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ORIGINAL PAPER

Cardiac-targeted RNA interference mediated by an AAV9 vector improves cardiac function in coxsackievirus B3 cardiomyopathy

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Abstract RNA interference (RNAi) has potential to be a novel therapeutic strategy in diverse areas of medicine. In this paper, we report on targeted RNAi for the treatment of a viral cardiomyopathy, which is a major cause of sudden cardiac death or terminal heart failure in children and young adults. RNAi therapy employs small regulatory RNAs to achieve its effect, but in vivo use of synthetic *small interfering* RNAs is limited by instability in plasma and low transfer into target cells. We instead evaluated an RNAi strategy using *short hairpin* RNA (shRdRp) directed at the RNA polymerase (RdRP) of coxsackievirus B3 (CoxB3) in

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HeLa cells, primary rat cardiomyocytes (PNCMs) and CoxB3-infected mice in vivo. A conventional AAV2 vector expressing shRdRp protected HeLa against virus-induced death, but this vector type was unable to transduce PNCMs. In contrast, an analogous pseudotyped AAV2.6 vector was protective also in PNCMs and reduced virus replication by >3 $\log_{10}$ steps. Finally, we evaluated the intravenous treatment of mice with an AAV2.9-shRdRp vector because AAV9 carries the most cardiotropic AAV capsid currently known for in vivo use. Mice with CoxB3 cardiomyopathy had disturbed left ventricular (LV) function with impaired parameters of contractility (dP/dt_{max}=3,006±287 vs. 7,482±487 mmHg/s, $p<0.01$) and diastolic relaxation (dP/dt_{min}=−2,224±195 vs. −6,456±356 mmHg/s, $p<0.01$ and Tau=16.2±1.1 vs. 10.7±0.6 ms, $p<0.01$) compared to control mice. AAV2.9-shRdRp treatment significantly attenuated the cardiac dysfunction compared to control vector-treated mice on day 10 after CoxB3 infection: dP/dt_{max}=3,865±354 vs. 3,006±287 mmHg/s ($p<0.05$), dP/dt_{min}=−3,245±231 vs. −2,224±195 mmHg/s ($p<0.05$) and Tau=11.9±0.5 vs. 16.2±1.1 ms ($p<0.01$). The data show, for the first time, that intravenously injected AAV9 has the potential to target RNAi to the heart and suggest AAV9-shRNA vectors as a novel therapeutic approach for cardiac disorders.

Keywords Virus infections · Coxsackievirus · RNA interference · Gene silencing · Gene therapy

Abbreviations

AAV	adeno-associated virus
AAV2.6	pseudotyped AAV2.6 vector
CoxB3	coxsackievirus B3
dP/dt_{max}	maximal rate of pressure increase over time
LV	left ventricle
moi	multiplicity of infection
pfu	plaque-forming unit
PNCM	primary neonatal cardiomyocyte
RdRp	RNA-dependent RNA polymerase
RNAi	RNA interference
shRNA	short hairpin RNA
siRNA	short interfering RNA
Tau	isovolumetric relaxation time constant
vg/c	vector genomes per cell

Introduction

Recent investigations suggest that cardiac viral infections are important factors in the pathogenesis of cardiac diseases such as dilated cardiomyopathy (DCM). Infections by cardiotropic viruses may be the underlying cause of DCM or at least negatively influence the course of the disease. Coxsackievirus B3, a single-stranded RNA virus with genomic RNA in plus-strand orientation belongs to the genus *Enterovirus* of the *Picornaviridae*, which is one of the largest and most important families of human pathogenic viruses. CoxB3 is a major agent of viral myocarditis, which can be mild with complete recovery but often takes a fulminant course with sudden death or terminal heart failure [1, 2]. CoxB3 may directly damage the myocardium, and its genomes are detectable during all stages of acute and chronic myocarditis until end-stage DCM [3]. Some cases of DCM may also result from autoimmunity secondary to previous CoxB3 exposure [4]. For the treatment of CoxB3 infections, no specific anti-viral drugs are established so far. Therefore, molecular therapy has come into the focus of research. New approaches encompass soluble cellular virus receptor isoforms [5, 6], antisense oligonucleotides and morpholino oligomers inhibiting CoxB3 replication via steric blocking of viral RNA [7].

RNA interference (RNAi) is increasingly evaluated as a novel therapeutic strategy. In contrast to gene therapy, which acts via recombinant protein expression, the therapeutic effects of RNAi are mediated by regulatory RNAs, which affect cell functions without encoding any protein. Recently, small interfering (si) RNA mediating RNA interference (RNAi) against the coxsackievirus–adenovirus–receptor (CAR) [8] or CoxB3-encoded proteins [9, 10] have been shown to inhibit CoxB3 infections. Due to high target specificity and efficacy, RNAi-based methods holds promise for the treatment of viral infections [11], and siRNAs targeting respiratory viruses have entered clinical trials. For experimental and therapeutic application, siRNA can be chemically presynthesised or expressed from a vector as short hairpin (sh) RNAs subsequently processed by the enzyme Dicer into siRNAs [12]. In most studies, chemically synthesised siRNAs were employed, which undergo rapid degradation and thus allow only *transient* target silencing. Furthermore, for in vivo studies in mice, a rapid high-volume injection procedure was used, which cannot be adapted for humans and leads to siRNA uptake into the liver whereas the heart as the actual target organ in CoxB3 cardiomyopathy was refractory [9, 13]. RNAi-based protocols with possible clinical potential could be based on viral vectors with appropriate organ targeting. With respect to the treatment of cardiac diseases, adeno-associated virus (AAV) vectors have advantages over others vector systems. First, they support long-term gene expression as already shown in primates [14–16] without chromosomal integration of the vector genome. Second, pseudotyped AAV vectors with serotype 8 or 9 capsids efficiently transduce the heart after intravenous application [14, 17, 18]. Third, AAV vectors with self-complementary “dimeric” vector genomes enable fast and strong expression of transgenes

[19]. These three properties make them promising for the treatment of cardiac diseases by RNAi strategies.

In the current study, we have developed pseudotyped, self-complementary AAV vectors generating *short hairpin* RNAs (shRdRp) directed at the RNA polymerase (RdRP) of CoxB3 and evaluated their potential against CoxB3 infections in permissive HeLa cells, primary neonatal rat cardiomyocytes (PNCMs) and mouse hearts in vivo. CoxB3 replication was reduced >3 $\log_{10}$ steps in vitro and intravenous treatment with a cardiotropic AAV9 pseudotype vector significantly reduced the cardiac dysfunction induced by CoxB3 in vivo.

