulation or hepatotoxicity during these RNAi therapies.

**Conclusions**—Our data show for the first time the high efficacy of an RNAi therapeutic strategy in a cardiac disease.

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**Key Words:** heart failure ■ RNA interference ■ gene therapy ■ hypertrophy ■ microRNAs

RNA interference (RNAi) has been investigated as a new treatment option in infectious diseases and cancer. We report here on a strategy for the treatment of a cardiac disease by locally induced RNAi. Heart failure (HF) remains a leading cause of mortality in the developed world. Current drug treatment has limited efficacy, and in advanced HF, left ventricular (LV) assist devices or heart transplantation are the ultimate options. For long-term treatment of HF, novel approaches are currently being explored, including gene and cell therapies, whereas the use of RNAi to modulate cardiac gene functions has not yet been evaluated. Whereas gene therapy rests on recombinant protein expression as its basic principle, RNAi therapy instead uses small regulatory RNAs to achieve its effect. Targeting, kinetics, and toxicity of these RNAs in vivo are grossly different from those of recombinant proteins and not yet well characterized.

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Although HF may result from multiple causes, defective cardiac Ca\(^{2+}\) homeostasis has been identified as an important...
nal common pathway. In the present study, we show successful treatment of HF by RNAi targeting of a key regulator of cardiac Ca\(^{2+}\) homeostasis. Malfunction of the failing heart is due in part to dysfunction of the phospholamban-controlled sarcoplasmic reticulum Ca\(^{2+}\) ATPase pump (SERCA2a), which results from reduced SERCA2a expression and/or phospholamban phosphorylation.\(^1\) Unphosphorylated phospholamban keeps the Ca\(^{2+}\) affinity of SERCA2a low, which results in decreased sarcoplasmic reticulum (SR) Ca\(^{2+}\) uptake, slowed relaxation, and decreased SR Ca\(^{2+}\) load, whereas phospholamban phosphorylation in response to ß-adrenergic stimulation relieves this inhibition.\(^2\) Germ line ablation of the phospholamban gene, gene transfer for dominant-negative phospholamban mutants,\(^3,4\) phospholamban antisense RNAs,\(^5\) and intracellular inhibitory phospholamban antibodies\(^6,7\) have been used to increase SERCA2A activity and to rescue HF models.\(^8\) RNAi mediated by chemically synthesized small interfering RNAs in cardiomyocytes showed very low efficacy and stability even in vitro,\(^9\) and pharmacological approaches to phospholamban modulation have failed thus far. Fundamental limitations of synthetic small interfering RNAs are their rapid degradation in plasma and target cells and the unsolved problem of achieving adequate transfer and targeting in vivo. Viral vectors have the potential to overcome these limitations, and we previously showed highly efficient phospholamban ablation in primary neonatal rat cardiomyocytes (NRCMs) by an adenoviral RNAi vector.\(^10\) No change in the expression of other cardiac proteins, including Ca\(^{2+}\)-handling proteins, occurred, which indicates high target specificity.

In the present study, we evaluated the principle of RNAi against phospholamban for short-term and long-term treatment of HF in vivo. Functional characterization of a series of vectors and the determinants of their efficacy was followed by investigation of both an optimized recombinant adenoviral phospholamban antisense virus pseudotype 9 vector (rAAV9) alongside a traditional adenoviral vector in an animal model of HF and cardiomyocyte microRNAs (miRNAs) during RNAi therapy.

### Methods

#### Development of Recombinant Adenoviral and Adeno-Associated Virus Vectors

rAAV vectors were developed for the in vitro studies as pseudotyped rAAV6 and for the in vivo work as rAAV9. Throughout all in vitro and in vivo studies, we used only self-complementary adenoviral vector genomes owing to their enhanced performance compared with single-stranded adeno-associated virus vectors. Vector maps are shown in Figure 1A. For details of all methods used, see the online-only Data Supplement.

#### Vector Production and Purification, Quality Assessment, and Titration

rAAV9-shGFP (rAAV9 generating small hairpin RNA [shRNA] to silence green fluorescent protein [GFP]) and rAAV9-shPLB (rAAV9 generating shRNA to silence phospholamban) were produced by a 2-plasmid protocol described previously\(^11\) with the following modifications: 293T cells were grown in triple flasks for 24 hours (DMEM, 10% FBS) before the addition of calcium phosphate precipitate. After 72 hours, the virus was purified from Benzonase-treated (Merck, Darmstadt, Germany) cell culture lysates over an iodixanol density gradient, followed by heparin-agarose type I affinity chromatography. Finally, viruses were concentrated and formulated into lactated Ringer solution with a Vivaspin 20 centrifugal concentrator (ISC BioExpress, Kaysville, Utah) with 50K molecular weight cutoff and stored at \(-80^\circ\text{C}\). Vector stock biochemical purity (>95%) was assessed by silver staining after electrophoresis. Genome-containing particles were determined by a real-time polymerase chain reaction approach.

#### Vector Evaluation in Primary Cardiomyocytes

Primary NRCMs are suitable to pretest any RNAi-based cardiac therapy before its definitive test in vivo because although developmentally regulated, the SERCA2a/phospholamban system functions well in NRCMs, and adenoviral gene transfer strategies that target the SERCA2a/phospholamban system have been successful in both neonatal and adult cardiomyocytes. Although both cell types are suited for in vitro pretesting, a number of other differences between cultured cardiomyocytes and the intact heart in vivo render any in vitro study of RNA-based therapies in cultured cells preliminary.

NRCMs were prepared from ventricular tissue of 1- to 3-day-old Wistar rat pups and grown in 6-well dishes. Phospholamban, troponin I, sodium-calcium exchanger, and SERCA2A mRNA or protein expression were determined by Northern and Western blot analyses, as described previously.\(^12\) Transients were measured during electrical stimulation at 1 Hz after loading of NRCMs with 8 \(\mu\text{mol/L Fluo-4 AM for 20 minutes (image capture at 120 Hz, 8.3 ms per image). Five treatment groups of NRCMs were studied: AAV9-shPLB (n=26 cells), AAV9-shGFP (n=26 cells), adenovirus (AdV)-shPLB (n=71 cells), AdV-shGFP (n=49 cells), and untreated control cells (n=32). The amplitude of the transient (systolic [Ca\(^{2+}\)]; F/F\(_0\)), its time to peak, and the time constant (\(\tau\)) of its decay were measured.

#### Induction of Hypertrophy

Phenylephrine at a concentration of 100 \(\mu\text{mol/L}\) was used in portions of the in vitro studies as a hypertrophic stimulus. TaqMan assays (Applied Biosystems, Foster City, Calif) to quantify the cellular miRNAs were performed in NRCMs, either under baseline conditions or in the presence of phenylephrine, or in rat hearts. The agent was added on day 2 of culture, either alone or together with the respective RNAi vector.

#### miRNA Assays

In search of possible influences of vector-derived shRNAs on cardiomyocyte miRNAs, we used TaqMan assays to quantify 2 miRNAs with known cardiac functions.\(^12\) Transsaortic Banding and Serial Echocardiographic Assessment

Four-week-old Sprague Dawley rats (weight 70 to 80 g) were anesthetized with pentobarbital (65 mg/kg IP) and placed on a ventilator. A suprasternal incision was made to expose the aortic root, and a tantalum clip with an internal diameter of 0.58 mm was placed on the ascending aorta. Animals in the sham group underwent a similar procedure without insertion of a clip. In the animals that were aortic-banded, we waited 25 to 30 weeks for the animals to develop LV dilatation and a decrease in ejection fraction by 25% before cardiac gene transfer. Of the initial 56 that underwent pressure-overload hypertrophy, only 40 animals survived; these were further grouped to receive either AdV-shGFP (n=10), AdV-shPLB (n=10), rAAV9-shGFP (n=10), or rAAV9-shPLB (n=10). Operators performing the echocardiographic studies were blinded in terms of the animal groups they were studying.

