RESEARCH ARTICLE

In vivo knock-down of multidrug resistance transporters ABCC1 and ABCC2 by AAV-delivered shRNAs and by artificial miRNAs

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ABSTRACT

ABC transporters export clinically-relevant drugs and their over-expression causes multidrug resistance. In order to knock-down ABC transporters, ABCC1 and ABCC2, 13 shRNAs were developed. Four shRNA candidates were tested *in vivo* using self-complementary adeno-associated virus serotype 8. A strong, specific knock-down of *Abbc2* was observed in mice liver, but at the cost of toxicity caused by oversaturation of the RNAi machinery due to high shRNA expression. Subsequent generation of artificial miRNAs showed better efficacy profile. These results demonstrate the feasibility of knocking down *Abbc2* via AAV-delivered shRNAs to the liver, and encourage the use of miRNA in further therapeutics development.

KEYWORDS: shRNA, miRNA, AAV, *Abbc1*, *Abbc2*, multidrug resistance, hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer worldwide, with about 750,000 patients reported globally each year (International Agency for Research on Cancer, website: http://globocan.iarc.fr). Poor survival of HCC patients has several causes, frequently including resistance to chemotherapy. Many ATP-binding cassette (ABC) transporter family members can decrease the intracellular concentration of toxic compounds (Cole et al, 1994; Hopper-Borge et al, 2004; Tian et al, 2006; Hopper-Borge et al, 2009; Lagas et al, 2009). These transmembrane pumps are over-expressed in tumor cells, hence causing the multidrug resistance phenotype. ABCB1 and ABCC3 have been shown to be up-regulated in HCC (Grude et al, 2002; Mizukoshi et al, 2008) and the up-regulation of ABCC1 has been associated with a more aggressive HCC phenotype (Vander et al, 2008). Thus far ABCB1 inhibitors have failed to show benefit during clinical trials, but more are being tested (Kuppens et al, 2007). Decreasing the expression of other ABC transporters is a desirable alternative as it could potentially reverse the multidrug resistance phenotype.

RNA interference (RNAi) is an attractive approach to achieve this goal, as it would allow combinatorial, possibly patient-tailored targeting of ABC transporters. RNAi is a naturally-occurring post-transcriptional gene silencing mechanism, which can induce sequence-specific degradation of a messenger RNA (mRNA), thus

reducing gene expression. RNAi can be induced by synthetic small interfering RNAs (siRNAs), or by intracellular expression of short hairpin RNAs (shRNAs) and artificial microRNAs (miRNAs). shRNAs and miRNAs are processed by Dicer into siRNAs, which are loaded onto the RNA-induced silencing complex (RISC), where they mediate sequence-specific mRNA recognition, ultimately causing its degradation. *In vivo* siRNA-mediated knock-down of *Abbc1* has been demonstrated after intratumoral siRNA injection followed by *in situ* electroporation of the tumor; this led to a reduction in tumor weight in response to epirubicin (Wu et al, 2011). In addition, *Abbc2* was inhibited *in vitro* and *in vivo* by plasmid-, adenovirus- and lentivirus-delivered shRNAs, which respectively resulted in reversed cisplatin and paclitaxel resistance (Materna et al, 2006), decreased bilirubin transport (Narvaiza et al, 2006), and reduced growth of cisplatin-treated tumors (Xie et al, 2008). Despite these findings, the major problem of RNAi applications still lies in sustained and tissue-specific delivery of the effector molecules *in vivo*. Recombinant adeno-associated virus (AAV) has emerged as the vector of choice for gene therapy and for RNAi-mediated therapy, as it yields long-term, tissue-specific expression without any apparent pathogenicity (Daya et al, 2008). In the current study we assessed the feasibility of *in vivo* AAV-mediated knock-down of two murine endogenous ABC transporters: ABCC1 and ABCC2. Initially, we verified the knock-down activity of 13 shRNA constructs and selected two candidates targeting *Abbc2* for further *in vivo* testing. A single injection of self-complementary AAV8 (scAAV8) carrying shAbcc2 resulted in efficient *Abbc2* knock-down in murine liver. Concomitant signs of toxicity including elevated transaminases were attributed to high levels of shRNAs being processed into siRNAs, causing oversaturation of the RNAi machinery. Nevertheless, subsequent translocation of the validated shRNA sequences into a miRNA scaffold offers the perspective of safe AAV-mediated *in vivo* knock-down.

MATERIALS AND METHODS

DNA constructs

Six shRNA constructs targeting murine *Abbc1* and seven targeting murine *Abbc2*, and control shRNAs targeting eGFP (shGFP) and Luciferase (shLuc) and a scramble sequence (shScr) were made by annealing of complementary oligonucleotides and ligating them into the pSuper vector containing the H1 Pol III promoter (OligoEngine, Seattle, WA). The sequence for constructing the negative control hairpin shScr has been described previously (Doege et al, 2008). The sequences of oligonucleotides used in this study are listed in Table 1. Luciferase reporters *Luc-Abbc11/17* and *Abbc22/28* were made by cloning of *Abbc1/2* sequences behind renilla luciferase in the siCheck vector (Promega, Madison, WI).

For cloning in the AAV backbone, the H1-shRNA expression cassettes were subcloned in pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA), and then ligated in the pVD287 vector. pVD287 contains the *egfp* gene under the control of the liver-specific LP-1 promoter and generates scAAV due to a mutation in one terminal repeat (McCarty et al, 2003).

Cell culture and transfections

Human embryonic kidney (HEK) 293T and murine hepatoma (Hepa1-6) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% (v/v) fetal calf serum, 100U/ml penicillin and 100U/ml streptomycin, at 37^{o}C and 5% (v/v) CO_2. Cells were plated in 6-, 24- or 96-well plates one day prior to transfection. Transfections were performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. For the interferon response experiment we transfected 2µg of a synthetic analog of dsRNA, polyinosinic:polycytidylic acid (poly I:C), as a positive control.