Materials and methods

Cell cultures and coxsackievirus B3

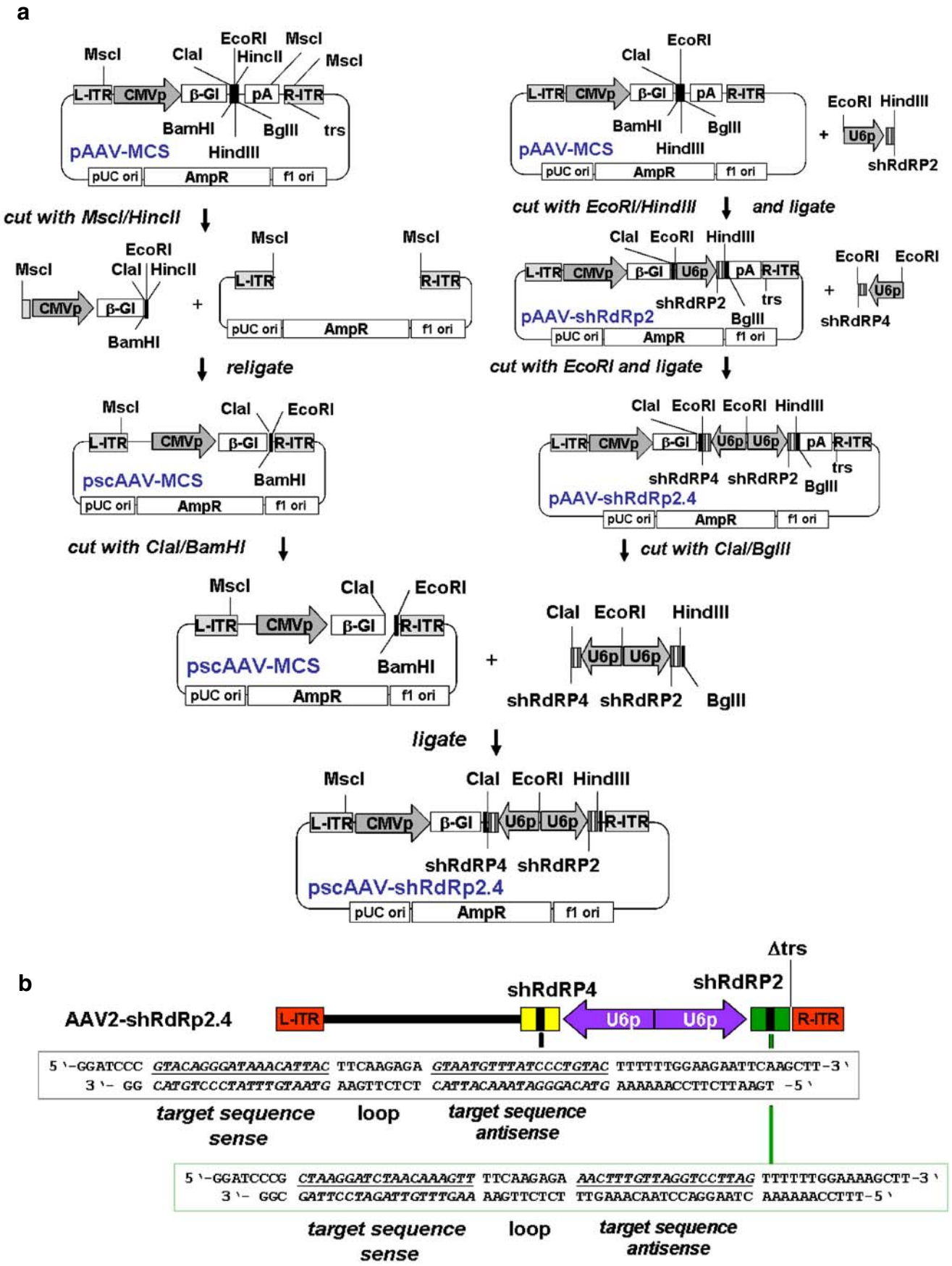
HeLa (human cervix carcinoma) and HEK 293T (human embryonal kidney) cells were cultured in DMEM (Gibco BRL) supplemented with 10% FCS (Gibco BRL) and 1% of each penicillin (Sigma) and streptomycin (Sigma). Primary neonatal rat cardiomyocytes (PNCMs) were isolated from neonatal rats and cultured in CMRL 1415 Medium (Biochrom) supplemented with 2.8 mM glucose, 5.4 mM KCl, 1.26 mM $CaCl_2$ and 2 μM 5′ Fluoro-2′-deoxyuridine (Sigma) 10% FCS and 2 μg/ml of gentamycin (Biochrom) as described [20]. Coxsackievirus B3 used in this study was of the Nancy strain (ATCC VR-30) in second passage.

AAV vector development

For the generation of self-complementary AAV2-shRdRp, AAV2-shGFP and AAV2-GFP vectors and their pseudotyped AAV2.6 and AAV2.9 analogues (AAV2.6-shRdRP, AAV2.6-shGFP, AAV2.6-GFP, AAV2.9-shRdRP, AAV2.9-shGFP, AAV2.9-GFP), basic plasmids pscAAV-shRdRP2.4 and pscAAV-shGFP with deletion of the terminal resolution site and the D-sequence at the 3′-ITR of the AAV genome (nucleotide positions 114 to 141) were constructed as shown in Fig. 1a. Plasmid pAAV-MCS (Stratagene) was digested with *Hin*cII and *Msc*I and the 2,832- and 1,234-bp fragments were harvested. The 2,832-bp fragment was dephosphorylated and ligated to the 1,234-bp fragment, thus generating the basic plasmid pscAAV-MCS. The pscAAV-shRdRP2.4 was constructed as follows: the plasmid SiDEx [21] was digested with *Eco*RI and *Hin*dIII. The 400 bp fragment containing the murine U6 promoter and the CoxB3-shRNA shRdRP2 was harvested and ligated into the *Eco*RI/*Hin*dIII-digested pAAV-MCS, thus generating the plasmid pAAV-shRdRP2. The fragment containing the U6 promoter and the shRdRP4 was harvested after *Eco*RI digestion of SiDEx and ligated into the plasmid pAAV-shRdRP2, which was digested with *Eco*RI and dephosphorylated, thus generating plasmid pAAV-shRdRP2.4. The approximative 800-bp fragment comprising both shRdRP2 and shRdRP4 expression cassettes was obtained after cutting pAAV-shRdRp2.4 with *Cla*I/*Bgl*II and ligated into the *Cla*I/*Bam*HI-digested (*Bam*HI compatible with *Bgl*II) plasmid pscAAV-MCS, giving rise to the basic plasmid pscAAV-shRdRp2.4. For the construction of the plasmid pscAAV-shGFP, a 400-bp fragment comprising the U6 promoter and GFP-shRNA was obtained after digestion of the plasmid pSilencer-siGFP (generated by the insertion of GFP-shRNA into the plasmid pSilencer [Ambion]) with *Hin*dIII and *Eco*RI and ligated in the *Eco*RI/*Hin*dIII-digested pscAAV-MCS.