#### Cardiac Distribution of rAAV9 Vectors After Intravenous Injection

Rats were transduced either with a vector rAAV9-GFP that expresses the marker protein GFP or with saline. One month after delivery of rAAV9-GFP or saline, hearts were removed and visualized under a fluorescence system (Maestro In Vivo Imaging, Cambridge Research & Instrumentation, Inc, Woburn, Mass) at 510 nm with a single excitation peak at 490 nm of blue light. In addition to this...
Figure 1. RNAi vectors for HF therapy. A, Maps of the RNAi vector genomes. Self-complementary “dimeric” adenovirus genomes (rAAV2) were pseudotyped into rAAV6 or rAAV9 capsids for studies in cardiomyocytes in vitro or HF rats in vivo, respectively. rAAV-shPLB has the same U6-shRNA transcription system as AdV-shPLB and the corresponding shGFP control vectors. Two further RrAAV vectors carried CMV-GFP or CMV-β-intron expression cassettes in head-to-head orientation with the U6-shPLB sequence and separated from them by a bovine growth hormone (bgH) terminal signal. To assess the influence of the CMV promoter on vector function, the CMV-free variants rAAV-shPLB-GFP and rAAV-shPLB-β-intron were constructed. Note that the genomes in the rAAV6 vectors for in vitro work are identical to those shown here for the rAAV9 vectors. B, Comparison of the target silencing efficacy of shRNA vectors in NRCMs. Cells were harvested 5 (top) or 10 (bottom) days, respectively, after treatment with the respective vector at the dose (in particles per cell [p/c]) given above the lanes. Northern blots were then performed with a rat phospholamban (PLB)-specific probe. To confirm equal RNA loading, blots were stripped and rehybridized with a β-actin–specific probe. Lanes 1 to 18 show dose dependency of RNAi-mediated phospholamban mRNA downregulation for the rAAV-based vectors rAAV-shPLB (lanes 1 to 6), rAAV-shPLB-CMV-β-intron (lanes 7 to 12), and rAAV-shPLB-CMV-GFP (lanes 13 to 18). As a control for unspecific shRNA effects, lanes 19 to 24 show phospholamban-mRNA expression after treatment with rAAV-shGFP, which generates an shRNA sequence targeting GFP (lanes 19 to 24). No difference was found from untreated cells (lanes 29 and 30). For comparison with rAAV, the adenovector AdV-shPLB (lanes 25 to 28) was used. Phospholamban mRNA was ~98% ablated until day 10 by rAAV-shPLB at the lowest dose of 4×10^3 p/c (lanes 1 and 2), similar to AdV-shPLB (lanes 25 to 28). Incorporation of a CMV-GFP expression cassette in the rAAV-shPLB vector (lanes 7 to 12) to provide this vector with a tag that is easily detectable by in vivo imaging led to strong GFP expression in infected cells (not shown) but unexpectedly abolished its phospholamban gene-silencing effect. Incorporation of a CMV-β-intron cassette (lanes 12 to 18) had a similar but less pronounced effect. We therefore used only rAAV9-shPLB vs rAAV9-shGFP and AdV-shPLB vs AdV-shGFP for in vivo therapy (Figures 2A through 2G and 3A through 3F). C, Cellular shRNA levels produced by vectors from Figure 1B. In the presence of a CMV-GFP cassette, shPLB production was abolished (lanes 13 to 18), whereas the U6-shPLB vector without additional sequences showed stable expression over 5 (lanes 1 to 3) and 10 (lanes 4 to 6) days. AdV-shPLB generated very high shPLB levels on day 5, which then declined rather rapidly in NRCMs. Studies with further vectors (online-only Data Supplement Figures Ia through Ic) showed that interaction of the CMV promoter with the shRNA-transcribing polymerase type III U6 promoter apparently disturbs...
Figure 1 (Continued). shRNA transcription from the respective adeno-associated virus vectors. D, Left, Western blot analysis of phospholamban protein during treatment of NRCMs; right, quantitation on days 3, 5, and 7 after vector addition. AdV-shPLB and rAAV6-shPLB resulted on day 7 in downregulation of cellular phospholamban to 9% and 13%, respectively, of baseline. TnI indicates troponin I. E, [Ca^{2+}]_i transients in NRCMs during AAV9-shPLB treatment showed significantly higher amplitudes and accelerated transient kinetics (shortened time to peak and τ) compared with the AAV9-shGFP group, with transients indistinguishable from untreated cells. AdV-shPLB treatment also resulted in a significantly higher amplitude than in AdV-shGFP controls. In contrast to the AAV9 groups, time to peak was prolonged in the AdV-shPLB vs AdV-shGFP group, which displayed no difference in τ. F, Statistical evaluation of the [Ca^{2+}]_i transients. *P < 0.05 and **P < 0.01. CTRL indicates control. Additional studies of SR Ca^{2+} loading in the adenovirus groups (measured by rapid caffeine addition) were performed (online-only Data Supplement Figure Id and le) and showed increased SR Ca^{2+} loading and fractional Ca^{2+} release from the SR in the AdV-shPLB vs AdV-shGFP group.
Figure 2. Protocol for RNAi therapy of HF. A, Animals for the in vivo RNAi therapy study were divided into 2 groups: 1 group of 56 animals with aortic banding and a second group of 12 sham-operated animals. In the aortic-banded animals, we waited 25 to 30 weeks for them to develop LV dilatation and a decrease in fractional shortening by 25% (echocardiography) before cardiac RNAi vector transfer. Of 56 aortic-banded animals, 40 survived and were further divided into groups that received AdV-shGFP (n=10), AdV-shPLB (n=10), rAAV9-shGFP (n=10), or rAAV9-shPLB (n=10). A total of $3 \times 10^{10}$ pfu of each adenovirus was injected in 200 μL of solution. For experiments with rAAV9, tail-vein injection was done with $5 \times 10^{11}$ genomes of either vector. Outcome evaluation by echocardiography, tip catheter, morphometry, and histology was performed after 1 month in the adenoviral group and after 3 months in the rAAV9 groups (Figures 3A through 3F). In the adenoviral groups, 8 of 10 and 9 of 10 animals survived after 1 month. After 3 months, 9 of 10 in the rAAV-shPLB group and 6 of 10 in the rAAV-shGFP group survived. B, Rats were injected intravenously with an rAAV9-GFP vector expressing GFP or with saline. One month later, hearts were removed and visualized by GFP imaging, which showed a grossly homogeneous signal in cardiac cross sections in the rAAV9-GFP group (bottom) and no signal in the saline group (top). C, Overview of GFP fluorescence in hearts from rAAV9-GFP-treated rats reaching 90% of surface area at 1 month. D, Immunohistochemical staining of GFP in different organs 1 month after intravenous injection of rAAV9-GFP. Although after intravenous injection of an adenoviral vector (AdV-GFP), no GFP was detected in the heart (a), rAAV9-GFP treatment resulted in strong GFP expression (b and c), which was grossly homogeneous. Few areas were completely devoid of GFP immunoreactivity (encircled yellow areas); others showed homogeneous cytoplasmic staining (red circles). Staining was particularly dense at sites where high expression over 1 month had obviously resulted in the formation of precipitates (white arrows) of GFP, which is stable in cells, in contrast to shRNA generated from RNAi vectors.
visualization of GFP expression, GFP immunohistological staining was performed 1 month after intravenous injection of rAAV9-GFP to evaluate vector distribution on a microscopic scale.

**Experimental Protocol for RNAi Therapy In Vivo**

The adenoviral delivery system has been described in detail previously by our group. Briefly, after the rats were anesthetized and a thoracotomy was performed, a 22-gauge catheter containing 200 μL of adenoviral (3×10^10 pfu) solution was advanced from the apex of the LV to the aortic root. The aorta and main pulmonary artery were clamped for 40 seconds distal to the site of the catheter, and the solution was injected; then, the chest was closed, and the animals were allowed to recover. For experiments with rAAV9, a simple tail-vein injection was performed with 5×10^11 genomes of either rAAV9-shRNA vector. Animals in the sham group were injected with saline.

**Hemodynamics and Cardiac Histology During RNAi Therapy**

Rats in the different treatment groups and at different stages after adenoviral gene transfer were anesthetized with pentobarbital 40 mg/kg and mechanically ventilated. A small incision was then made in the apex of the LV, and a 2.0F high-fidelity pressure transducer (Millar Instruments, Houston, Tex) was introduced into the LV. Pressure measurements were digitized at 1 kHz and stored for further analysis. The operators performing the hemodynamic studies were blinded in terms of the animal groups they were studying.

**Statistical Analyses**

Data in Figures 1C through 1F, 2G, and 3A through 3F and in online-only Data Supplement Figures Ia through Ie, IIIa through IIIe, and IVa through IVc are presented as mean (columns)±SD (error bars). We analyzed the groups of rats that received interventions using a 2-step procedure. First, we performed an overall F test to determine whether any significant difference existed among any of the means. We then selected 2 means and performed a Tukey test for each mean comparison. We next determined whether the Tukey score was statistically significant with Tukey probability/critical values. Statistical significance was accepted at the level of P<0.05.

