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Restoring the natural tropism of AAV2 vectors for human liver

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One sentence summary:

Adeno-associated viruses isolated from the human liver are tropic for human hepatocytes in vivo but this phenotype becomes attenuated after passage in culture.

Editor's Summary

Abstract

Recent clinical successes in gene therapy applications have intensified interest in using adeno-associated viruses (AAV) as vectors for therapeutic gene delivery. Although prototypical AAV2 shows robust in vitro transduction of human hepatocyte-derived cell lines, it has not translated into an effective vector for gene therapy in vivo. This is consistent with observations made in the *Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}* (FRG) humanized liver xenograft mouse showing that AAV2 functions poorly in this model. Here, we derived naturally hepatotropic AAV capsid sequences from primary human liver samples. We demonstrated that capsid mutations, likely acquired as an unintentional consequence of tissue culture propagation, attenuated the intrinsic human hepatic tropism of natural AAV2 and related human liver AAV isolates. These mutations resulted in amino acid changes that increased binding to heparan sulfate proteoglycan (HSPG), which has been regarded as the primary cellular receptor mediating AAV2 infection of human hepatocytes. Propagation of natural AAV variants in vitro showed tissue culture adaptation with resulting loss of tropism for human hepatocytes. In vivo re-adaptation of the prototypical AAV2 in FRG mice with a humanized liver resulted in restoration of the intrinsic hepatic tropism of AAV2 through decreased binding to HSPG. Our results challenge the notion that high affinity for HSPG is essential for AAV2 entry into human hepatocytes and suggest that natural AAV capsids of human liver origin are likely to be more effective for liver-targeted gene therapy applications than culture-adapted AAV2.

Introduction

Adeno-associated viruses (AAVs) were discovered in the 1960s as contaminants of adenoviral isolates(1) and subsequently shown to be endemic in both human(2) and non-human primate populations(3). The vectorization of AAV2, a human isolate, in 1984(4) set in motion the development of an increasingly powerful and modular gene transfer and editing system that is now delivering therapeutic success in the treatment of genetic diseases such as spinal muscular atrophy(5), RPE65 deficiency(6) and hemophilia B (7). These successes have hinged on continuing development of the AAV vector system, driven primarily by advances in AAV capsid technology, the principal determinant of vector tropism. The liver, in particular, is a key target for the development of more efficient AAV vector delivery given its direct involvement in an extensive set of genetic and acquired diseases. Successes in liver-targeted gene therapy have thus far primarily been for conditions in which the secretion of functional proteins is impaired and where gene transfer to a relatively low proportion of hepatocytes confers therapeutic benefit, such as hemophilia A and B(7-9). Substantially higher targeting efficiencies will be required to bring the majority of liver disorders, such as the cell-autonomous ornithine transcarbamylase deficiency (OTCD)(10) within technological reach of AAV-mediated gene transfer. This necessitates a deeper understanding of the determinants of human liver tropism and the development of AAV vectors with increased liver transduction efficiency.

We have recently shown that the prototypical human isolate, AAV2(11), contains a liver-specific enhancer-promoter element in the 3' untranslated region (UTR) between the *cap* stop codon and the right-hand inverted terminal repeat (ITR) (12). This 124-nucleotide sequence contains binding sites for human master hepatic transcription factors including HNF1 α , HNF4 α , HNF6 and GATA6, providing direct evidence that AAV2 is evolutionarily associated

with infection of the human liver (12). Although little is known about the life-cycle of AAV2, membrane-associated heparan sulfate proteoglycan (HSPG) is widely accepted as a critical determinant of the ability of AAV2 to attach to and transduce cells (13). In support of AAV2's dependence on HSPG for cell entry, soluble heparin was shown to compete for AAV2 binding to permissive cells, leading to decreased transduction (13). The determination of the AAV2 crystal structure(14) together with extensive mutagenesis analysis(15), have localized the HSPG binding domain in AAV2 to a patch of basic amino acids residues on the capsid surface(16), with five basic residues (R484, R487, K532, R585 and R588) being essential for the interaction of AAV2 with HSPG(17). These residues facilitate high efficiency transduction of human hepatocyte-derived cell lines(18, 19).