Luciferase assays

For luciferase assays, cells were co-transfected with 10ng *Luc-Abbc* reporter that contains both firefly and renilla luciferase genes, and 0, 0.5, 2.5, 10 or 50ng of the corresponding shAbcc expression construct. Transfected cells were assayed at 48hr post-transfection and firefly and renilla luciferase were measured with the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated as the ratio between the renilla and firefly luciferase activities, and transfection with shScr was set at 100%. Data are represented as mean values ±SD from a representative experiment conducted with three technical replicates.

RNA isolation, quantitative RT-PCR (RT-qPCR), siRNA and miRNA Taqman assays

For *in vitro* experiments, total RNA was isolated from cells 18hr or 72hr post-transfection using the Nucleospin kit (Clontech, Mountain View, CA). For *in vivo* experiments, total RNA was isolated from frozen liver sections at two weeks post-injection (p.i.) using Trizol (Invitrogen) according to the manufacturer's protocol. DNAse-treatment and RT-qPCR were performed as described previously (Koornneef et al, 2011) and data are represented as mean values ±SD from a representative experiment conducted with three technical replicates. siRNA and miRNA expression was quantified with custom-made siRNA assays or miRNA-specific Taqman assays (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

AAV vector production and *in vivo* experiments

Self-complementary AAV8 vectors were produced and purified as previously described (Zolotukhin et al, 1999; Gao et al, 2002; Koornneef et al, 2011) with an added fractionation step yielding higher virus concentration. Final concentration was determined by qPCR with LP1 primers. All animal experiments were conducted according to the guidelines of the local animal welfare committee. Six-to-eight-week-old male C57BL/6 mice received $2.2x10^{11}$gc AAV-shScr, $2.6\text{-}3x10^{12}$gc AAV-shAbcc22 or -shAbcc28 per animal intravenously via the tail vein. Heparinized blood samples were taken by retro-orbital bleeding at 1 and 2 weeks p.i. for plasma analysis. Mice were sacrificed on day 15 p.i. and livers were examined for *Abbc1* and *Abbc2* knock-down. Plasma levels

Table 1. Oligonucleotide sequences used in this study. mmu-*Abcc1* (NM_008576), mmu-*Abcc2* (NM_013806), *luc* and *gfp* sequences in the shRNA oligos are in bold.

