Gene Therapy (2011) 18, 199–209

www.nature.com/gt

ORIGINAL ARTICLE

microRNA122-regulated transgene expression increases specificity of cardiac gene transfer upon intravenous delivery of AAV9 vectors

A Geisler[1,5], A Jungmann[2,5], J Kurreck[3], W Poller[1], HA Katus[2], R Vetter[4], H Fechner[1,3,5] and OJ Müller[2,5]

Adeno-associated virus (AAV) vectors with capsids of AAV serotype 9 enable an efficient transduction of the heart upon intravenous injection of adult mice but also transduce the liver. The aim of this study was to improve specificity of AAV9 vector-mediated cardiac gene transfer by microRNA (miR)-dependent control of transgene expression. We constructed plasmids and AAV vectors containing target sites (TSs) of liver-specific miR122, miR192 and miR148a in the 3′ untranslated region (3′UTR) of a luciferase expression cassette. Luciferase expression was efficiently suppressed in liver cell lines expressing high levels of the corresponding miRs, whereas luciferase expression was unaffected in cardiac myocytes. Intravenous injections of AAV9 vectors bearing three repeats of miR122 TS in the 3′UTR of an enhanced green fluorescent expression (EGFP) expression cassette resulted in the absence of EGFP expression in the liver of adult mice, whereas the control vectors without miR TS displayed significant hepatic EGFP expression. EGFP expression levels in the heart, however, were comparable between miR122-regulated and control vectors. The liver-specific de-targeting *in vivo* using miR122 was even more efficient than transcriptional targeting with a cardiac cytomegalovirus (CMV)-enhanced myosin light chain (MLC) promoter. These data indicate that miR-regulated targeting is a powerful new tool to further improve cardiospecificity of AAV9 vectors.
Gene Therapy (2011) **18,** 199–209; doi:10.1038/gt.2010.141; published online 4 November 2010

Keywords: AAV vector targeting; microRNA; heart

INTRODUCTION

Heart diseases are one of the most important causes of death worldwide. Despite development of novel therapeutic modalities in the last decades, options for treatment of many heart diseases are still limited. Gene transfer to the myocardium has become a promising therapeutic strategy, as it allows highly specific modulation of singular genes or gene networks. Its suitability for employment in heart diseases has been demonstrated in several animal models of myocardial ischemia,[1] heart failure[2–4] and genetic disorders.[5] More recently, a first clinical trail for treatment of patients with advanced heart failure has been initiated.[6]

As cardiac gene transfer efficiency of plasmid DNA is low even after local injection[7] viral vector systems have gained increasing interest. Among them the adeno-associated virus (AAV) vectors are currently the most potent and promising vectors used for delivery of transgenes to the heart. AAV vectors have several advantages over other viral vector systems as they are not associated with any disease in humans. Furthermore, they allow long-term gene transfer in humans.[8] Improvements in vector development resulted in so-called self-complementary (sc) AAV vectors harboring double-stranded genomes.[9] These vectors allow an extremely rapid and efficient expression of transgenes enabling even treatment of acute virus infections of the heart in a murine model.[10] Most importantly, identification of novel AAV serotypes[11] resulted in the development of AAV vectors suitable for an efficient cardiac gene transfer upon systemic application in mice as shown for AAV9.[3,10,12–15] However, AAV9 vectors exhibit a broad tissue tropism and allow also transduction of the liver upon intravascular administration.[3,12–14,16,17] Reduction of AAV-mediated transgene expression in the liver, however, may be a desirable aim to reduce unwanted side effects in cardiac gene therapy. Transcriptional control of gene expression is a promising approach to overcome this limitation.[18] A heterologous promoter containing the strong cytomegalovirus (CMV) enhancer and a cardiac myosin light chain (MLC)-2v promoter allowed an efficient and predominant gene transfer into the rodent heart, but could not completely prevent a low level hepatic gene expression.[19,20]

MicroRNAs (miRs) are a group of endogenous, short and non-coding RNA molecules that have a central role as key post-transcriptional regulators of gene activity including development, differentiation, apoptosis and proliferation.[21,22] The molecules are transcribed by polymerase II as long hairpin precursor transcripts. After sequential processing steps, a double-stranded 18–24 nucleotide (nt) long miR is incorporated into the RNA-induced silencing complex. Only one strand (the guide strand) of the miR duplex remains stably associated with RNA-induced silencing complex and forms the mature miR, whereas the opposite strand is disposed.[23] By pairing

[1]Department of Cardiology and Pneumology, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany; [2]Department of Internal Medicine III, University Hospital Heidelberg, Heidelberg, Germany; [3]Institute of Biotechnology, University of Technology, Berlin, Germany and [4]Institute of Clinical Pharmacology and Toxicology, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany
Correspondence: Dr H Fechner, Department of Cardiology and Pulmology, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, Berlin 12200, Germany.
E-mail: henry.fechner@charite.de
[5]These authors contributed equally to this work.
Received 4 May 2010; revised 9 August 2010; accepted 9 August 2010; published online 4 November 2010

with partially complementary sites in 3′ untranslated regions (3′UTRs) mature miRs mediate post-transcriptional silencing of genes.[24]

Previous studies have demonstrated that the insertion of miR target sites (TSs) into the 3′UTR of a gene expression cassette reduces expression levels of the transgene in cells and organs with high levels of corresponding miR expression.[25,26] More recently, this approach was successfully introduced in gene therapeutic applications for prevention of oncolytic picornavirus-induced myositis[27] and to de-target replication deficient and oncolytic adenoviruses from the liver *in vivo*.[28–30]

In this study, we analyzed the ability to restrict AAV9 vector-mediated gene transfer to the heart through miR-mediated suppression of the transgene delivery in the liver. We show that cardiospecificity of AAV9 vectors can be drastically improved by insertion of three tandemly inserted TS with perfect complementarity to liver-specific miR122 into the 3′UTR of a enhanced green fluorescent expression (EGFP) reporter gene expression cassette. Moreover, this approach was more efficient than a transcriptional targeting strategy using the cardiac CMV-MLC0.26 promoter.

RESULTS

Confirmation of liver tissue and liver cell-specific expression of miR122, miR192 and miR148a

Recently, miR array analysis has been shown that miR122, miR192 and miR148a are highly or abundantly expressed in the liver, whereas their expression is rare or absent in the heart,[31,32] To verify these data, an *in vivo* expression profiling analysis was carried out, considering the levels of cognate miR expression in the liver and the heart. As shown in Figure 1a, miR122 showed the absolute highest expression levels in mouse liver, whereas miR192 and miR148a expression levels reached only 8.4 and 2.4% relative to miR122 expression levels. The expression of miR122 in the heart was drastically (about 53.000-fold) and the expression of miR192 moderately (about 71-fold) lower compared with the liver, whereas the expression of miR148a differed only slightly between heart and liver. Compared with miR122, however, the expression levels of miR192 and miR148a were 63-fold and 426-fold higher in the heart, respectively. Comparison of miR expression in liver cell lines demonstrated that human liver cell line Huh7 showed a similar miR expression profile as observed in mouse liver. AML12 mouse liver cells and more distinctly human HepG2 cells showed a different expression profile, with almost no expression of miR148a in AML12 and very low levels of miR122 in HepG2.

Furthermore, we determined miR122, miR192 and miR148a expression in primary human cardiac myocytes, in neonatal rat cardiac myocytes (NRCM) and in HL-1 cells, a cell line established from the murine atrium. miR122 and miR192 were actually rarely expressed in all cultured cardiac myocytes with up to 11.000-fold and 360-fold lower expression compared with miR122 expression determined in Huh7 cells. miR148a expression, however, was abundantly found in cardiac cells similar to the murine heart (Figure 1b).

De-targeting of transgene expression in liver cells by liver-specific miRs

To investigate whether vector-mediated transgene expression can be prevented by liver-specific miR, luciferase reporter gene expressing