Despite the evolutionary relationship with human liver, clinical use of the AAV2 capsid resulted in unexpectedly low efficacy in a liver-directed gene therapy trial (20). While this early study demonstrated that AAV2-based vectors could transduce human hepatocytes in vivo, peak therapeutic values of 3-11% of factor IX were observed only in subjects infused with the highest dose tested of 2×10^{12} vg/kg via the hepatic artery. These results underpinned the clinical evaluation of alternative AAV serotypes, such as the non-human primate-derived AAV8(21), in liver-targeted clinical trials. However, despite the fact that AAV8 mediated over 10-fold higher gene transfer into the liver in animal models, including non-human primates(8, 9, 22-24), in human studies the peak concentrations of vector-encoded human factor IX were similar for individuals treated with AAV2 and AAV8 (9, 20). These results are consistent with subsequent observations made by ourselves and others in a xenograft mouse model of human liver, the *Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}* (FRG)(25), where AAV2 and AAV8 each only transduced around 3% of human hepatocytes (19).

More recently, directed evolution-based selection of AAV capsid libraries on this model has yielded AAV vectors with improved human hepatotropism, such as AAV-LK03(19) and AAV-NP59 (26). By studying the correlation between capsid sequence/structure of AAV-NP59, which differs from prototypical AAV2 in only eleven amino acid positions, we have recently reported that attenuation of the heparan sulfate proteoglycan binding affinity enhances transduction performance of AAV2 in vivo in the hFRG model, most likely due to reduced vector sequestration on HSPG-rich matrixes, leading to overall improved biodistribution and human hepatocyte transduction (27).

A key question arising from these observations relates to the mechanistic understanding behind AAV2's high affinity for HSPG, given the observed detrimental effect on in vivo vector function. In this study we postulated that high affinity to HSPG could be an end result of tissue culture replicative adaptation, rather than a natural property of AAV2. We hypothesized that tissue culture replication of naturally occurring AAV variants would ultimately lead to selection of clones that acquired mutations leading to stronger HSPG binding. Finally, we hypothesized that the opposite phenomenon would occur upon in vivo replication of AAV2 in primary human hepatocytes, where the viral population would shift to HSPG de-targeted variants.

Results

The human liver is a rich source of AAV variants

In order to identify AAV capsid residues that were evolutionarily selected to support efficient targeting of primary human liver we performed an in silico analysis of capsid sequences of natural hepatotropic AAV variants. To do so, in addition to analyzing published sequences(28, 29) we undertook a screen of primary human liver samples to identify naturally hepatotropic wildtype AAV variants. A survey of 72 liver samples for the presence of proviral AAV sequences using multiplex PCR identified 30 samples (42 %) that were AAV-positive. Of those, seven (designated AAV-hu.Lvr01-07) yielded amplicons encompassing the entire *cap* open reading frame when amplified with a *rep2*-specific primer and a reverse primer placed at the AAV2 3'UTR region. From among the seven novel isolates, the *rep* coding sequence was determined for four. A phylogeny of deduced capsid amino acid sequences revealed that two of the identified sequences clustered with AAV2 (clade B), while the remaining five were intermediate between AAV2 and AAV13 (**Fig. 1A**), and likely corresponded to the previously described hybrid sequences comprising AAV clade C (2, 30). Consistent with the previously postulated origin of AAVs in clade C (2), the *rep* sequences of hu.Lvr01, hu.Lvr02, hu.Lvr05 and hu.Lvr07 clustered phylogenetically with AAV2 (**fig. S1**).

