Selection and evaluation of clinically relevant AAV variants in a xenograft liver model

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Recombinant adeno-associated viral (rAAV) vectors have shown early promise in clinical trials1–5. The therapeutic transgene cassette can be packaged in different AAV capsid pseudotypes, each having a unique transduction profile. At present, rAAV capsid serotype selection for a specific clinical trial is based on effectiveness in animal models. However, preclinical animal studies are not always predictive of human outcome4–6. Here, in an attempt to further our understanding of these discrepancies, we used a chimaeric human–murine liver model to compare directly the relative efficiency of rAAV transduction in human versus mouse hepatocytes in vivo. As predicted from preclinical and clinical studies6,8, aAAV2 vectors functionally transduced mouse and human hepatocytes at equivalent but relatively low levels. However, rAAV8 vectors, which are very effective in many animal models, transduced human hepatocytes rather poorly—approximately 20 times less efficiently than mouse hepatocytes. In light of the limitations of the rAAV vectors currently used in clinical studies, we used the same murine chimaeric liver model to perform serial selection using a human-specific replication-competent viral library composed of DNA-shuffled AAV capsids. One chimaeric capsid composed of five different parental AAV capsids was found to transduce human primary hepatocytes at high efficiency in vitro and in vivo, and provided species-selected transduction in primary liver, cultured cells and a hepatocellular carcinoma xenograft model. This vector is an ideal clinical candidate and a reagent for gene modification of human xenotransplants in mouse models of human diseases. More importantly, our results suggest that humanized murine models may represent a more precise approach for both selecting and evaluating clinically relevant rAAV serotypes for gene therapeutic applications.

rAAV clinical trials have been hampered by unanticipated immunological responses and lower than expected levels of transgene product1–3. For a single serotype there is little correlation between in vitro and in vivo transduction of primary cells. Between two serotypes, AAV8 and AAV2, the former provides >10-fold higher levels of liver-mediated gene transfer in animals, including non-human primates9–11. An exception may be humans, where in the case of haemophilia B the peak level of factor IX transgene product was similar in rAAV2- and rAAV8-treated individuals5. There are many potential reasons for the observed discordance in gene transfer efficiency among species, but relatively small differences in capsid sequence can affect both cellular uptake and post-cell-entry processing between species, ultimately affecting the level of gene transfer22.

To establish if murine and human hepatocytes contained within the context of an intact liver are themselves differentially transduced, we injected Fas−/−/Rag2−/−/Il2rg−/− (FRG) mice13 partially repopulated with primary human hepatocytes (hFRG) with single-stranded or self-complementary rAAV2 and rAAV8 vectors expressing enhanced green fluorescent protein (eGFP) (Fig. 1a, b). rAAV2 administration resulted in a low but equal number of eGFP-positive mouse and human hepatocytes. However, the rAAV8 vector resulted in a ~20-fold higher transduction efficiency in mouse compared with human hepatocytes, consistent with differences observed in preclinical and clinical studies published so far8,9,10,11. The block to functional rAAV8 transduction in human cells was not due to a lack of viral binding/uptake in the human hepatocytes because vector genomes were near equal in both human and mouse hepatocytes, as measured by quantitative polymerase chain reaction (qPCR) after laser capture microscopy (LCM) (Fig. 1c). By contrast, the rAAV2 vector genomes were preferentially taken up by human hepatocytes even though gene expression was similar in both the mouse and human cells. These results strongly suggest that differential functional transduction (measured as transgene expression) between capsid serotypes and species can be dependent on post-uptake factors.

Many different approaches have been used to alter the viral capsid and hence vector transduction properties14–21. As our goal was to identify new capsids with improved human tissue transduction, we created and screened a replicating AAV capsid library in the humanized mouse liver model. Our library screens are different from most in that selection is dependent not only on viral uptake and internalization, but also on viral replication, allowing us to select for these important post-uptake parameters that can affect vector-mediated gene transfer (reviewed in ref. 22).

Ten AAV capsid genes (AAV1, 2, 3B (ref. 23), 4, 5, 6, 8, 9, avian and bovine AAV) were used to generate an AAV-shuffled library (see Methods). To perform virus selection in vivo we used FRG mice partially repopulated with primary human hepatocytes (see Methods). Because the AAV libraries co-infected with wild-type human adenovirus 5 (hAd5) do not replicate in mice14, we have a stringent simultaneous positive and negative selection between the human and murine cells, respectively.