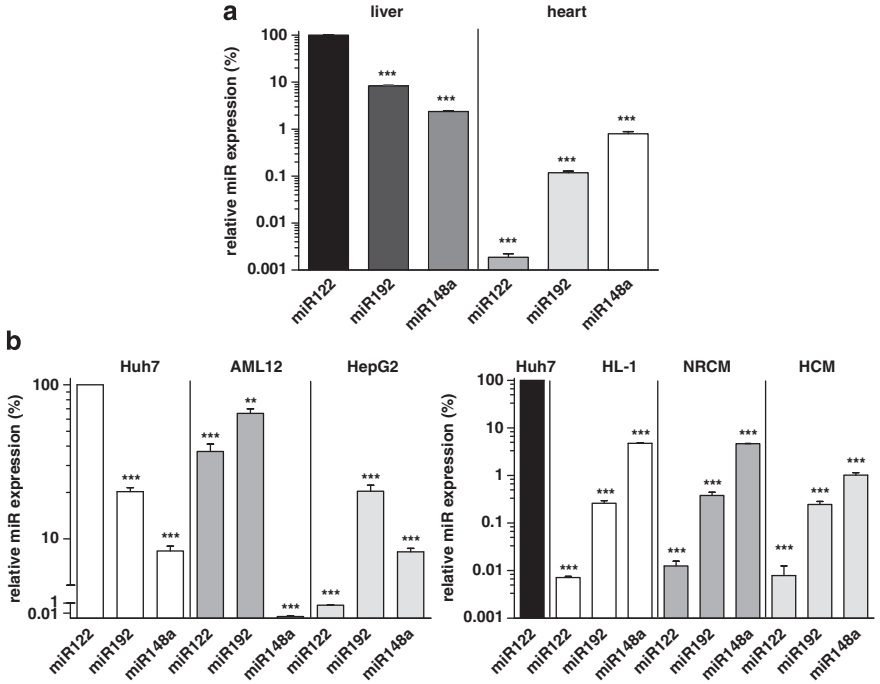


Figure 1 Relative miR122, miR192 and miR148a expression levels determined by quantitative RT–PCR. (**a**) Comparison of liver- and heart-specific expression *in vivo*. Expression levels of miR192 and miR148a in mouse liver and miR122, miR192 and miR148a in mouse heart are shown as relatives to miR122 expression levels measured in the liver (=100%). ***$P<0.001$ for each column versus control (first column). (**b**) Comparison of miR expression levels in liver cells and cardiac myocytes. Expression levels of miR122, miR192 and miR148a in the human liver cell lines Huh7 and HepG2, mouse liver cell lines AML12, permanent mouse cardiac cell line HL-1, NRCM and primary human cardiac myocytes (HCM) are shown as relatives to miR122 expression levels measured in Huh7 cells (=100%, first white column in the left diagram, black column in the right diagram). miR expression profile in Huh7 cells was most similar to expression profile determined in mouse liver. All cardiac cells showed similar miR expression pattern as found in mouse heart. **$P<0.01$; ***$P<0.001$ for each column versus control (first column). Note: Although miR122 and miR192 are preserved among mouse, rat and human, miR148a sequence has been described for mouse and human but not for rat in the literature. However, a sequence complementary to mouse and human miR148a is located in the rat chromosome 4 and may represent the putative miR148a.

b

miR122(1x)TS	5′-TAAttctagatcgcgaa**caaacaccattgtcacactcca**gtacta-3′
miR192(1x)TS	5′-TAAttctagatcgcct**ggctgtcaattcataggtcag**tctaga-3′
miR148a(1x)TS	5′-TAAttctagatcgcct**acaaagttctgtagtgcactga**gtttaactctaga-3′
miR122(3x)TS	5′-TAAttctagatcgcgaa**caaacaccattgtcacactcca**gtatac**acaaacaccattgtcacactcca** gatatca**caaacaccattgtcacactcca**aggcta-3′
miR122(6x)TS	5′-TAAttctagatcgcgaa**caaacaccattgtcacactcca**gtatac**acaaacaccattgtcacactcca**gatatca**caaacaccattgtcacactcca** aggcgaa **caaacaccattgtcacactcca**gtatac**acaaacaccattgtcacactcca**gatatca**caaacaccattgtcacactcca**aggcta-3′
miR122(3x)TS/ miR192(3x)TS	5′-TAAttctagatcgcgaa**caaacaccattgtcacactcca**gtatac**acaaacaccattgtcacactcca**gatatca**caaacaccattgtcacactcca** aggcct**ggctgtcaattcataggtcag**tctagg**ggctgtcaattcataggtcag**tctagg**ggctgtcaattcataggtcag**tctaga-3′
miR122(3x)TS/ miR148a(1x)TS	5′-TAAttctagatcgcgaa**caaacaccattgtcacactcca**gtatac**acaaacaccattgtcacactcca**gatatca**caaacaccattgtcacactcca** aggcct**acaaagttctgtagtgcactga**gtttaactctaga-3′

Figure 2 Scheme of luciferase reporter gene vector with miR122, miR192 and miR148a TS. (**a**) Schematic illustration of *Firefly* luciferase reporter gene construct containing the (CMV)-enhanced 0.26 kb MLC promoter (CMV-MLC0.26), the luciferase reporter gene and miR TS in the 3′UTR of the expression cassette. (**b**) Sequences of miR122, miR192 and miR148a TS including the number of repeats and combinations of different miR TS used in the present investigation. miR TS are in bold. Restriction sites flanking miR TS are underlined. TAA represents the translational stop codon of the *Firefly* luciferase cDNA.

plasmid vectors were constructed each containing a single TS with perfect complementarity to the mature miR122, miR192 and miR148a in the 3′UTR of the expression cassette, respectively. Additionally, two constructs were produced containing either three or six tandem repeats of the miR122 TS, one construct that contains three tandem repeats of miR122 TS and three of miR192 TS, and one construct containing three tandem repeats of miR122 TS and one miR148a TS (Figures 2a and b). Three different liver cell lines (Huh7, AML12 and HepG2), the cardiac cell line HL-1 and NRCM were transfected with these plasmids and the strength of miR-mediated suppression of luciferase reporter gene activity was determined. The extent of suppression of luciferase activity (Figures 3a and b) showed a clear correlation to the miR expression levels (Figure 1b) determined for each miR and cell line investigated. All tested constructs containing a single TS showed a reduction of luciferase activity in the investigated liver cell lines. An exception was seen for the miR148a TS construct, which was not suppressed in AML12 cells, which may be explained by lack of miR148a expression in this cell line (Figure 1b). Highest suppression of luciferase expression with a reduction of about 81% for the miR122 TS construct was observed in Huh7 cells representing the cell line with absolutely highest miR122 expression. No significant change of luciferase activity was visible in NRCM. Only the construct carrying the miR148a TS resulted in distinctly suppressed reporter activities in HL-1 cells.

An increase of miR TS repeats for miR122 TS from one (up to 81% reduction in Huh7) to three repeats (up to 92.2% reduction in Huh7) but also from three to six repeats (up to 94.8% reduction in Huh7) as well as combination of three miR122 TS repeats with three miR192 TS repeats (up to 94.6% reduction in Huh7 cells) significantly increased suppression of luciferase activity (Figure 3a). This additive effect was only seen for miRs that were highly or moderately expressed in the corresponding cell line. In cardiac cells that express miR122 and miR192 at very low levels, these effects were negligible or absent (Figure 3b).

miR-dependent regulation of AAV vectors

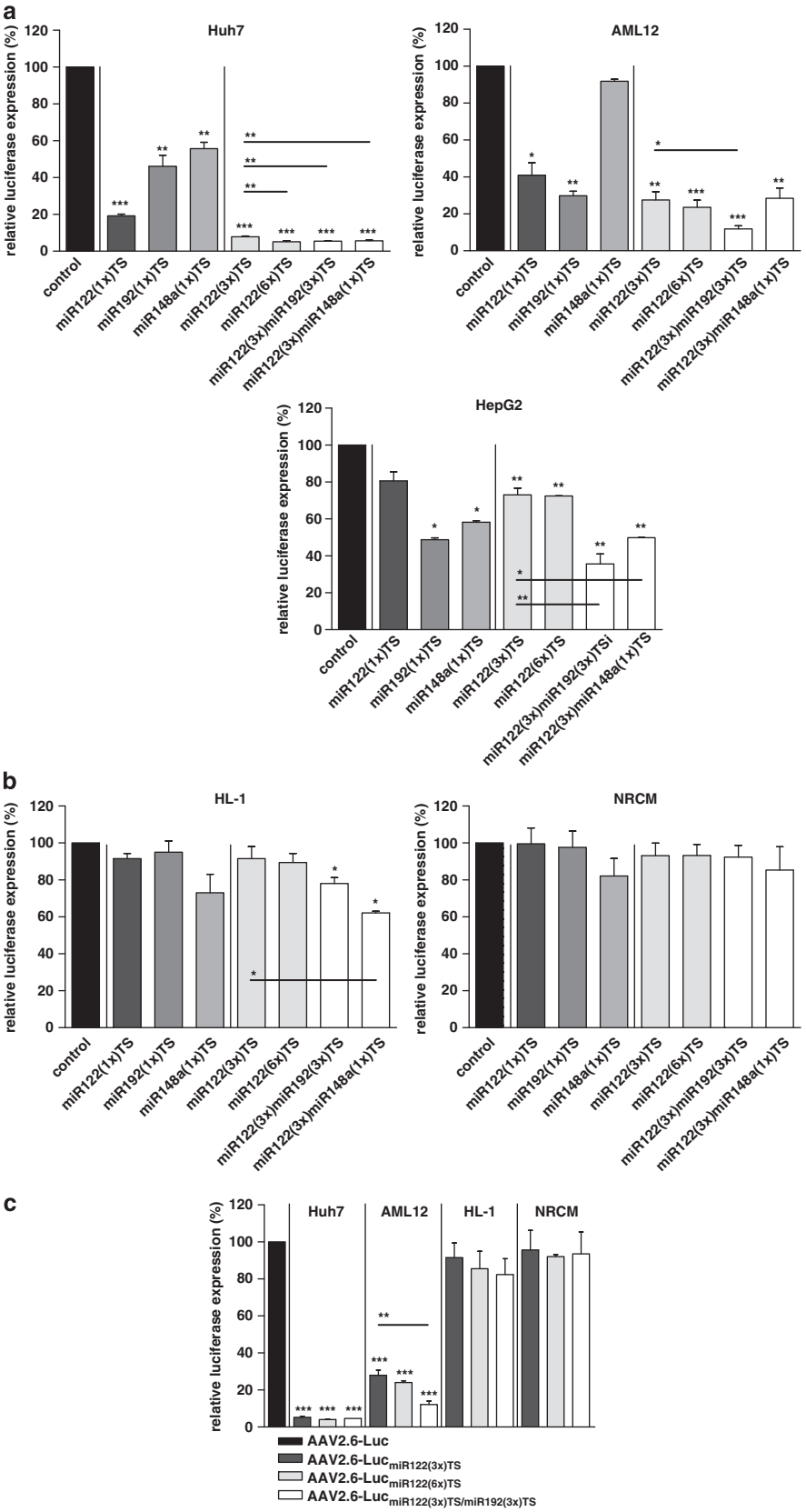
To verify plasmid data in the context of AAV vectors, we constructed three AAV6 pseudotyped vectors with sc vector genomes, bearing a luciferase reporter gene expression cassette with $miR122_{(3\times)TS}$, $miR122_{(6\times)TS}$ and the combination of $miR122_{(3\times)TS}$/$miR192_{(3\times)TS}$. Corresponding to the results obtained after plasmid transfection, the luciferase expression was reduced up to 95% in Huh7 cells for all AAV constructs. A reduction of luciferase activity of about 90% for vectors containing the $miR122_{(3\times)TS}$/$miR192_{(3\times)TS}$ was observed in AML12 cells. The luciferase expression in HL-1 and NRCM, however, was not affected (Figure 3c).

