LETTERS

Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways

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RNA interference (RNAi) is a universal and evolutionarily conserved phenomenon of post-transcriptional gene silencing by means of sequence-specific mRNA degradation, triggered by small double-stranded RNAs[1,2]. Because this mechanism can be efficiently induced *in vivo* by expressing target-complementary short hairpin RNA (shRNA) from non-viral and viral vectors, RNAi is attractive for functional genomics and human therapeutics[3,4]. Here we systematically investigate the long-term effects of sustained high-level shRNA expression in livers of adult mice. Robust shRNA expression in all the hepatocytes after intravenous infusion was achieved with an optimized shRNA delivery vector based on duplex-DNA-containing adeno-associated virus type 8 (AAV8). An evaluation of 49 distinct AAV/shRNA vectors, unique in length and sequence and directed against six targets, showed that 36 resulted in dose-dependent liver injury, with 23 ultimately causing death. Morbidity was associated with the downregulation of liver-derived microRNAs (miRNAs), indicating possible competition of the latter with shRNAs for limiting cellular factors required for the processing of various small RNAs. *In vitro* and *in vivo* shRNA transfection studies implied that one such factor, shared by the shRNA/miRNA pathways and readily saturated, is the nuclear karyopherin exportin-5. Our findings have fundamental consequences for future RNAi-based strategies in animals and humans, because controlling intracellular shRNA expression levels will be imperative. However, the risk of oversaturating endogenous small RNA pathways can be minimized by optimizing shRNA dose and sequence, as exemplified here by our report of persistent and therapeutic RNAi against human hepatitis B virus *in vivo*.

To allow simple, efficient and persistent shRNA expression in entire mouse liver, we constructed a vector from AAV8. It carried a 'stabilized double-stranded' (sds) DNA genome, flanked by packaging signals derived from two heterologous AAV genotypes (see Supplementary Information)[5–7]. We evaluated liver-directed shRNA expression by co-injecting firefly luciferase-expressing sdsAAV8 into mice together with five individual vectors encoding distinct anti-luciferase shRNAs (Supplementary Table 1). Complete liver transduction was achieved with doses as low as 2×10^{11} particles. Comparisons of bioluminescence in shRNA-injected and control animals showed efficient luciferase knockdown with two vectors for more than one month after injection (Supplementary Fig. 1).

Several mice treated with high doses of shRNA vector developed signs of severe toxicity (for example weight loss) and ultimately died within one month. A transient increase in luciferase expression in the liver of moribund mice indicated that acute events occurred in this organ (Supplementary Fig. 1). Numerous controls proved that toxicity was shRNA-specific and not related to vector components (not shown).

To confirm these findings we treated mice transgenic for the human α-1 antitrypsin (*hAAT*) gene with a series of anti-*hAAT* shRNA-expressing sdsAAV8 (Supplementary Table 1; representative results in Fig. 1a). Again, several shRNAs caused toxicity and morbidity, particularly the 25-mer at high doses, usually killing treated animals within one month. In most other mice RNAi was transient and reached a peak after two weeks, before hAAT levels rose sharply, often to surpass initial values. A notable exception was the low dose of 19-mer, which gave efficient and persistent hAAT knockdown for more than one year (Fig. 1a).

The data overall indicated that *in vivo* toxicity was frequent and not restricted to a particular shRNA or target. For further confirmation we created a battery of sdsAAV8 vectors encoding 40 additional distinct shRNAs (total 49; Supplementary Table 1). Infusion of high particle doses (10^{12}, ensuring complete liver transduction) into wild-type C57/BL6 or FVB mice ($n \geq 3$ each; both strains gave identical results) revealed that 36 of 49 shRNAs (73%) were severely toxic. Nearly half of all vectors (23 of 49; 47%) killed treated mice within two months. We concluded that lethality did not require the presence of an shRNA target (see also Supplementary Fig. 2).