We performed four rounds of selection (Fig. 2a) and monitored progress by sequencing >100 clones after each round (Fig. 2b). Library selection in non-humanized FRG animals in the presence of hAd5 served as a negative control and confirmed that rescued AAV was derived from the human cells (Fig. 2b), whereas a non-humanized FRG animal injected with hAd5 only served as a control to ensure that AAV capsid-specific PCR signals were not caused by wild-type/AAV contamination of the hAd5 preparation (Fig. 2a, b). After four rounds, the three most frequent variants, AAV-LK01, -LK02 and -LK03, accounted for 22.7%, 4.54% and 2.3% of the isolates, respectively. From the 19 most abundant variants, 15 were successfully used to package vectors, rAAV-RSV-eGFP (RSV, Rous sarcoma virus) (Fig. 2c). Reconstruction of the genealogical relationship at the DNA and amino acid level between the isolates and parental AAVs used to generate the library is shown in Fig. 2d.

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We compared the in vitro transduction of these 15 AAV vector variants against standard AAV serotypes on a variety of cell types from different species (Extended Data Table 1). In comparison to rAAV-DJ, which efficiently transduces most mouse and human cells in culture\(^4\), rAAV-LK03 preferentially transduced cells of human origin, suggesting that this variant has species-restricted specificity. Importantly, rAAV-LK03 transduced primary human hepatocytes 100-fold better than AAV8. AAV-LK03 is closely related to AAV3B, with lesser contributions from AAV1, 2, 4, 6, 8 and 9 (Figs 2d and 3a, Extended Data Table 2, Supplementary Table 1 and Supplementary Figs 1 and 2). rAAV-LK03 transduced human hepatocytes in culture 3, 67 and 6.5 times more efficiently than rAAV-DJ, rAAV8 and rAAV3B, respectively (Fig. 3b).

A second isolate, AAV-LK19, differed from AAV-LK03 by a single amino acid, S262C. This isolate transduced mouse cell lines and was less efficient than rAAV-LK03 at transducing most human cells, with the exception of primary human keratinocytes, where AAV-LK19 resulted in 5 and 7.6 times higher levels compared with rAAV-LK03 and rAAV6, the serotype reported to transduce human keratinocytes\(^4\), respectively (Extended Data Table 1).

As pre-existing humoral immunity can block the clinical efficacy of a vector\(^5\), we tested selected AAVs in a pooled human immunoglobulin-G (IVIG) neutralization assay (Extended Data Fig. 1 and Methods). Compared with AAV2, AAV3B, AAV-LK03 and AAV-LK19 were much more resistant to IVIG neutralization (Fig. 3c), suggesting that these vectors could transduce a large population of humans if used in a clinical trial\(^5,6\).

Owing to the similarity between AAV-LK03, -LK19 and AAV3B, we wanted to establish whether the human hepatocyte growth factor (hHGF) receptor c-MET, previously identified as the AAV3 co-receptor\(^7\), was involved in AAV-LK03 and -LK19 transduction. Transduction of HuH-7 cells with rAAV-hPGK-GFP-P2A-Luc2 (Luc2, luciferase 2) in the presence of increasing concentrations of hHGF (used for c-MET competition) demonstrated that rAAV-LK03, but not rAAV-LK19, was competitively inhibited by hHGF (Fig. 3d). These results suggest that the single amino acid difference (S262C) changes the receptor/co-receptor entry of AAV-LK19. Although the AAV capsid structure is available for several serotypes, the bulk of the sequence variation in these two new pseudotypes was in VP1 (amino acids 1–125), the one region of the capsid for which the structure has yet to be determined, making it difficult to speculate on specific structure–function relationships.

To compare the relative effectiveness of selected AAVs for transgene expression in vivo, we selected eight serotypes to package the hFIX expression cassette used in the rAAV2-FIX clinical trial\(^8\). In mice, the rAAV-LK03 and rAAV-LK19 vectors expressed very low levels of hFIX (Fig. 3e). By contrast, rAAV-LK01 and rAAV-LK02, which are closely related to AAV8 and AAV9, and AAV1 and AAV6, respectively (Fig. 2d), provided similar levels of expression as that observed with rAAV-DJ and rAAV8. Liver vector copy number (VCN) analysis performed 54 days after vector administration (Extended Data Fig. 2a) correlated with the hFIX expression data: rAAV8 > rAAV-LK02 ≈ rAAV-DJ ≈ rAAV-LK03. Furthermore, liver AAV VCN determined at an early time point (Extended Data Fig. 2b) also correlated with transgene expression (Extended Data Fig. 2c), suggesting that rAAV-LK03 did not enter murine hepatocytes and/or was rapidly degraded upon cell entry.

To verify the strong species preference, we tested rAAV-LK03 in a hepatocellular carcinoma xenograft model\(^9\). Animals injected with rAAV3B showed no Luc expression in the liver or tumour, whereas animals injected with rAAV8 showed Luc expression from the tumour and the liver (Fig. 4a and Supplementary Figs 3, 4). Consistent with the data obtained with rAAV-hFIX in normal mice (Fig. 3e and Extended Data Fig. 2c), animals treated with rAAV-LK03-RSV-Luc2 did not show a detectable Luc signal from the liver but only from human tumours. In addition, the onset of transgene expression from AAV-LK03 was slower by about 48 h compared to AAV8 (Extended Data Fig. 3).