AAVs isolated from human liver transduced primary human hepatocytes in vivo with high efficiency

We next investigated the ability of the new human liver AAV isolates to transduce primary human hepatocytes in a xenograft mouse model of human liver, the humanized *Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-} (hFRG) mouse (25). To do so, six barcoded (BC) reporter constructs expressing eGFP under the control of a liver-specific promoter (LSP) (ssAAV-LSP1-eGFP-BC-WPRE-BGHpA)(31) were independently packaged into each of the AAV-hu.Lvr01-07 variants, reference serotypes AAV2 and AAV13, as well as AAV8 and AAV-LK03 as positive controls for functional transduction of murine and human hepatocytes, respectively(19). Two hFRG

mice were injected intravenously with 5.5×10^{11} vector genomes (vg) containing an equimolar mix of the individual variants (5×10^{10} vg of each capsid). Next-generation sequence (NGS) analysis of the barcoded transgene was performed to measure the ability of each variant to transduce murine liver cells and human hepatocytes. Specifically, NGS at the DNA level was used to quantify the cell entry (physical transduction), while NGS at the RNA/cDNA was used to quantify transgene expression (functional transduction). Interestingly, all the human liver-derived variants were more efficient at transduction of primary human hepatocytes than the respective closest reference serotype (AAV2 or AAV13), at both DNA and RNA levels (**Fig. 1B-C**). As expected, based on the known ability of AAV8 to functionally transduce murine hepatocytes with high efficiency(*19*), the majority of cDNA barcodes recovered from the murine liver mapped to this capsid (**fig. S2**).

Immunohistochemical analysis of all AAV-hu.Lvr variants and controls in individual hFRG mice showed that six out of the seven liver-isolated variants were more efficient at functional transduction of primary human hepatocytes than AAV-LK03, the positive control (**Fig. 1D-E**).

Natural AAV isolates were deficient in HSPG binding

Comparison of the two AAV2-like sequences, AAV-hu.Lvr02 and AAV-hu.Lvr06, revealed that they lacked two critical arginine residues associated with AAV2 HSPG binding (R585 and R588, **fig. S3A**), which is in keeping with most previously published clade B variants detected in human tissues (*2, 28-30, 32*). Similarly, all AAV13-like variants identified by us and others(*29*) lacked the structural equivalent of the key lysine 528 residue required for HSPG binding (**fig. S3B**).

Given that all isolated variants lacked key residues of the HSPG binding motif, the heparin binding capacity (a surrogate for HSPG binding(*17*)) of the isolated AAV capsids hu.Lvr01 to 07 was analyzed. In contrast to the prototypical AAV2 and AAV13, which bound to and eluted

from a HiTrap Heparin Column with an elution peak maximum at ~454 mM and ~332 mM NaCl, respectively, all seven liver-derived isolates were unable to efficiently bind to the column (**Table 1 and figs. S4-S11**). In vitro competition assays, using soluble heparin as an analogue of HSPG, provided further evidence that the human liver isolates did not interact with heparin at the tested concentration (**Fig. 1F**). Notably, prototypical AAV2 transduced HuH-7, a human hepatoma-derived cell line, at a considerably higher efficiency than the primary human liver variants, and the transduction was significantly ($p = 0.0079$) inhibited by heparin.

AAVs rapidly acquired mutations leading to amino acid changes, enhancing HSPG binding during in vitro replication

The substantial difference in the in vivo and in vitro performance of the human liver isolates and prototypical AAV2 (**Fig. 1 B-C, Fig. 1F**), combined with the differential interactions with heparin, led us to hypothesize that strong HSPG binding might not be a common property among natural AAVs, but rather a characteristic acquired during in vitro propagation as a result of viral adaptation to a new environment.