Pathologically, the moribund mice often had severe ascites and widespread subcutaneous oedema. Gross and histological analyses of all major organs revealed that the main changes were in liver (Fig. 1b), with chronic hepatopathy characterized by lobular collapse, multifocal hepatocyte necrosis and an apparent regenerative effort demonstrated by marked anisocytosis and presence of numerous megalocytes (Fig. 1c). Additional signs of liver failure included elevated total and direct bilirubin levels (1.2–5.6 mg dl^{-1}, normal 0.65–0.85; direct bilirubin more than 85% of total), correlating with icteric sera. The prominent change in blood parameters was a decrease in total protein, albumin and globulin to nearly undetectable levels (less than 0.5 g dl^{-1}), probably causing the oedema and ascites. Liver enzymes were typically elevated in moribund mice at about one month after vector administration (alanine aminotransferase: 232–2665 IU l^{-1}, normal 76–160; aspartate aminotransferase: 444–5723 IU l^{-1}, normal 192–388; alkaline phosphatase: 192–894 IU l^{-1}, normal 171–183). All other parameters, including complete blood counts and electrolyte panels, were normal. Together with the fact that more than 90% of AAV vector DNA resided in the liver (not shown), these findings indicated shRNA-induced liver dysfunction as the cause of morbidity.

We found no evidence in ailing mice for activation of the RNA-dependent protein kinase/interferon pathway (previously controversially discussed as an adverse effect of shRNA expression[8]) or for cell cycle perturbation. ELISA-based screening of 45 sera (25 from ailing mice, 20 from healthy) showed normal interferon-α/β/γ, tumour necrosis factor-α and interleukin-6 levels (not shown), and

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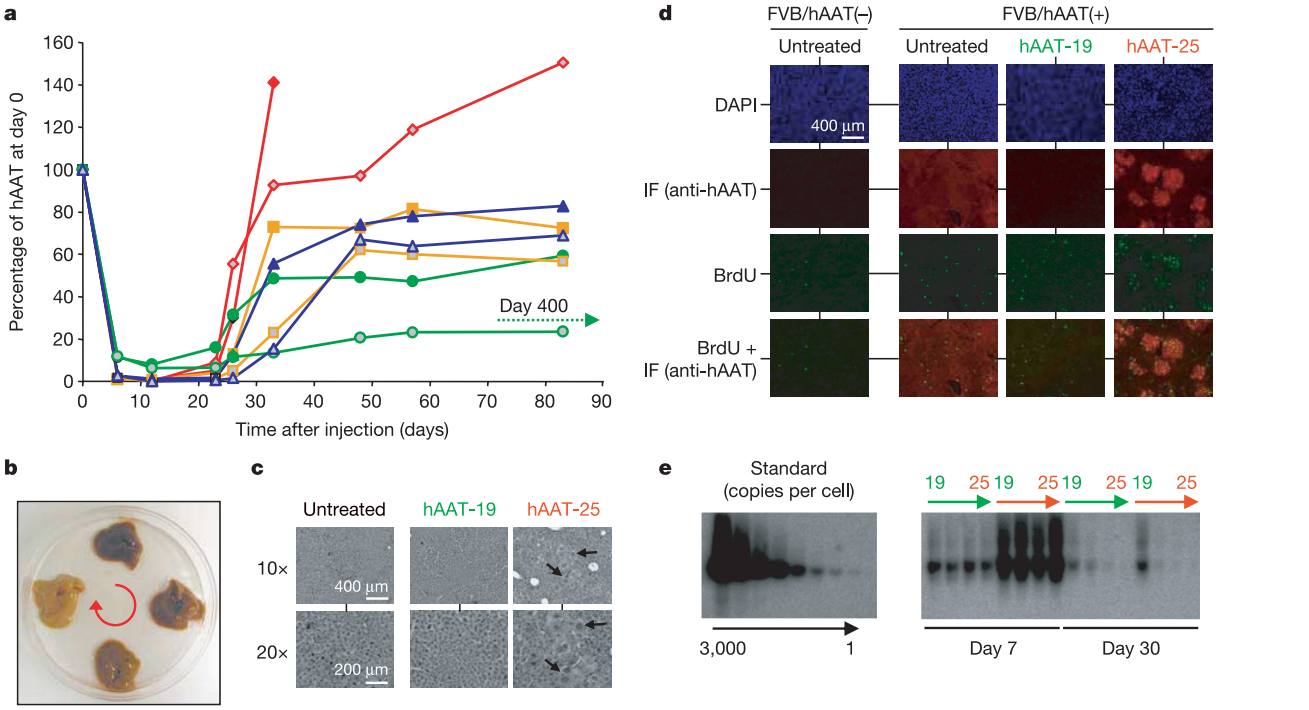