Name	Sequence (5'-3')	Target
shAbcc11-f	GATCCCC**GATGACACACCTCAACAAA**TTCAAGAGA**TTTGTTGAGGTGTGTCATC**TTTTTA	Abcc1 207-225
shAbcc11-r	TCGAGAAAAAGATGACACACCTCAACAAATCTCTTGAATTTGTTGAGGTGTGTCATCGGG	
shAbcc12-f	GATCCCC**TTACTACAAGGCAGTTATG**TTCAAGAGA**CATAACTGCCTTGTAGTAA**TTTTTA	Abcc1 2214-2232
shAbcc12-r	TCGAGAAAAATTACTACAAGGCAGTTATGTCTCTTGAACATAACTGCCTTGTAGTAAGGG	
shAbcc15-f	GATCCCC**TCTACTTCTTCTATCTCTC**TTCAAGAGA**GAGAGATAGAAGAAGTAGA**TTTTTA	Abcc1 169-182
shAbcc15-r	TCGAGAAAAATCTACTTCTTCTATCTCTCTCTCTTGAAGAGAGATAGAAGAAGTAGAGGG	
shAbcc16-f	GATCCCC**TGTTCAATATCTTGCGCTT**TTCAAGAGA**AAGCGCAAGATATTGAACA**TTTTTA	Abcc1 1175-1793
shAbcc16-r	TCGAGAAAAATGTTCAATATCTTGCGCTTTCTCTTGAAAAGCGCAAGATATTGAACAGGG	
shAbcc17-f	GATCCCC**GAAGCTAATGGAAGCAGAC**TTCAAGAGA**GTCTGCTTCCATTAGCTTC**TTTTTA	Abcc1 2832-2850
shAbcc17-r	TCGAGAAAAAGAAGCTAATGGAAGCAGACTCTCTTGAAGTCTGCTTCCATTAGCTTCGGG	
shAbcc18-f	GATCCCC**CCTGGACCTGCTATACAAT**TTCAAGAGA**ATTGTATAGCAGGTCCAGG**TTTTTA	Abcc1 3147-3165
shAbcc18-r	TCGAGAAAAACCTGGACCTGCTATACAATTCTCTTGAAATTGTATAGCAGGTCCAGGGGG	
shAbcc22-f	GATCCCC**CTTGGCTAGGAGGCAGTAC**TTCAAGAGA**GTACTGCCTCCTAGCCAAG**TTTTTA	Abcc2 1221-1239
shAbcc22-r	TCGAGAAAAACTTGGCTAGGAGGCAGTACTCTCTTGAAGTACTGCCTCCTAGCCAAGGGG	
shAbcc23-f	GATCCCC**CCATGTTCTGGATTCTCTC**TTCAAGAGA**GAGAGAATCCAGAACATGG**TTTTTA	Abcc2 395-413
shAbcc23-r	TCGAGAAAAACCATGTTCTGGATTCTCTCTCTCTTGAAGAGAGAATCCAGAACATGGGGG	
shAbcc24-f	GATCCCC**TGACCAACTACTACACTT**TTCAAGAGA**AAGTGTAGTAGTTGGTCA**TTTTTA	Abcc2 1295-1313
shAbcc24-r	TCGAGAAAAATGACCAACTACTACACTTTCTCTTGAAAAGTGTAGTAGTTGGTCAGGG	
shAbcc25-f	GATCCCC**CATCTATATTCTAGACGAC**TTCAAGAGA**GTCGTCTAGAATATAGATG**TTTTTA	Abcc2 2334-2352
shAbcc25-r	TCGAGAAAAACATCTATATTCTAGACGACTCTCTTGAAGTCGTCTAGAATATAGATGGGG	
shAbcc26-f	GATCCCC**TTACTTGTAACATCAAGAG**TTCAAGAGA**CTCTTGATGTTACAAGTAA**TTTTTA	Abcc2 3953-3971
shAbcc26-r	TCGAGAAAAATTACTTGTAACATCAAGAGTCTCTTGAACTCTTGATGTTACAAGTAAGGG	
shAbcc27-f	GATCCCC**ATACTGGACAAGCCACAAT**TTCAAGAGA**ATTGTGGCTTGTCCAGTAT**TTTTTA	Abcc2 266-284
shAbcc27-r	TCGAGAAAAAATACTGGACAAGCCACAATTCTCTTGAAATTGTGGCTTGTCCAGTATGGG	
shAbcc28-f	GATCCCC**TCTCTACCTATGCACTTGG**TTCAAGAGA**CCAAGTGCATAGGTAGAGA**TTTTTA	Abcc2 312-330
shAbcc28-r	TCGAGAAAAATCTCTACCTATGCACTTGGTCTCTTGAACCAAGTGCATAGGTAGAGAGGG	
shScr-f	GATCCCC**GATCGAATGTGTACTTCGA**TTCCAAGAGA**TCGAAGTACACATTCGATC**TTTTTGCATGCC	Doege et al. 2008
shScr-r	TCGAGGCATGCAAAAAGATCGAATGTGTACTTCGATCTCTTGGAATCGAAGTACACATTCGATCGGG	
shGFP-f	GATCCCC**AGCTGGAGTACAACTACAA**CCTTCCTGTCAG**TTGTAGTTGTACTCCAGCT**TTTTGCATGCC	eGFP
shGFP-r	TCGAGGCATGCAAAAAGCTGGAGTACAACTACAACTGACAGGAAGGTTGTAGTTGTACTCCAGCTGGG	
shLuc-M-f	GATCCCC**CTGCCCTGCTGGTGCCCACAC**TTCCAAGAGA**GTGTGGGCACCAGCAGGGCAG**TTTTTGCATGCC	Luc
shLuc-M-r	TCGAGGCATGCAAAAACTGCCCTGCTGGTGCCCACACTCTCTTGGAAGTGTGGGCACCAGCAGGGCAGGGG	
Luc-Abcc11-f	TCGAG**TCTCTCTCGCCATGACCGGGGCTACATCCAGATGACACACCTCAACAAAACCAAAACTGCCTTAGGATTCTTTCT**GC	Abcc11
Luc-Abcc11-r	GGCCGC**AGAAAGAATCCTAAGGCAGTTTTGGTTTTGTTGAGGTGTGTCATCTGGATGTAGCCCCGGTCATGGCGAGAGAGA**C	
Luc-Abcc17-f	TCGAG**GCAGAAGGCTGGAGCTAAGGAGGAGACGTGGAAGCTAATGGAAGCAGACAAGGCCCAGACAGGGCAGGTGCAGCT**GC	Abcc17
Luc-Abcc17-r	GGCCGC**AGCTGCACCTGCCCTGTCTGGGCCTTGTCTGCTTCCATTAGCTTCCACGTCTCCTCCTTAGCTCCAGCCTTCTGC**C	
Luc-Abcc22-f	TCGAG**TATATAAGAAGGCACTAACCCTATCTAACTTGGCTAGGAGGCAGTACACGATTGGAGAGACGGTGAACTTGAT**GC	Abcc22
Luc-Abcc22-r	GGCCGC**ATCAAGTTCACCGTCTCTCCAATCGTGTACTGCCTCCTAGCCAAGTTAGATAGGGTTAGTGCCTTCTTATATA**C	
Luc-Abcc28-f	TCGAG**ACCTCTCACAGAAGATACTGGACAAGCCACAATTCCTCCTGTTAAATATACAAATCCAATTCTCTACCTATGCACTTGGCTCCTGGTGTTGGTGGCAG**GC	Abcc28
Luc-Abcc28-r	GGCCGC**CTGCCACCAACACCAGGAGCCAAGTGCATAGGTAGAGAATTGGATTTGTATATTTAACAGGAGGAATTGTGGCTTGTCCAGTATCTTCTGTGAGAGGT**C	
Abcc1-f	GAGTCAAAGCCGGTGGAAAAT	Abcc1 qRT-PCR
Abcc1-r	TTAGCTCCAGCCTTCTGCAGTT	
Abcc2-f	TGAAAAACAGAATGGGACCGA	Abcc2 qRT-PCR
Abcc2-r	TTGGATGCATTTCTGCAAGC	
Actin-f	ACGGCCAGGTCATCACTATTG	βactin qRT-PCR
Actin-r	CAAGAAGGAAGGCTGGAAAAGA	
Gapdh-f	ACATGTTCCAGTATGACTCCACTCA	Gapdh qRT-PCR
Gapdh-r	GCCTCACCCCATTTGATGTT	
Hprt-f	AAGACTTGCTCGAGATGTCATGAAG	Hprt qRT-PCR
Hprt-r	TCCAGCAGGTCAGCAAAGAA	
Ifnb1-f	CTGCCTTTGCCATCCAAGAG	Ifnb1 qRT-PCR
Ifnb1-r	ACTGTCTGCTGGTGGAGTTC	
Igs56-f	TGCACAACCTCCTGGCCTAC	Isg56 qRT-PCR
Igs56-r	TCGCCAGGCTTCTCTTGCTC	
Oas1b-f	TGATGTGCTGCCAGCCTATG	Oasb1b qRT-PCR
Oas1b-r	GATAACTTGCCCTCCTTCCC	
Stat1-f	GTGAGAGCCAGTCGTTTCAG	Stat1 qRT-PCR
Stat1-r	GCAGGTTCGGGATTCAACAC	
LP1-f	CCCTGTTTGCTCCTCCGATAA	LP1 qRT-PCR
LP1-r	GTCCGTATTTAAGCAGTGGATCCA	

of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin were analyzed on Modular Analytics P800 analyzer (Roche Diagnostics, Basel, Switzerland). Data are represented as mean values ±SEM (n = 4-5).

