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Supplemental Information

Sustained Inhibition of HBV Replication In Vivo after Systemic Injection of AAVs Encoding Artificial Antiviral Primary MicroRNAs

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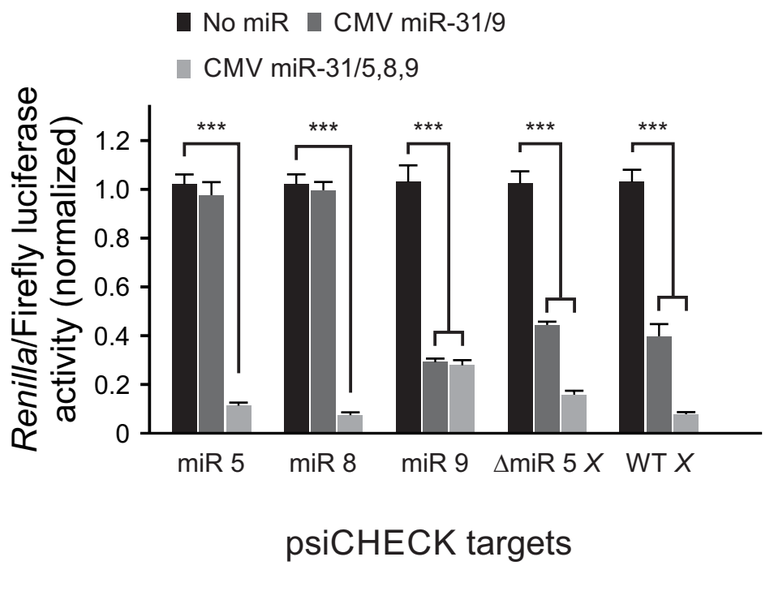
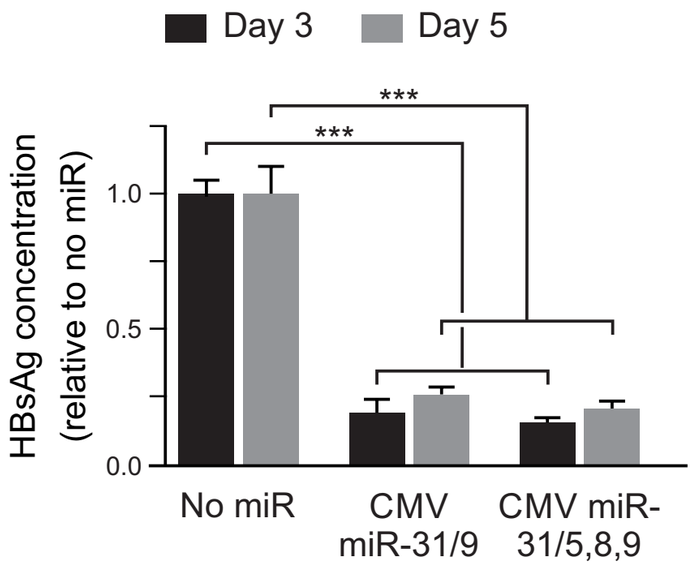
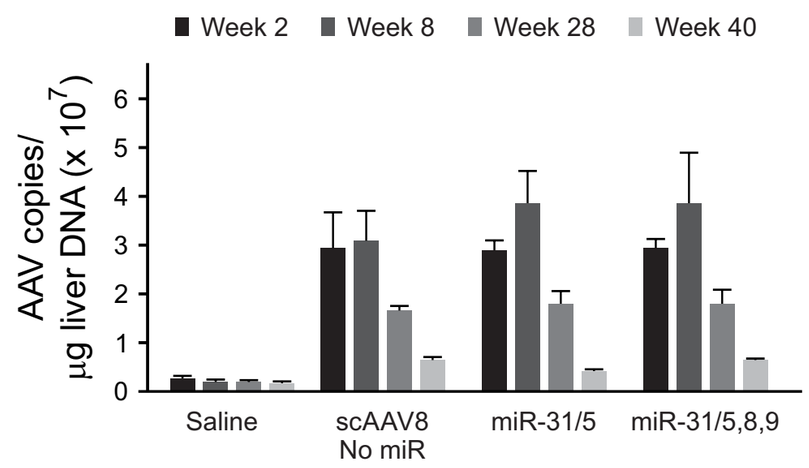


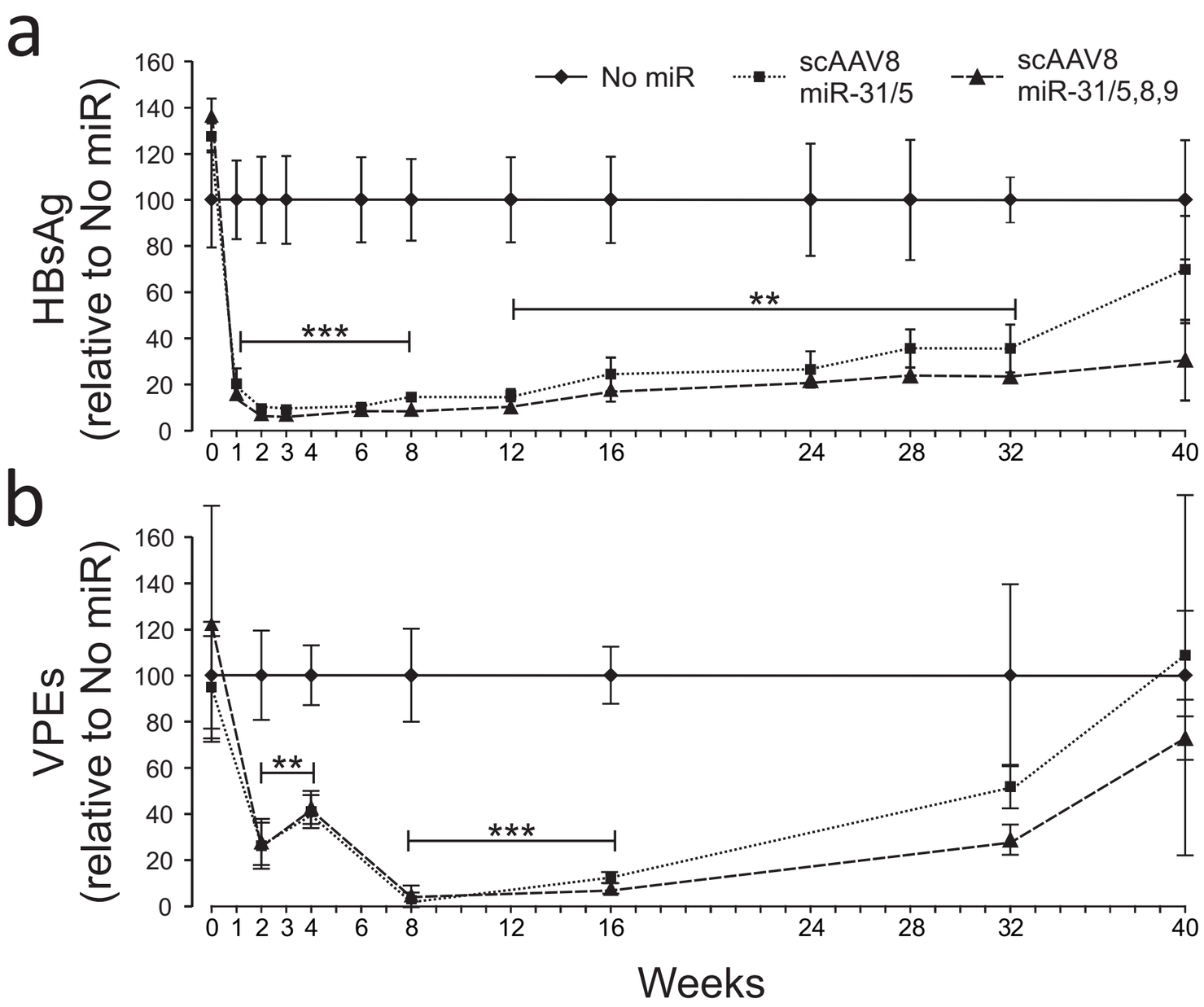
Supplementary Figure 1. Evaluation of target-specific silencing using reporter gene assay following transfection of cultured cells. Cultured Huh7 cells were transfected with psiCHECK 2.2 plasmids that contained targets for miR-5, miR-8, miR-9, *X* with a mutant cognate for miR-5 (ΔmiR 5 *X*) and wild-type *X* (WT *X*). These target sequences were inserted downstream of the *Renilla* open reading frame of the dual luciferase plasmid. Plasmids comprising the pCI-neo backbone with no miR, with miR-9 or miR-31/5,8,9 sequences, both under control of a CMV promoter/enhancer, were co-transfected with the reporter target plasmids. The ratio of *Renilla* to Firefly luciferase activity was measured as an indicator of knockdown efficiency. CMV miR-31/5,8,9 significantly silenced *Renilla* activity following co-transfection with each of the five target plasmids, while the CMV miR-31/9 plasmid diminished *Renilla* expression of target plasmids bearing the miR-9 target alone or in the context of ΔmiR 5 *X* or WT *X*. Statistically significant differences are indicated following analysis using the Student t-test (***: $p<0.005$).