miRs suppress luciferase expression by degradation of the TS-containing transcripts

In order to analyze whether miR-mediated inhibition of luciferase protein expression resulted from luciferase mRNA degradation or from inhibition of protein translation, we isolated total RNA from Huh7 cells transfected with miR122 TS, miR192 TS and miR148a TS-containing luciferase reporter plasmids, respectively and carried out northern blot hybridization to detect luciferase mRNA expression. The luciferase mRNA expression was distinctly decreased for all miR TS carrying constructs, indicating that regulation of reporter gene expression may preferentially occur through degradation of luciferase mRNA. Corresponding to the differences in miR expression levels measured in Huh7 cells, miR TS constructs containing multiple TS of miR122 or combination of $miR122_{(3\times)TS}$ and $miR192_{(3\times)TS}$ as well as single miR122 TS showed very strong suppression of luciferase mRNA expression, whereas expression of transcripts bearing single miR148a TS and miR192 TS was distinctly less suppressed (Figure 4).

5′ Deletion of miR122 TS abrogates miR122 suppression activity

Having shown that luciferase reporter expression regulated by liver-specific miRs is efficiently suppressed in liver cells, we were interested in identifying whether there are regions of the miR TS that are essential to ensure miR-mediated suppression. For this reason, we generated luciferase-expressing vectors bearing a single miR122 TS shortened stepwise at the 5′ part to a total length of 21–7 nt. All miR122 TS variations, however, contained sequences complementary to the miR122 seed sequence. As shown in Figures 5a and b, luciferase expression was not suppressed for the constructs containing miR TS with 7–15 nt complementary to miR122, whereas strong suppression was visible for all miR TS with $\geqslant$17 nt. The strength of inhibition for the constructs with an miR TS length of 17–21 nt, were in the range of the suppression level of the original miR122 TS. In summary, these data demonstrate that the 3′ part of miR122 TS including sequences complementary to the seed sequence of the miR122, the potential Argonaute 2 cleavage site (between the nt paired to bases 10 and 11 of


a
Huh7
AML12
HepG2
relative luciferase expression (%)
control
miR122(1x)TS
miR192(1x)TS
miR148a(1x)TS
miR122(3x)TS
miR122(6x)TS
miR122(3x)miR192(3x)TS
miR122(3x)miR148a(1x)TS
b
HL-1
NRCM
c
AAV2.6-Luc
AAV2.6-Luc miR122(3x)TS
AAV2.6-Luc miR122(6x)TS
AAV2.6-Luc miR122(3x)TS/miR192(3x)TS

the miR) and some nt upstream are essential for miR122-mediated silencing. Nevertheless, deletions of about one fourth of the miR122 TS on its 5′ end are well tolerated and do not affect miR122-mediated silencing.

miR122-regulated AAV9 vectors efficiently de-target the liver without affecting the heart

Encouraged by the *in vitro* data, we next analyzed whether regulation of transgene expression by liver-specific miRs can specifically suppress transgene expression in the liver following AAV vector-mediated gene transfer without affecting the heart *in vivo*. Therefore, we constructed two AAV9 vectors containing three tandem repeats of the miR122 TS in the 3′UTR of an EGFP expression cassette. EGFP expression of the vector AAV9-CMV-EGFP$_{miR122(3\times)TS}$ was driven by a CMV promoter and EGFP expression of the vector AAV9-MLC0.26-EGFP$_{miR122(3\times)TS}$ was driven by the cardiac CMV-MLC0.26 promoter. Efficiency and specificity of these vectors were compared with corresponding vectors without miR TS (AAV9-CMV-EGFP and AAV9-CMV-MLC0.26-EGFP). Prior to generation of AAV vectors, we tested the respective AAV-shuttle plasmids *in vitro* in Huh7 and AML12 cells. As shown in Figure 6a, the miR122 TS-regulated constructs were strongly suppressed in Huh7 cells as expected and at less degree also in AML12 cells. Interestingly, EGFP expression was lower in case of the CMV promoter driven and miR122-regulated construct than in the CMV-MLC0.26 construct without miR TS. This suggests that miR-dependent de-targeting may be more efficient than transcriptional targeting using the CMV-MLC0.26 promoter.

The two miR122-regulated and two control AAV9 vectors were applied in each three animals per group at 7.5×10^{11} AAV9 vector genomes per animal *via* tail vein injection. After 4 weeks, mice were killed and tissue slices of the heart and liver were directly imaged for EGFP expression (Figure 6b). There was a very strong EGFP expression in the heart of all animals transduced with AAV9 vectors harboring the CMV promoter irrespective of the presence or absence of miR122 TS. Similar expression levels were observed for AAV9 vectors containing the CMV-MLC0.26 promoter. No difference was observed in the cardiac transduction pattern between all groups (Supplementary Figure 1). In skeletal muscle, expression levels were close to background in all groups (Supplementary Figure 2). In the liver, all three animals transduced with AAV9-CMV-EGFP and two of three animals transduced with AAV9-CMV-MLC0.26-EGFP showed markedly EGFP expression. However, EGFP expression was distinctly lower for the CMV-MLC0.26 promoter compared with the CMV promoter driven constructs. In sharp contrast, hepatic EGFP expression was completely absent in all animals transduced either with AAV9-CMV-EGFP$_{miR122(3\times)TS}$ or AAV9-CMV-MLC0.26-EGFP$_{miR122(3\times)TS}$ (Figures 6b and c). To exclude that differences in EGFP expression resulted from differences in transduction efficiency of distinct AAV vectors, the amount of vector DNA in the liver and the heart was determined and found to be similar (Supplementary Figure 3). This supports that liver-specific suppression of EGFP expression in the miR122 TS-containing AAV vectors did not result from lower liver transduction of the respective vector batches. These data clearly demonstrate that AAV9 vector-mediated transgene expression can efficiently be suppressed in the liver by insertion of miR122 TS into AAV9 vector genomes. Most importantly, abolishing transgene expression in the liver occurred without affecting the transgene expression in the heart. Moreover, our data indicate that de-targeting of the liver by miR122 regulation was even more efficient than utilization of a cardiac promoter.

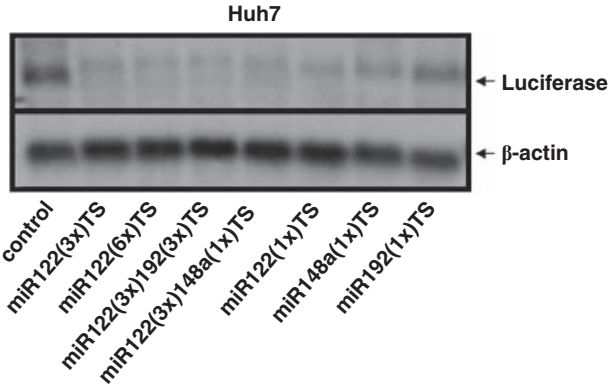


Figure 4 Investigated miRs inhibit luciferase expression by target mRNA degradation. Huh7 were transfected with *Firefly* luciferase expression plasmids containing the indicated miR TS in the 3′UTR of *Firefly* luciferase expression cassette. Total RNA was isolated from cells 48 h after transfection and expression levels of *Firefly* luciferase and cellular β-actin mRNA determined by northern blotting using ^{32}P-labeled single-stranded antisense probes directed against luciferase and β-actin mRNA, respectively. Compared with the control (*Firefly* luciferase vector without miR TS), luciferase mRNA expression levels were reduced for all constructs containing liver-specific miR TS. The experiments were carried out three times with similar results. A representative blot is shown.