For the generation of the sc reporter vectors AAV2-GFP and AAV2.6-GFP, a basic plasmid termed pscAAV-GFP was constructed as follows: For the construction of the GFP expression cassette, a 0.5-kb fragment of human β-globin intron was removed from the plasmid pAAV-hrGFP (Stratagene) by digestion with *Sac*II and *Cla*I. The 5′-overhangs were filled-in by use of T4-polymerase, and the plasmid was religated resulting in the generation of pAAV-GFP. The fragment containing the CMV promoter and the GFP cDNA was removed from pAAV-GFP after *Nde*I/*Bgl*II digestion and ligated in the *Nde*I/*Bam*HI-restricted plasmid pCMV-MCS (Stratagene) to generate the plasmid pCMV-GFP. A fragment containing the murine U6 promoter and CoxB3 RdRP2-shRNA was amplified from the pSiR2 [22] and ligated in *Bgl*II/*Hin*dIII-digested pCMV-GFP leading to the generation of the plasmid pCMV-GFP-R2. The bovine growth hormone polyadenylation signal sequences was harvested by *Nhe*I/*Xba*I digestion of pAd5TRE-Luc [23] and ligated in the *Xba*I-digested and dephosphorylated pCMV-GFP-R2 to yield the plasmid pCMV-GFP-R2. After the digestion of plasmid pCMV-GFP-R2 with *Mlu*I and *Bgl*II, the CMV-GFP-R2 fragment was harvested and finally ligated into *Bam*HI/*Mlu*I-digested pscAAV giving rise to the final construct pscAAV-GFP.

For the large-scale production of self-complementary AAV2-shRdRp, AAV2-shGFP and AAV2-GFP, HEK 293T cells were co-transfected with the AAV basic plasmids pscAAV-shRdRp2.4, pscAAV-shGFP and pscAAV-GFP and the packaging plasmid pDG (kindly provided by Dr. Kleinschmidt, Deutsches Krebsforschungszentrum, Heidelberg, Germany). Pseudotyped AAV2.6-shRdRp, AAV2.6-shGFP and AAV2.6-GFP were generated by co-transfection of these AAV basic plasmids with plasmid pDP6rs (kindly provided by Dr. Kleinschmidt). Pseudotyped self-complementary AAV2.9-shRdRp, AAV2.9-shGFP and AAV2.9-GFP vectors were generated by co-transfection of the basic plasmids with p5E18-VD2/9 (kindly provided by Dr. Wilson, University of Pennsylvania,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MscI
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5'-GGATCCC GTACAGGGATAAACATTAC TTCAAGAGA GTAATGTTTATCCCTGTAC TTTTTTGGAAGAATTCAAGCTT-3'
3'- GG CATGTCCCTATTTGTAATG AAGTTCTCT CATTACAAATAGGGACATG AAAAAACCTTCTTAAGT -5'
target sequence sense
loop
target sequence antisense
5'-GGATCCCG CTAAGGATCTAACAAAGTT TTCAAGAGA AACTTTGTTAGGTCCTTAG TTTTTTGGAAAAGCTT-3'
3'- GGC GATTCCTAGATTGTTTGAA AAGTTCTCT TTGAAACAATCCAGGAATC AAAAAACCTTT-5'
target sequence sense
loop
target sequence antisense

◀ **Fig. 1** Development of RNAi vectors. **a** Self-complementary "dimeric" RNAi vectors were developed by removing the terminal resolution site (*trs*) from the "monomeric" plasmid pAAV-MCS. The resulting plasmid pscAAV-MCS was ligated to a bidirectional transcription cassette, which generates two shRNAs (shRdRp2 and shRdRp4) active against the RNA-dependent RNA polymerase of coxsackievirus B3. The resulting plasmid pscAAV-RdRp2.4 carried the vector genome, which was then packaged in AAV2, AAV6 or AAV9 capsids by co-transfection of HEK 293T cells with the plasmid pDG (AAV2), pDP6rs (AAV6) or p5E18-VD2/9 (AAV9). **b** The self-complementary RNAi vector AAV2-shRdRp contains, in head-to-head orientation, transcription cassettes for shRdRp2 and shRdRp4. shRNA transcription is driven by a U6 promoter, the terminal repeats (*TR*) are from AAV2. The 3′-TR carries a deletion of trs and D-sequence (positions 118–141) that leads to the packaging of self-complementary AAV genomes

USA) and pHelper (Stratagene). Two days after transfection, the cells were harvested and the vectors were purified using Iodixanol gradients and concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore) according to the instructions of the supplier. AAV vector titers were determined by slot blot analysis using P^{32}-labelled probes directed against the U6 promoter and normalised to a vector genome standard by densitometry as described [24].

Virus plaque and cell viability assays

HeLa cells were cultured in six-well cell culture plates as confluent monolayers at a density of 1.2×10^6 cells per well and incubated at 37°C in Eagle's minimal essential medium (MEM) with 5% (*v*/*v*) fetal calf serum under 5% (*v*/*v*) CO_2. Twenty-four hours later, cells were overlaid with 1 ml diluted supernatant from HeLa cells or PNCMs, which were treated by three freeze/thaw cycles to release intracellular CoxB3 virus. The HeLa cell culture was then incubated at 37°C for 30 min, the supernatant removed and the cells overlaid with 2 ml agar containing Eagle's MEM. Three days later, cells were stained with 0.025% (*w*/*v*) neutral red. The virus titer was determined by plaque counting 4 h after staining. The mean and standard deviation were calculated from two independent experiments, each performed in duplicate. The cell viability was carried out by use of the Cell Proliferation Kit II (Roche) following the manufacturer's instructions. The absorbance measured at 492 nm is proportional to the number of metabolic active cells and, therefore, to cell viability.