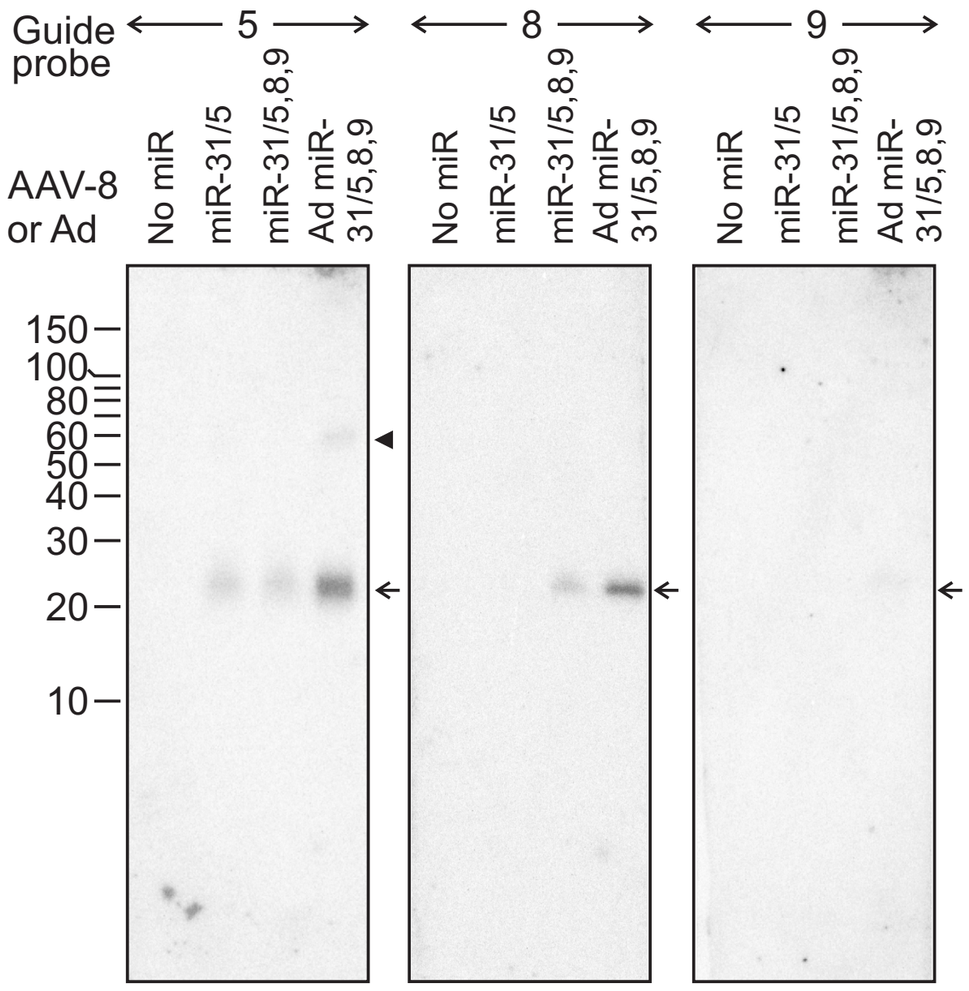
Supplementary Figure 2. Hydrodynamic injection of mice to evaluate silencing efficacy of miR-31/9 in vivo. Serum concentrations of HBsAg were measured at days 3 and 5 after hydrodynamic injection of mice with HBV replication-competent vector (pCH-9/3091) and plasmids expressing CMV miR-31/9 and CMV miR-31/5,8,9. A plasmid encoding Firefly luciferase was co-administered and bioluminescence imaging used to confirm equivalent transfection of mice in each of the groups. Statistically significant differences are indicated following analysis using the Student t-test (***: $p<0.005$).