Having demonstrated that AAV-LK03 selectively transduced human cells, we next evaluated its transduction efficiency in FRG mice...
with and without human hepatocyte reconstitution (Fig. 4b). In agreement with previously published data\textsuperscript{27}, rAAV3B did not transduce murine hepatocytes (control animals in Fig. 4b and Extended Data Fig. 4). Surprisingly, rAAV3B, previously shown to transduce human hepatocytes in culture\textsuperscript{27} (Fig. 3b), did not lead to detectable Luc expression in engrafted human hepatocytes in this \textit{in vivo} model. By contrast, a strong Luc signal was detected in non-humanized FRG animals injected with rAAV8-RSV-Luc (Fig. 4b and Extended Data Fig. 4), which efficiently transduced mouse hepatocytes \textit{in vivo} (Fig. 3e and Extended Data Fig. 2). Most importantly, as predicted, the Luc signal was detected in humanized FRG mice injected with rAAV-LK03, but not in non-humanized FRG controls. These data showed that rAAV-LK03 selectively transduces human hepatocytes \textit{in vivo}.

To quantify further the transduction efficiency of rAAV-LK03, we injected an equal dose of rAAV-LK03 or rAAV8-eGFP into humanized FRG mice. One week later, the number of transduced cells was compared (Fig. 4c). The fraction of GFP-positive human hepatocytes was 43.3 ± 11% and 3.6 ± 1.1% with rAAV-LK03 and AAV8 vector infusion, respectively. By contrast, almost all (>99%) mouse hepatocytes were eGFP positive with rAAV8, whereas ≤1% of mouse hepatocytes were eGFP positive after rAAV-LK03 vector infusion. Thus, rAAV-LK03 transduction, as measured by the number of transgene-positive cells, was about ten times higher than AAV8 in human hepatocytes \textit{in vivo}. This experiment may underestimate the differences in AAV-mediated human hepatocyte transduction because of the variation in the rate of capsid uncoating and peak transgene expression among different serotypes. Whereas rAAV8 is rapidly uncoated\textsuperscript{29}, longer uncoating for rAAV-LK03 can be inferred from the slower rise in transgene expression in the tumour xenotransplant transduction studies (Extended Data Fig. 3).

The fact that there was not always a correlation between vector uptake and transgene expression between species (Fig. 1) strongly suggests that post-receptor entry factors (such as intracellular transport, nuclear entry and/or uncoating) influence the final level of functional transduction (reviewed in ref. 12). By making replication in human cells a condition for selection, we placed selection pressure on many of these post-entry parameters. Moreover, the human primary hepatocytes were in an environment that emulated their native

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**Figure 2 | In vivo AAV-shuffled library screen.** a, Graphic representation of the screen. N = 2 for passages 1 (1°) and 2 (2°), n = 1 for passages 3 (3°) and 4 (4°). Percentage of human hepatocyte repopulation is listed. Selection was performed once (single biological repeat). No lib., no library control. b, PCR analysis of liver lysates after each selection step. NTC, no template control. c, Dot blot titre comparison between parental AAVs, AAV-DJ and novel isolates for two transduction experiments. Error bars show s.d.; n = 3. d, Genealogical relationship of isolates at protein (left) and DNA (right) levels.
Figure 3 | Functional analysis of selected isolates. a, Contribution comparison for the top three isolates in graphic form. Residues with unknown parental origin are indicated with a question mark. b, Transgene expression in primary human hepatocytes in culture (see Methods). Fluc, firefly luciferase; RLU, relative light unit. Error bars represent s.d.; n = 4 from a single biological repeat. c, IVIG neutralization assay; n = 4 (±15% variation). d, hHGF competition assay. Data represent averages from two biological repeats with n = 3 per repeat. e, Comparison of hFIX expression in C57Bl6 mice. N = 5 animals per group up to day 54, n = 2 at day 80. Single biological repeat (see also Extended Data Fig. 2c).