Absence of side effects after miR122-regulated AAV vector transduction *in vivo*

Exclusion of side effects following vector transduction is a major intention of gene therapy. To exclude possible side effects, we analyzed histological sections of liver tissue of mice transduced with AAV9-CMV-EGFP$_{miR122(3\times)TS}$ and AAV9-CMV-MLC0.26-EGFP$_{miR122(3\times)TS}$ vectors. Additionally, expression of endogenous miR122 levels and expression of Gys1, representing a protein that is potentially regulated by miR122,[33] were determined. Liver sections did not reveal any signs of necrosis or inflammation (Figure 6d). In fact, no differences were seen in comparison to liver sections of wild-type mice. Moreover,

Figure 3 Effects of miR TS on transgene expression in liver and cardiac cells. (**a**) miR-dependent regulation of *Firefly* luciferase expression in liver cells. The ratio for cells transfected with unmodified *Firefly* luciferase construct (control) was set as 100% for each cell line transfected, and the other values for the same cell type were given relative to this reference. miR TS including the number of TS repeats and TS combinations are given below the corresponding columns. *Firefly* luciferase expression levels were normalized against *Renilla* luciferase expression levels in each sample. (**b**) miR-dependent regulation of transgene expression in cardiac myocytes. Measurement of *Firefly* luciferase expression levels and calculation of inhibition of *Firefly* luciferase expression were performed as in (**a**). (**c**) miR TS-mediated regulation of AAV vector delivered transgenes. Liver and cardiac cells were transduced with 2000 vg per cell of pseudotyped AAV6 vectors carrying *Firefly* luciferase reporter expression cassette without miR TS (AAV6-Luc), with 3 (AAV6-Luc$_{miR122(3\times)TS}$) or 6 (AAV6-Luc$_{miR122(6\times)TS}$) tandem repeats of miR122 TS or three tandem repeats of miR122 TS in combination with three tandem repeats of miR192 TS (AAV6-Luc$_{miR122(3\times)TS/miR192(3\times)TS}$). Levels of significance are indicated: ***$P<0.001$; **$P<0.01$; *$P<0.05$ versus the first column (control) or between columns of miR122(3×)TS and TS repeats/combinations. vg, vector genomes.

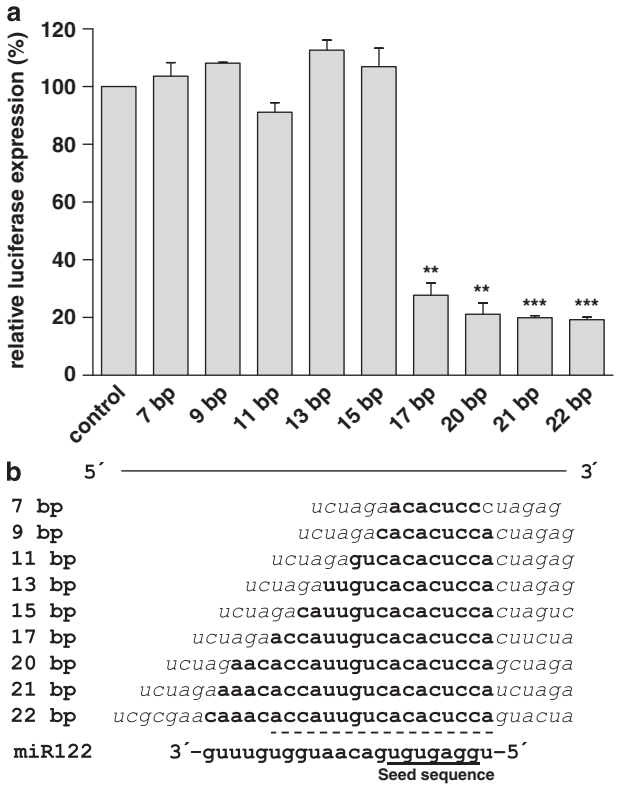


Figure 5 5′ Deletion of miR122 TS abrogates miR122 suppression activity. (**a**) Huh7 cells were transfected with *Firefly* luciferase expression plasmids containing the reporter gene alone (without TS, control) or containing a reporter gene with single miR122 TS shortened stepwise at 5′ part to a total length of 21 to 7 nt and the complete miR122 TS respectively. The *Firefly* to *Renilla* ratio for cells transfected with unmodified *Firefly* luciferase construct (control) was set as 100% and the other values were given relative to this reference. Inhibition of luciferase expression was visible for constructs containing 17 to 22 nt of miR122 TS. $^{**}P<0.01$; $^{***}P<0.001$ for each column versus control (first column). (**b**) Sequences of miR122 TS variants used for analysis (bold letters) and miR122 sequence. Dashed line marks nt present in miR122 TS essential to achieve miR122 silencing. The seed sequence of miR122 is underlined.

miR122 expression profile did not differ to the wild-type mice (Figure 6e) and Gys1 expression was not changed (Figure 6f), indicating that miR122 was not saturated by miR122 TS expressed by AAV9 vectors. In summary, these data reveal the absence of any side effects or dysregulations of genes that represent potential targets of miR122 after transduction of the liver with miR122 TS bearing AAV9 vectors.

DISCUSSION

Cardiac-specific delivery of a transgene is an important prerequisite for gene therapy of cardiac diseases especially if the transduction of extracardiac tissue may be a potential risk of side effects. A breakthrough in cardiac gene transfer was achieved with the finding that AAV vectors based on AAV serotypes 6 and 9 allow an efficient cardiac transduction in rodents.[3,10,12–15,34] By selection of an AAV *cap* gene library generated by DNA shuffling of different AAV serotype capsid genes, a novel AAV vector (AAVM41) was identified whose capsid is composed by elements of AAV1, 6, 7 and 8. This vector revealed a similar transgene expression efficiency in murine myocardium as AAV9 but significantly reduced gene transfer into the liver.[17]

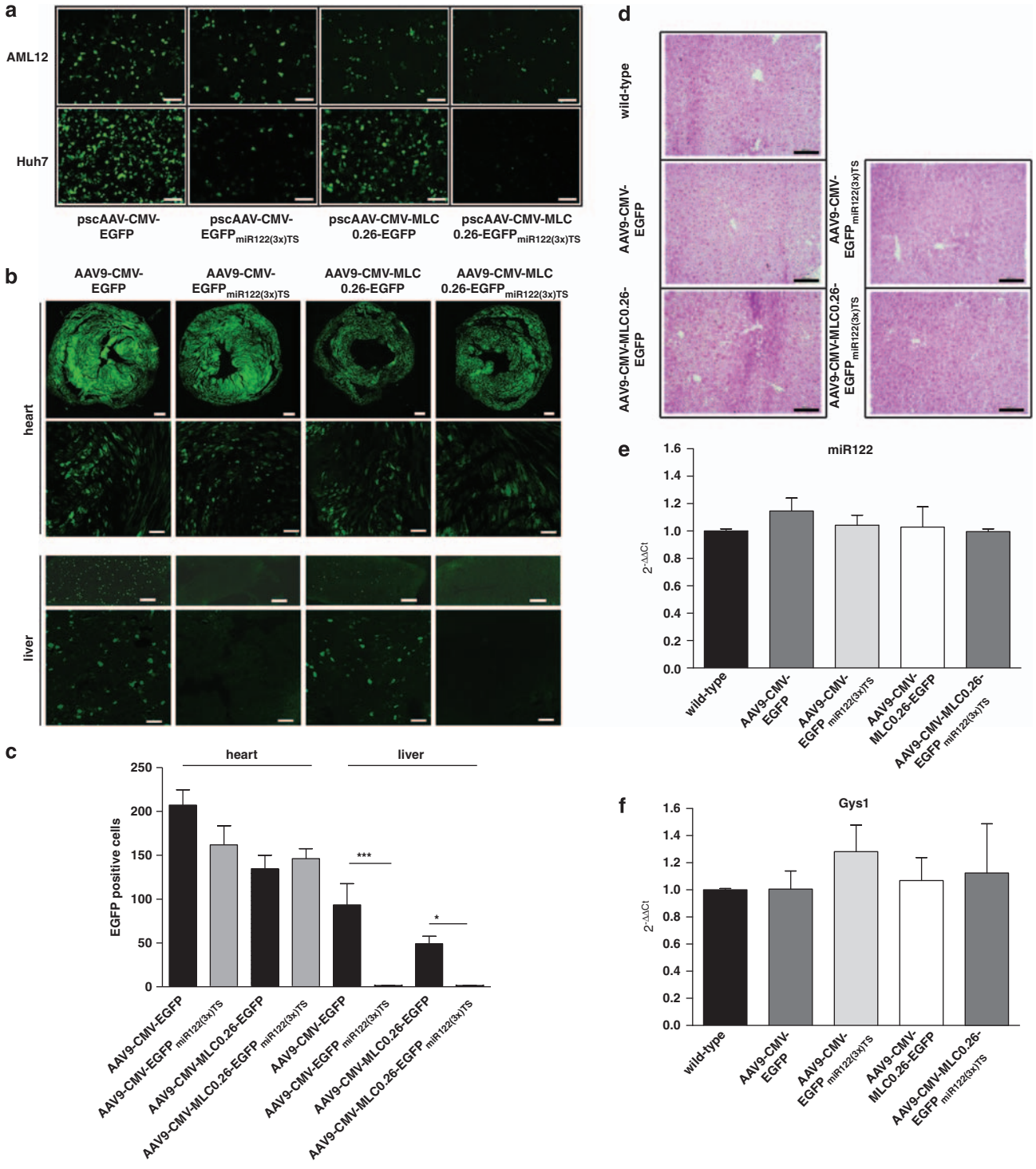
Transcriptional targeting with cardiac-specific promoters has been described as another valuable strategy to improve specificity of cardiac transgene delivery. We could recently show that expression of a transgene driven by a CMV-MLC1.5-hybrid promoter is suitable to increase specificity of cardiac transgene expression of AAV vectors.[19] However, despite significant improvements by employment of cardiotropic AAV vectors and use of heart-specific promoters residual expression of transgenes stayed still detectable in several non-targeted tissues following AAV vector application.[19]

miR-dependent regulation has recently emerged as a powerful new tool to improve specificity of transgene delivery[25] and has been used in gene therapy approaches with lentiviral[35] and adenoviral vectors[28,29,36] as well as oncolytic picornaviruses.[27] In a proof of concept approach we have aimed to investigate the potential of this technique to improve the specificity of AAV vectors to the heart by de-targeting AAV9 vectors from the liver. Initial evaluation of three miR candidates (miR122, miR192 and miR148a) that have recently been described to be selectively expressed in the liver[31,32] confirmed that miR122 was most abundantly and selectively expressed in murine liver.