Drs Poller, Hajjar, Fechner, and Suckau designed and performed research, analyzed data, and wrote the article. The remaining authors performed research or contributed analytical tools. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Optimization of RNAi Systems**

The determinants of the silencing efficacy of viral RNAi vectors were investigated because we observed that rAAV-shPLB vectors with apparently minor structural differences (Figure 1A) had grossly different shRNA production rates and target silencing in NRCMs in vitro (Figure 1B and 1C). For these initial studies in NRCMs, the rAAV6 pseudotype was used, which has higher transduction efficacy than rAAV9 in vitro. For the later RNAi therapeutic investigations reported in Figures 2 and 3, only rAAV9 was used, which displays superior cardiac transduction in vivo.

The in vitro experiments showed that coexpression of GFP marker to tag cells harboring the shPLB vector nearly abolished shPLB production (Figure 1C) and phospholamban silencing (Figure 1B). The presence of a cytomegalovirus (CMV) promoter in the expression cassette that contained the U6 promoter used for shRNA transcription reduced shRNA production strongly if GFP was driven by the CMV, but also if CMV was linked to a β-intron (online-only Data Supplement Figure I). Figure 1 shows that by far the highest efficacy was displayed by an rAAV construct previously considered too short for efficient packaging. Comparison of the shRNA transcription by AdV-shPLB versus rAAV6-shPLB in NRCMs showed a decline to one third by day 10 for the adenoviral vector but constant expression for the rAAV vector (Figure 1C). Ablation of phospholamban expression at the mRNA level was >98% for both vectors at a dose of 4×10^10 vector particles per cell. Interestingly, incorporation of a CMV-GFP cassette to allow detection by in vivo imaging unexpectedly led to a vector unable to silence its target. CMV promoter-driven marker gene expression is apparently unsuitable for use in U6-shRNA vectors, and we therefore chose only the simplest and most efficient U6-shRNA vectors (Figure 1) for in vivo RNAi. rAAV9-shRNA was used for long-term therapy in vivo because of its highly stable shRNA production compared with AdV-shPLB and its long-term stability in vivo. For short-term therapy, the adenoviral vector was used. In vitro, a lag of several days in phospholamban ablation was found at the protein level compared with the mRNA level, with protein leveling off at 9% (AdV-shPLB) and 12% (rAAV6-shPLB) of baseline, respectively, on day 7 (Figure 1D).

Measurement of [Ca^{2+}], transients during AAV9-shPLB treatment of NRCMs (Figures 2E and 1F) showed that this vector led to significantly higher amplitude and accelerated transient kinetics (shortened time to peak and τ) compared with the AAV9-shGFP group, with transients indistinguishable from untreated cells. AdV-shPLB treatment also resulted in a significantly higher amplitude than with AdV-shGFP. In contrast to the AAV9 groups, time to peak [Ca^{2+}], transient was prolonged in AdV-shPLB versus AdV-shGFP, and no difference was found in τ. Studies of SR Ca^{2+} loading in the adenoviral groups showed increased SR Ca^{2+} loading and fractional Ca^{2+} release from the SR in the AdV-shPLB versus AdV-shGFP group (online-only Data Supplement Figure Id). With respect to SERCA2a was decreased in the shGFP groups that were in HF compared with sham, whereas SERCA2a was increased significantly in both shPLB groups. G, Statistical evaluation of Western blots from the different treatment groups. *P<0.05 compared with AdV-shGFP; **P<0.05 compared with rAAV9-shGFP.
cell-to-cell variability of transduction in vitro, online-only Data Supplement Figure Ib shows grossly homogeneous GFP expression in NRCMs treated with rAAV-GFP marker vector, which serves as the best possible approximation of a direct demonstration of homogeneous shPLB expression in vitro. With current technology, the latter cannot be visualized directly, because coexpression of GFP together with shPLB extinguishes its silencing capacity (Figure 1B and 1C). Homogeneous spatial and temporal distribution of the RNAi vectors in rat hearts in vivo is also indirectly inferred by fluorescent imaging (Figure 2B and 2C) of a GFP vector of the same type (rAAV9) as used for RNAi therapy (Figure 3). Figure 2D and online-only Data Supplement Figure IIa through IIc show GFP immunohistochemical staining of the heart and other organs after intravenous injection of rAAV9-GFP. Strong and grossly homogenous expression in the heart contrasts with weak staining of liver and skeletal muscle and no visible staining of the lungs (for quantitation, see online-only Data Supplement Figure IIc).
Efficacy of RNAi Therapy In Vivo

Transaortic banding led to HF in rats after 30 weeks. The experimental protocol for in vivo RNAi therapy is outlined in Figure 2A. Fluorescent imaging and immunohistological analysis of a GFP vector of the same type (rAAV9) as used for the RNAi therapies showed grossly homogeneous cardiac GFP expression 1 month after intravenous injection on the macroscopic and microscopic scale and may be assumed to approximate the cardiac shRNA expression levels generated by the RNAi vectors (Figures 2B through 2D). Direct measurement of shPLB production in vivo is unfeasible with current technology. Figures 2F and 2G show significantly decreased cardiac phospholamban protein after treatment with either AdV-shPLB or rAAV9-shPLB. SERCA2a protein was decreased in failing hearts, whereas shPLB therapy was accompanied by an increase in cardiac SERCA2a protein. The sodium-calcium exchanger was not changed significantly. Treatment by aortic root injection of AdV-shPLB compared with AdV-shGFP control vector (generating an irrelevant shRNA sequence directed at xenogenic GFP) served as a model of short-term treatment of acute (and potentially reversible) HF. One month after injection, the LV end-diastolic pressure, rate of LV pressure decrease (−dP/dt), and isovolumetric relaxation time constant (τ) as measures of diastolic function (Figure 3A and online-only Data Supplement Figure IIIa) were significantly (P<0.05) better in the AdV-shPLB group than in the control group. LV systolic pressure, rate of LV pressure increase (+dP/dt), and fractional shortening (FS) as parameters of systolic function (Figure 3B; online-only Data Supplement Figure IIIb) were likewise improved. Beyond these beneficial effects on hemodynamics, the massive cardiac hypertrophy and dilation after transaortic banding were reduced significantly at 1 month (Figure 3C; online-only Data Supplement Figure IIIc). Postmortem morphometric data (LV−body weight ratio, LV−tibia length ratio) correlated with echocardiography (Figure 3D). Histology showed reduced cardiomyocyte size after 1 month of AdV-shPLB therapy, whereas cardiac collagen content was unchanged (Figures 3E and 3F). Survival rates were 8 of 10 versus 9 of 10.

Treatment by intravenous injection of the most efficient rAAV9-shPLB compared with the rAAV9-shGFP vector served as a model of long-term therapy of chronic HF. Three months after injection, diastolic function (LV end-diastolic pressure, −dP/dt, τ; Figure 3A; online-only Data Supplement Figure IIIa) was improved significantly by rAAV9-shPLB therapy compared with control and was no longer significantly different from the sham-operated non-HF group. Systolic function (LV systolic pressure, +dP/dt, fractional shortening; Figure 3B; online-only Data Supplement Figure IIIb) was also restored, although less so than diastolic functional parameters. Beyond hemodynamics, this treatment reduced LV hypertrophy (LV−body weight ratio) and dilation (LV−tibia length ratio) at 3 months (Figure 3C; online-only Data
Supplement Figure IIIc). Echocardiography corroborated the reduction of LV wall thickness and dilation (Figure 3D; online-only Data Supplement Figure IIIId). Histology showed reduction of both cardiomyocyte size and cardiac collagen after 3 months of rAAV9-shRNA therapy (Figures 3E and 3F). Survival of rAAV9-shPLB–treated animals after 3 months was 9 of 10 versus 6 of 10 in the control group.