RESULTS AND DISCUSSION

Design and validation of shRNAs targeting murine *Abcc1* and *Abcc2*

Six shRNAs were designed against murine *Abcc1* mRNA (shAbcc11-shAbcc17) (Figure 1A and 1C), and seven against murine *Abcc2* mRNA (shAbcc22-shAbcc28) (Figure 1B and 1D). The ability of these constructs to knock-down endogenous *Abcc1* and *Abcc2* was assessed *in vitro* in Hepa1-6 cells using shGFP and shLuc as negative controls. shAbcc11, shAbcc17, and all 7 shAbcc2 constructs, respectively inhibited endogenous *Abcc1* and *Abcc2* expression by more than 50% (Figure 1E and 1F). For each target we selected the 2 most efficient shRNAs: shAbcc11 and shAbcc17, shAbcc22 and shAbcc28.

The specificity of *Abcc1* and *Abcc2* knock-down by these 4 constructs was tested on luciferase reporters containing the *Abcc1* or *Abcc2* target sequences. For all 4 constructs increasing concentrations of the shRNA construct induced dose-dependent inhibition of the specific luciferase reporter (Figure 1G). These results support the sequence-specificity of endogenous *Abcc1* and *Abcc2* knock-down by these shRNAs.

siRNA processing was examined *in vitro* in two cell lines and was compared with the siRNA processing of a validated active shRNA, shApoB (Koornneef et al, 2011). Efficient processing of the shRNA would indicate efficacy of the shRNA *in vivo*. HEK293T and Hepa1-6 cells were transfected with increasing amounts of plasmids encoding shAbcc22, shAbcc28 and shApoB. All shRNAs expressed similar amounts of siRNAs in HEK293T (Figure 1H) and Hepa1-6 cells (Figure 1I), indicating that they do not suffer from any misprocessing that could impair the anticipated *in vivo* knock-down.

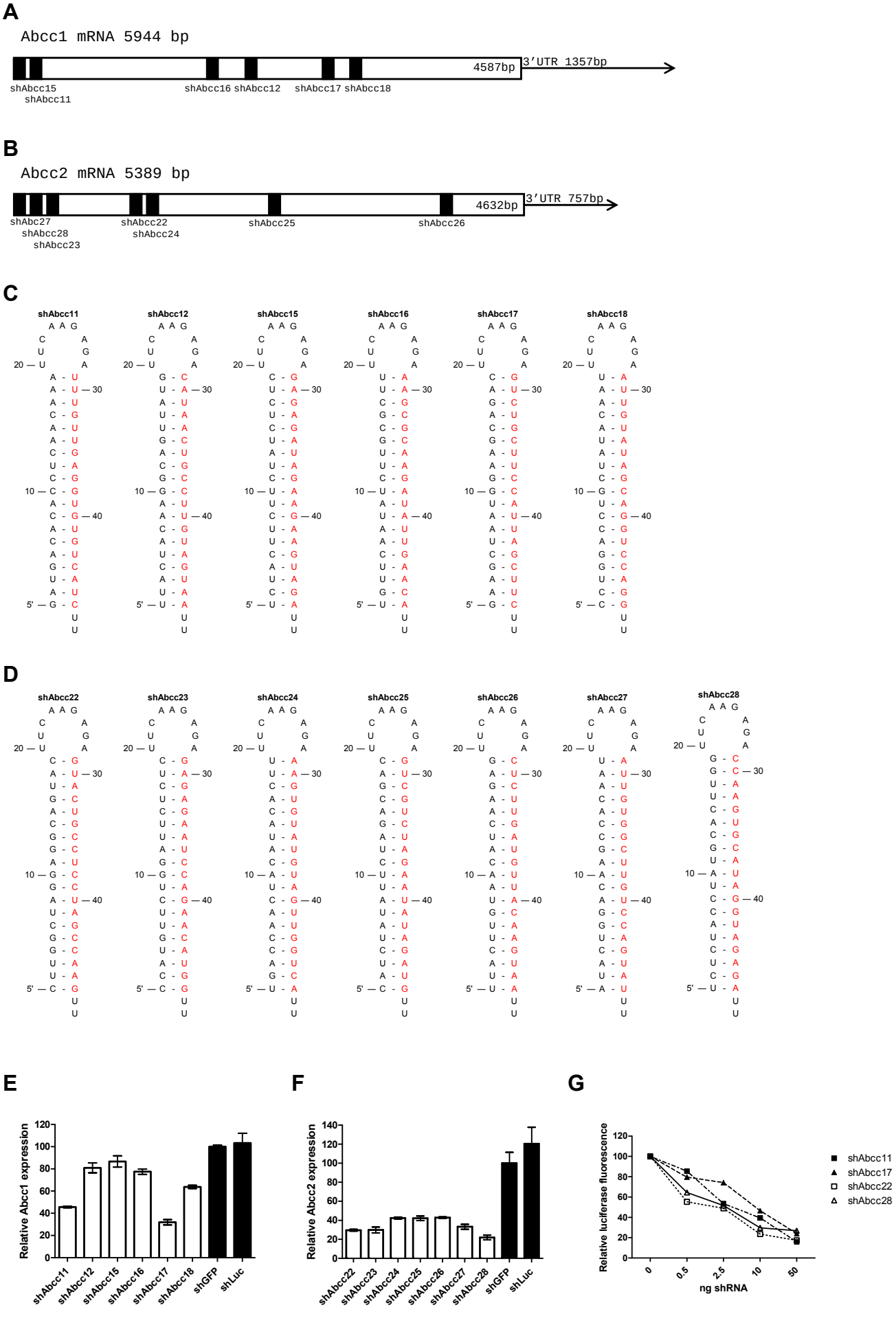
Double-stranded RNA (dsRNA), including siRNAs, can induce the interferon response (Sledz et al, 2003). We determined that none of the 4 selected shRNAs induced expression of marker genes of the interferon response *in vitro* following transfection of Hepa1-6 cells (Figure 2).