Given the similarly poor in vitro transduction performance of AAV-hu.Lvr03, hu.Lvr04 and hu.Lvr07 on HuH-7 cells (**Fig. 1F**), AAV-hu.Lvr07 was selected to test the hypothesis of tissue culture adaptation. To do so, a replication-competent (RC) version (ITR2-rep2-cap.hu.Lvr07-ITR2) of AAV-hu.Lvr07, referred to as RC-AAV-hu.Lvr07, was subjected to an in vitro adaptation protocol on HuH-7 cells in the presence of human adenovirus 5 (hAd5) (**Fig. 2A**). No AAV was detected in HuH-7 cells infected only with hAd5 (**fig. S12**). After four rounds of tissue culture replication, 98.57 % of the isolated capsids harbored a glutamate to lysine change at position 530 (E530K) and 1.14 % of clones had acquired an arginine at position 593 (G593R). Both these amino acids have positively charged side-chains and have been shown to facilitate heparin binding at corresponding structural regions in AAV13 and AAV3B,

respectively (**Table 2**)(16, 33). In-depth analysis of the capsid gene region containing both modified residues using high-throughput NGS demonstrated that the original AAV-hu.Lvr07 was displaced rapidly after the initial appearance of E530K and G593R in round 1 and 2, respectively (**Fig. 2B-C**). The same phenomenon was observed upon hAd5-supported iterative replication of a RC version of AAV-hu.Lvr02 (ITR2-rep2-cap.hu.Lvr02-ITR2) in HuH-7 cells. The initial single-variant population was rapidly dominated by a variant that acquired two point mutations that resulted in substitution of acidic amino acids at positions 469 (D469N) and 555 (E555K) (**fig. S13**).

In vitro adaptation improved cell culture vector function and attenuated in vivo performance

To test whether the observed changes in the AAV-hu.Lvr07 capsid sequence affected transduction characteristics, the two new AAV-hu.Lvr07 variants, harboring solely the individual amino acid substitutions (E530K or G593R), were used to package the AAV-LSP1-eGFP-WPRE-BGHpA vector and were evaluated in vitro on HuH-7 cells. FACS analysis revealed that culture-adapted (ca)AAV-hu.Lvr07^{E530K} and caAAV-hu.Lvr07^{G593R} functionally transduced HuH-7 cells more efficiently than AAV-hu.Lvr07 (**Fig. 2D**). Furthermore, co-incubation with soluble heparin reduced the observed improvement in transduction to the extent observed for natural AAV-hu.Lvr07 (**Fig. 2D**). In contrast to the natural AAV-hu.Lvr07, the tissue culture-adapted variants (E530K and G593R) were shown to efficiently bind to the HiTrap Heparin Column (**Table 1, figs. S14-S15**). This was anticipated, as structural analysis revealed that the E530K change was adjacent to residues R487, K527, and K532, which are part of the AAV13 HSPG binding region(16), and created a basic patch on the capsid surface in close proximity to R484 at base of the 3-fold protrusions (**Fig. 2E-F, Table 2**). An identical phenotype was observed for caAAV-hu.Lvr02^{D469N+E555K}, which in contrast to the natural

serotype was found to efficiently bind to the HiTrap Heparin column (**fig. S16**) and to transduce HuH-7 with significantly ($p = 0.0286$) higher efficiency (**fig. S17**).

We next wanted to investigate whether change in HSPG-binding properties would have a detrimental effect in vivo. For that, six barcoded reporter constructs were packaged into reference serotypes AAV2, AAV8 and AAV13, as well as AAV-hu.Lvr07 and its tissue culture adapted version (caAAV-hu.Lvr07^{E530K}). Two hFRG mice were injected intravenously with a total of 2.5×10^{11} vg containing an equimolar mix of the individual variants (5×10^{10} vg of each capsid). Interestingly, the single amino acid substitution at the corresponding structural region in AAV13 (E530K) sufficed to significantly ($p = 0.0022$) attenuate the in vivo performance of the otherwise highly human hepatotropic AAV-hu.Lvr07 to an AAV13-like phenotype (**Fig. 2G-H**).