Coxsackievirus myocarditis model

Six-week-old C57/bl6J mice (Charles River, Sulzfeld, Germany) were injected intravenously via vena jugularis with AAV2.9-shRdRp (treatment group) or AAV2.9-shGFP (control group), respectively. Ten days later, the mice were inoculated intraperitoneally with 2×10^5 plaque-forming units of CoxB3 (Nancy strain, VR-30, Manassas, USA) in 0.2 ml of buffered saline (infected mice, $n=16$). Control C57/bl6J mice matched for age and sex received 0.2 ml of buffered saline intraperitoneally ($n=12$).

Haemodynamic measurements and cardiac histology

Before killing the animals and the gathering of myocardial tissue, anaesthetised (thiopental 125 μg/g i.p.) and artificially ventilated mice underwent haemodynamic evaluation with a 1.2-F Milar tip catheter introduced into the left ventricle as described [25]. The maximal rate of pressure increase over time (dP/dt_{max}), as an index for cardiac contractility, and the minimal rate of pressure decrease over time (dP/dt_{min}) and the isovolumetric relaxation constant Tau, as indices of cardiac diastolic relaxation, were recorded.

To assess the myocardial injury and inflammation, heart tissues from CoxB3-infected mice transduced with RNAi vectors were stained with haematoxylin/eosin. To quantify myocardial damage comprising cardiac cell necrosis, inflammation and scarring, we applied a score from 0 to 4 according to Eriksson et al. [26]. To assess cardiac fibrosis, the cardiac interstitial collagen was stained with Sirius Red and quantified under circularly polarised light. Collagen type I and III was detected by immunohistochemistry and quantified by digital image analysis.

Statistical analysis

To test for significance, Student's *t* test was applied, and $p<0.05$ was considered statistically significant.

Results

Anti-viral RNAi in permissive cells

Self-complementary AAV vectors [19] support faster and stronger therapeutic transgene expression than otherwise identical AAV vectors with traditional single-stranded genomes. Because RNA viruses are particularly prone to develop escape mutants after treatment with a single siRNA, we co-expressed two highly efficient RdRp-shRNAs [27] from the self-complementary vector AAV2-shRdRp (Fig. 1b). Figure 2a shows that 4×10^3 vector genomes per cell (vg/c) of self-complementary AAV2-GFP led to GFP expression in all HeLa cells 24 h after transduction, whilst only few cells expressed GFP after treatment with ssAAV2-GFP. GFP expression then further increased in scAAV2-GFP-infected cells but remained very low for ssAAV2-GFP. We, therefore, subsequently used only scAAV vectors for the anti-viral work. First, we studied its anti-viral efficacy as a function of dose and time in permissive HeLa cells. Cells pre-treated with a dose of 4×10^3 vg/c of AAV2-shRdRP were efficiently protected against CoxB3-induced cell lysis