Figure 1 | Hepatocellular toxicity and liver regeneration from shRNA overexpression. **a**, hAAT-transgenic mice ($n = 6$) were injected with 10^{11} (low, filled symbols) or 10^{12} (high, open symbols) anti-hAAT shRNA-expressing sdsAAV8. Means of hAAT serum levels are relative to day 0 (error bars are omitted for clarity). Red, 25-mer; blue, 23-mer; orange, 21-mer; green, 19-mer. **b**, **c**, Transient RNAi coincided with liver toxicity, evident from abnormal morphology (**b**; arrow indicates increasing liver damage over time) and histology (**c**; arrows indicate megakaryocytes, a sign of liver regeneration). **d**, Stable silencing with the 19-mer and abundant hAAT-/bromodeoxyuridine (BrdU)-positive liver nodules with the 25-mer. DAPI, 4,6-diamidino-2-phenylindole; IF, immunofluorescence. **e**, Southern blot showing that hepatocyte death results in the loss of AAV vector DNA. Numbers indicate shRNA stem lengths; arrow colours show vector doses (green, low; red, high; see **a**).

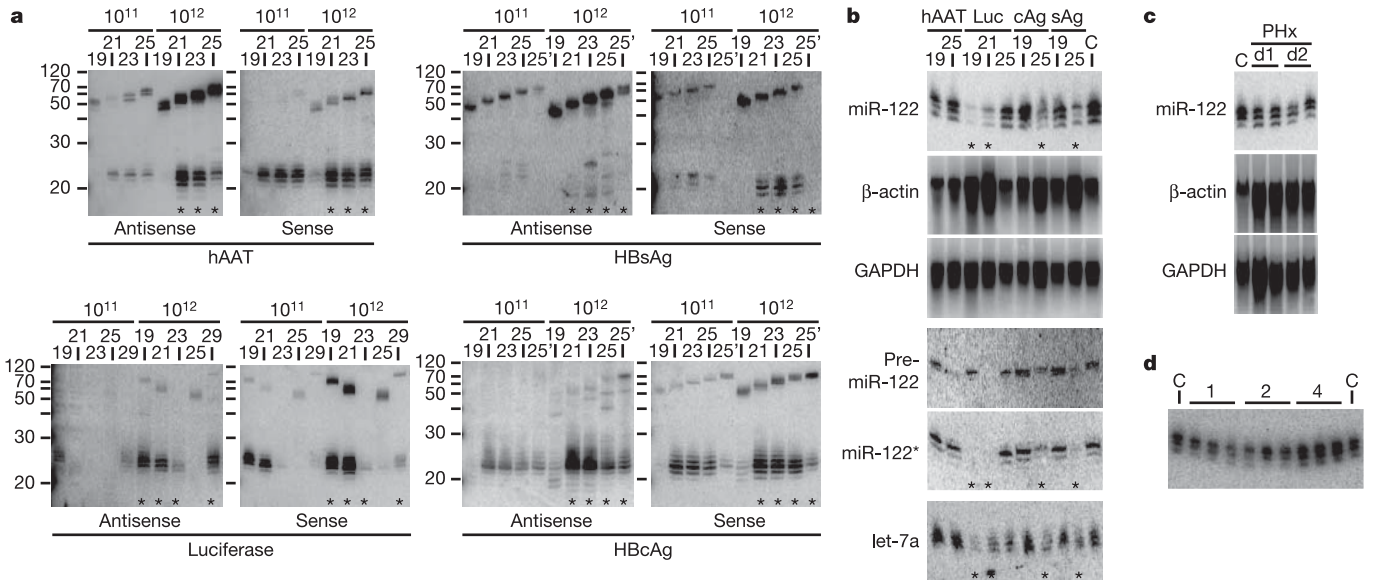

Figure 2 | shRNA overexpression saturates cellular miRNA pathways. **a**, Northern blot shows dose-dependent shRNA expression from a representative subset of vectors (Supplementary Table 1). Stem lengths are indicated above each lane, and marker sizes (in nucleotides) at the left. Asterisks indicate toxic shRNAs. **b**, Decrease in hepatocellular *miR-122* levels (asterisks) in mice overexpressing toxic shRNAs (northern blot with representative samples). The hAAT-25 mouse exemplifies rare recovery and return to normal *miR-122* levels. **c**, After partial hepatectomies (PHx) to induce cell division[9], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin mRNA were typically elevated for two days, whereas *miR-122* levels remained stable. **d**, Continuous