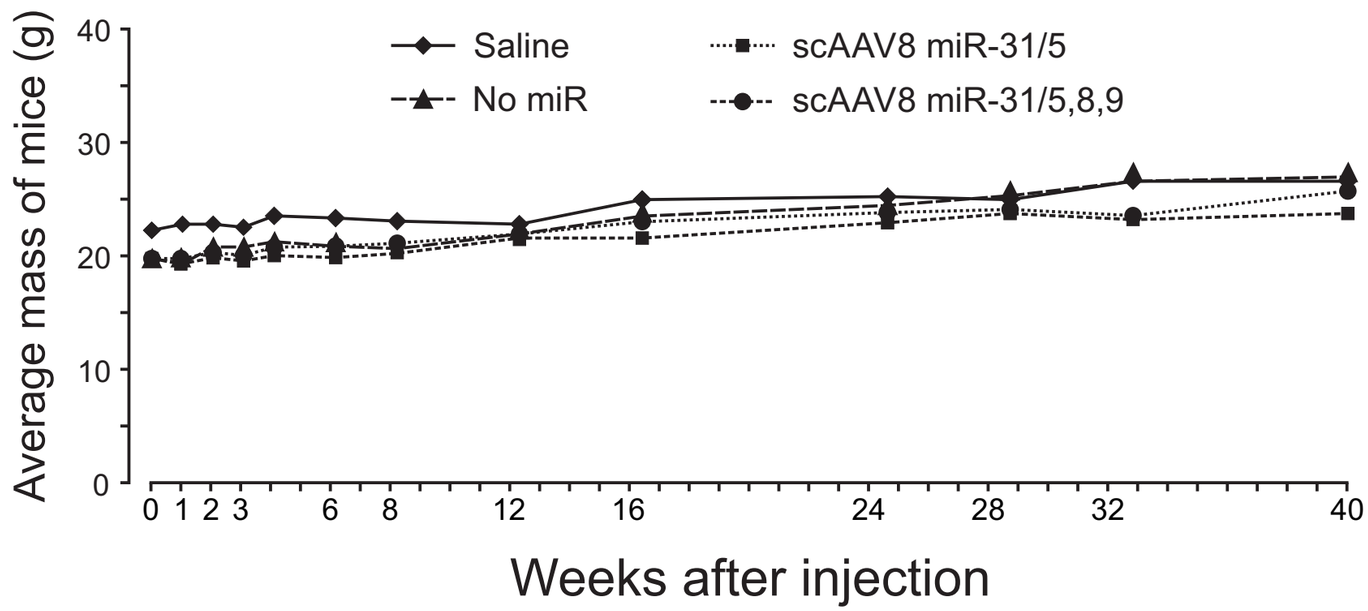
Supplementary Figure 3. Quantitation of DNA of scAAVs in liver cells after injection of vectors. Quantitative PCR analysis was undertaken to measure the copies of scAAVs per µg of total hepatic DNA extracted from mice killed at weeks 2, 8, 28 and 40 after injection with the indicated vectors. The peak of scAAV copies was reached at week 8 and declined over the period until week 40.



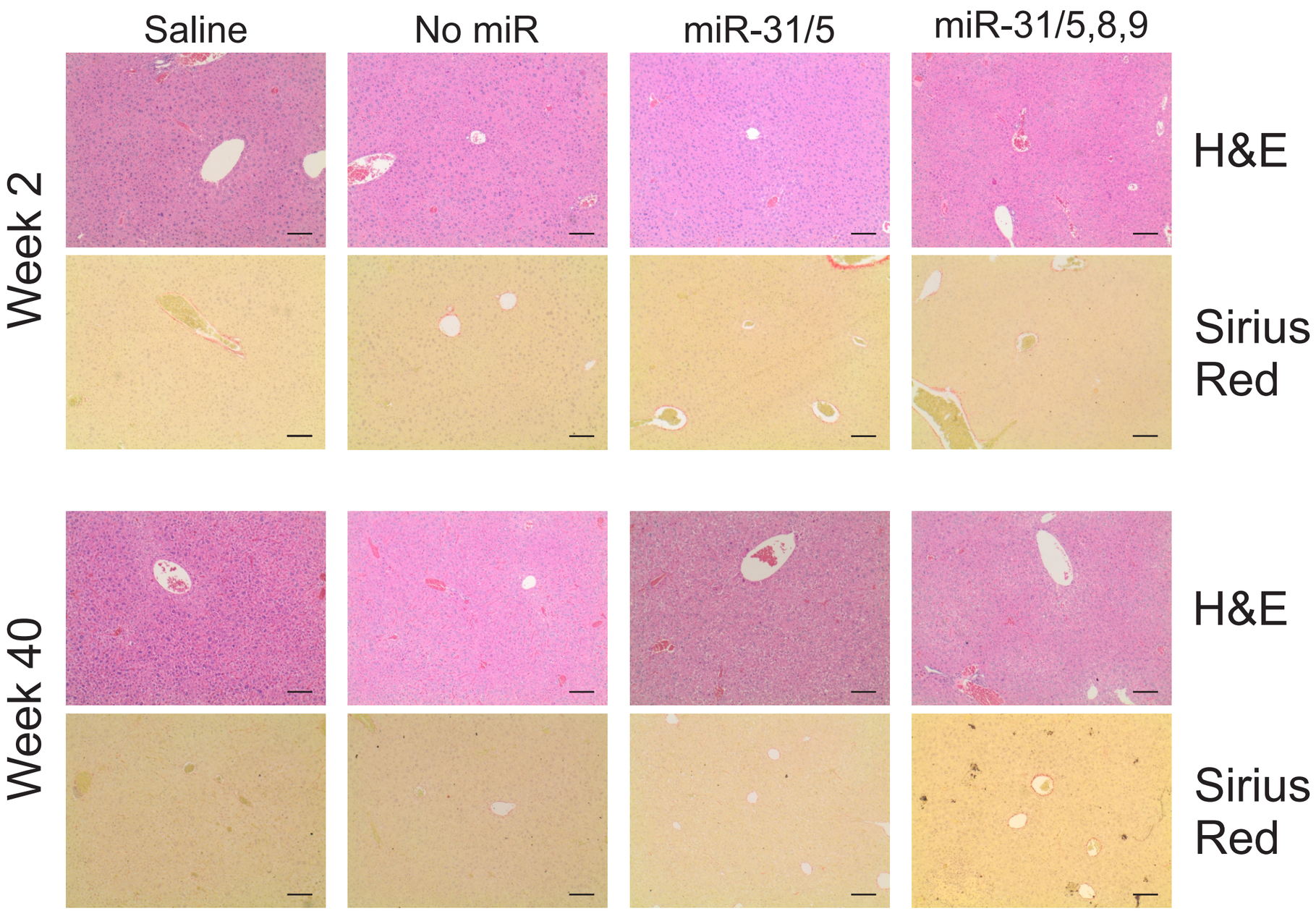
Supplementary Figure 4. Evaluation of long-term effects in vivo of HBV-targeting scAAVs on concentrations of HBV surface Ag (HBsAg) and circulating viral particle equivalents (VPEs) in HBV transgenic mice. a. HBV transgenic mice were intravenously injected with scAAV8 with no artificial miR (diamond), scAAV8 pri- miR-31/5 (square) or scAAV8 pri- miR-31/5,8,9 (triangle). Each animal received a dose of 1×10^{11} vector particles. HBsAg in serum samples was measured thereafter for a 40 week period. Data are presented relative to the normalized values obtained from the group of animals that was treated with the scAAV that did not produce miR. **b.** Circulating VPEs were measured using real-time quantitative PCR and the data are also given relative to the normalized values obtained from animals that were treated with the scAAV that did not produce miR. Statistically significant differences between the control and experimental samples, determined using the student's two tailed paired t-test, are indicated by asterisks (**: $p<0.01$, ***: $p<0.005$).