Figure 6 miR122-regulated AAV9 vector is de-targeted from the liver but unaffected in the heart. (**a**) Analysis of EGFP-expressing AAV-shuttle plasmids in liver cells. Huh7 and AML12 cells were transfected with AAV-shuttle plasmids expressing EGFP under the control of a CMV or CMV-MLC0.26 promoter either without miR TS (pscAAV-CMV-EGFP and pscAAV-CMV-MLC0.26-EGFP) or with 3 repeats of miR122 TS in the 3′UTR (pscAAV-CMV-EGFP$_{miR122(3\times)TS}$ and pscAAV-CMV-MLC0.26-EGFP$_{miR122(3\times)TS}$). Strong suppression of EGFP expression with miR122 TS-regulated constructs occurred in Huh7 cells and at less degree in AML12 compared with the non-regulated constructs. Images of GFP-expressing cells were taken by fluorescence microscopy 48 h after transfection. Bar: 200 μm. (**b**) Regulation of AAV9 vector-mediated expression by miR122 in the heart and the liver *in vivo*. Each three mice per group were injected with 7.5×10^{11} vg of AAV9-CMV-EGFP, AAV9-CMV-EGFP$_{miR122(3\times)TS}$, AAV9-CMV-MLC0.26-EGFP and AAV9-CMV-MLC0.26-EGFP$_{miR122(3\times)TS}$ via tail vein injection. After 4 weeks, animals were killed and organs were dissected and rapidly frozen in liquid nitrogen. In all, 10 μm cryosections of heart and liver were evaluated for EGFP expression by fluorescence microscopy using direct imaging of tissue slices. Representative microphotographs are shown for heart (upper row overview, lower row magnification) and liver. EGFP expression in the heart was similar in mice having received vectors with the CMV promoter compared with the CMV-MLC0.26 promoter. Cardiac EGFP expression was similar between miR122 TS-regulated vectors and corresponding control vectors. Both AAV9 control vectors containing the CMV-MLC0.26 and CMV promoter without miR122 TS sequences showed EGFP expression in the liver, whereas both miR122-regulated AAV9 vectors did not show any EGFP expression in the liver. Bar heart and liver: upper row 500 μm, lower row 100 μm. (**c**) Quantification of EGFP-expressing cells in heart and liver in the presence and absence of miR122 TS. Counting of EGFP-expressing cells in the heart using the software Image J reveals similar EGFP-positive cells irrespective of the presence of the miR122 TS. In contrast, addition of the miR122 TS significantly reduced EGFP expression in the liver both in CMV- and CMV-MLC0.26-driven vectors. $^{*}P<0.05$; $^{***}P<0.001$. (**d**) Histological analysis of liver tissues. In all, 6 μm hepatic cryosections transduced with AAV vectors as indicated were stained with hematoxilin and eosin and analyzed under a light microscope. No differences were observed between livers transduced with AAV9 vectors and untreated wild-type controls. Bar: 100 μm. (**e**) Transduction with miR122-regulated AAV9 vectors does not alter endogenous miR122 expression in the liver. RNA from liver was isolated and miR122 expression was determined by quantitative RT–PCR. No difference between livers transduced with AAV9 vectors and untreated wild-type controls was observed. Analysis was carried out using the $2^{-\Delta\Delta Ct}$ method. (**f**) Expression of predicted miR122-regulated liver proteins is not altered following transduction with miR122-regulated AAV9 vectors. Expression of Gys1, a predicted target protein of miR122 regulation, was determined by real-time RT–PCR in livers transduced with AAV9 vectors as indicated. No differences were found in livers transduced with AAV9 vectors compared with untreated wild-type controls. vg, vector genomes.

Nevertheless, also miR192 showed high and liver specific expression, whereas only low levels in the liver and no cardiac specificity were found for miR148a. *In vitro* analysis revealed that among tested liver cell lines only Huh7, but all cardiac myocyte cell lines reflected the *in vivo* expression pattern of these three miRs. Based on these data, we considered miR122 as the most suitable potential regulator molecule for AAV vectors.

Carrying out a step-by-step analysis of functional mechanisms of miR-dependent regulation of reporter transgene expression *in vitro* confirmed this hypothesis. Moreover, our results exhibit several findings with general and specific impact for utilization of miR-dependent regulation of AAV vectors. Our data reveal that expression levels of all three investigated miRs inversely correlated with their suppression activity. On the first view, this seems to be obvious as an increase of miR concentration per cell might result in a higher probability to bind to a corresponding miR TS, resulting in increased suppression of the transgene.[37] Others, however, have described some miR that obviously do not follow this relationship and suppress the



targets independently from their expression levels.[25] The mechanism is currently not completely understood, but factors that directly or indirectly influence the miR or the cell may be involved therein.[38,39] Nevertheless, miR122 TS-regulated reporter followed a clear relationship between expression levels and suppression activity. Interestingly, efficient suppression was not only observed when the miR122 TS perfect complementary to mature miR122 was used in the constructs, but also when 5′ deleted miR122 TS variants with 17–21 bp length were used. Moreover, a simple nt substitution from G to T immediately following the 3′ end of the miR122 TS significantly influenced miR122 suppression activity of the 20 nt miR122 TS construct (results not shown). This demonstrates on the one hand that the 5′ part of the miR122 TS is not required for miR122-mediated silencing. On the other hand, it suggests that structural events outside the miR TS might not obligatory be connected with miR122 binding to its TS but still influence silencing.

In agreement with earlier reports,[25,40,41] our data confirm that the number of repetitive miR TS can aggravate miR-mediated suppression of the transgene expression. We observed that the degree of suppression measured for constructs with three miR122 TS repeats was markedly increased to about 92% compared with constructs bearing only a single miR TS, which results only in a reduction of about 81% in Huh7 cells. This could be explained by serial binding of miR to the target mRNA that subsequently may result in increased cleavage events after introduction of the miR–mRNA complex into the miR silencing pathway. Nevertheless, a simple additional steric inhibition by forming stable miR—miR TS duplexes has also been taken into account. The concatamerization of miR122 TS to six tandem repeats resulted in a significant but only negligible further improvement of suppression *in vitro* when compared with constructs carrying three elements. This observation is in line with a report showing that 12 copies of miR122 TS did not further improve suppression activity of an oncolytic adenovirus vector compared with the vector containing six copies of the miR122 TS.[28] In general, it seems that already three to six copies of the miR TS are sufficient to achieve maximal suppression by a highly expressed miR. It is important to note that also the combination of miR122 TS and miR192 TS acted in an additive manner and increased suppression of gene expression after transfer of vectors in liver cell lines *in vitro*. However, an advantage of this combination over utilization of miR122 TS alone was only seen in cells with comparable high or moderate expression levels of the corresponding miR. In Huh7 cells, AAV6 vectors bearing miR122 TS/miR192 TS with each three TS repeats did not result in higher reporter gene suppression than AAV6 with only three repeats of miR122 TS. For this reason, we did not choose this combination variant for our *in vivo* applications. In other application as for example control of viral replication, a combination of different miR TS may have an advantage, as it might help to prevent occurrence of escape mutants emerged from mutation in one miR TS.[27] Importantly, both expression of concatamerized miR122 TS and combination of miR122 TS and miR192 TS from AAV6 vectors *in vitro* did not affect the transgene expression in cardiac cells. This in fact underlines the specificity of the liver-specific miR approach *in vitro*.

In previous studies, we have used cardiotropic AAV9 vectors[3,10,20] and cardiac-specific promoters in combination with AAV vectors[19] with the intention to improve specificity of cardiac transgene delivery *in vivo*. Although this approach resulted in a markedly increase in cardiac specificity, some transgene expression could still be detected in the liver of transduced animals. In the present study, we could show that EGFP reporter expression of AAV9 vectors bearing three miR122 TS were completely abolished within the liver, whereas expression was unaffected in the heart. This was observed irrespective of whether EGFP was driven by a CMV or cardiac CMV-MLC0.26 promoter. It demonstrates that regulation by miR122 is an efficient method to de-target AAV vectors from the liver *in vivo* and confirms that three miR122 TS inserted into the AAV9 vector genome are sufficient to achieve this aim. Moreover, according to a recent study,[28] the lack of any EGFP expression in the liver indicates that miR122-mediated suppression *in vivo* is much stronger than *in vitro*. Most likely, this discrepancy may result from several fold higher miR122 expression in primary hepatocytes *in vivo* compared with Huh7 cells.[28]

Importantly, miR122-dependent hepatic de-targeting of AAV9 vectors seems to be *per se* superior to transcriptional targeting of the vector to myocardium using the cardiac CMV-MLC0.26 promoter. These findings may now open a novel way to increase specificity of AAV-mediated transgene delivery to the heart and other organs. Especially in cases when no tissue-specific promoter is available, its activity is too low to induce expression at sufficient levels or it is only necessary to protect few selected organs from the transgene, miR-dependent AAV vector regulation may be employable. Furthermore, it was shown that tissue-specific promoters and miR act in an additive manner leading to improvement of vector specificity.[28] Although direct detection of EGFP on histological sections in our study was too insensitive to reveal such possible additive effects, a similar effect might occur.