In vivo propagation of the prototypical AAV2 led to rapid acquisition of HSPG de-targeting substitutions

In view of the rapid kinetics of mutagenesis observed in vitro and the resultant change in vector performance following tissue culture adaptation, we investigated whether it was possible to “re-adapt” the prototypical AAV2 on primary human hepatocytes in order to improve in vivo function. To this end, a replication-competent version of AAV2 (RC-AAV2, ITR2-*rep2-cap2*-ITR2) was used to perform an iterative in vivo adaptation experiment on primary human hepatocytes in hFRG mice in the presence of hAd5 (**Fig. 3A**). Naïve FRG mice and an hFRG mouse injected with hAd5 only were used as negative controls (**Methods, fig. S26**). Sequence analysis of random full-length *cap* genes ($n = 24$) recovered after the first round of the in vivo adaptation experiment revealed substantial enrichment (16.6 % of the population) of an AAV2 clone harboring a point mutation leading to a R588I substitution, affecting one of the key arginine residues involved in HSPG binding. Interestingly, 12.0 % of clones analyzed after

round 2 harbored an R487Q amino acid substitution. While this clone was selected against, or outcompeted by R588I, in the subsequent rounds of selection, R487Q affected another key arginine residue involved in heparin binding and was therefore included as a variant in subsequent analysis (**Table 1**). Importantly, clones harboring other random DNA point-mutations, including silent ones, were observed in the three rounds of selection, as detailed in **tables S7-S9**.

To gain a better understanding of the kinetics of the population genetic shift, Illumina high-throughput NGS of the 150-bp region surrounding the affected positions G1460A (amino acid 487) (**Fig. 3B**) and G1763T (amino acid 588) (**Fig. 3C**) was performed in the initial virus stock and after each round of in vivo selection. As shown in **Fig. 3C**, the R588 present in AAV2 was rapidly replaced by R588I within two rounds of selection. NGS analysis of the pre-adapted RC-AAV2 preparation suggested that the identified variants resulted from random mutagenesis events that gave rise to AAV2 variants with improved biodistribution and overall in vivo tropism for primary human hepatocytes. Supporting this hypothesis, a third variant, R588T, was detected after the second round of selection and accounted for 1.37 % of the total reads. However, AAV2-R588T was no longer detectable after the subsequent round of selection.

To further validate the hypothesis that the observed variants resulted from random mutagenesis events occurring during viral replication, we repeated the in vivo adaptation study using a new preparation of viral RC-AAV2. During the second independent in vivo adaptation experiment, AAV2-R588T rapidly expanded over other capsid variants and accounted for 88 % of the population following the second round of selection (**Fig. 3D**). As mentioned earlier, the interaction between the 3-fold protrusions of AAV2 and HSPG has been reported to be mediated mainly by interactions of the sugar sulfates with positively charged basic residues located on the face of the 3-fold protrusions(34). The three amino acid substitutions, R487Q and R588I/T, disrupt and reduce the positive charge of AAV2 (**Fig. 3E-F**), and would therefore

reduce the strength of the AAV2-HSPG interaction, consistent with our observations using the HiTrap column (**Table 1, figs. S20-S22**).

Single amino acid changes acquired during in vivo readaptation restored the intrinsic human hepatotropism of AAV2

To evaluate the functionality of the new AAV variants, the three clones AAV2-R588I, AAV2-R588T, and AAV2-R487Q were vectorized and used to package the AAV-LSP1-eGFP-BC-WPRE-BGHpA cassette. To more comprehensively understand the effect of the amino acid substitutions on vector function, the three variants were tested in vitro on HuH-7 cells (**Fig. 3G**) and in vivo on primary human hepatocytes in hFRG mice (**Fig. 4A-B**). As expected, all variants showed reduced transduction of HuH-7 at the MOIs tested when compared to the prototypical AAV2 (**Fig. 3G**) and consistent with natural AAV liver isolates (**Fig. 1F**), performance was not affected by soluble heparin at the concentration tested (**Fig. 3G**). In striking contrast to the in vitro results, all three variants were found to outperform AAV2 and the human hepatotropic bioengineered capsid AAV-LK03 in both entry (DNA) and expression (cDNA) analyses in vivo using primary human hepatocytes (**Fig. 4A-B**). As observed previously (**Supplementary Figure S2**), AAV8-derived barcodes accounted for the majority of signal from murine liver cDNA (**fig. S23**). NGS results were confirmed by immunohistochemical analysis of the three AAV2 variants in individual hFRG mice (**Fig. 4C-D**).