In vivo knock-down of *Abcc1* and *Abcc2* via scAAV-delivered shRNAs

C57BL/6 mice were injected with $4x10^{12}$gc/kg scAAV8 encoding shAbcc11, shAbcc17, shAbcc22, shAbcc28 and the shScr and PBS as controls. Animals were sacrificed at two weeks p.i. and *Abcc1*, *Abcc2* knock-down and siRNA expression in liver was determined, as well as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in plasma and interferon pathway genes expression in white blood cells. Transduction efficiency by scAAV8-shAbcc was greater than 90% as determined by fluorescence microscopy (data not shown). However, *Abcc1* and *Abcc2* mRNA expression was not affected significantly, and we could determine only very low levels of siRNA (data not shown). We concluded that at this viral dose, these shRNAs were not expressed at a level sufficient to show a detectable effect. We subsequently narrowed our focus on the highly-expressed *Abcc2* gene and injected C57 BL/6 mice with a 10-30 fold higher dose of $1.2\text{-}1.3x10^{14}$gc/kg AAV-shAbcc22, AAV-shAbcc28, and AAV-shScr and PBS as controls. Mice injected with AAV-shAbcc22 and AAV-shAbcc28 were therefore sacrificed on day-13p.i. in agreement with the guidelines of the local animal welfare committee as the animals presented some signs of physiological stress and one AAV-shAbcc22-injected mouse died. Animals from AAV-shScr and PBS groups were sacrificed on day-15p.i. Gene expression analysis by RT-qPCR in the livers revealed a profound knock-down of *Abcc2* mRNA up to 83% by shAbcc22 and shAbcc28 (Figure 3A). Since there were signs of toxicity, we determined whether the *Abcc2* knock-down was sequence-specific rather than due to general toxicity. Therefore, the expression of the housekeeping genes, *β-Actin*, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine phosphoribosyl transferase (*Hprt*), was determined in RNA isolated from mouse livers. Our results indicated no detectable down-regulation of expression of the housekeeping genes between the 3 AAV-injected groups (data not shown), thus indicating the sequence-specificity of *Abcc2* knock-down by AAV-shAbcc22 and AAV-Abcc28 *in vivo*. Further quantification of the amount of siAbcc22 and siAbcc28 by Taqman revealed a higher number of siRNA in the AAV-shAbcc22-injected mice than in the AAV-shAbcc28-injected mice (Figure 3B).

Interestingly, the mouse injected with AAV-shAbcc22, which first showed signs of toxicity and died was indeed the one presenting the highest levels of siRNA. While *in vitro* the amount siRNA produced by shAbcc22 and shAbcc28 was similar, *in vivo* there were differences between mice injected with the same dose of AAV-shAbcc22 and AAV-shAbcc28. We conclude that our *in vitro* studies were insufficient predictors of the shRNAs processing *in vivo*. Nevertheless, *in vitro* efficacy correlated well with *in vivo* results, which, following highly-efficient transduction of hepatocytes with AAV8, achieved 83% knock-down of the target mRNA.

During the first week p.i. the mice from the AAV-shAbcc22 and AAV-shAbcc28 groups did not gain any weight, while the AAV-shScr and the PBS groups did (data not shown). It appears that the over-expression of shAbcc22 and shAbcc28 *in vivo* resulted in severe toxicity. Mice presented several indications of physiological stress. Analysis of plasma sampled on day-8p.i. revealed elevated levels of AST, ALT and total bilirubin indicating liver toxicity in the AAV-shAbcc groups (Figure 4A). On day-13p.i. one of the AAV-shAbcc22-injected mice died and subsequently all mice from both AAV-shAbcc22 and AAV-shAbcc28 groups were sacrificed on the same day. Plasma analysis at two weeks p.i. revealed highly-elevated AST and ALT levels in the AAV-shScr group (data not shown), and weight loss was also observed during the second week p.i., indicating a slower onset of the toxicity, most likely due to the lower viral dose that was administered to this group.


A
Abcc1 mRNA 5944 bp
4587bp
3′UTR 1357bp
shAbcc15
shAbcc11
shAbcc16 shAbcc12
shAbcc17 shAbcc18
B
Abcc2 mRNA 5389 bp
4632bp
3′UTR 757bp
shAbc27
shAbcc28
shAbcc23
shAbcc22
shAbcc24
shAbcc25
shAbcc26
C
shAbcc11
shAbcc12
shAbcc15
shAbcc16
shAbcc17
shAbcc18
D
shAbcc22
shAbcc23
shAbcc24
shAbcc25
shAbcc26
shAbcc27
shAbcc28
E
Relative Abcc1 expression
shAbcc11
shAbcc12
shAbcc15
shAbcc16
shAbcc17
shAbcc18
shGFP
shLuc
F
Relative Abcc2 expression
shAbcc22
shAbcc23
shAbcc24
shAbcc25
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shGFP
shLuc
G
Relative luciferase fluorescence
ng shRNA
shAbcc11
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shAbcc28