treatment with carbon tetrachloride to induce liver damage (demonstrated by elevated alanine aminotransferase (573–786 IU l^{-1}), icteric sera and abnormal morphology (not shown)) did not affect *miR-122*. Each lane represents one mouse per time point (weeks after first injection). Lanes marked C represent untreated control FVB mice.

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western blots confirmed stable p21/p53 levels and a lack of eIF2-α phosphorylation (Supplementary Fig. 3). Although it was technically impossible to rule out off-targeting[8], this seemed unlikely because 23 different shRNAs gave a similar lethal phenotype.

We proposed that overexpressing shRNAs led to hepatocyte death, often severe enough to cause liver failure and morbidity. We assume that in mice that escaped morbidity but showed transient RNAi, the intrinsic ability of liver to regenerate itself[9] allowed dying hepatocytes to be replaced with new cells lacking shRNA. Direct proof for this idea was obtained by injecting hAAT-transgenic mice to induce either persistent hAAT silencing (19-mer, low dose) or toxicity (25-mer, high dose). When the latter seemed morbid and RNAi became transient (day 26), all livers were collected and stained (Fig. 1d). As predicted, mice treated with the 19-mer showed no hAAT expression, whereas the 25-mer group displayed manifold liver nodules of hAAT-positive (and thus shRNA-negative) hepatocytes, identified as actively dividing cells repopulating the liver by bromodeoxyuridine co-staining. This was corroborated by quantification of AAV vector DNA in livers early or late after injection: only the vector expressing the 19-mer persisted in the long term, whereas all other AAV genomes were almost completely lost during the process of liver damage and repopulation (Fig. 1e).