Discussion

During the sixteen years between isolation of prototypical AAV2 and its sequencing and genome annotation, the replicating virus was propagated in vitro on KB and HeLa cells in the presence of Ad12 and Ad2, respectively, for an unknown period of time (11, 35). Here we showed that during replication, AAVs that acquire mutations leading to capsid amino acid changes that markedly enhance interaction with HSPG, can rapidly achieve population dominance in vitro. Importantly, while advantageous in tissue culture, clinical(20) and preclinical data in chimeric mouse-human livers(19) indicate that this property is detrimental to human liver transduction.

Our observation that human liver AAV isolates demonstrate strikingly opposite performance to prototypical AAV2 in vitro and in vivo supports the previously proposed hypothesis(36) that the ability to bind HSPG with high affinity might not be a requirement for natural infection in humans, as recently reviewed in the context of AAV and other viruses (37). Importantly, while deficient HSPG binding has been shown to markedly reduce AAV2 transduction of the murine liver(15, 38), we showed that reduction of heparin binding strength improves functional transduction of human hepatocytes in vivo in a xenograft model.

There are some caveats in the interpretation of these findings. The first is dictated by the difficulty in isolating heparan sulfate (HS) from natural systems and thus the use of structurally similar heparin as a surrogate for HSPG in the binding and competition assays (39). Heparin might not perfectly recapitulate biological interactions between HS and AAVs, and it is reasonable to speculate that a transient interaction would be preferential for viral attachment, which is not easily replicated with HiTrap Heparin chromatography. This could explain why a small proportion of the AAV-hu.Lvr variants was detected in the elution fraction. Further

studies will be required to understand if this residual binding could be indicative of a low affinity interaction of AAVs with HS.

Another caveat is extrapolating AAV biology and AAV-liver interactions from studies in a xenograft model. Although the hFRG model allows study of the interactions of AAV with human hepatocytes in vivo, these grow in clusters on the murine scaffold, where the nature of the extracellular environment remains of murine origin. Other limitations associated with this model include the potential effect of cluster size and levels of engraftment on AAV-transduction. In the present study we attempted to minimize these effects by using similarly engrafted hFRGs for vector comparison and analyzing multiple barcoded variants in the same animal. However, barcoded NGS analysis of vector function provides information on the average net transduction in the tested sample and not the vector biodistribution within a hepatic lobule.

We hypothesize that the observed in vivo attenuation of AAVs could be caused by impaired hepatic biodistribution. Under our current proposed model, the culture-adapted variants retain the ability to transduce human cells, as evident from in vitro studies, but excessive HS attachment prevents the vectors from reaching the human clusters. In the context of non-human primates or humans, we hypothesize that acquisition of strong HS binding would direct vector transduction to periportal hepatocytes. In fact, we have recently observed this phenomenon for AAV8 using murine liver (27).

The fast kinetics for both the in vitro and in vivo AAV adaptations resembles the mutation rates of the canine parvovirus(40) and the human B19 erythrovirus (41). This could relate to the fact that AAV replication depends on, and induces, the DNA damage response signaling pathway(42), which comprises error-prone DNA polymerases (43). It is reasonable to assume that during the replicative cycle of infection numerous viral distributions or clouds, rather than

a uniform population, coexist in infected cells and organisms, a phenomena known as viral quasispecies (3, 44). Thus, any AAV variant isolated from an infected host does not necessarily represent a wild-type AAV, but rather a single random individual that coexisted within a pool of other genetically related variants. Importantly, this finding presents novel opportunities for AAV engineering, a method we propose to designate as Quasispecies-Enabled (QE) AAV engineering.