**Cardiac miRNAs and RNAi Treatment**

ShRNAs exploit the cellular machinery of RNAi to mediate therapeutic effects by mimicking the endogenous process but may disturb cellular miRNA pathways. Because miRNAs play important roles in cardiac morphogenesis, hypertrophy, arrhythmogenesis, and failure, we searched for possible side effects of the RNAi vectors at the miRNA level in NRCMs, under standard culture conditions and in the presence of the hypertrophy-inducing drug phenylephrine. In the absence of phenylephrine, no significant effects of any vector on miRNA-1 or miRNA-133 were found. In the presence of phenylephrine, a marked reduction was found on day 5 that was reversed in NRCMs treated with shPLB vectors. Rat hearts treated with shPLB vector had higher miRNA levels than the shGFP group (online-only Data Supplement Figure IIa through IIc). With respect to possible side effects of the RNAi vectors, hematoxylin-and-eosin stains of the liver (Figure 2E) and other organs after vector injection revealed no pathological findings.

**Discussion**

**RNAi Therapy of HF**

Building on recent landmark articles by other groups describing highly cardiotropic vector systems and after work on rAAV-mediated downregulation of phospholamban, the present study demonstrates for the first time the in vivo restoration of cardiac function and reduction of pathological hypertrophy and dilation in an HF animal model by RNAi. Comparison of the 2 vector systems used here suggests that for intermediate time scales, adenoviral vectors may suffice and even provide advantages over long-term stable rAAV, because RNAi may be desirable only temporarily in acute and potentially reversible HF. In fact, the significant improvement of diastolic and systolic function and LV morphology 1 month after AdV-shPLB treatment is evidence of at least a functional therapeutic benefit from the adenoviral vector on miRNA-1 or miRNA-133 were found. In the presence of the hypertrophy-inducing drug phenylephrine. In the absence of phenylephrine, no significant effects of any vector on miRNA-1 or miRNA-133 were found. In the presence of phenylephrine, a marked reduction was found on day 5 that was reversed in NRCMs treated with shPLB vectors. Rat hearts treated with shPLB vector had higher miRNA levels than the shGFP group (online-only Data Supplement Figure IIa through IIc). With respect to possible side effects of the RNAi vectors, hematoxylin-and-eosin stains of the liver (Figure 2E) and other organs after vector injection revealed no pathological findings.

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We have developed a method for gene delivery by recombinant adenovirus using cross-clamping of the aorta and the pulmonary arteries in rat hearts, which yields homogeneous transgene expression and has been used by several groups during the past 10 years. In terms of rAAV9, we now show by fluorescent imaging and immunohistochemistry that a single intravenous injection of an rAAV9-GFP marker vector induces strong and grossly homogeneous cardiac GFP expression 1 month after treatment (Figures 2B through 2D). The important finding that coexpression of GFP together with shPLB extinguishes its silencing capacity (Figures 1B and 1C) prevents, at the present time, direct demonstration of spatial and temporal uniformity of cardiac shRNA synthesis in vivo by GFP coexpression. However, uniformity may be deduced by inference from our analogous in vivo imaging of GFP expression from adenoviral and rAAV9 vectors. Using rAAV9-GFP, we further show that the transduction rate increases over time and reaches 90% at 1 month. Improvement of HF by both RNAi therapeutic protocols is mediated via ablation of phospholamban protein, which occurs in cultured NRCMs and in rat hearts in vivo (Figures 2F and 2G).

Cardiac phospholamban protein was decreased significantly after RNAi therapy. Although sodium-calcium exchanger expression was unchanged, SERCA2a expression was more complex. In failing hearts treated with control vectors, SERCA2a expression was decreased, as expected in any experimental HF model compared with sham. After RNAi therapy, SERCA2a expression was found to be increased compared with HF groups, consistent with the fact that RNAi therapy normalized LV function. Because SERCA2a expression is a well-known marker of the degree of HF, its increase after RNAi therapy by shPLB reflects the improved status of cardiac function. Both RNAi therapies induced a decrease in cardiomyocyte size (Figure 3E). In contrast, adenovirus-based therapy over 1 month did not influence the fibrosis induced in failing hearts, whereas long-term rAAV9-based treatment resulted in significantly reduced fibrosis after 3 months (Figure 3F). This could be a consequence of basic differences between the traditional adenoviral and the new AAV9 vectors, as suggested by the calcium transient studies in vitro. Adenovirus per se could induce changes in the transcriptional program of target cells different from those of AAV9 (see discussion of possible side effects below). Irrespective of such differences in vitro, however, the effect of the RNAi vectors in vivo on phospholamban ablation, hemodynamics, and morphology confirm that the doses chosen for both systems are within the therapeutic range.

The AAV9 vector used in the present study fulfills 1 initial requirement for application in human HF, because it is cardiotropic in primates. Beyond its long-term stability, rAAV9 offers further advantages of clinical interest through cardiac targeting after intravenous injection (Figures 2B through 2D) and low immunogenicity. In contrast to rodents, regulatable phospholamban modulation is most likely required in humans, because permanent phospholamban de-
iciency or phospholamban dysfunction due to genomic mutations has been associated with cardiomyopathies. Drug-regulatable RNAi appears possible, however, on both vector platforms used here. Transcriptional control of shRNA expression is technically more demanding than the use of the tissue-specific promoters employed for traditional gene therapy, because these promoters are unable to support proper formation and cleavage of shRNA. New promoters compatible with shRNA biosynthesis have been developed and may add an additional safety feature to organ-targeted RNAi therapy.

Independent of its therapeutic potential, organ-targeted RNAi may be of use to identify novel gene functions in that organ by functional ablation, analogous to classic tissue-specific inducible knockout models. The extent of ablation observed in the present study may suffice to recognize unknown gene functions, and the efficacy of this novel analytical approach is likely to increase with the advent of more sophisticated RNAi delivery systems. Importantly, gene ablation by RNAi may be induced at any desired age or disease state and would be more rapid and inexpensive than the traditional models.

Possible Side Effect of RNAi Therapy

The cellular machinery of RNAi evolved over millions of years and is the most efficient and versatile mechanism known for specific gene silencing. shRNAs exploit this machinery to mediate therapeutic effects by mimicking the endogenous process and achieve silencing at far lower concentrations than antisense RNAs, but they may disturb cellular miRNA pathways and thereby cause hepatotoxicity. When using a cardiotropic rAAV9 serotype with low affinity for the liver, we observed no histological evidence of acute or chronic liver damage. We also studied the cardiac-expressed miRNAs 1 and 133 during RNAi treatment. Because malignant arrhythmias are important complications in HF, deregulation of an arrhythmia-related miRNA such as miRNA-1, by a novel treatment should be considered as a possibly serious adverse effect. None of the RNAi vectors changed miRNA-1 levels in NRCMs, but interestingly, shPLB treatment was instead associated with rescue of the miRNA-1 depression induced by phenylephrine in these cells, and rat hearts that underwent shPLB therapy had higher miRNA levels than controls (online-only Data Supplement Figure IVa through IVc). In conjunction with the trend toward improved survival in the AAV-shPLN treatment group, no evidence was thus found for arrhythmogenic side effects.

Although improved contractile function during shPLB therapy results immediately from its influence on excitation-contraction coupling via the cytoplasmic Ca$^{2+}$ transients, the marked reduction of LV hypertrophy and dilation (Figures 3C, 3D, and 3F) is not deduced as easily, because it involves major reprogramming of the cardiac transcriptome. The RNAi-induced changes of Ca$^{2+}$ homeostasis may affect not only the cytoplasmic Ca$^{2+}$ transients but also the separate and insulated Ca$^{2+}$ signals generated in the perinuclear space that influence transcription. With respect to hypertrophy, it is of interest that shPLB treatment was associated with rescue of the phenylephrine-induced miRNA-133 depression in NRCMs and higher levels in rat hearts on shPLB treatment (online-only Data Supplement Figures IVb and IVc). The rescue of miRNA-133, which plays a critical role in the control of cardiomyocyte size, appears to be linked to RNAi at the cell level, because it also occurs in vitro, where hemodynamic stress, neurohumoral, or cytokine activation, as in HF in vivo, cannot play any role.

Although our rationale to measure miRNA expression was to test whether shPLB may “poison” the RNAi machinery, differential miRNA induction can also be viewed as “master switches” that control reinduction of fetal genes during HF. A clear distinction between side effect and master-switch aspects of miRNA regulation is very difficult and certainly beyond the scope of the present report. In addition, clarification of the mechanism by which shPLB-RNAi is linked to the observed changes in miRNA-1 and miRNA-133 (passive association or component of the therapeutic process) requires future studies.