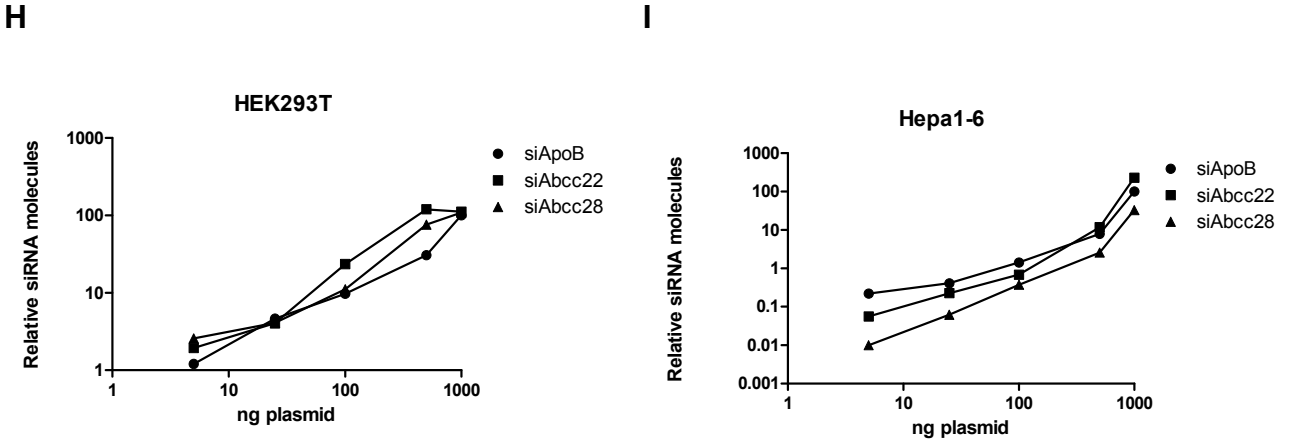

Figure 1. shRNAs design and *in vitro* studies on endogenous *Abcc* mRNA and *Abcc* luciferase reporters. Schematic representation of *Abcc1* and *Abcc2* mRNA and target sequences of the shRNAs. **(A)** shAbcc11, shAbcc12, shAbcc15, shAbcc16, shAbcc17, shAbcc18 targeting *Abcc1* mRNA (NM_013806.2) and **(B)** shAbcc22, shAbcc23, shAbcc24, shAbcc25, shAbcc26, shAbcc27, shAbcc28 targeting *Abcc2* mRNA (NM_008576.2). Predicted stem-loop structure with guide strand in red of **(C)** shAbcc1 and **(D)** shAbcc2. **(E)** Endogenous *Abcc1* mRNA knock-down by shAbcc1 constructs and **(F)** endogenous *Abcc2* mRNA knock-down by shAbcc2 constructs in Hepa1-6 cells (shGFP-treated cells were set at 100%). **(G)** *Luc-Abcc1* and *Luc-Abcc2* knock-down by shAbcc1 and shAbcc2 constructs in HEK293T cells (shScr-treated cells were set at 100%). Relative amount of siRNA molecules after transfection of HEK293T **(H)** and Hepa1-6 cells **(I)** with shAbcc22, shAbcc28 and shApoB (the highest siApoB value was set at 100%).

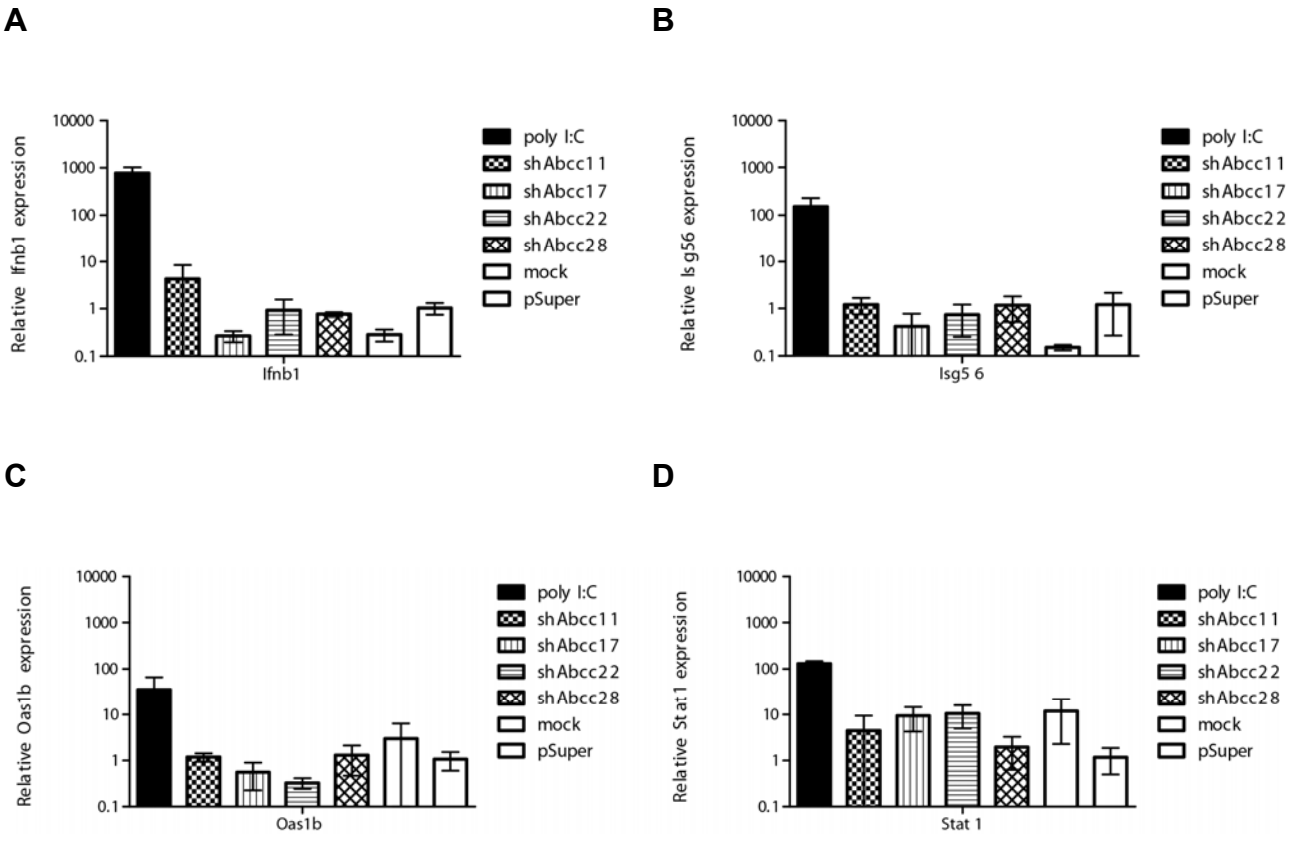

Figure 2. Four interferon pathway genes are not activated by the shAbcc constructs. Expression of interferon pathway genes. **(A)** Interferon beta 1 (*Ifnb1*), **(B)** Interferon-stimulated gene 56 (*Isg56*), **(C)** 2'-5' oligoadenylate synthetase 1B (*Oasb1b*), **(D)** Signal transducer and activator of transcription 1 (*Stat1*). Gene expression was quantified by RT-qPCR and normalized to *β-Actin* expression (pSuper was set at 1).