We observed no hepatic side effects *in vivo* by application of AAV vectors harboring miR122 TS underlining the safety of our approach. In this regard, it should be noted that several vectors bearing miR TS have successfully been used as competitive inhibitors to repress miRs. Repression of miR by corresponding miR TS is possible, but only when the miR TS are expressed from a strong promoter at very high levels and/or the transgene contains multiple tandem miR TS ($\geqslant 6$), which preferentially contain a bulge at position 9–12 to prevent RNA interference type endonucleolytic cleavage of the miR—miR TS duplex by Argonaute 2.[26,42,43] None of these features are characteristics of the miR-regulated AAV vectors used here. Therefore, AAV vectors with miR122 TS are obviously unable to deregulate endogenous miR122 expression levels and induce side effects.

Finally, based on its small genome size, AAV vectors have a comparable low packaging capacity for foreign DNA. This, however, may not limit the utilization of miR TS concatamers to control transgene expression. In general, miR TS have sizes below 100 bp. Several different miR TS regulator units may be inserted into a single AAV vector genome without oversizing the genome, even when AAV vectors with sc genomes are generated. Therefore, miR mediating de-targeting seems to be ideal for improving specificity of AAV vector-mediated transgene expression.

In summary, our proof of concept study demonstrates that miR122 TS bearing cardiotropic AAV9 vectors can efficiently de-target gene expression from the liver. Because of its efficiency, specificity, absence of side effects, and high homology between different species, miR-dependent regulation techniques have the potential to be used in the context of AAV vectors in a wide panel of applications.

MATERIALS AND METHODS

Cell cultures

Huh7 cells (kindly provided by Christian Freise, Department of Gastroenterology, Infectiology and Rheumatology, Charité, Berlin, Germany) and HepG2 cells (both representing human hepatocellular cell lines) as well as 293 (human embryonal kidney) cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (Invitrogen GmbH, Karlsruhe, Germany). AML12, a mouse

liver immortalized cell line (kindly provided from Anna Foryst-Ludwig, Center for Cardiovascular Research, Charité, Berlin, Germany) was cultured in high glucose Dulbecco's Modified Eagle's Medium/Ham's F12 with glutamine (Sigma-Aldrich Chemie, Steinheim, Germany) supplemented with 0.005 mg ml^{-1} insulin, 0.005 mg ml^{-1} transferrin, 5 ng ml^{-1} selenium (Sigma-Aldrich) and 40 ng ml^{-1} dexamethasone (Sigma-Aldrich). Media of permanent cell lines Huh7, HepG2, 293, AML12 were supplemented with 10% fetal calf serum and 1% of each penicillin and streptomycin. The HL-1 cell line, a cardiac muscle cell line established from an AT-1 mouse atrial cardiomyocyte tumor lineage, was a kind gift from William C Claycomb (LSU Health Center, New Orleans, LA, USA). The cells were maintained in Claycomb medium (SAFC Biosiences, Lanexa, KS, USA) supplemented with 10% fetal calf serum, 1% of each penicillin and streptomycin, 0.1 mM norepinephrine (Sigma-Aldrich) and 2 mM L-glutamine (Invitrogen). Before culturing HL-1 cells, tissue culture flasks were coated with fibronectin/0.02% gelatine (Sigma-Aldrich). Primary NRCM were isolated from neonatal rats and cultured in CMRL 1415 Medium (Biochrom, Berlin, Germany) supplemented with 2.8 mM glucose, 5.4 mM KCl, 1.26 mM $CaCl_2$, 2 μM 5′ fluoro-2′-deoxyuridine (Sigma-Aldrich), 10% fetal calf serum and 2 μg ml^{-1} of gentamycin (Biochrom) as described.[44] Human cardiac myocytes from normal human ventricle tissue of the adult heart (PromoCell, Heidelberg, Germany) were cultured in myocyte growth medium (PromoCell) supplemented with SupplementMix (PromoCell).

Plasmid construction

To generate AAV-shuttle plasmids containing miR TS, a DNA fragment containing three repeats of miR122 TS and one of miR148a TS (5′-tct aga tcg cga aca aac acc att gtc aca ctc cag tat aca caa aca cca ttg tca cac tcc aga tat cac aaa cac cat tgt cac act cca agg cct aca aag ttc tgt agt gca ctg agt tta act cta ga-3′) was synthesized, inserted into pBluescript II SK (+) (ATG:biosynthetics, Merzhausen, Germany), and amplified from the plasmid with primers M13 Forward and M13 Reverse (Invitrogen). The fragment was inserted into a single *Xba*I site at the 3′UTR of luciferase expression cassette of AAV-shuttle plasmid pUF-CMV$_{enh}$/MLC0.26-Luc containing a cardiac (CMV)-enhanced 0.26 kb rat MLC promoter (MLC0.26) driving a luciferase reporter gene. The resulting plasmid was termed pUF-Luc$_{miR122(3\times)TS/miR148a(1\times)TS}$. To exchange the miR148a TS by the miR192 TS, oligonucleotides 5′-cct ggc tgt caa ttc ata ggt ca gt-3′ and 5′-cta gac tgac cta tga att gac agc cag g-3′ were annealed and ligated into *Stu*I and *Xba*I digested pUF-Luc$_{miR122(3\times)TS/miR148a(1\times)TS}$ resulting in pUF-Luc$_{miR122(3\times)TS/miR192(1\times)TS}$. Plasmids containing single miR122 (pUF-Luc$_{miR122(1\times)TS}$), miR148a (pUF-Luc$_{miR148a(1\times)TS}$) or miR192 (pUF-Luc$_{miR192(1\times)TS}$) TS as well as a triplet of the miR122 TS (pUF-Luc$_{miR122(3\times)TS}$) were derived from pUF-Luc$_{miR122(3\times)TS/miR148a(1\times)TS}$ and pUF-Luc$_{miR122(3\times)TS/miR192(1\times)TS}$. Plasmids containing tandems of three miR122 TS and three miR192 TS (pUF-Luc$_{miR122(3\times)TS/miR192(3\times)TS}$) and six miR122 TS (pUF-Luc$_{miR122(6\times)TS}$) were generated by inserting two additional miR192 TS into the plasmid pUF-Luc$_{miR122(3\times)TS/192(1\times)TS}$ and three miR122 TS into the plasmid pUF-Luc$_{miR122(3\times)TS}$, respectively. pUF-Luc$_{miR122(1\times)TS}$ plasmids with a single miR122 TS having homology to miR122 fragments ranging from 7 to 21 nt were generated by insertion of appropriate DNA fragments obtained by oligonucleotide annealing into the 3′UTR of pUF-CMV$_{enh}$/MLC0.26-Luc via a single *Xba*I site. Two AAV vector-shuttle plasmids with sc vector genomes containing an enhanced green fluorescent protein (EGFP) reporter gene under control of either the CMV or the CMV-MLC0.26 promoter were generated by insertion of a DNA fragment containing three repeats of miR122 TS into the *Bsr*GI site of the 3′UTR of the EGFP expression cassette in the plasmids pscAAV-CMV-EGFP[20] and pscAAV-CMV-MLC0.26-EGFP[20] resulting in pscAAV-CMV-EGFP$_{miR122(3\times)TS}$ and pscAAV-CMV-MLC0.26-EGFP$_{miR122(3\times)TS}$, respectively. Plasmids were controlled by sequence analysis using an ABI 310 Genetic Analyzer (Applied Biosystems Inc., Forster City, CA, USA).

Generation of AAV vectors

AAV6 pseudotyped vectors were generated by co-transfection of 293 cells with the respective AAV-shuttle plasmid containing a luciferase reporter gene expression cassette and the AAV packaging plasmid pDP6-rs providing the AAV2 *rep* and AAV6 *cap* genes and adenoviral helper functions. AAV9 pseudotyped vectors were generated by co-transfection of AAV-shuttle plasmids containing an enhanced EGFP expression cassette with the plasmids p5E18-VD2/9[45] providing AAV2 *rep* and AAV9 *cap* genes as well as pDGdelVP[46] providing the adenoviral helper sequences. All AAV-shuttle plasmids contain AAV2-ITRs flanking the AAV vector genome at its 5′ and 3′ end. AAV-shuttle plasmids packaged into AAV9 pseudotyped vectors provide an sc vector genome, whereas AAV-shuttle plasmids packaged into AAV6 pseudotyped vectors provide a monomeric vector genome because the *Firefly* luciferase cDNA is too big in size to become packaged into sc vectors. Generation of AAV6 and AAV9 vectors was carried out as described previously[10] with some variations. Briefly, for AAV6 production, 293 cells were seeded to 14.5 cm (10 plates were used for preparation of each AAV vector) and transfected at next day at a confluence of about 70%. For transfection of one plate 61.92 μg Polyethylenimine (Polysciences Inc., Warrington, PA, USA) was diluted in 1 ml of 150 mM NaCl and 6 μg AAV-shuttle plasmid and 18 μg of pDP6-rs were diluted in 1 ml of 150 mM NaCl. Polyethylenimine solution was then added dropwise to the plasmid solution and incubated at room temperature for 15 min. The transfection mixture was added dropwise to the plate, and cells were incubated at 37 °C. After an incubation period of 72 h, cells were harvested and the cell supernatant was collected. Cells were pelleted by centrifugation at 3000 r.p.m. in a centrifuge 5810 R (Eppendorf, Hamburg, Germany), than washed three times with phosphate-buffered saline and resuspended in phosphate-buffered saline. After three freeze-thaw cycles, Benzonase (Merk KGaA, Darmstadt, Germany; final concentration of 250 U ml^{-1}) was added, and the lysates were incubated for 1 h at 37 °C. Cell debris was pelleted and supernatant stored at −80 °C.