Figure 4 | In vivo vector specificity analysis. a, Luc expression in human hepatocellular carcinoma xenograft model 6 days after intravenous vector injection (n = 5 per group). Controls, naive animals. The same results were observed in two independent biological repeats. b, Luc expression in humanized FRG animals (hFRG) or naive controls (Ctr). Serum human albumin (hAlb) levels and estimated percentages of human hepatocyte (hep.) repopulation are given. All animals used in the study are shown: n = 1 + 2 (rAAV3B), n = 1 (rAAV8), n = 2 + 2 (rAAV-LK03). c, In vivo comparisons between hFRG animals transduced with 5 × 10¹¹ vg rAAV8 or rAAV-LK03. Representative histological images are shown. Cell counting was as in Fig. 1a (see Methods). hFAH, human fumarylacetoacetate hydratase. Scale bar, 100 μm. The graph represents quantification of in vivo data from three animals.
setting and thus maintained a more physiological gene expression profile compared with an in vitro setting. This made selecting a candidate useful in humans more likely. A summary of the transduction efficiencies of standard and newly selected AAV capsids described here is shown in Extended Data Table 3. Taken together, the rAAV-LK03-based vector is not only a highly promising clinical candidate, but will also prove useful when restricted genetic manipulation of xenotransplanted cells/tissues is required.

Most importantly, our studies suggest that the use of a human primary cell xenotransplant model compared with commonly used mouse and non-human primate models may more accurately predict potential transduction efficiency in humans. The approach provided herein may accelerate the ability to identify and establish clinically useful AAV vector candidates for clinical trials in which current serotype selection is in large part based on gene transfer efficiency in animal models.

METHODS SUMMARY

An AAV capsid library derived from ten different capsids was generated as previously described14, with minor modifications. The library was subsequently injected into FRG mice reconstituted with primary human hepatocytes13. Twenty-four hours later, wild-type hAd5 was injected to induce replication of the AAV library in the human hepatocytes. Forty-eight hours after hAd5 injection, livers were harvested and analysed for capsid sequences by PCR and Sanger sequencing. These liver lysates were used for subsequent rounds of selection. Computer algorithms were designed for sequence comparisons and capsid of origin contribution analysis.

After four passages of the library, selected capsid clones were cloned into standard AAV vector production plasmids and then used to package various vector expression cassettes. rAAV-RSV-gGFP, RSV-Luc and hPGK-GFP-P2A-Luc expression cassettes packaged in various capsids were used in cell culture studies14. AAV-RSV-Luc and rAAV-LSP1-eGFP were used in tumour xenotransplant and FRG in vivo studies, respectively. In the FRG mice, 1–2 weeks after vector infusion the number of human hepatocytes was determined by anti-human-albumin staining, and the transduced mouse and human cells were quantified by eGFP fluorescence12. In some animals, the number of proviral vector genomes in mouse versus human hepatocytes was determined by ICM and qPCR15.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions L.L. helped with study design, performed experiments and data analysis, prepared figures and the manuscript. A.D. performed some of the experiments and data analysis, and assisted in figure preparation and manuscript editing; K.C. helped in performing some of the experiments. Y.Z. performed some of the vector sequencing analysis. S.C.C. performed some of the animal studies and assisted in manuscript editing. E.M.W. generated the human transplanted FRG mice in Fig. 4c. S.C.C. performed some of the animal studies and assisted in manuscript editing. All authors reviewed and commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.A.K. (markay@stanford.edu).

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METHODS

AAV library generation. The AAV library was generated as previously described with minor modifications. AAV capsid genes (cap) from AAV1, 2, 3, 4, 5, 6, 8, 9, avian AAV and bovine AAV were PCR amplified and cloned into TOPO AAV Cloning Kit (Life Technologies) and sequenced. cap genes were cut out using PacI and Ascl, mixed at a 1:1 ratio and digested using Dnase (Roche) for 1, 5, 10, 15, 20, 25 and 30 min. All reactions were separated on 1% (w/v) agarose gel, and fragments <1,000 bp were cut out and used in a primer-less PCR reassembly step, followed by a second round of PCR including primers binding outside the cap gene (forward primer 5'-GTCGAGTGAATGTCGAGT and reverse primer 5'-GCTTACTGTAAGCTCAGTGG) final. Reassembled shuffled cap genes were cloned into the AAV recipient plasmid based on wild-type AAV2. The final ligations were transformed into independent competent DH5α cell aliquots and plated on 100 µg/ml Luria broth (LB)/agar plates. One-hundred clones were picked and sequenced to confirm library variability. The remaining clones were scrapped, pooled and expanded in liquid LB plus ampicillin culture. Finally, plasmid DNA was extracted using EndoFree Megaprep Kit (Qiagen) and used for AAV library production.

AAV vectors and AAV library production. AAV library and AAV vectors were packaged as previously described using a CaPO4 transfection protocol followed by CaCl2 gradient purification as described. Vectors used in studies shown in Figs 1 and 4c were titrated by qPCR as described, whereas all other vectors used in the study were titrated by quantitative dot blot as described. All AAV productions were performed using HEK293 cells (ATCC, catalogue no. CRL-1573). AAV library yielded a preparation with a particle titre of 2×1011 vg per animal AAV library via i.v. injection followed by i.v. injection of wild-type human AAVS (VR-5; ATCC) 24 h later. Cells were harvested 48 h after transduction, as described.