Conclusions

The present study demonstrates for the first time the high efficacy of a locally targeted RNAi therapeutic strategy in a cardiac disease. Whereas classic gene therapy rests on recombinant protein expression as its basic principle, RNAi therapy instead uses regulatory RNAs with grossly different targeting, kinetics, and toxicity. We provide evidence that under the precondition of careful vector adaptation to the specific requirements of RNAi, and provided that regulatory RNA with high intrinsic activity and target specificity is selected, RNAi therapy may achieve long-term cardiac benefit without apparent toxicity. When functionally optimized RNAi vectors were used, and aortic root vector injection or cardiotropic rAAV9 capsids were used to target RNAi to the heart, no evidence was found of side effects. Short-term phospholamban silencing led to improved cardiac function 1 month after aortic root injection of an adenoviral RNAi vector. Long-term RNAi after simple intravenous injection of an optimized rAAV9 vector resulted in restored cardiac function and reduction of cardiac dilation, hypertrophy, and fibrosis after a period of 3 months. The rAAV9 approach uses a vector known to target the heart in primates, thus offering potential for clinical translation. Specifically, for targets such as phospholamban in which pharmacological approaches have failed thus far, the RNAi approach may enhance the therapeutic repertoire for cardiac diseases.

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References


**CLINICAL PERSPECTIVE**

RNA interference (RNAi) has the potential to be a novel therapeutic strategy in diverse areas of medicine. Whereas gene therapy (already used for cardiac disorders) rests on recombinant protein expression as its basic principle, RNAi therapy instead uses regulatory RNAs to achieve its effect. Fundamental limitations of the use of chemically synthesized small interfering RNAs to mediate RNAi are their rapid degradation in plasma and target cells and the unsolved problem of adequate targeting in vivo. The present study shows for the first time the high efficacy of an RNAi therapeutic strategy in a cardiac disease in vivo. It demonstrates successful treatment of heart failure in a rat model by cardiac-targeted RNAi ablating phospholamban, a key regulator of cardiac Ca²⁺ homeostasis. A novel vector was developed based on a cardiotropic adeno-associated virus (rAAV9) that carries RNAi activity to the heart after intravenous injection. Over a period of 3 months, this therapy restored cardiac function, reversed cardiac dilation and hypertrophy, and reduced cardiac fibrosis. In recent years, adeno-associated virus–based vectors have overcome key challenges to gene therapy, such as stability, safety, and host immune response. The present study shows that under the precondition of careful adeno-associated virus vector adaptation to the specific requirements of RNAi, if regulatory RNA sequences with high intrinsic activity and target specificity are selected, they may also serve as valuable tools for cardiac RNAi therapy and offer clinical potential. Specifically, for targets such as phospholamban, for which pharmacological approaches have failed so far, the RNAi approach may enhance the therapeutic repertoire.
Long-Term Cardiac-Targeted RNA Interference for the Treatment of Heart Failure
Restores Cardiac Function and Reduces Pathological Hypertrophy


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SUPPLEMENTAL MATERIAL

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Chronic Cardiac-Targeted RNA Interference for the Treatment of Heart Failure
Restores Cardiac Function and Reduces Pathological Hypertrophy
SUPPLEMENTAL METHODS

Development of Recombinant Adenoviral and AAV Vectors

Recombinant Adeno-Associated Virus (rAAV) vectors were developed for the in vitro studies as pseudotyped rAAV6 and for the in vivo work as rAAV9. Both contained identical AAV2 vector genomes with the indicated shRNA expression cassettes and were processed identically with the exception of using the plasmid AAV6cap for rAAV6 and AAV9cap for rAAV9, respectively. Throughout all in vitro and in vivo studies we used only self-complementary (“dimeric”) rAAV genomes due to their enhanced performance compared to single-stranded (“monomeric”) rAAV vectors. Starting from an shRNA expression cassette previously used in an adenoviral (AdV) vector AdV-shPLB, which efficiently and stably silenced PLB expression in cultured primary neonatal rat cardiomyocytes (NRCMs) 1, we sought to develop AdV and rAAV vectors co-expressing shRNA and GFP as a marker which would allow vector tracking during the experiment with heart failure (HF) animals by in vivo imaging. However, a construct rAAV-shPLB-CMV-GFP displayed very low silencing activity compared to the original AdV-shPLB vector which contained no CMV-GFP component (Fig. 1a). The rAAV vector produced GFP under CMV control with transcription in the CMV-GFP cassette running opposite to the U6-promotor-shRNA cassette. To test if the CMV promoter itself and/or the GFP sequence were responsible for the loss of silencing activity, we constructed the vector rAAV-shPLB-CMV-β-intron with an intronic “stuffer” sequence instead of the GFP. Both rAAV-shPLB-CMV-GFP (size 2.40 kb) and rAAV-shPLB-CMV-β-intron (size 2.14 kb) had sizes considered necessary for packaging into the capsid. A third vector which contained neither CMV nor GFP or β-intron, but only the original U6-shPLB cassette from AdV-PLB, rendered at total vector genome size of only 0.98 kb. Nevertheless this “minimalistic” rAAV-shPLB was efficiently packaged during the standard procedure, probably as a concatemer (data not shown). The maps of these 4 and of 2 additional control vectors (rAAV-shPLB-GFP and rAAV-shPLB-β-intron) are summarized in Fig. 1a.

Virus Vector Production and Purification

rAAV9-shGFP and rAAV9-shPLB were produced using the two-plasmids protocol described by Zolotukhin et al. 1999 2 with the following modifications: 293-T cells (ATCC, Manassas, VA) were grown in triple flasks for 24 h (DMEM, 10% FBS) prior to adding the calcium phosphate precipitate. After 72 hours, the virus was purified from benzonase-treated cell crude lysates over an iodixanol density gradient (Optiprep, Greiner Bio-One Inc., Longwood, FL), followed by heparin-agarose type I affinity chromatography (Sigma-Aldrich Inc., St. Louis, MO). Finally viruses were concentrated and formulated into lactated Ringer’s solution (Baxter Healthcare Corporation, Deerfield, IL) using a Vivaspin 20 Centrifugal concentrators 50K MWCO (Vivascience Inc., Carlsbad, CA), and stored at –80°C.
Vector Quality Assessment and Titration

Vector stock biochemical purity (>95%) was assessed by silver staining after electrophoresis. Genome containing particles (gcp) were determined by a real-time PCR approach (LightCycler, Roche Diagnostics) using the SYBR™ Green Taq ReadyMix™ (SIGMA, Saint-Louis, MO) and primers CMV-F and CMV-R.

Vector Evaluation in Primary Cardiomyocytes

Primary neonatal cardiomyocytes (NRCMs) are suitable to pre-test any RNAi-based cardiac therapy before its definitive test in vivo since, although developmentally regulated, the SERCA2a/PLB system functions well in NRCMs and adenoviral gene transfer strategies targeting the SERCA2a/PLB system were successful in both neonatal and adult cardiomyocytes. Although both cell types are well suited for in vitro pre-testing, a number of other differences between cultured cardiomyocytes and the intact heart in vivo render any in vitro study of RNA-based therapies in cultured cells rather preliminary.

Cell cultures: Primary neonatal cardiomyocytes (NRCMs) were prepared from ventricular tissue of 1-3 day-old Wistar rat pups. NRCMs were grown in 6-well dishes.

Evaluation of phospholamban silencing: PLB mRNA and protein expression in NRCMs and rat hearts, as well as SERCA2a and NCX protein in rat hearts, was determined by Northern and Western blot analyses as described 1, 3, 4, 5.

Calcium Transients During RNAi Treatment: 

$[Ca^{2+}]_i$ transients were measured during electrical stimulation at 1 Hz after loading of NRCMs with 8 μM Fluo-4/AM for 20 min (image capture at 120 Hz, 8.3 ms per image). Five treatment groups of NRCMs (number of cells) were studied: AAV9-shPLB (n=26), AAV9-shGFP (n=26), AdV-shPLB (n=71), AdV-shGFP (n=49), and untreated control cells (n=32). The amplitude of the transient (systolic $[Ca^{2+}]_i (F/F_0)$), its time to peak (TTP) (ms), and the time constant $\tau$ of its decay (ms) were measured. The measurement of $[Ca^{2+}]_i$ transients during AAV9-shPLB treatment of NRCMs (Fig. 1e,f) showed that this vector led to significantly higher amplitude and accelerated transient kinetics (with shortened TTP and $\tau$) compared to the AAV9-shGFP group with transients indistinguishable from untreated cells. AdV-shPLB treatment also resulted in a significantly higher amplitude compared to the AdV-shGFP group. In contrast to the AAV9 groups, however, the TTP was prolonged in AdV-shPLB vs. AdV-shGFP and there was no difference in $\tau$. Therefore additional studies of sarcoplasmic reticulum (SR) Ca$^{2+}$ loading in the AdV groups (Suppl. Fig. 1d) were performed as follows: $[Ca^{2+}]_i$ transients were again measured during electrical stimulation at 1 Hz after loading of NRCMs with 8 μM Fluo-4/AM for 20 min, but followed by rapid addition of 20 mM caffeine which blocks re-uptake of Ca$^{2+}$ into the SR via SERCA2a. Electrically stimulated $[Ca^{2+}]_i$ transients were compared with the caffeine-induced and the fractional release of Ca$^{2+}$ was calculated (see Kockskämper et al. 2008 6 and references therein).