The cell supernatant (20 ml) of one plate was mixed with 0.4 ml of 2.5 mM $CaCl_2$ and incubated on ice for 1 h. After centrifugation at 4000 r.p.m. for 30 min, 6.5 ml of 24% PEG/NaCl (1.86 M) solution was added to the supernatant and incubated at 4 °C for at least 24 h and centrifuged again. Pellets were resuspended in a buffer containing 50 mM Hepes, 150 mM NaCl, 25 mM EDTA and centrifuged at 8000 r.p.m. for 30 min. The supernatant was incubated with Benzonase (final concentration 250 U ml^{-1}) for 1 h at 37 °C and stored at −80 °C. Vector solutions from the cell pellets and supernatant were pooled and vectors were purified using Iodixanol step gradients.[47]

AAV9 vectors were produced and purified as described previously.[19,20] Purification of virus was carried out by a filtration cascade followed by an iodixanol step gradient centrifugation.[19,20] Vector titers were determined by quantitative real-time PCR using the primers 5′-tgc cca gta cat gac ctt atg g-3′ and 5′-gaa atc ccc gtg agt caa acc-3′ and the 6-fam-agt cat cgc tat tac cat gg-MGB-labeled probe directed against a target sequence located in the CMV promoter enhancer present in all AAV vectors genomes by use of the StepOnePlus Realtime PCR System (Applied Biosystems Inc.).

Plasmid transfection

Cells were seeded in 24-well plates. After 24 h, cells were transfected with 80 ng of plasmids expressing *Firefly* luciferase with or without miR TS, 10 ng of a plasmid encoding *Renilla* luciferase for standardization and 700 ng of an unrelated carrier plasmid containing a green fluorescent protein cDNA. To analyze EGFP expression of AAV-shuttle plasmids, Huh7 cells were transfected with 1 μg and AML12 cells with 2 μg plasmid DNA. Transfection of AML12 and NRCM was carried out with Lipofectamine LTX + Plus Reagent (Invitrogen GmbH). Huh7, HL-1 and HepG2 were transfected with Lipofectamine 2000 (Invitrogen) according to the recommendation of the supplier. Analysis of reporter gene expression was carried out 48 h after transfection.

In vitro gene transfer

Cells seeded in 24-well plates were incubated with serum-free medium containing 2×10^3 AAV6 vector genome equivalents per cell. After 2 h, medium was replaced by culture medium, and cells were further incubated. Because of low transduction efficiency of AML12 cells, vector incubation time was extended to 12 h. Reporter gene expression was analyzed 48 h after transduction.

Quantitation of luciferase expression

Firefly luciferase activity of the AAV reporter vectors was measured using the Luciferase Reporter Gene Assay (Roche Diagnostics GmbH, Mannheim,

Germany) according to the manufacturer's instructions. In cells co-transfected with *Firefly* and *Renilla* luciferase-expressing plasmids, the luciferase activity was determined with the Dual-Luciferase Reporter Assay (Promega GmbH, Mannheim, Germany). Luciferase activity was measured in a Lumat LB 9501 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Quantitation of AAV vector DNA

Genomic DNA was extracted from heart and liver tissue using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. AAV vector DNA was quantified by real-time PCR using the TaqMan Gene Expression Master Mix (Applied Biosystems Inc.) and oligonucleotide primers/probe as follows: EGFP forward primer 5′-acg taa acg gcc aca agt tc-3′, EGFP reverse primer 5′-aag tcg tgc tgc ttc atg tg-3′ and EGFP probe 6fam-cga ggg cga tgc cac cta cg-TAMRA. Data were normalized to the genomic DNA (TaqMan Gene Expression Assay mGAPDH_g1). PCR reactions were carried out in triplicate with the StepOnePlus Realtime PCR System (Applied Biosystems Inc.).

Analysis of endogenous miR and gene expression

miR-enriched total RNA was isolated from cultured cells or tissues with *mir*Vana miRNA Isolation Kit according to the recommendation of the supplier (Applied Biosystems Inc./Ambion, Austin, TX, USA). In all, 50 ng of isolated RNA was reverse transcribed using miR122, miR192 and miR148a specific primers (TaqMan MicroRNA Reverse Transcription Kit, Applied Biosystems Inc.). Expression levels were determined by real-time PCR (TaqMan MicroRNA Assays hsa-miR122, hsa-miR192, hsa-miR148a, Applied Biosystems Inc.). For normalization, U6snRNA expression was used (Applied Biosystems Inc.). To analyze Gys1 expression in the liver, 2 μg of RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc.). Expression levels were determined by real-time PCR (TaqMan Gene Expression Assay mouse Gys1, Applied Biosystems Inc.). Mouse HPRT expression was used for normalization (TaqMan Gene Expression Assay mHPRT1, Applied Biosystems Inc.). PCR reactions were carried out in triplicate with the StepOnePlus Realtime PCR System (Applied Biosystems Inc.).

Northern blot hybridization

Total RNA was isolated with *mir*Vana miRNA Isolation Kit (Applied Biosystems Inc./Ambion) following the manufacturer's instruction. In all, 10 μg of isolated RNA was electrophoretically separated on a 1% formaldehyde agarose gel. After transfer to a Hybond N nylon transfer membrane (GE Healthcare, Buckinghamshire, UK), the membranes were hybridized with a single-stranded *antisense Firefly* luciferase-specific DNA probe, which was amplified with Luc-sense (5′-ccg gcg cca ttc tat cc-3′) and Luc-antisense (5′-cga gaa tct gac gca gg-3′) and labeled with ^{32}P-dCTP in a PCR-like reaction[48] using the Luc-antisense primer. Loading of equal amounts of RNA was verified by rehybridization with a single-stranded *antisense* β-actin-specific DNA probe as previously described.[49] Hybridized filters were exposed to Kodak BioMax MR film (Sigma-Aldrich).

In vivo gene transfer

All procedures involving the use and care of animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the German animal protection code. Approval was also granted by the local ethics review board.

In all, 7.5×10^{11} vector genome equivalents of AAV9 vectors were intravenously injected into the tail vein of 2-month-old NMRI mice (Charles River Laboratories, Sulzfeld, Germany) as 150 μl bolus using a sterile syringe and 29-gauge needle. After 4 weeks, animals were euthanized by cervical dislocation. Organs were dissected and rapidly frozen in liquid nitrogen. For histological analyses, tissue was embedded individually in tissue freezing medium (Jung, Nussloch, Germany) and frozen in liquid nitrogen.

Histological and fluorescence analysis

To analyze EGFP reporter gene activity within mouse heart and liver, 10 μm cross-sections of fixed and cryoconserved tissue were performed (CM3050S; Leica, Wetzlar, Germany) and evaluated for EGFP expression (FITC, 515–555 nm) in combination with Dapi staining by fluorescence microscopy (Nikon Eclipse 90i upright automated microscope, Düsseldorf, Germany) using the D1QM camera (Nikon). Optical overview images were obtained by the 'scan large image' function of the microscope. To rule out relevant inflammation, 6 μm cryoslices were stained with hematoxylin/eosin. The tissue was evaluated by transmission microscopy (digital microscopy research biology (DMRBE); Leica) using the 3CCD-XC-003P color-camera (Nikon). To determine EGFP expression of AAV-shuttle plasmids, *in vitro* images of EGFP-expressing cells were taken 48 h after transfection by fluorescence microscopy (microscope Axio Observer.D1; Carl Zeiss AG, Jena, Germany) using the AxioCam camera MRc and Axiovision Rel. 4.7 software (Carl Zeiss AG). Counting of EGFP-expressing cells in microphotographs was carried out using the software 'Image J' (http://rsbweb.nih.gov/ij/) with the function 'analyze particles'. In each mouse, three different sections per organ were analyzed followed by calculation of the mean and standard error of mean (s.e.m.).

Statistical analysis

All data were expressed as mean ± standard error of mean (s.e.m.). To test for statistical significance, an unpaired Student's *t*-test was applied.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Barbara Leuchs and the DKFZ vector core production unit for their support in generating high titer AAV vector stocks and Stefanie Schinkel and Georg Zingler for support in histological analyses. In addition, we thank Ulrike Gärtner for skillful intravenous injections and the Nikon Center Heidelberg for support in microscopy. This work was supported through *SFB Transregio 19* by project grants C1 to HF and RV and by a grants of the Deutsche Forschungsgemeinschaft (FE785/2-1 and FE785/3-1 to HF, KU1436/6-1 to JK, MU 1654/3-2 to OJM); and the Bundesministerium für Bildung und Forschung (01GU0527 to OJM and HAK).