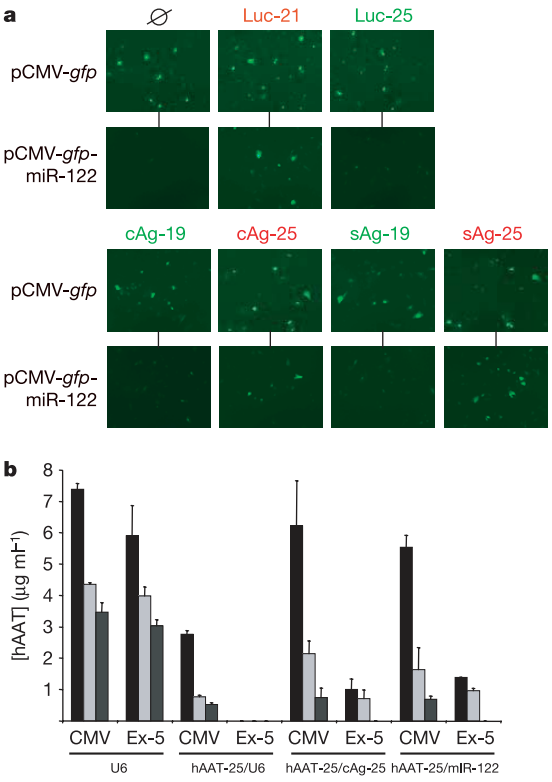

Figure 3 | shRNA overexpression inhibits *miR-122* functionality in liver cells. a, Human Huh7 liver cells were co-transfected with the shown shRNA plasmids (green, non-toxic; red, toxic; ϕ, empty plasmid control) and a *gfp*-encoding vector with or without a *miR-122*-binding site[12]. GFP expression from the fusion construct was marginal in the presence of no or non-toxic shRNAs, but elevated with toxic shRNAs, indicating possible inhibition of *miR-122* function (up to 50% positive cells, in comparison with non-fused *gfp* controls). **b**, Wild-type FVB mice ($n = 3$ per group) were hydrodynamically transfected with 8 μg of a Rous sarcoma virus (RSV)-promoter-driven *hAAT* gene and 12 μg of a human U6 promoter plasmid (lacking shRNA), or 2 μg of the hAAT-25 and 10 μg of the empty U6, the cAg-25, or a *miR-122* plasmid. In addition, all mice received 5 μg of a cytomegalovirus (CMV)-promoter-driven exportin-5 gene or an empty CMV control plasmid (pBKCMV; Stratagene). Plasma hAAT levels (means ± s.d.) were determined 1, 2 or 3 days after transfection (black, light grey and dark grey bars, respectively).

To understand the differences between various shRNAs, we studied their expression in injected mice. The shRNA precursors occasionally dominated the mature shRNAs, probably as a result of inefficient processing. Moreover, shRNA levels sometimes varied drastically between molecules differing in only two nucleotides. These findings might explain some historical controversies[8] and highlight the necessity of experimentally controlling for shRNA expression. We frequently found a direct correlation of high shRNA levels with toxicity (Fig. 2a), indicating that a possible reason for hepatocyte death was oversaturation of the endogenous shRNA processing machinery. The latter is shared by miRNAs, cellular RNAs about 22 nucleotides long with central regulatory functions in mRNA translation, the cell cycle and development, for example, but also in cancer or virus replication[10–12]. We therefore speculated that highly expressed 'toxic' shRNAs competed with miRNAs for intracellular processing, to such an extent that affected cells died.

Further RNA analyses supported this model, because toxicity and morbidity were correlated with decreased (by 54–81%) levels of the miRNAs *miR-122* (representing about 70% of all liver miRNAs[13]) or *let-7a* (Fig. 2b). Typically for repopulating liver, β-actin transcription was greater (unlike that of other cellular RNAs; Supplementary Fig. 4). When we induced liver regeneration by surgery (Fig. 2c) or chemical injury (Fig. 2d) we found that miRNA inhibition specifically resulted from shRNA overexpression and not from general liver injury. The correlation between toxicity and premature miRNAs was less clear, although the latter were frequently also present in lower concentrations like the processed forms (Fig. 2b, and not shown).

To assess whether shRNA overexpression compromised miRNA functionality, we co-transfected Huh7 cells (abundantly expressing *miR-122* (ref. 12)) with a *gfp* reporter fused to a *miR-122* target site, together with various shRNAs (Fig. 3a). Expression of green