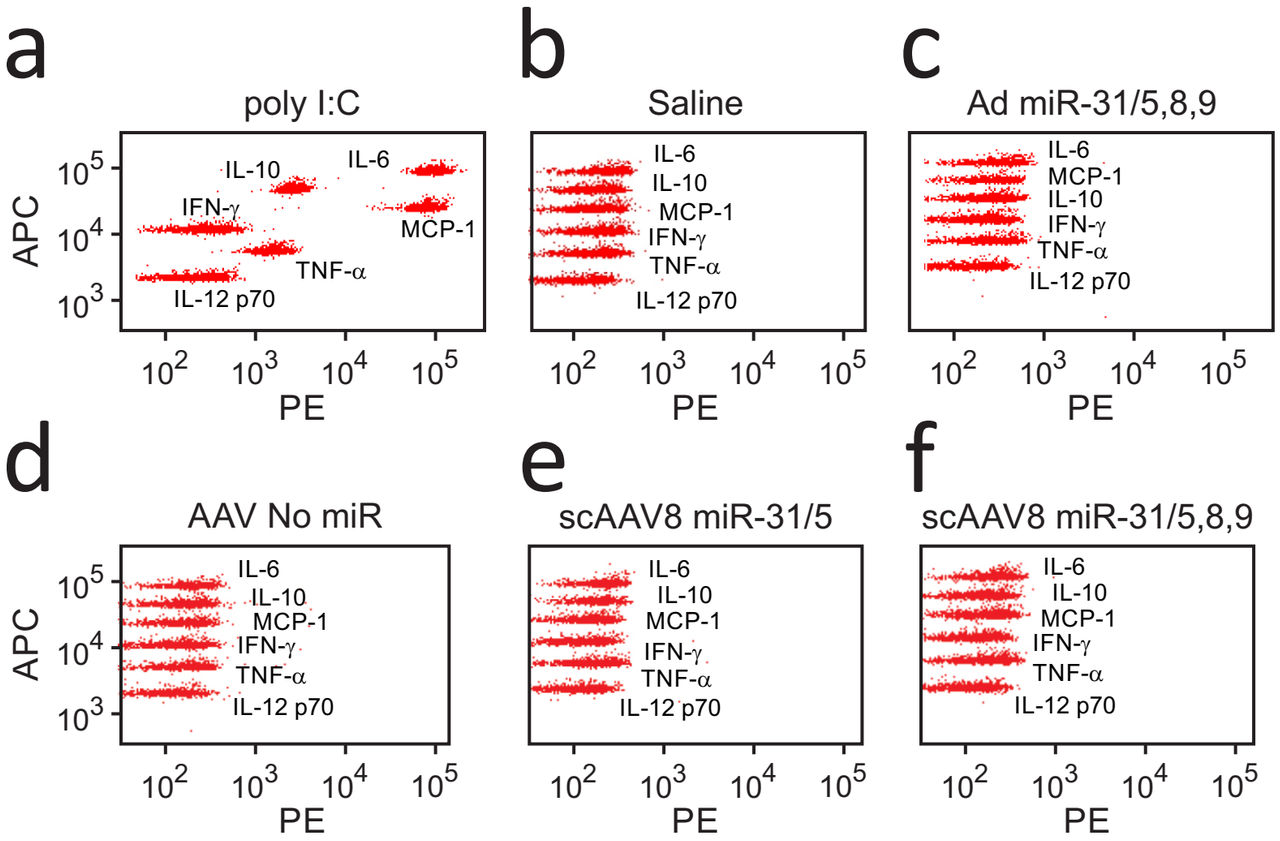
Supplementary Figure 5. Northern blot hybridization was carried out on total RNA extracted from livers of mice that were treated with scAAV containing no artificial miR, scAAV8 pri- miR-31/5 or scAAV8 pri- miR-31/5,8,9. As an additional control RNA was extracted from cells that had been transduced with an adenoviral vectors (Ad pri- miR-31/5,8,9) that delivered the trimeric cassette. Blots were hybridized to probes complementary to the sequences of guides 5, 8 or 9. Animals were killed at week 2 after injection. Positions of bands from the RNA molecular weight markers are indicated on the left. The data from the blot are uncropped to indicate presence of precursors (arrowhead) and the mature guide sequences (arrows). Putative precursors were only present in extracts from the Ad pri- miR-31/5,8,9-treated mice analyzed with the guide 5 probe.



Supplementary Figure 6. Weight of mice following administration of saline or indicated scAAVs. Average mass of groups of the animals receiving the vectors is indicated for the period of the experimental investigation.



Supplementary Figure 7. Liver histology (hematoxylin and eosin staining or Sirius red staining) obtained from liver sections of representative mice that had been injected with saline, scAAV8 without miR, scAAV8 miR-31/5 and scAAV8 pri-miR-31/5,8,9. Livers were harvested at 2 and 40 weeks after injection of the animals. Low power fields are shown with the scale bar indicating 100 μm. Sections were evaluated by assessing evidence of inflammatory infiltration, fibrosis, malignancy, hepatocyte death, cholestasis and macro- or microvesicular steatosis.



Supplementary Figure 8. Assessment of cytokine induction in HBV transgenic mice following intravenous administration of viral vectors or saline. Representative data from the assay using the cytometric bead array to quantify interleukin 6 (IL-6), interleukin 10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-a) and interleukin 12p70 (IL-12p70) concentrations. Measurements were made 6 hours after injection with (**a**) poly(I:C) (**b**) saline, (**c**) Adenovirus miR-31/5,8,9, (**d**) scAAV8 without miR, (**e**) scAAV8 miR-31/5 and (**f**) scAAV8 miR-31/5,8,9. APC: Allophycocyanin channel and PE: phycoerythrin channel.