Induction of hypertrophy: Phenylephrine (PE) at a concentration of 100 μM was employed in part of the in vitro studies as a hypertrophic stimulus. TaqMan assays to quantitate the cellular miRNAs were performed in NRCMs under baseline conditions (Suppl. Fig. 4a) or in the presence of PE (Suppl. Fig. 4b). The agent was added on day 2 of culture, either alone or together with the respective RNAi vector. NRCM cultures were continued in the presence of 10% FACS.
Quantitation of Gene Expression and shRNA Production by TaqMan™ Assays

miRNA assays: miRNAs were isolated from cells and tissues by using the Mirvana RNA isolation kit according to the instructions of the manufacturer (Ambion, Austin, TX, USA). In a search for possible influences of vector-derived shRNAs on cardiomyocyte miRNAs we used TaqMan™ assays to quantitate (Suppl. Fig. 4a-c) two miRNAs (miRNA-1, miRNA-133a) known to be functionally expressed in the heart.7-9 BNP assay: Cardiac BNP gene expression was likewise quantitated by TaqMan™ in the hearts of vector-treated rats (Suppl. Fig. 3e). shPLB production: The quantitation of short hairpin RNA transcription by the different RNAi vectors (Fig. 1c) followed a previously published protocol.1,10

Transaortic Banding

Four-week old Sprague Dawley rats (70-80 g) were anesthetized with intraperitoneal pentobarbital (65 mg/kg) and placed on a ventilator. A suprasternal incision was made exposing the aortic root and a tantalum clip with an internal diameter of 0.58 mm (Weck, Inc.) was placed on the ascending aorta. Animals in the sham group underwent a similar procedure without insertion of a clip. The supraclavicular incision was then closed and the rats were transferred back to their cages. The supraclavicular approach was performed because during gene delivery a thoracotomy is necessary and by not opening the thorax during the initial aortic banding, avoids adhesions when gene delivery is performed.

Animals were initially divided into two groups: one group of 56 animals with transaortic aortic banding (TAB) and a second group of 12 animals which were sham-operated (10 of the sham operated animals survived). In the animals which were aortic banded we waited 25-30 weeks for the animals to develop left ventricular dilatation and a decrease in ejection fraction by 25% prior to cardiac gene transfer. From the initial 56 who underwent pressure overload hypertrophy only 40 animals survived and were further divided to receive either Ad-shGFP (n=10) or Ad-shPLB (n=10), or rAAV9-shGFP (n=10) or rAAV9-shPLB (n=10).

Serial Echocardiographic Assessment

After twenty-two weeks of banding, serial echocardiograms were performed on a weekly basis in lightly anesthetized animals (pentobarbital 40 mg/kg intra-peritoneally). Transthoracic M-mode and two-dimensional echocardiography was performed with a GE Vivid-7 ultrasound machine and a 12 MHz broadband transducer. A mid-papillary level left ventricular short axis view was used and measurements of posterior wall thickness, left ventricular diastolic dimension and fractional shortening were collected. Gene transfer was performed in all animals within 3 days of detection of a drop in fractional shortening (FS) of > 25% compared to FS at 12 weeks post-banding. The operators performing the echocardiographic studies were blinded in terms of the animal groups they were studying.

Cardiac Distribution of AAV9 Vectors After Intravenous Injection

Rats were injected intravenously (i.v.) either with a marker vector rAAV9-GFP which expresses the marker protein GFP, or with saline. 1 month following delivery of rAAV9-GFP or saline the hearts were
removed and visualized under a fluorescent system (*Maestro In Vivo Imaging, Woburn, MA*) at 510 nm with single excitation peak at 490 nm of blue light (**Fig. 2b,c**) shows the images observed.

In addition to this visualization of GFP expression at the macroscopic scale, GFP immunohistochemical staining was performed 1 month after i.v. injection of rAAV9-GFP to evaluate its distribution at microscopic dimensions (**Fig. 2d, Suppl. Fig. 2a,b**). After blockade of endogenous peroxidase with 30% hydrogen peroxide, washing in PBS buffer, a polyclonal rabbit-anti-hrGFP antibody (*Vitality™, catalogue #240142, Stratagene*) was added in 1:1.000 dilution for 120 min. After washing, as secondary a polyclonal goat-anti-rabbit immunoglobulins/HRP antibody (*catalogue# P0448, Dako, Glostrup, Denmark*) was used in 1.50 dilution for 60 min. Staining and counterstaining with haemalaun was as described.

For Western blot analysis of GFP expression in the different organs the polyclonal rabbit-anti-hrGFP antibody (*Vitality™, catalogue #240142 Stratagene*) was used in 1:5.000 dilution at 4°C overnight. As secondary the polyclonal goat-anti-rabbit immunoglobulins/HRP antibody (*catalogue# P0448, Dako, Glostrup, Denmark*) was used in 1:2.000 dilution at RT for 60 min. For detection of GAPDH as loading control a monoclonal mouse-anti-GAPDH antibody (*catalogue# MAB374, Chemicon /Millipore*) was used as primary in 1:5.000 dilution for 1 hr at RT, and a polyclonal goat-anti-mouse immunoglobulins/HRP antibody (*catalogue# P0447, Dako, Glostrup, Denmark*) as secondary in 1:10.000 dilution at 1 hr at RT. The GFP Western blots all organs were processed equally as described, then developed by use of a chemiluminescence kit (*USB Corp., Cleveland, OH*). Blots were then exposed to X-ray film for 5 min each. For densitometric quantitation of the films *TINA 2.09* software and a *Raytest™* system were used.

**Experimental Protocol for RNA Interference Therapy in vivo**

The adenoviral delivery system has previously been described by our group in detail **4, 5, 12-14**. Briefly, after anesthetizing the rats and performing a thoracotomy, a 22 G catheter containing 200 µl of adenoviral (3x10¹⁰ pfu) solution was advanced from the apex of the left ventricle to the aortic root. The aorta and main pulmonary artery were clamped for 40 sec distal to the site of the catheter and the solution injected, then the chest was closed and the animals were allowed to recover. For experiments with rAAV9, a simple tail vein injection was performed using 5x10¹¹ genomes of either rAAV9-shRNA vector. Animals in the sham group were injected with saline.

**Hemodynamics Evaluation during RNA Interference Therapy**

Rats in the different treatment groups and at different stages following adenoviral gene transfer were anesthetized with 40 mg/kg of pentobarbital and mechanically ventilated. The chest was then opened through a mid-line incision and the heart exposed. A small incision was then made in the apex of the left ventricle and a 2.0 French high fidelity pressure transducer (*MILAR Instruments Inc., TX*) introduced into the left ventricle. Pressure measurements were digitized at 1 KHz and stored for further analysis. Left ventricular systolic pressure (LVSP), end-diastolic left ventricular pressure (LVEDP), the maximal rates of pressure rise (+dP/dt) and of pressure fall (-dP/dt), and the time constant of relaxation (t) were measured or derived in the different groups. The time course of isovolumetric relaxation was measured using the equation: P=P₀e⁻⁴⁺Pₜ, where P is the left ventricular isovolumic...
pressure, \( P_0 \) is pressure at the time of peak \(-\frac{dP}{dt}\) and \( P_b \) is residual pressure. The operators performing the hemodynamic studies were blinded in terms of the animal groups they were studying.