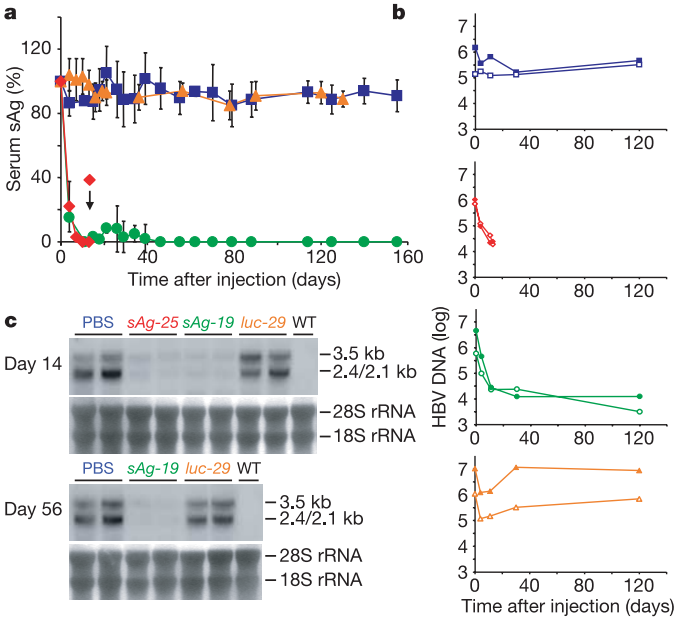

Figure 4 | AAV/shRNA-mediated persistent HBV inhibition in transgenic mice. a, HBV-transgenic mice ($n = 6$) were treated with anti-HBV (or anti-luciferase control (orange)) shRNA-expressing sdsAAV8 (3×10^{11} particles per mouse). HBsAg knockdown is shown as a percentage of day 0 (means ± s.d.). The 25-mer anti-HBV shRNA (red) caused early death, whereas the 19-mer (green) persistently and efficiently repressed HBsAg. Blue, PBS. **b**, Serum HBV DNA level (virus load) confirms long-term HBV knockdown *in vivo*. Colours as in **a**. **c**, Northern blots show nearly complete and persistent elimination of viral mRNAs in mice treated with the non-toxic 19-mer. No effect was seen in negative controls. Shown are two representative mice per group. WT, HBV-negative wild-type FVB mouse; kb, kilobases.

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fluorescent protein (GFP) was marginal in the presence of a non-lethal shRNA or no shRNA, because of efficient *miR-122*-mediated cleavage of the fusion mRNA[12]. In contrast, co-delivery of highly expressed, toxic shRNAs inhibited *miR-122* processing, demonstrated by greater expression of GFP. These results corroborate a recent study *in vitro*[14].

To assess shRNA/miRNA competition *in vivo*, we performed hydrodynamic plasmid injections in wild-type FVB mice (Fig. 3b). We co-expressed the *hAAT* gene with a limiting amount of the anti-hAAT 25-mer shRNA and an excess of the toxic cAg-25 shRNA or *miR-122*. As expected, hAAT-25 shRNA functionality was reduced in the presence of cAg-25 or *miR-122* in comparison with controls without competitor.

The nuclear karyopherin exportin-5 was identified as an essential and shared component of the shRNA and miRNA pathways[15–17]. We therefore overexpressed exportin-5 in the transfected mice and found that it enhanced hAAT silencing, in the absence or presence of shRNA/miRNA competitors. The finding of exportin-5 as a limiting factor in the RNAi pathway is consistent with our observation that toxicity did not require an shRNA target, hinting at saturation of one or more early components. It also corroborates the current theory that the main function of exportin-5 is the nuclear export and stabilization of shRNAs/miRNAs, and not other critical macromolecules involved in translation, because we did not observe a global protein synthesis shutdown in ailing mice (see also Supplementary Fig. 2). To conclusively unravel all the molecules involved, future work will have to focus on selective inhibition or overexpression of other factors shared by shRNAs/miRNAs, such as Dicer or other parts of the RNA-induced silencing complex[18]. Our sdsAAV8/RNAi technology will prove useful for such approaches.

If the pathway can be oversaturated, efficient and persistent RNAi *in vivo* should be possible with the use of minimal vector doses and/or inherently weakly expressed shRNAs. We tested this in a therapeutically relevant model of AAV/RNAi-mediated gene silencing, by using hepatitis B virus (HBV).

We injected mice, transgenic with the full-length HBV genome and producing viral particles[19], with low doses of sdsAAV8 expressing the non-toxic sAg-19 shRNA. A lethality control group received the sAg-25 vector, whereas luc-29 (Supplementary Table 1) sdsAAV8 (non-lethal at this dose) or PBS served as negative controls. Blood was collected to quantify serum HBsAg and viral loads, and livers were harvested for RNA analyses.

Within two weeks, HBsAg decreased rapidly to below 10% of starting levels with both anti-HBV vectors, before sAg-25-treated mice died (Fig. 4a). The sAg-19 group showed no signs of morbidity, and HBsAg stabilized six weeks after injection at nearly undetectable levels for the entire duration of the experiment (more than 5 months). Efficient HBV suppression was corroborated by quantification of viral loads (Fig. 4b) and transcripts (Fig. 4c). Both anti-HBV vectors caused a more than 32-fold decrease in serum viral DNA within two weeks, and at four months the decrease with the sAg-19 shRNA stabilized at 148 $\pm$ 3-fold ($n = 3$). HBV mRNA had decreased in amount by more than 90% two weeks after sAg-19 injection relative to controls, and was barely traceable at week 8.