For studies shown in Figs 2a and 4b, only 8–12-week-old mice were used for repopulation, whereas for studies shown in Figs 1 and 4c, both repopulated males and females were used. Twenty-four hours before hepatocyte transplantation, mice received intravenous (i.v.) injection of 5×107 plaque-forming units (p.f.u.) of human urokinase (uPA) adenoviral vector. Animals received wild-type human AD5 (VR-5; ATCC) 24 h later. Cells were harvested 48 h after transduction, as described.

The progress of liver repopulation was monitored by biweekly human albumin ELISA using Human Albumin ELISA Quantitation Kit (Bethyl Labs, catalogue no. HMCPSI, lot no. H80089) while mice used in studies shown in Fig. 4c were transplanted with cells isolated from psa-diatric donors or purchased from Invitrogen (catalogue no. CC-2591S, lot no. 9F3097 and 9F3003) (1×106 viable cells per mouse) as described. This mouse model has been used for testing other specific human processes related to drug metabolism and infectious diseases.

In vitro transduction analysis and transduction titre calculation. Cells were plated in 24-well tissue-culture-coated plates in appropriate media 16 h before transduction. Cells were transduced with AAV-RSV-GFP vectors at four tenfold dilutions for 16 h. GFP levels were analysed using fluorescence-activated cell sorting (FACS) using BD LSR II. The percentage of GFP-positive cells was used to calculate transduction titre (TU ml-1) by using the following formula: TU ml-1 = (%GFP/100)×(cell number at transduction)×(dilution factor). Transduction titre was normalized to dot blot titre (vg ml-1). For each cell line, the vector with the lowest normalized transduction (TU vg-1) was assigned the value 1 and used as a basis for normalization of all other vectors.

Primary human hepatocyte culture and transduction. Fresh primary human hepatocytes were plated on plates coated with rat tail Collagen Type II (BD Biosciences) in HMM Hepatocyte Maintenance Medium with UltraGlutamin-1 (Lonza). Cells were transduced with AAV-RSV-Luc (Extended Data Table 1) or AAV-PKG-GFP-P2A-Luc2-pA (Fig. 3b) vectors for 16 h. For the experiment shown in Fig. 3b, cells were transduced at the same multiplicity of infection (MOI) as the dot blot titre. Seventy-two hours after transduction, cells were washed in PBS and analysed using Dual-Luciferase Assay System (Promega) and Tescan M-1000 luminescent.

Mycoplasma testing. All cell lines and primary cells used in the study underwent mycoplasma testing before use and after passage. Mycoplasma contamination status was verified using LookOut Mycoplasma PCR Kit (Sigma, catalogue no. MP0035) and MycoAlert Mycoplasma Detection Kit plus Assay Control (Lonza, catalogue no. LT07-318 and LT07-518).

IVIG neutralization assay. IVIG neutralization assay was adopted from the previously described method, with modifications. Two IVIG preparations, Gammagard S/D (Baxter/Hyland) (IgA content of 1.6 µg ml-1 in 5% solution) and Gamunex (Bayer) (IgA content of 46 µg ml-1), were initially compared side by side using Huh-7 cells (gift from J. Glenn). rAAV-RSV-Luc2-pA vector was used as a positive control to establish IVIG neutralization assay. Two protocols were compared. AAV pre-incubation with IVIG at 4°C overnight and 37°C for 60 min. As shown in Extended Data Fig. 1, both preparations showed a similar profile of AAV2 neutralization. For all future experiments, 60 min AAV pre-incubation with IVIG at 37°C was used.

For the rAAV variants IVIG neutralization assay, an identical number of vector particles of the eight selected rAAV variants were incubated with increasing concentrations of Gammagard/Gamunex at 37°C before Huh-7 cell transduction in DMEM media without FBS. Cells were washed 5 h later and allowed to grow for 72 h in DMEM plus 10% FBS (v/v). Cells were harvested and analysed for Luc expression (see earlier).

hHGF competition assay. hHGF competition assay was performed as described previously. Huh-7 cells were transduced with rAAV-PKG-GFP-P2A-Luc2 at MOI 2×104. Vectors were premixed with increasing concentrations of recombinant HGF (Life Technologies). Cells were analysed for Luc levels 72 h after transduction (see earlier).

In vivo hFIX expression and analysis. Six-to-eight-week-old female C57BL6 mice (Jackson Laboratory) received retro-orbital injection of 5×105 vg rAAV-hFIX-16 vectors. Blood was collected at the indicated time points via retro-orbital bleeding, and plasma hFIX levels were determined using ELISA, as described.