**Histology of Hearts after RNA Interference Therapy**

*Cardiomyocyte size and cardiac fibrosis:* Histological analyses were performed on a subset of animals to evaluate myocyte size (CMD) and collagen content (CAP). LV specimens were fixed with 10% formalin and embedded in paraffin. Sections (3 µm-thick) were stained with hematoxylin-eosin to determine CMD or with Azan-Mallory to assess CAP. In longitudinally oriented cardiomyocytes, transnuclear width was measured as CMD (Fig. 3f). Digital photographs were taken at six sites on each Azan-Mallory section. Interstitial/perivascular collagen area and myocyte area were determined separately by counting the computerized pixels using an NIH imager (Fig. 3e).

**Statistical Analysis**

Data in Fig. 1cf, 2g, 3a-f and in Suppl. Fig. 1ae, 3a-e, 4a-c are presented as mean (columns) ± SD (error bars).

We analyzed the groups of rats intervened on by using a two step procedure. First, we carried out an over-all F test to determine if there is any significant difference existing among any of the means. We then selected two means and calculated Tukey's test for each mean comparison. We then checked to see if Tukey's score is statistically significant with Tukey's probability/critical values.

Statistical significance was accepted at the level of \( p<0.05 \).

**References**


6. Kockskämper J, Seidlmayer L, Walther S, Hellenkamp K, Maier L, Pieske B. Endothelin-1 enhances nuclear \( \text{Ca}^{2+} \) transients in atrial myocytes through Ins(1,4,5)P3-dependent \( \text{Ca}^{2+} \) release from perinuclear \( \text{Ca}^{2+} \) stores. *Journal of Cell Science.* 2008;121:186-195.


**Supplemental Table**

<table>
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<tr>
<th>Treatment</th>
<th>PW, mm</th>
<th>LVDD, mm</th>
<th>LVSD, mm</th>
<th>FS, %</th>
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<td>Sham</td>
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<td>20-23 wk</td>
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<td>9.44 ± 0.28</td>
<td>6.36 ± 0.10</td>
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PW, posterior wall thickness during diastole; LVDD, left ventricular diameter during diastole; LVSD, left ventricular systolic diameter during systole; FS, fractional shortening; *, P < 0.05 compared to sham at similar time period; ′, P < 0.05 compared to values at 6 wk. From (Miyamoto et al, PNAS; 2000; Table 1; 97, Issue 2, 793-798)
Legends to Supplemental Figures

Supplemental Fig. 1
Interference of CMV promoter with short hairpin RNA Production

A: In addition to the recombinant adenoviral and rAAV vectors functionally characterized in Fig. 1a-c, a direct comparison of the RNAi vectors rAAV6-shPLB-CMV-GFP (which contains a functional GFP expression cassette) vs. rAAV6-shPLB-GFP (carrying the GFP sequence but lacking a CMV promoter), and rAAV6-shPLB-CMV-β-intron (carrying β-intron plus CMV sequence) vs. rAAV-shPLB-β-intron (lacking the CMV promoter). Obviously, the presence of the CMV promoter per se reduced the shRNA production rate markedly, although shRNA suppression in the CMV-GFP marker vector was more pronounced.

B: Phase contrast (upper) and GFP fluorescence (lower) microphotograph of a cultured cardiomyocyte monolayer on day 5 after incubation with the rAAV6-shPLB-CMV-GFP marker vector, conforming a very high transduction rate for the rAAV6 pseudotype. Marker expression was obviously strong, so that transcription in CMV-GFP-bHG direction appears unaltered.

C: Synopsis of the structure of the vectors (see also Fig. 1a) suggests as a possible cause for the disturbed shRNA production in the U6-shPLB-bGH direction a read-through from CMV through the termination signal into the very short shRNA sequence which then impairs proper formation of the short hairpins which mediate RNAi.

D and E: Measurement of [Ca$^{2+}$]$_i$ transients during AAV9-shPLB treatment of NRCMs (Fig. 1e,f) showed that this vector led to significantly higher amplitude and accelerated transient kinetics (with a shortened TTP and $\tau$) compared to the AAV9-shGFP group with transients indistinguishable from untreated cells. AdV-shPLB treatment also resulted in a significantly higher amplitude compared to AdV-shGFP. In contrast to the AAV9 groups, TTP was prolonged in AdV-shPLB compared to the AdV-shGFP and there was no difference in $\tau$. Additional studies of the [Ca$^{2+}$]$_i$ transients induced by a caffeine pulse (upper panel D) revealed increased significantly (p<0.05) SR Ca$^{2+}$ loading and fractional Ca$^{2+}$ release (FR) from the SR in the AdV-shPLB compared to the AdV-shGFP group (lower panel E).

Supplemental Fig. 2
Expression of rAAV-GFP Vector After Intravenous Injection

A: Immunohistochemical staining for GFP in different organs 1 month after i.v. injection of rAAV9-GFP in rats. Whereas after i.v. injection of an adenoviral vector (AdV-GFP) no GFP was detectable within the heart (Fig. 2d), rAAV9-GFP treatment resulted in strong cardiac GFP expression in 40-80% of cells (upper four photomicrographs) which is grossly homogeneous over large areas. Few areas are completely devoid of GFP immunoreactivity (encircled yellow), others show homogeneous cytoplasmic staining (encircled red). Staining is particularly dense at sites where high expression over 1 month has obviously resulted in the formation of precipitates (white arrows) of GFP which is stable in cells, in contrast to shRNA generated from RNAi vectors. AAn average of about 70% of cardiomyocytes was positive as assessed by GFP immunohistochemistry with variability of expression among individual
cells. The lower two photomicrographs document specificity of the GFP signal by lack of staining if the primary (GFP) antibody was omitted.

**B:** Immunohistochemical staining for GFP shows skeletal muscle (middle left) with faint staining of a major fraction of cells, whereas the liver (upper left) showed prominent signals of individual cells only. No signal was visible in the lungs (lower left). The photomicrographs on the right document specificity of the GFP signal by lack of staining without primary antibody.

**C:** This panel shows on the left side representative GFP Western blots of heart, liver, skeletal muscle and lung and respective GAPDH loading controls for liver and muscle. Side by side the results from two rats and two mice injected i.v. with rAAV9-GFP are shown. First, the high cardiac expression rate in both species is illustrated in contrast to the very faint expression in the lungs. Second, there was a difference in the GFP ratio heart/liver between rats (ratio ≈ 14.2) and mice (ratio ≈ 0.8), with relatively low hepatic GFP expression in rats. Third, there was a low level of skeletal muscle GFP expression in the rats, whereas no signal was detectable in mice. GFP Western blots processed and exposed by identical protocols were quantified by densitometric analysis as shown by the bar graphs below. In summary the data show that rAAV9-GFP resulted in a significantly higher heart/liver GFP expression ratio in rats than in mice. Cardiac affinity of rAAV vectors may apparently differ between species and appears to be favourable in rats over mice.

**Supplemental Fig. 3**

**Influence of RNAi Treatment on Hemodynamics and Cardiac Morphology**

**A:** *Influence of RNAi Therapy on Diastolic Function.* The figure summarizes the influence of the RNAi therapies upon hemodynamic parameters of diastolic LV function. The high LV filling pressure (LVEDP) in HF rats after TAC was significantly lowered by the shPLB vectors (lanes 3, 5) compared to the shGFP control vector groups (lanes 2, 4). The maximal rate of pressure fall (-dP/dt) was significantly increased by the shPLB vector therapies, as was also the isovolumetric relaxation time constant Tau. Values were restored to within the normal range (lanes 1) after three months of rAAV-shPLB therapy (lanes 5) (addendum to Fig. 3a).

¶ denotes p<0.05 for animals treated with AdV-shPLB vs. control vector AdV-shGFP, ‡ denotes p<0.05 for treatment with rAAV9-shPLB vs. rAAV9-shGFP control group.

**B:** *Influence of RNAi Therapy on Systolic Function.* The figure summarizes RNAi treatment effects on systolic LV function analogous to panel A. Both the LV systolic pressure (LVSP) and the maximal rate of pressure rise (+dP/dt) were significantly improved by shPLB vector therapy (lanes 3, 5) compared to shGFP control vectors (lanes 2, 4). Echocardiography revealed an essentially normalized fraction shortening (FS) after 3 months of rAAV9-shPLB therapy (lane 5), whereas the improvement of FS was also significant but less pronounced for rats treated with AdV-shPLB (lane 3)(addendum to Fig. 3b).

¶ denotes p<0.05 for animals treated with AdV-shPLB vs. control vector AdV-shGFP, ‡ denotes p<0.05 for treatment with rAAV9-shPLB vs. rAAV9-shGFP control group.