There is increasing evidence that introducing siRNAs into, or expressing shRNAs in, mammalian cells can have non-specific effects, including off-target silencing and activation of the interferon system[8]. However, here we have linked *in vivo* shRNA overexpression to tissue damage and frequent lethality in adult animals. We detected an intracellular shRNA threshold for toxicity, causally linked to fatal competition with endogenous miRNA processing and functionality. On the basis of this and published data[14,15], we speculate that the competition involves the overloading of exportin-5 (whose main function is nuclear export and stabilization of shRNAs/miRNAs) and perhaps other common factors (Supplementary Fig. 5). Individual miRNAs were recently blocked in mice by using 'antagomirs'[20]. The authors found minor non-lethal phenotypes, substantiating our model that shRNA-induced toxicity requires global miRNA inhibition, with *miR-122* and *let-7a* merely being examples. Additional support comes from studies with siRNAs to trigger silencing *in vivo*, in which lethality was also absent (although it is uncertain whether the same high siRNA concentrations were achieved)[8]. We can explain this by the fact that siRNAs enter the RNAi pathway at a late step[21], thus avoiding the saturation of exportin-5 or other early factors. Last, our competition model could explain why efforts to establish transgenic mice expressing multicopy shRNA sequences at high levels have remained unsuccessful[22].

We believe that by identifying the risk of unintentional miRNA inhibition by shRNA overexpression, our study will help to pave the way for future *in vivo* RNAi applications. We believe that monitoring and controlling intracellular shRNA levels is imperative for guaranteeing stable *in vivo* gene silencing while mitigating adverse effects. Fortunately, there are several options, such as shRNA expression from inducible or tissue-specific RNA polymerase II promoters[23,24]. Until those advances are in place, the present and previous work[25–28] show that, under optimized conditions, the current generation of shRNA vectors already permits an effective and stable *in vivo* silencing of endogenous and exogenous genes. This fuels hope that the wide use of RNAi for functional genomics, or as a new therapeutic in humans, will become a reality.

METHODS

See refs 29, 30 and Supplementary Information for more details.

ShRNAs were cloned into the sdsAAV vector and were expressed from the human U6 or H1 promoter. MicroRNA cleavage was measured by transfecting a plasmid containing a *gfp* gene with a *miR-122* site into Huh7 hepatoma cells; co-transfection with the shRNA expressing plasmids resulted in an enhanced fluorescence signal when miRNA-mediated cleavage was inhibited. Experiments *in vivo* were performed in FVB or C57/BL6 mice.

Received 26 September 2005; accepted 7 April 2006.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank J. Wilson for providing the AAV8 packaging plasmid, P. Sarnow for the *gfp* fusion and miR-122 expression plasmids, H. Doege and A. Stahl for the H1-driven shRNA cassettes, I. Macara for the exportin-5 vector, D. Haussecker and B. Garrison for critically reading the manuscript, and J. S. Lee and H. Xu for technical assistance. This work was supported by grants from the National Institutes of Health (to M.A.K.) and the Anna Ng Charitable Foundation (to M.A.K.).

Author contributions D.G. performed and designed (with M.A.K.) most of the included studies. K.L.S. maintained the hAAT-transgenic mice and performed all injections and histological liver analyses. C.L.J. performed crucial steps of the small RNA Northern blot analyses and provided the *gfp* plasmids used in Fig. 4a. T.A.S. and K.P. generated the sdsAAV8 preparations and helped with the DNA, RNA and protein analyses. C.R.D. performed the complete mouse pathologies. P.M. and F.S. provided and maintained the HBV-transgenic mice. M.A.K. supervised the research project, and assisted in the experimental design. All authors discussed the experimental results and had input into the writing of the final manuscript.

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