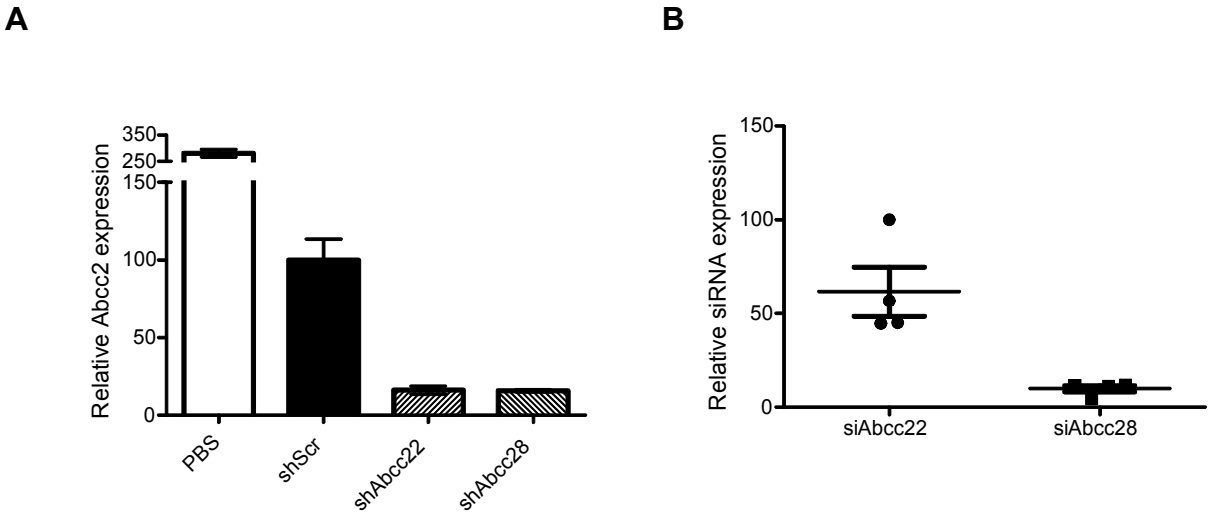

Figure 3. *In vivo* knock-down of *Abcc2* and quantification of siAbcc2. **(A)** Relative *Abcc2* expression was quantified by RT-qPCR and normalized to *β-Actin* (shScr group was set at 100%). **(B)** Relative expression of siAbcc22 and siAbcc28 molecules was quantified by RT-qPCR and normalized to *β-Actin* expression (the highest value was set at 100%).

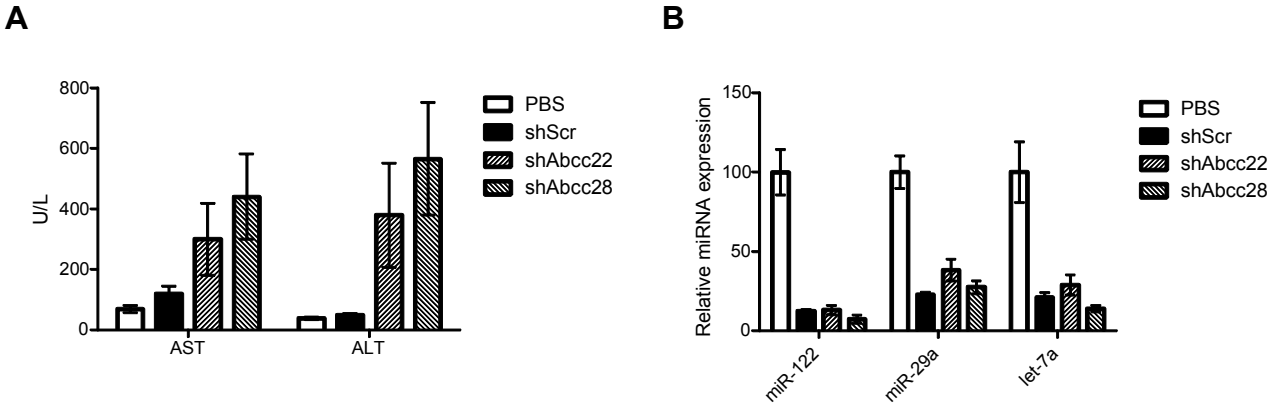

Figure 4. Overexpression of shAbcc2 induces liver toxicity. **(A)** Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in plasma at 8-days p.i. **(B)** Relative expression of three cellular miRNAs, miR-122, miR-29a and let-7a was quantified by RT-qPCR and normalized to *β-Actin* expression (PBS group was set at 100%).

It has been reported that high siRNA expression from shRNA cassettes containing a strong promoter can lead to lethal toxicity by over-saturating the RNAi machinery and affecting endogenous miRNA processing (Grimm et al, 2006). We determined the expression levels of 3 cellular miRNAs, namely miR-122, miR-29a and let-7a, in the livers of the injected animals. If saturation of the RNAi machinery occurred to the point where endogenous metabolic processes are expected to be significantly affected, expression of these liver miRNAs should be significantly decreased. Taqman assays for the 3 cellular miRNAs revealed down-regulation in the mouse livers injected with AAV-shScr, AAV-shAbcc22 and AAV-shAbcc28 compared to the PBS group (Figure 4B), indicating oversaturation of the RNAi machinery.

Circumventing shRNA toxicity

Expression of shRNA from a weaker promoter and from a miRNA scaffold can circumvent toxicity problems (McBride et al, 2008). Therefore, shAbcc11 and shAbcc28 sequences were expressed from a miRNA backbone (Figure 5A and 5B) and their ability to knock-down *Luc-Abcc11* and *Luc-Abcc28* luciferase reporters was assessed *in vitro* in HEK293T cells. Comparison of knock-down induced by shAbcc11 and miAbcc11, and by shAbcc28 and miAbcc28 showed that both constructs had a similar efficiency – the miRNA being slightly better (Figure 5C and 5D). Furthermore, quantification of the amount of siRNA molecules necessary to achieve this knock-down effect by Taqman assay showed that miAbcc28 produced 55%-75% less siAbcc28 molecules than shAbcc28 (Figure 5E). Since these miRNAs were expressed from a significantly weaker Pol II promoter, less siRNA molecules were apparently being produced while retaining equal inhibitory properties compared to the shRNA. This indicates that miRNAs may be more potent molecules for induction of RNA silencing than shRNAs. Considering the toxicity issues raised in this study the possibility of expressing siRNA sequences from a miRNA backbone, which would render similar or increased efficiency but