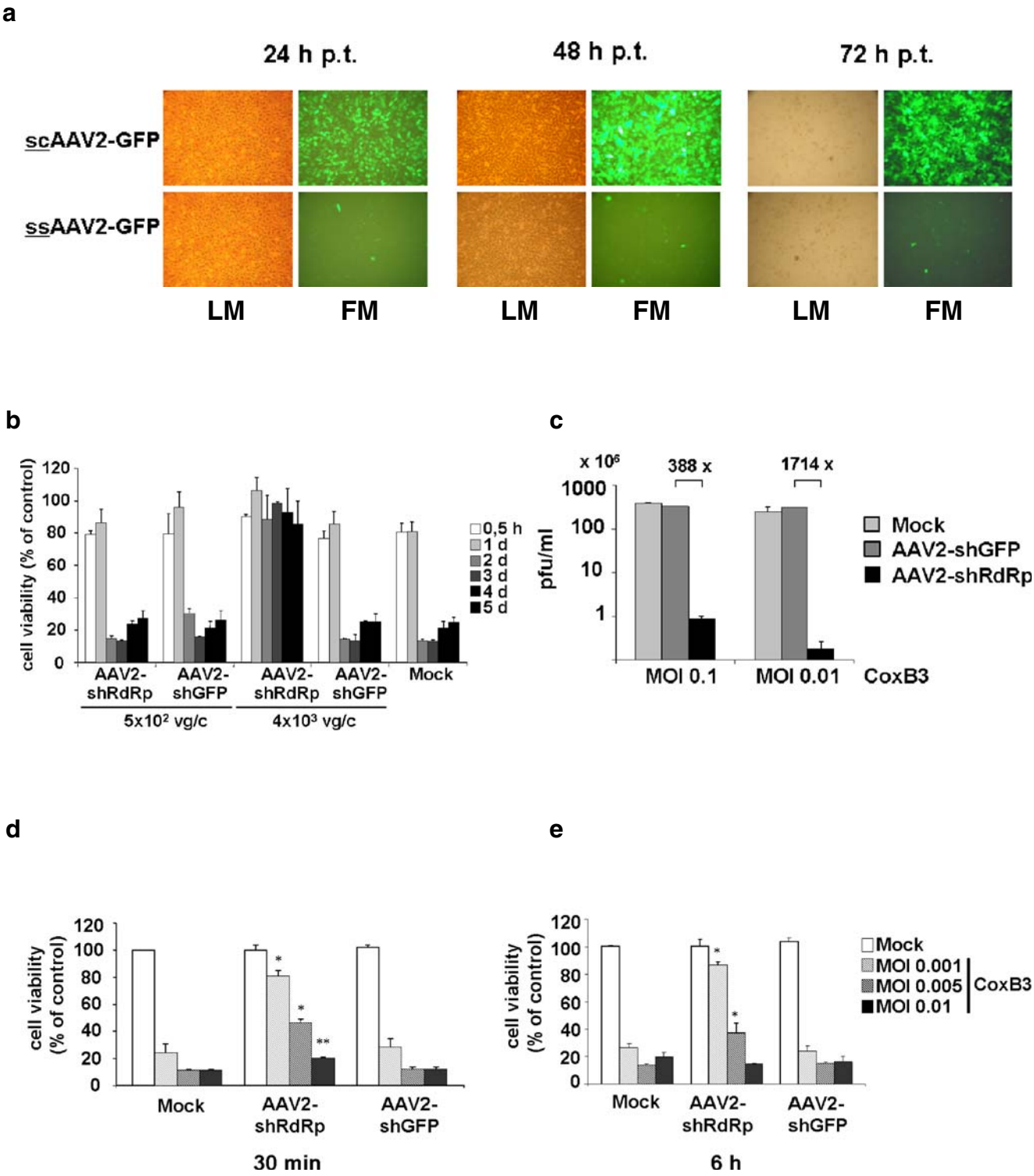

Fig. 2 Anti-viral RNAi in permissive HeLa cells. **a** HeLa cells were transduced with 4×10^3 vg/c of single-stranded (*ss*) or self-complementary (*sc*) AAV2-GFP. After 24, 48 and 72 h, GFP-expressing cells were visualised by fluorescence microscopy. The sc vector supported GFP expression in >90% of cells, whilst the ss vector supported GFP expression in <5%. Only vectors of the sc type were used in all further experiments. *LM* light microscopy, *FM* fluorescence microscopy with FITC filter. **b** RNAi vector AAV2-shRdRp protected HeLa cells against CoxB3-induced cytotoxicity in a dose- and time-dependent manner; 4×10^3 vg/c of vector kept cell viability at baseline level over 5 days compared to control vector AAV2-shGFP. **c** This protection was due to 388-fold and 1,714-fold, respectively, reduction of virus plaque titers after infection with CoxB3 at mois of 0.1 or 0.01, respectively. The RNAi vector also inhibited ongoing CoxB3 infections. HeLa cells were infected with CoxB3 at the indicated mois. Either 30 min (**d**) or 6 h (**e**) later, the medium was then replaced and cells transduced with RNAi or control vector was added at 4×10^3 vg/c. Cell viability was determined 72 h later and is shown relative to that of untreated cells. *$p<0.001$; **$p<0.05$

over the whole study period, whilst strong cytopathic effects occurred in all other samples early after infection (Fig. 2b). Additional plaque reduction assays (Fig. 2c) provided further measures of CoxB3 replication. Determination of CoxB3 titers 72 h after infection showed a strong protective effect of the RNAi treatment with titers reduced by >2 $\log_{10}$ steps (99.75%) at a CoxB3 dose of 0.1 moi and >3 $\log_{10}$ steps (99.94%) at a dose of 0.01 moi. Obviously, the reduction of CoxB3 cytotoxicity by AAV2-shRdRP treatment results from the inhibition of virus replication. Having shown protective efficacy, we next investigated if the treatment was able to inhibit ongoing infections (Fig. 2d and e). Cells were infected with CoxB3 at moi 0.001, 0.005 or 0.01 and then treated with 4×10^3 vg/c of anti-viral or control vector 30 min (Fig. 2d) or 6 h (Fig. 2e) later. The low CoxB3 dose reduced cell viability only by 15% to 20% in AAV2-shRdRP-treated cells, whereas viability was reduced by 70% to 80% in control vector-treated cells after 72 h. This protective effect was reduced at medium and absent at the high CoxB3 dose (Fig. 2d and e). When applied 24 h after CoxB3 infection, the RNAi vector was no longer able to protect the HeLa cells, not even at the lowest CoxB3 dose of moi 0.001 (data not shown). The RNAi vector is capable of inhibiting ongoing CoxB3 infections, too, but its efficacy depends strongly on the infectious dose and the time lag between infection and RNAi treatment. Expectedly, the anti-viral protection in these treatment experiments (Fig. 2d) was lower than in the CoxB3 challenge studies (Fig. 2e), but in addition to increased cell viability, a reduction of CoxB3 titer by 1 $\log_{10}$ step was achieved by the RNAi vector at low CoxB3 virus loads such as encountered in chronic human CoxB3 cardiomyopathy.

Comparison of different RNAi vectors in primary cardiomyocytes

Because cardiomyocytes are the actual target cells of CoxB3 in human cardiomyopathies, any RNAi-based treatment of possible clinical relevance needs to be efficient in primary cardiac cells. After the above preliminary evaluation in a permissive cell line, we, therefore, investigated transduction rates and anti-viral efficacy of standard AAV2-shRdRp and pseudotyped AAV2.6-shRdRP vector in primary cardiomyocytes (PNCMs). It has been reported that AAV2 vectors are unable to transduce the heart with high efficacy, whilst AAV vectors pseudotyped with capsid proteins of AAV6, AAV8 or AAV9 allow efficient cardiac gene transfer in vivo. To decide which AAV pseudotype would allow the most efficient shRNA expression in PNCMs, the cells were treated with 3×10^3 or 6×10^3 vg/c of native AAV2 or pseudotyped AAV2.6 or AAV2.9 marker vectors expressing GFP. Highest transduction rates were achieved with AAV2.6 with the majority of PNCMs expressing GFP as early as 48 h after transduction and a further increase in the expression level at 72 and 96 h (Fig. 3a). In sharp contrast, only few PNCMs expressed the marker after treatment with AAV2 (Fig. 3a) and AAV2.9 (not shown). We subsequently used only AAV2.6-shRdRp for anti-viral studies in PNCMs. Cells were treated with 1×10^3 or 6×10^3 vg/c of AAV2.6-shRdRp or control vector and 24 h later infected with CoxB3 at moi 1. CoxB3 replication was inhibited in PNCMs by about 3 $\log_{10}$ steps (99.76% at 1×10^3 vg/c and 99.89% at 6×10^3 vg/c of RNAi vector; Fig. 3a). Ongoing CoxB3 infection was also significantly inhibited if infected PNCMs were treated 30 min or 6 h later with the RNAi vector (Fig. 3b). The data indicate that, in these primary cells, the AAV2.6 vector was at least as efficient against CoxB3 as AAV2 in permissive HeLa cells.