**C:** *Morphometric Changes During RNAi Therapy.* The figure summarizes the *post mortem* morphometry with marked LV hypertrophy induced by TAB (lanes 2, 4). LV weight and the LV/body weight (LV/BW) ratio rose ≈ 2/3 thirds above baseline (lanes 1). Treatment with either RNAi vector (lanes 3,
5) reduced this hypertrophy compared to the controls (lanes 2, 4) without reaching, however, the normal range. There was also a marked LV dilation as measured by the LV diameter/tibia length ratio. By morphometry the latter was reduced to within the normal range after 1 or 3 months of therapy with AdV-shPLB or rAAV9-shPLB (lanes 3, 5), respectively (addendum to Fig. 3c).

¶ denotes p<0.05 for animals treated with AdV-shPLB vs. control vector AdV-shGFP, ‡ denotes p<0.05 for treatment with rAAV9-shPLB vs. rAAV9-shGFP control group.

D: Echocardiographic Changes During RNAi Therapy. The figure shows the echocardiographic data on cardiac morphology and function which corroborate the morphometric findings. The LV end-diastolic diameter (LVEDD) was significantly enhanced by TAB. This dilation was reduced to within the normal range after 3 months of treatment with rAAV9-shPLB (lane 5), whereas the effect of 1 month of therapy with AdV-shPLB was also significant but less pronounced. Corresponding to the morphometric changes noted in panel C, the LV anterior and posterior wall thickness measured by echocardiography were significantly reduced (lanes 3, 5) compared to the heart failure groups after TAB and treatment with the control vectors (lanes 2, 4) (addendum to Fig. 3d).

¶ denotes p<0.05 for animals treated with AdV-shPLB vs. control vector AdV-shGFP, ‡ denotes p<0.05 for treatment with rAAV9-shPLB vs. rAAV9-shGFP control group.

E: Cardiac BNP mRNA expression levels quantitated by TaqMan™ PCR in the different groups were consistent with the hemodynamics as shown in panels A and B.

¶ denotes p<0.05 for animals treated with AdV-shPLB vs. control vector AdV-shGFP, ‡ denotes p<0.05 for treatment with rAAV9-shPLB vs. rAAV9-shGFP control group.

Supplemental Fig. 4
Cardiomyocyte miRNAs during RNAi Treatment

A: There was no evidence of significant alteration of the levels of miRNAs 1 or 133a during treatment of NRCMs with any of the RNAi vectors described in this study at the given doses.

B: In contrast, several cellular miRNAs were strongly reduced in the presence of an hypertrophy-inducing drug despite otherwise unchanged culture conditions. In the presence of phenylephrine (PE) there was a marked reduction of miRNA-1 and miRNA-133a on day 5 of treatment with PE ± RNAi vector, consistent with previous studies by others. Treatment with the RNAi vectors AdV-shPLB or rAAV6-shPLB, respectively, was associated with elevation of these miRNA levels to baseline range.

¶ denotes p<0.05 for untreated (NT) vs. PE-treated cells, ‡ p<0.05 of cells treated with PE alone vs. PE + AdV-shPLB, † p<0.05 of PE vs. PE + rAAV6-shPLB.

C: Quantitation of cardiac miRNA1 and miRNA-133a levels in rats during RNAi treatment with either the adenoviral or the rAAV9 vector system showed a significant decrease of both miRNAs in the HF groups (treated with the shGFP control vectors) compared to sham, whereas both miRNA-1 and 133a were significantly elevated as compared to these groups in TAB rats treated with shPLB vector.
Supplemental Table

We have extensively used this model of pressure-overload hypertrophy in transition to heart failure over the last decade and have characterized the time course of heart failure development in previous publications (Miyamoto et al. Proc Natl Acad Sci USA 2000; 97: 793-798 - Table 1). The same model was used in this specific study on RNA interference therapies. The table shows the development of echocardiographic parameters up to 23 weeks after transaortic banding (TAB) compared to sham surgery. We have definitely observed the phenomenon that animals with TAB that survive beyond 22-24 weeks seem to stabilize somewhat at these low hemodynamic numbers. Probably they can only survive if they are able to sustain this level of hemodynamic numbers, otherwise they die.

A steady state of cardiac morphology and function was apparently also reached within the short-term follow-up phase of 1 month after injection of the RNAi vectors, since no significant further deterioration of cardiac function occurred between the control group of the long-term experiment (rAAV9-shGFP) vs. the control group of the short-term study (AdV-shGFP).
Suppl. Fig. 1a-c: Suppression of shRNA Production by CMV Promotor in RNAi Vectors Carrying a GFP Expression Cassette

**A**

- 55%
- 85%

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**B**

Day 5

AAV6-shPLB-CMV-GFP

AAV 6-shPLB-CMV-GFP

**C**

AAV9-shPLB-CMV-GFP  2401 bp

5' ITR  CMV  GFP  bgH  shPLN  U6  3' ITR
Suppl. Fig. 1d,e: Alteration of Calcium Homeostasis in NRCMs During RNAi Treatment

D

AdV-shPLB

Caffeine (20 mmol/L)

\[ [Ca^{2+}] \text{ (FF)} \]

1 10

AdV-shGFP

Caffeine (20 mmol/L)

\[ [Ca^{2+}] \text{ (FF)} \]

1 10

E

Electrically-stimulated [Ca^{2+}], transient

Caffeine-induced [Ca^{2+}], transient

Fractional release (%)

peak [Ca^{2+}] (FF)
Suppl. Fig. 2a: Cardiac GFP expression 1 month after intravenous (i.v.) rAAV9-GFP injection

Four upper panels (rAAV9-GFP) with primary GFP antibody, two lower panels (Cont) without.
Suppl. Fig. 2b : GFP expression in other organs 1 month after i.v. rAAV9-GFP injection

Left panels (rAAV9-GFP) with primary GFP antibody, right panels (Cont) without.
Suppl. Fig. 2c: GFP quantitation by immunoblot in different organs after i.v. rAAV9-GFP injection
Suppl. Fig. 3a: Influence of RNAi Therapy on Diastolic Function

- **LVEDP**
  - sham
  - AdV-shGFP
  - AdV-shPLB
  - AAV9-shGFP
  - AAV9-shPLB

- **-dp/dt**
  - sham
  - AdV-shGFP
  - AdV-shPLD
  - AAV9-shGFP
  - AAV9-shPLB

- **Tau**
  - sham
  - AdV-shGFP
  - AdV-shPLB
  - AAV9-shGFP
  - AAV9-shPLB

‡: p < 0.05 for AAV9-shGFP vs. AAV9-shPLB
¶: p < 0.05 for AdV-shGFP vs. AdV-shPLB
Suppl. Fig. 3b: Influence of RNAi Therapy on Systolic Function

LVSP

+dp/dt

Fractional shortening

††: p < 0.05 for AAV9-shGFP vs. AAV9-shPLB
†: p < 0.05 for AdV-shGFP vs. AdV-shPLB
Suppl. Fig. 3c: Morphometric Changes During RNAi Therapy

LV weight

LV/body weight

LV diameter/tibia length

†: p < 0.05 for AAV9-shGFP vs. AAV9-shPLB
‡: p < 0.05 for Adv-shGFP vs. Adv-shPLB

1 month 3 months

1 month 3 months

1 month 3 months
Suppl. Fig. 3d: Echocardiographic Changes During RNAi Therapy

LV end-diastolic diameter

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LV anterior wall

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LV posterior wall

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‡: p < 0.05 for AAV9-shGFP vs. AAV9-shPLB
¶: p < 0.05 for AdV-shGFP vs. AdV-shPLB
Suppl. Fig. 3e: Cardiac BNP mRNA level during RNAi therapy

\[ \text{sham} \quad \text{AdV-shGFP} \quad \text{AdV-shPLB} \quad \text{AAV9-shGFP} \quad \text{AAV9-shPLB} \]

‡: p < 0.05 for AAV9-shGFP vs. AAV9-shPLB
¶: p < 0.05 for AdV-shGFP vs. AdV-shPLB
Suppl. Fig. 4a,b: miRNA Levels in Cardiomyocytes Treated with RNAi Vectors

A

miRNA.1

B

miRNA-1

miRNA-133
Suppl. Fig. 4c: Cardiac miRNA-1 and 133 levels during RNAi therapy

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‡ : p < 0.05 for AAV9-shGFP vs. AAV9-shPLB
¶ : p < 0.05 for AdV-shGFP vs. AdV-shPLB