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**In vivo VCN analysis.** Genomic DNA was extracted from indicated tissues using DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer’s instructions. Multiplex real-time TaqMan PCR was performed on BioRad CFX384 using: hFIX probe, 5'-6FAM-CCACTGGAATTTGACACTGCCTGT; hFIX forward primer, 5'-GTACCTGAGGATATCGACCTG; hFIX reverse primer, 5'-GCCGGGTTAGGTTTGAAGTTTGT; β-actin probe, 5'-VIC-GCTGTGTTCTTGCACCTCTTGATG; β-actin forward primer, 5'-TGAGACTCCAGCACACTGAATT; β-actin reverse primer, 5'-ACACCTAGGCGGATTGAAATGTA.

**In vivo Luc assay.** For in vivo Luc imaging, animals received i.v. injections of 5 × 10^9 vg (FRG mice; Fig. 4b) or 1 × 10^11 vg (NOD/SCID mice; Fig. 4a) of AAV-RSV-Luc variants. Naïve animals injected with the same doses of rAAV vectors served as controls. Animals were imaged every 48 h using Xenogen IVIS Lumina imaging system (Caliper LifeSciences) after intraarterial injection of luciferin substrate (120 mg kg⁻¹). Living image software was used for bioluminescent image analysis. Detailed analysis of images shown in Fig. 4a, b, including region of interest (ROI) quantification (total flux (p s⁻¹)), and average radiance (p s⁻¹ cm⁻² sr⁻¹) is shown in Extended Data Figs 3, 4 and Supplementary Figs 3, 4.

**Analysis of VCN in mouse and human hepatocytes using LCM and qPCR.** Liver sections from engrafted mice transduced with rAAV, and harvested 2 weeks later, were immunostained for human albumin as described earlier. The PALM MicroBeam System (Carl Zeiss GmbH) was used to collect areas of mouse and human hepatocytes. All the clusters of human cells in 2–4 0.5-μm liver sections per mouse, and 2–4 samples of mouse hepatocyte areas (roughly equivalent to the area of human cells collected) were collected by LCM. Samples were collected into 30 μL digestion buffer (10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.5% (v/v) Tween 20) and incubated overnight at 56°C with 20 U of lysed sample, with no further purification, using primers and probe to the woodchuck hepatitis post-transcriptional regulatory element (WPRE) (single-stranded vectors) or eGFP (self-complementary vectors) as described for viral titration. The reactions were carried out on 5 μl of sample or standards (plasmid or human/mouse genomic DNA). To account for potential cross-contamination of samples with cells from the other species, and to normalize for sample size, mouse and human GAPDH qPCR was performed on each sample. Reactions using 2× QuantTect Sybr Green Master mix (Qiagen) and 0.5 μM forward primer (for mouse GAPDH, 5'-ACGGCAAAATCAGGCGGCA; for human GAPDH, 5'-GCTCTCTGCTCTGCTCTGTCG) and 0.5 μM reverse primer (for mouse GAPDH, 5'-TATGGGGTGTCGTCAGGGC; for human GAPDH, 5'-GGAAACATCCGGGCTTG). Standards consisted of genomic DNA prepared from mouse NIH3T3 cells or human HEK 293 cells (0.0625–100 ng per reaction). Total GAPDH per LCM sample was determined and therefore the proportion of the sample that was human (or mouse) calculated. The vector genome amount was adjusted accordingly and normalized.

**Hepatocellular carcinoma xenograft model.** Hepatocellular carcinoma xenograft model was established as described, with modifications. Six-to-eight-week-old female NOD/SCID mice were obtained from Jackson Laboratory. Mice were γ-irradiated at 3 Gy 24 h before cell transplantation. Mice received subcutaneous injection of Huh-7 cells on the dorsal side of the neck between shoulder blades. 1 × 10^8 Huh-7 cells mixed 1:1 with matrigel (BD Biosciences) were injected (200 μl total). Animals were kept in separate sterile cages until the end of the experiment.

**Immunohistochemistry, microscopy, image processing and fluorescent cells counts.** To avoid any bias, all samples were blinded before immunohistochemical analysis, so that the person processing the samples did not know what vectors were used to inject given animals. Only after the final samples were analysed and described, were the samples unblinded and matched to vectors used.

For the detection of human albumin, human FAH and eGFP, as shown in Figs 1 and 4c, hFRG mice with human albumin levels between 1.2–3.2 nmol ml⁻¹ (equivalent to 24–64% repopulation) were injected with 5 × 10^10 vg (Fig. 1) or 5 × 10^11 vg (Fig. 4c) of rAAV-LSP1-eGFP. Tissues were harvested 10–14 days after AAV administration and livers were fixed in 4% (w/v) PFA after harvest and processed through a sucrose gradient (10–30% w/v) before freezing in Optimum Cutting Temperature (O.C.T.; Tissue-Tek, Sakura) freezing media. Frozen liver sections (5 μm) were permeabilized in ice-cold methanol, blocked with 13% (v/v) donkey serum (Sigma) and 8.7% (v/v) PBS in PBS without calcium and magnesium then reacted with a goat polyclonal anti-human albumin primary antibody (1:200 dilution; Bethyl Labs, catalogue no. A80 299P) or a rabbit polyclonal anti-FAH primary antibody (1:1200 dilution; Sigma-Aldrich, catalogue no. AV14681).