Anti-viral RNAi treatment in an animal model

Initial in vivo experiments comparing the efficacies of AAV2, AAV2.6 and AAV2.9 showed that the cardiac transduction rate was highest for the recently described new AAV2.9 pseudotype [19]. AAV2.9 was far more efficient in vivo than the AAV2.6 pseudotype, which was used for the in vitro work. Figure 4a shows a vector-tracking experiment with an AAV2.9-GFP vector demonstrating that, even at the very low dose of 1×10^{10} vector genomes per animal (vg/a), a significant fraction of cardiac cells expressed GFP as assessed by immunohistochemistry. Based on the work of Inagaki et al. [18], we used 2×10^{11} vg/a for the RNAi treatment experiments shown in Fig. 4b. AAV2.9-shRdRp or AAV2.9-shGFP were injected via the jugular vein in C57/bl6J mice, followed 9 days later by intraperitoneal CoxB3 infection with a dose of 2×10^5 pfu of CoxB3 per mouse. Haemodynamics were measured by tip catheter on day 10 after infection. In animals with CoxB3 cardiomyopathy, cardiac function was significantly disturbed with impaired systolic contractility (dP/dt_{max}= 3,006±287 vs. 7,482±487 mmHg/s, $p<0.01$) and diastolic relaxation (dP/dt_{min}=−2,224±195 vs. −6,456±356 mmHg/s, $p<0.01$ and Tau 16.2±1.1 vs. 10.7±0.6 ms, $p<0.01$) compared to controls without CoxB3 infection. AAV2.9-shRdRp treatment significantly attenuated the CoxB3-induced cardiac dysfunction compared to control vector-treated mice on day 10 after the CoxB3 infection: dP/dt_{max}=3,865± 354 vs. 3,006±287 ($p<0.05$) and dP/dt_{min}=−3,245±231 vs. −2,224±195 mmHg/s ($p<0.05$) and Tau 11.9±0.5 vs. 16.2± 1.1 ms ($p<0.01$). By histology and immunohistochemistry, there was no significant difference between AAV2.9-shRdRp- and control vector-treated mice on day 10 after infection.

Discussion

Whereas the potential of RNAi to inhibit CoxB3 in vitro was investigated by several groups, only two studies so far

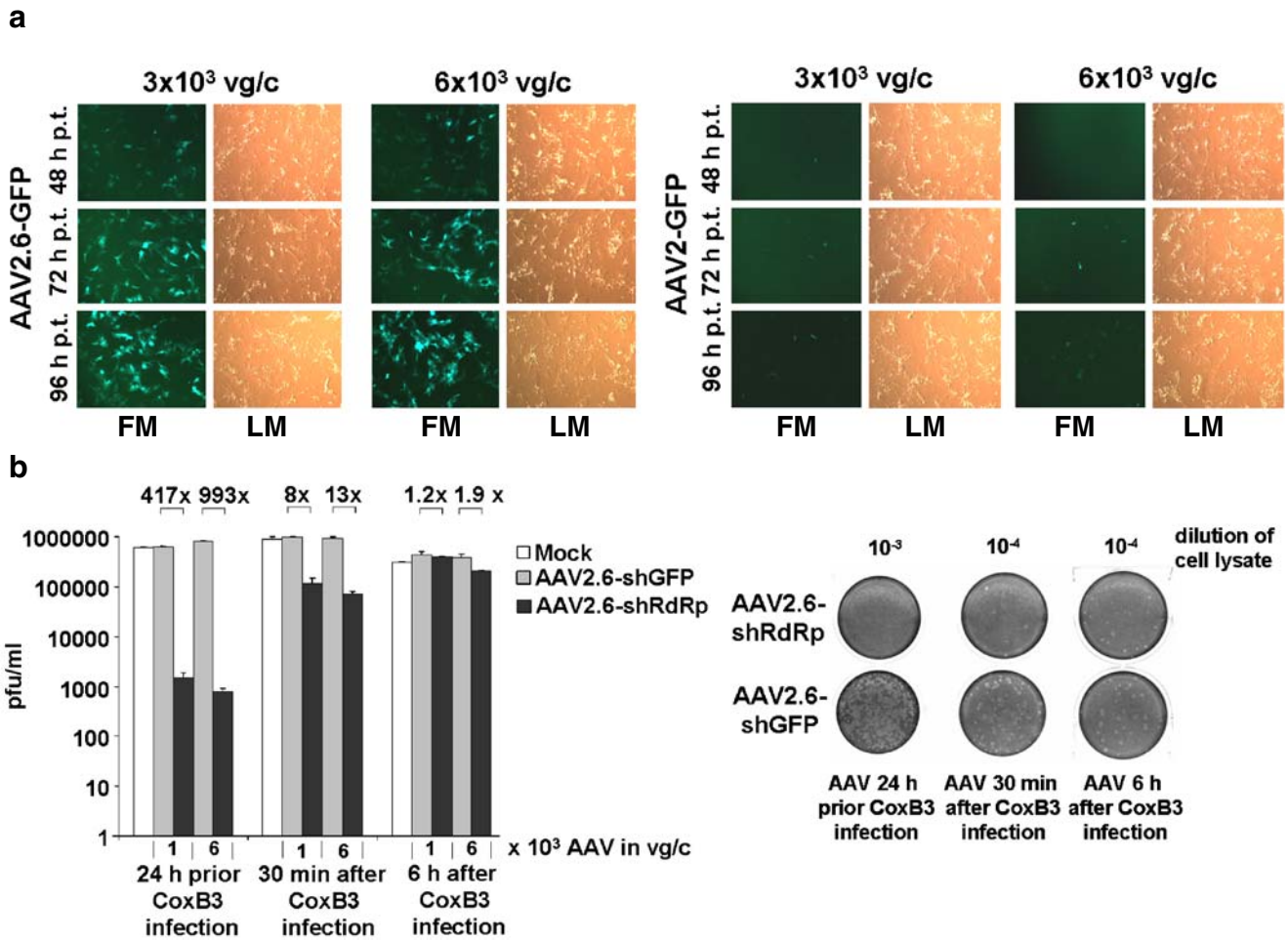

Fig. 3 Comparison of different RNAi vectors in primary cardiomyocytes. **a** The transduction efficacy of conventional AAV2 and pseudotyped AAV2.6 GFP marker vectors in PNCMs is grossly different with <5% for AAV2-GFP compared to >90% at 96 h expressing GFP after transduction, at both 3×10^3 and 6×10^3 vg/c. For this reason, for all subsequent experiments in PNCMs, only AAV2.6 pseudotyped vectors were used. *LM* light microscopy, *FM* fluorescence microscopy with FITC filter. **b** Pretreatment of PNCMs with AAV2.6-shRdRp 30 min before CoxB3 infection resulted in a 417-fold or 993-fold reduction in virus plaque titers in the cells at doses of 1×10^3 or 6×10^3 vg/c, respectively (*bars on the left*). When treatment with the RNAi vector was initiated 30 min after infection, the two vector doses resulted in 8-fold or 13-fold reduction in plaque titers (*bars in the middle*). Initiation of RNAi treatment 6 h after CoxB3 infection was no longer efficient with respect to virus titer

examined it in vivo. In one of these [9] chemically synthesised siRNA and in the other [10] plasmids transcribing shRNA were applied by rapid high-volume intravenous injection in mice. For the treatment of CoxB3 cardiomyopathies in humans where the virus is located in the myocardium itself, however, this approach is unsuitable because neither siRNAs nor plasmids are able to transfect the heart in situ. Furthermore, the hydrodynamic injection procedure used cannot be adapted to human patients. A new generation of pseudotyped AAV vectors containing capsids from serotype 8 or 9 AAV offers a possible alternative to overcome these hurdles because they enable efficient cardiac gene transfer after simple intravenous vector administration in small volumes [14, 17, 18]. AAV vectors in general enable long-term transgene expression without genomic integration, which obviates the need to re-administer the therapeutic agent and at the same time minimises the risk of insertional mutagenesis.