1 Su H, Joho S, Huang Y, Barcena A, Rakawa-Hoyt J, Grossman W *et al*. Adeno-associated viral vector delivers cardiac-specific and hypoxia-inducible VEGF expression in ischemic mouse hearts. *Proc Natl Acad Sci USA* 2004; **101**: 16280–16285.
2 del Monte F, Williams E, Lebeche D, Schmidt U, Rosenzweig A, Gwathmey JK *et al*. Improvement in survival and cardiac metabolism after gene transfer of sarcoplasmic reticulum Ca(2+)-ATPase in a rat model of heart failure. *Circulation* 2001; **104**: 1424–1429.
3 Suckau L, Fechner H, Chemaly E, Krohn S, Hadri L, Kockskamper J *et al*. Long-term cardiac-targeted RNA interference for the treatment of heart failure restores cardiac function and reduces pathological hypertrophy. *Circulation* 2009; **119**: 1241–1252.
4 Most P, Seifert H, Gao E, Funakoshi H, Volkers M, Heierhorst J *et al*. Cardiac S100A1 protein levels determine contractile performance and propensity toward heart failure after myocardial infarction. *Circulation* 2006; **114**: 1258–1268.
5 Goehringer C, Rutschow D, Bauer R, Schinkel S, Weichenhan D, Bekeredjian R *et al*. Prevention of cardiomyopathy in delta-sarcoglycan knockout mice after systemic transfer of targeted adeno-associated viral vectors. *Cardiovasc Res* 2009; **82**: 404–410.
6 Jaski BE, Jessup ML, Mancini DM, Cappola TP, Pauly DF, Greenberg B *et al*. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID Trial), a first-in-human phase 1/2 clinical trial. *J Card Fail* 2009; **15**: 171–181.
7 Ma X, Glover C, Miller H, Goldstein J, O'Brien E. Focal arterial transgene expression after local gene delivery. *Can J Cardiol* 2001; **17**: 873–883.
8 Jiang H, Pierce GF, Ozelo MC, de Paula EV, Vargas JA, Smith P *et al*. Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. *Mol Ther* 2006; **14**: 452–455.
9 McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ. Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*. *Gene Ther* 2003; **10**: 2112–2118.
10 Fechner H, Sipo I, Westermann D, Pinkert S, Wang X, Suckau L *et al*. Cardiac-targeted RNA interference mediated by an AAV9 vector improves cardiac function in coxsackievirus B3 cardiomyopathy. *J Mol Med* 2008; **86**: 987–997.
11 Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* 2002; **99**: 11854–11859.

12 Inagaki K, Fuess S, Storm TA, Gibson GA, McTiernan CF, Kay MA *et al.* Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. *Mol Ther* 2006; **14**: 45–53.
13 Pacak CA, Mah CS, Thattaliyath BD, Conlon TJ, Lewis MA, Cloutier DE *et al.* Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction *in vivo*. *Circ Res* 2006; **99**: e3–e9.
14 Vandendriessche T, Thorrez L, Costa-Sanchez A, Petrus I, Wang L, Ma L *et al.* Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs lentiviral vectors for hemophilia B gene therapy. *J Thromb Haemost* 2007; **5**: 16–24.
15 Bish LT, Morine K, Sleeper MM, Sanmiguel J, Wu D, Gao G *et al.* AAV9 provides global cardiac gene transfer superior to AAV1, AAV6, AAV7, and AAV8 in the mouse and rat. *Hum Gene Ther* 2008; **19**: 1359–1368.
16 Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther* 2008; **16**: 1073–1080.
17 Yang L, Jiang J, Drouin LM, Gbandje-McKenna M, Chen C, Qiao C *et al.* A myocardium tropic adeno-associated virus (AAV) evolved by DNA shuffling and *in vivo* selection. *Proc Natl Acad Sci USA* 2009; **106**: 3946–3951.
18 Pacak CA, Sakai Y, Thattaliyath BD, Mah CS, Byrne BJ. Tissue specific promoters improve specificity of AAV9 mediated transgene expression following intra-vascular gene delivery in neonatal mice. *Genet Vaccines Ther* 2008; **6**: 13.
19 Muller OJ, Leuchs B, Pleger ST, Grimm D, Franz WM, Katus HA *et al.* Improved cardiac gene transfer by transcriptional and transductional targeting of adeno-associated viral vectors. *Cardiovasc Res* 2006; **70**: 70–78.
20 Muller OJ, Schinkel S, Kleinschmidt JA, Katus HA, Bekeredjian R. Augmentation of AAV-mediated cardiac gene transfer after systemic administration in adult rats. *Gene Ther* 2008; **15**: 1558–1565.
21 Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281–297.
22 Harfe BD. MicroRNAs in vertebrate development. *Curr Opin Genet Dev* 2005; **15**: 410–415.
23 Pushparaj PN, Aarthi JJ, Manikandan J, Kumar SD. siRNA, miRNA, and shRNA: *in vivo* applications. *J Dent Res* 2008; **87**: 992–1003.
24 Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005; **120**: 15–20.
25 Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A *et al.* Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol* 2007; **25**: 1457–1467.
26 Gentner B, Schira G, Giustacchini A, Amendola M, Brown BD, Ponzoni M *et al.* Stable knockdown of microRNA *in vivo* by lentiviral vectors. *Nat Methods* 2009; **6**: 63–66.
27 Kelly EJ, Hadac EM, Greiner S, Russell SJ. Engineering microRNA responsiveness to decrease virus pathogenicity. *Nat Med* 2008; **14**: 1278–1283.
28 Leja J, Nilsson B, Yu D, Gustafson E, Akerstrom G, Oberg K *et al.* Double-detargeted oncolytic adenovirus shows replication arrest in liver cells and retains neuroendocrine cell killing ability. *PLoS One* 2010; **5**: e8916.
29 Cawood R, Chen HH, Carroll F, Bazan-Peregrino M, van RN, Seymour LW. Use of tissue-specific microRNA to control pathology of wild-type adenovirus without attenuation of its ability to kill cancer cells. *PLoS Pathog* 2009; **5**: e1000440.
30 Suzuki T, Sakurai F, Nakamura S, Kouyama E, Kawabata K, Kondoh M *et al.* miR-122a-regulated expression of a suicide gene prevents hepatotoxicity without altering antitumor effects in suicide gene therapy. *Mol Ther* 2008; **16**: 1719–1726.
31 Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A *et al.* A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 2007; **129**: 1401–1414.
32 Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol* 2004; **5**: R13.
33 Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M *et al.* miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. *Cell Metab* 2006; **3**: 87–98.
34 Bish LT, Sleeper MM, Brainard B, Cole S, Russell N, Withnall E *et al.* Percutaneous transendocardial delivery of self-complementary adeno-associated virus 6 achieves global cardiac gene transfer in canines. *Mol Ther* 2008; **16**: 1953–1959.
35 Brown BD, Cantore A, Annoni A, Sergi LS, Lombardo A, Della VP *et al.* A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* 2007; **110**: 4144–4152.
36 Ylosmaki E, Hakkarainen T, Hemminki A, Visakorpi T, Andino R, Saksela K. Generation of a conditionally replicating adenovirus based on targeted destruction of E1A mRNA by a cell type-specific microRNA. *J Virol* 2008; **82**: 11009–11015.
37 Brown BD, Naldini L. Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications. *Nat Rev Genet* 2009; **10**: 578–585.
38 Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 2006; **125**: 1111–1124.
39 Forstemann K, Horwich MD, Wee L, Tomari Y, Zamore PD. Drosophila microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. *Cell* 2007; **130**: 287–297.
40 Doench JG, Petersen CP, Sharp PA. siRNAs can function as miRNAs. *Genes Dev* 2003; **17**: 438–442.
41 Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. *PLoS Biol* 2005; **3**: e85.
42 Ebert MS, Neilson JR, Sharp PA. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods* 2007; **4**: 721–726.
43 Sayed D, Rane S, Lypowy J, He M, Chen IY, Vashistha H *et al.* MicroRNA-21 targets Sprouty2 and promotes cellular outgrowths. *Mol Biol Cell* 2008; **19**: 3272–3282.
44 Fechner H, Suckau L, Kurreck J, Sipo I, Wang X, Pinkert S *et al.* Highly efficient and specific modulation of cardiac calcium homeostasis by adenovector-derived short hairpin RNA targeting phospholamban. *Gene Ther* 2007; **14**: 211–218.
45 Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X *et al.* Clades of Adeno-associated viruses are widely disseminated in human tissues. *J Virol* 2004; **78**: 6381–6388.
46 Dubielzig R, King JA, Weger S, Kern A, Kleinschmidt JA. Adeno-associated virus type 2 protein interactions: formation of pre-encapsidation complexes. *J Virol* 1999; **73**: 8989–8998.
47 Sipo I, Fechner H, Pinkert S, Suckau L, Wang X, Weger S *et al.* Differential internalization and nuclear uncoating of self-complementary adeno-associated virus pseudotype vectors as determinants of cardiac cell transduction. *Gene Ther* 2007; **14**: 1319–1329.
48 Fechner H, Haack A, Wang H, Wang X, Eizema K, Pauschinger M *et al.* Expression of Coxsackie adenovirus receptor and alphav-integrin does not correlate with adenovector targeting *in vivo* indicating anatomical vector barriers. *Gene Ther* 1999; **6**: 1520–1535.
49 Fechner H, Pinkert S, Wang X, Sipo I, Suckau L, Kurreck J *et al.* Coxsackievirus B3 and adenovirus infections of cardiac cells are efficiently inhibited by vector-mediated RNA interference targeting their common receptor. *Gene Ther* 2007; **14**: 960–971.

Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)