Primary antibody was detected with either an Alexa Fluor-594 donkey anti-goat IgG secondary antibody (1:1,000 dilution; Invitrogen, catalogue no. A11058) or an Alexa Fluor-594 donkey anti-rabbit IgG secondary antibody (1:800 dilution; Invitrogen, catalogue no. A21207). After immunolabelling, sections were analysed on an Olympus BUX1 upright microscope using filter sets D350/50 (excitation) and D460/30 (emission) for 4′,6-diamidino-2-phenylindole (DAPI) fluorescence; D480/30 (excitation) and D535/40 m (emission) for eGFP fluorescence; HQ560/55 (excitation) and HQ645/75 (emission) for Alexa Fluor-594 fluorescence. Images were captured with a Spot Enhancer black and white digital camera using Spot Version 4.0 imaging program (Diagnostic Instruments) and merged to determine colocalization of eGFP and human albumin.

The percentage of transduced human hepatocytes per field of view was determined by individually analysing and comparing images taken using filters D480/30 (excitation) and D535/40 m (emission) and HQ560/55 (excitation) and HQ645/75 (emission) to detect eGFP fluorescence and hFAH or hAlb immunostaining (Alexa-594), respectively, rather than direct analysis of merged images. The latter gives an underestimate due to variability of eGFP fluorescence intensity in individual hepatocytes.

**Statistical analyses.** Statistical analysis shown in Fig. 3b was done using one-way ANOVA with Sidak’s multiple comparison test using GraphPad Prism 6 software.

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Extended Data Figure 1 | IVIG neutralization assay optimization on Huh-7 cells using rAAV2-RSV-Luc2. Gamunex and Gammagard IVIGs were compared at two different temperatures. See Methods for experimental details.
Extended Data Figure 2 | In vivo vectors comparison in C57/BL6 animals.

a, b, In vivo average VCN analysis in tissues harvested on day 54 (a) from the first in vivo rAAV-hFIX experiment and on day 7 (b) from the second in vivo rAAV-hFIX experiment.

c, In vivo hFIX expression levels. hFIX levels obtained from the first in vivo rAAV-hFIX comparison (solid colour lines, from day 5 until day 80) are presented on the same graph with data obtained during the second in vivo experiment (dotted lines, days 2, 4 and 7).
Extended Data Figure 3 | Time course of Luc signal in animals shown in Fig. 4a. a, b. Data for days 2, 4 and 6 were collected and are shown. In a, all animals are shown with the same pseudo-scale, whereas in b, auto-scale was selected for each group.
Extended Data Figure 4 | Detailed analysis of bioluminescence for animals shown in Fig. 4b. The table represents detailed information on signal for each animal/ROI.

| Serotype   | Animal     | ROI | Total Flux (p/s) | Avg Radiance (p/s/cm²/sr) | Stdev Radiance | Min Radiance | Max Radiance | Area (cm²) |
|------------|------------|-----|------------------|---------------------------|----------------|--------------|--------------|------------|
| AAV3B      | hFRG       | ROI 1 | 7.2E+06          | 8.70E+04                  | 7.72E+04       | -1.51E+04    | 2.84E+05    | 6.61E+00   |
| AAV3B      | FRG control| ROI 2 | 5.03E+05         | 6.00E+03                  | 8.26E+03       | -1.63E+04    | 3.28E+04    | 6.61E+00   |
| AAV3       | FRG control| ROI 3 | 1.14E+06         | 1.38E+04                  | 9.54E+03       | -1.26E+04    | 5.30E+04    | 6.61E+00   |
| AAV8       | FRG control| ROI 4 | 9.02E+08         | 1.08E+07                  | 1.20E+07       | 5.71E+05     | 4.61E+07    | 6.61E+00   |
| AAV-LK03   | hFRG       | ROI 1 | 1.27E+07         | 1.52E+05                  | 1.20E+05       | -5.86E+03    | 6.39E+05    | 6.61E+00   |
| AAV-LK03   | hFRG       | ROI 2 | 2.12E+07         | 2.55E+05                  | 2.10E+05       | 4.70E+03     | 9.10E+05    | 6.61E+00   |
| AAV-LK03   | FRG control| ROI 3 | 1.77E+06         | 2.14E+04                  | 2.29E+04       | -2.16E+04    | 1.18E+05    | 6.61E+00   |
| AAV-LK03   | FRG control| ROI 4 | 8.73E+05         | 1.05E+04                  | 1.47E+04       | -2.23E+04    | 6.11E+04    | 6.61E+00   |
Extended Data Table 1 | Relative transduction efficiency of in vivo AAV isolates and wild-type AAV serotypes in tissue culture cell lines