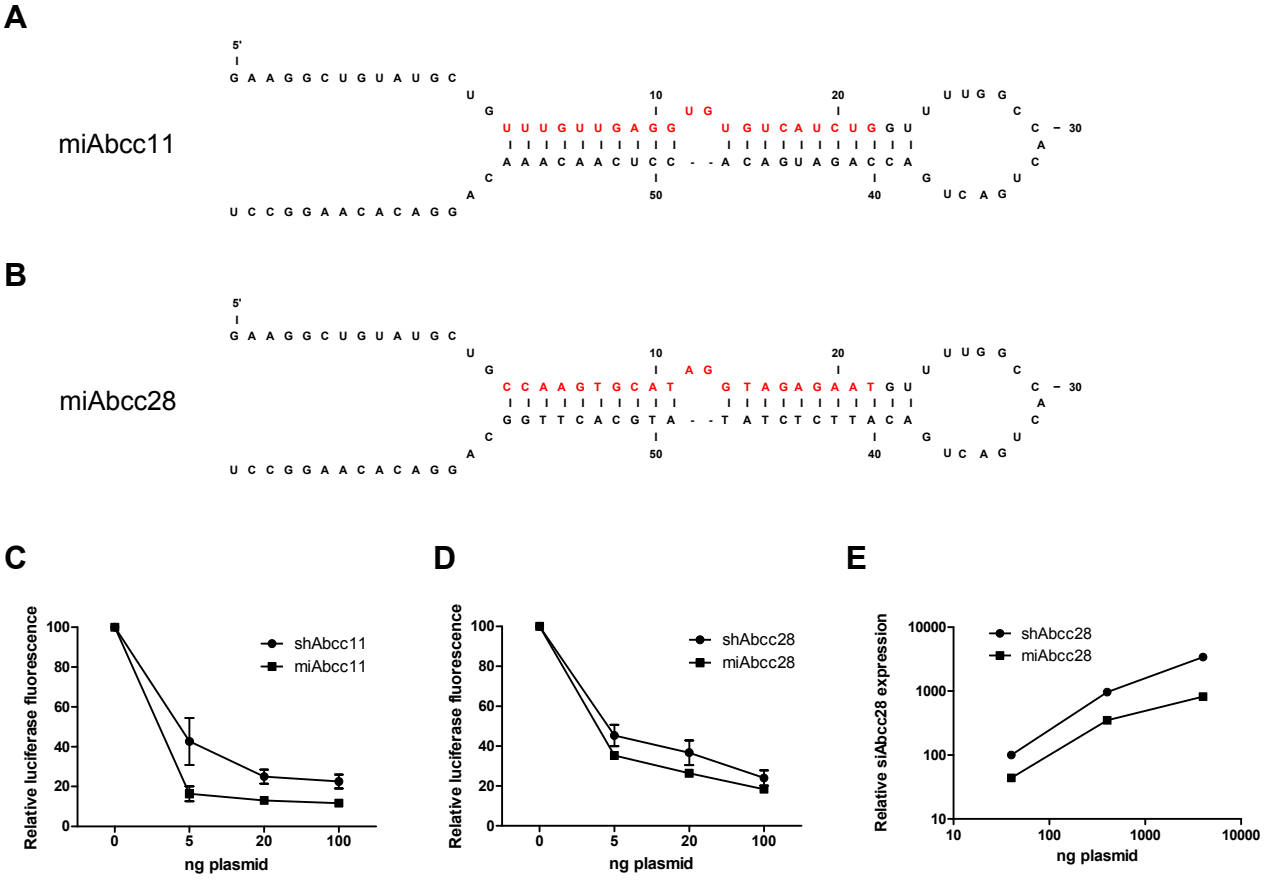

Figure 5. Expression of shAbcc11 and shAbcc28 from a miRNA scaffold and siRNA quantification. Predicted stem-loop structure with guide strand in red of **(A)** miAbcc11 and **(B)** miAbcc28. *Luc-Abcc1* knock-down by *Abcc1*-targeting **(C)** and *Luc-Abcc2* knock-down by *Abcc2*-targeting **(D)** shRNAs and miRNAs (shScr and miScr were set at 100%). **(E)** Relative amount of siRNA molecules produced by shRNA and miRNA constructs (the lowest shRNA dose was set at 100%).

would present reduced toxicity risks in *in vivo* studies is of considerable interest. We are currently comparing the long-term efficacy and toxicity profile of siRNAs expressed from AAV-shRNA and AAV-miRNA backbone *in vivo*. Future research will therefore focus on expressing siRNA from a miRNA backbone to achieve efficient and safe *in vivo* target knock-down.

To our knowledge, this is the first report to show AAV-mediated knock-down of *Abcc2 in vivo*. Concomitant toxicity was observed and was attributed to a previously described mechanism of oversaturation of the RNAi machinery (Grimm et al, 2006). Subsequent generation of miRNAs showed a better efficacy profile, *i.e.*, the same effect mediated by significantly less siRNA molecules. We therefore expect that these constructs will yield a strong and safe knock-down following liver transduction with AAV.

CONCLUSIONS

- Expression of multiple anti-*Abcc1* and anti-*Abcc2* shRNAs
- *In vitro* studies are insufficient predictors of the shRNAs processing *in vivo*
- Strong knock-down of *Abcc2 in vivo* following AAV-shAbcc delivery
- Concomitant toxicity due to oversaturation of the RNAi machinery
- Toxicity can be circumvented by siRNA expression from a natural miRNA scaffold

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COMPETING INTERESTS

The authors declared no conflict of interest. Amsterdam Molecular Therapeutics declared no commercial interest in the conclusions.

LIST OF ABBREVIATIONS

gc; Genomic copies
p.i.; Post injection
PBS; Phosphate-buffered saline

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