Our evaluation of anti-viral AAV vectors in a permissive cell line, primary cardiomyocytes and mouse hearts in vivo supports the view that this approach to therapeutic RNAi in the heart offers significant advantages. In agreement with the work of McCarty et al. [19], we observed that self-complementary AAV enabled faster and higher shRNA expression than single-stranded AAV also in cardiomyocytes, which would be a prerequisite for efficacy in acute infections such as fulminant CoxB3 myocarditis. To prevent or delay emergence of escape mutants [11], particularly relevant for RNA viruses with their error-prone replication machinery, we used co-expression of two shRNAs throughout the study. HeLa cells pre-treated with standard AAV2.2-shRdRp were protected against CoxB3-induced cytolysis by

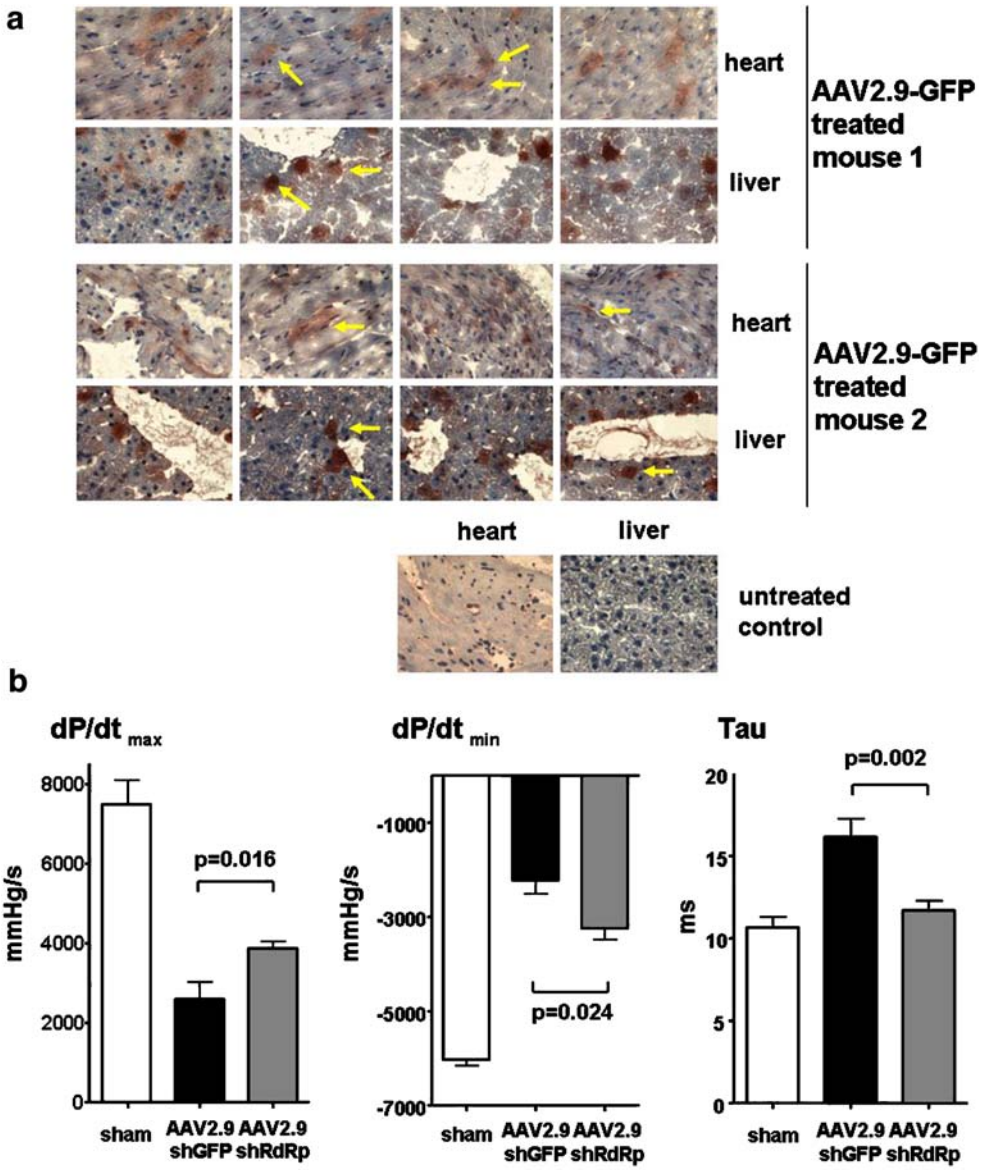

Fig. 4 Anti-viral RNAi in an animal model of CoxB3 cardiomyopathy. **a** The distribution of pseudotyped AAV2.9-GFP marker vector in mice in vivo after intravenous injection is shown. One week after jugular vein injection of 1×10^{10} vg/a of AAV2.9-GFP, the animals were killed and the distribution of GFP-expressing cells was determined by peroxidase immunohistochemistry using a primary antibody against GFP. Even at this very low vector dose, a significant fraction of cardiac cells expressed GFP. A 20-fold higher vector dose of 2×10^{11} vg/a was used for the experiment shown in **b**. *Arrows* show examples of GFP-expressing cells. **b** For anti-viral RNAi treatment, C57/bl6J mice were injected with 2×10^{11} vg/a intravenously. Ten days later, the mice were infected with 2×10^{5} pfu of CoxB3 and developed the typical cardiomyopathy after another 9 days. In infected mice, the LV function was severely disturbed with impaired contractility (dP/dt_{max}) and relaxation (dP/dt_{min}, Tau) compared to controls without CoxB3 infection. AAV2.9-shRdRp-treated mice had significantly improved systolic and diastolic LV function compared to control vector-treated mice on day 9 after the CoxB3 infection

inhibition of CoxB3 replication by >3 $\log_{10}$ steps. siRNAs of the same sequence reduced the CoxB3 titer by only one $\log_{10}$ step [27] even in HeLa cells, which in contrast to cardiomyocytes, are easy to transfect. Anti-viral RNAi by siRNAs or shRNA-plasmids has not yet been reported in PNCMs, which are very difficult to transfect with siRNA or plasmid, but it is unlikely that efficient anti-viral RNAi can be achieved at all by use of these tools. Because cardiomyocytes are the primary targets of CoxB3 in the heart [3], we compared different standard AAV2 with AAV pseudotype vectors in PNCMs. Whereas AAV2 was insufficient to transduce this cell type, the AAV2.6 vector type with identical AAV2 genome but a serotype 6 capsid efficiently entered the PNCMs.