| Cells      | Species | Origin | AAV1 | AAV10 | AAV10b | AAV11a | AAV11b | AAV2 | AAV8 | AAV9 | AAV12 | AAV12a |
|------------|---------|--------|------|-------|--------|--------|--------|------|------|------|-------|--------|
| FRhK4      | Rhesus monkey | Epithelial | 1.15±0.01 | 6.79±0.02 | 1.78±0.02 | 4.25±0.02 | 1.16±0.02 | 8.51±0.02 | 1.86±0.02 | 1.00±0.01 | 1.23±0.03 |
| MEF        | Mice    | Florigen | 3.28±0.02 | 2.18±0.02 | 9.43±0.00 | 1.23±0.01 | 3.65±0.02 | 1.85±0.01 | 1.48±0.02 | 1.68±0.01 | 1.78±0.04 |
| 3T3        | Mice    | Florigen | 1.46±0.02 | 1.46±0.02 | 4.02±0.05 | 2.35±0.01 | 1.56±0.02 | 1.53±0.02 | 1.52±0.02 | 2.58±0.01 | 7.32±0.04 |
| hiEF       | Rat     | Hepatocytes | 3.18±0.02 | 9.92±0.02 | 3.88±0.01 | 3.88±0.01 | 7.38±0.02 | 2.86±0.02 | 2.46±0.02 | 6.39±0.01 | 8.16±0.04 |

Transduction titre (TU ml⁻¹)(see Methods) was normalized to dot blot titre (vg ml⁻¹). For each cell line, the vector with the lowest normalized transduction (TU vg⁻¹) was assigned the value 1 and used as a basis for normalization of all other vectors. Vectors with the highest normalized transduction level for each cell line are shown in bold. A question mark indicates uncertainty of the cell type origin.
Extended Data Table 2 | AAV cap gene sequence comparison

|     | AAV-1E01 | AAV-1E02 | AAV-1E03 | AAV1 | AAV2 | AAV8 | AAV8B | AAV9 | AAV11 | AAVR | AAV20 | AAV5 | AAV6 | AAV7 | AAV9 | AAV11 | AAVR | AAV20 | AAV5 | AAV6 | AAV7 | AAV9 | AAV11 | AAVR | AAV20 | AAV5 | AAV6 | AAV7 | AAV9 | AAV11 | AAVR | AAV20 |
|-----|----------|----------|----------|------|------|------|-------|------|-------|------|-------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| a   |          |          |          |      |      |      |       |      |       |      |       |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| b   |          |          |          |      |      |      |       |      |       |      |       |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |

Extended Data Table 2

**a** Matrix representation of percentage identity between AAV cap genes on DNA level.

**b** Matrix representation of percentage identity between AAV Cap genes on protein level.
Extended Data Table 3 | Tabular representation of vector comparison data from different experiments presented in the study

|                          | AAV2 | AAV3B | AAV8 | AAV-DJ | AAV-LK01 | AAV-LK02 | AAV-LK03 | AAV-LK19 |
|--------------------------|------|-------|------|--------|----------|----------|----------|----------|
| Huh7 (2)                 | 46.3%| 2.3%  | 0.4% | 97.2%  | 0.1%     | 0.1%     | 100%     | 74.7%    |
| Hepatocytes in vitro     | 7.2% | 15.4% | 1.5% | 33.2%  | 0.2%     | 0.3%     | 100%     | 10.3%    |
| C57/BL6                  | 0.6% | 0.1%  | 100% | 29.0%  | 10.7%    | 37.0%    | 0.2%     | 0.3%     |
| hFRG (Fig 1)             |      |       |      |        |          |          |          |          |
| Human                    | 3.0% | 2.7%  |      |        |          |          |          |          |
| Mouse                    | 1.3% | 59.0% |      |        |          |          |          |          |
| hFRG (Fig 4c)            |      |       |      |        |          |          |          |          |
| Human                    |      |       |      |        |          |          |          |          |
| Mouse                    |      |       |      |        |          |          |          |          |
| Xenograft Model (Sup Fig. 3) | | | | | | | | |
| Human (Tumor)*           | 0.1% | 9.9%  |      |        |          |          |          | 100%     |
| Mouse (Liver)            | 0.3% | 100%  |      |        |          |          |          | 1.7%     |

For each experiment, the vector with the highest transduction was assigned a value of 100%.

*On the basis of average maximum radiance.

** The true origin of the signal (mouse versus human) cannot be determined.