Recently, it was discovered that AAV2.9 vectors are in vivo even more potent in transducing the heart than AAV2.6 or AVV2.8 [14, 18]. It was, therefore, quite unexpected that, in vitro, the pseudotyped AAV2.9 was not more efficient in entering PNCMs than the standard AAV2. This clearly was not the result of faulty AAV2.9 vector construction because in vivo in mice we observed the high cardiac transduction rate as reported previously [18]. This gross difference in AAV2.9 efficacy between rat PNCMs in vitro and mouse hearts in vivo is an interesting finding in itself. It may result from species- or age-dependent differences in AAV9 receptor expression, loss of receptors by the culturing procedure, serotype-dependent differences of virion stability in the blood, its capacity to cross anatomical barriers or its nuclear uncoating [18, 24, 28, 29]. From a practical point of view, we have used AAV2.6 for cardiomyocytes in vitro and AAV2.9 for the animal model in vivo. Easy packaging of the basic vector genome in different capsids allows tailoring for either purpose. AAV2.6-shRdRp showed high anti-viral activity in PNCMs with CoxB3 titers being reduced by >3 $\log_{10}$

steps. To our knowledge, this is the first demonstration of anti-viral RNAi in this cell type.

The kinetics of virus migration and replication in vivo are far more complex and multiple pathogenic processes are active (anti-viral immune response, inflammation and associated disturbances of cardiac morphology and function, induction of autoimmune process) during CoxB3 cardiomyopathy. To assess the overall effect of the RNAi vector in this very complex setting, we treated mice intravenously and evaluated the functional outcome 10 days after the initiation of an acute CoxB3 cardiomyopathy. Animals treated with a control shRNA vector came out of this acute phase of the disease with severely impaired systolic and diastolic function, which was significantly ameliorated after treatment with the anti-viral RNAi vector. This is consistent with the data from the primary cell cultures and demonstrates that, even in the complex setting of a CoxB3 infection in vivo, the haemodynamic course of the disease is favourably influenced by this RNA interference approach. No difference in cardiac fibrosis, which typically occurs at later time points in this model, was observed. The local cardiac immune response triggered by CoxB3 infection was expectedly not abolished by the RNAi treatment, which is effective only against intracellular virus *after* its systemic migration to the heart and thus unable to prevent initiation of the immune response. Reduction of the intracellular virus load within the heart and of the transcriptional activity of residual CoxB3 genomes in the cardiomyocytes apparently results in improved systolic and diastolic function.

In the clinical setting, the therapeutic challenge would almost always be the treatment of an ongoing acute or a chronically persistent cardiac CoxB3 infection. Approaches which require pre-emptive administration of any therapeutic agent before CoxB3 infection occurs, therefore, do not meet the actual clinical requirement. siRNA administered *after* CoxB3 inhibited viral spread in HeLa cells, but significantly less than siRNA transfected *before* virus challenge [30]. These previous studies were performed, however, in HeLa cells, which are irrelevant targets, when CoxB3 cardiomyopathies are to be treated. We have compared preventive vs. therapeutic RNAi in primary cardiomyocytes and expectedly found lower efficacy of the therapeutic approach. It takes several hours from vector entry into the cells to nuclear uncoating of the vector genome and initiation of anti-viral shRNA expression [24, 29]. We observed maximum shRdRp expression 48 h after transduction. Because one CoxB3 replication cycle takes ≈6 h, the virus passes through several rounds of replication during the 48 h investigation period with myriads of new virus progeny being produced. The RNAi approach employed in the current study may obviously be overwhelmed by very high virus loads in primary cardiomyocytes. From clinical studies, it is known, however, that during acute cardiac CoxB3 infections only ≈13% and during chronic infections only ≈0.01% of myocardial cells harbour CoxB3 genomes. Thus, the CoxB3 replicative activity in human myocardium appears to be very low compared to CoxB3-infected PNCMs in vitro. The capacity of AAV2.6-shRdRp to protect cardiomyocytes 6 h after low-level CoxB3 infections suggests that it has significant anti-viral potential under these circumstances. In contrast to the short-term efficacy of synthetic siRNAs, we previously found stable RNA interference from shRNA vectors in PNCMs [20], which would seem appropriate for the treatment of a chronic infection, too. It seems unlikely, however, that anti-viral RNAi alone would suffice to control life-threatening acute fulminant myocarditis where it would have to be combined with all other available supportive and additional anti-viral measures including virus receptor blockade to inhibit virus migration and spreading [5, 6, 8].

In conclusion, we developed a novel cardiac-directed approach for the treatment of CoxB3 cardiomyopathies, which takes advantage of cardiotropic RNAi vectors with high transduction rate and stable shRNA expression in primary cardiomyocytes. In contrast to synthetic siRNA or shRNA-plasmids, this approach has the potential to reach the myocardium in vivo after intravenous injection—as already shown for AAV9 in non-human primates—and to mediate therapeutic RNA interference in the heart. First data from a mouse cardiomyopathy model suggest that treatment with the RNAi vector results in functional protection of the heart against CoxB3-induced damage, consistent with the results of high anti-viral activity in cultured cardiomyocytes.

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Note added in proof An important recent study of AAV 1–9 tropism after intravenous injecton comprehensively demonstrated that AAV9 is superior to the other serotypes by 1–3 orders of magnitude with respect to both cardiotropism and CMV promotor-driven GFP expression level [31]. Although the current paper employs U6 promoter-driven shRNA transcription instead this new study suggests that currently only AAV9 is suitable to generate therapeutic RNAi levels within the heart.

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