In the absence of sequence data for the original 1966 AAV2 isolate(11) we may never know whether the prototypical AAV2 adapted to the tissue culture environment via *de novo* mutations or expanded from a naturally occurring rare variant harboring the HSPG binding motif. Interestingly, HSPG de-targeted variants resulted from single nucleotide mutations. This could represent an intrinsic mechanism to allow rapid adaptation to changing conditions with minimal corresponding changes to the genome. It can be further postulated that during an AAV infection, individual clones arising from random mutagenesis may acquire properties allowing infection of other tissues or surrounding cells. This could explain why not all natural AAV2-like variants published to date lack the HSPG binding motif (29).

The paradigm of AAV culture adaptation appears to extend to at least some other prototypical variants. The five liver-derived AAV clade C capsid sequences presented herein resembled AAV13 in terms of heparin-binding residues (16), with the key change of lysine 528 to a negatively charged glutamate (2, 30). We postulate that all the prototypical variants reported to bind to HSPG, such as AAV3B(45), AAV6(45) and AAV13(16), which like AAV2 were passaged in vitro in the presence of adenovirus before their initial sequencing, may represent culture-adapted variants. Importantly, the principle of preferential periportal zonation would also apply to engineered AAVs harboring HSPG binding regions from these variants, such as AAV-DJ (18), AAV-LK03 (19) and AAV-KP1 (46).

In summary, the studies reported here support the hypothesis that prototypical AAV2 is unlikely to be a primary human isolate, but rather an HSPG-binding variant that achieved population dominance in in vitro culture. The results provide a direct link between increased HSPG binding and resultant attenuation of tropism for primary human hepatocytes in vivo and suggest a mechanism explaining the low efficacy of the AAV2 capsid in the human liver in a clinical trial targeting hemophilia B (20). Our study shows that AAV vectors derived directly from human liver transduce primary human hepatocytes in vivo in a xenograft model of human liver with high efficiency and have therapeutic potential. Importantly, the findings presented herein impact the future of AAV-based liver-targeted gene therapy and warrant the re-evaluation of natural AAV human isolate-derived vectors in clinical applications.

Materials and Methods

Study design

The study was designed to investigate the likelihood of replicative adaptation for Adeno-Associated Viruses (AAV). The studies were performed using natural AAV variants isolated directly from human liver samples as well as previously published natural AAVs. For in vitro characterization assays, four replicates for each AAV variant were used to allow defining of statistical significance. Replicative adaptation of AAV in vitro was performed for two independent natural AAV variants, AAV-hu.lvr02 and AAV-hu.lvr07, through four and five passages in HuH-7 cells, respectively. Replicative adaptation of AAV2 in vivo was performed in biological duplicate using three and two humanized FRG mice per study, respectively, each starting from an independent viral preparation. Acquired mutations were first identified by Sanger Sequencing of no less than $n=20$ random clones, and population shift was then analyzed with Next Generation Sequencing by designing custom primers flanking the regions of interest. Evaluation of heparin affinity of all reported AAV variants was determined using the same 1-mL Heparin HP column, which was cleaned and reconstituted for binding after each sample.

The in vivo studies in humanized FRG mice were performed in 19- to 31-week-old FRG female mice engrafted with human hepatocytes. Animals with similar levels of engraftment were randomly assigned to experimental groups involving NGS and immunohistochemistry-based comparisons. Investigators were not blinded during monitoring and transduction evaluation of AAVs. For NGS-based studies, six random 6-mer barcoded transgenes were packaged per AAV variant, allowing us to statistically distinguish the performance of variants at both DNA and cDNA levels. For studies that employed immunochemical analysis, AAV transduction efficiency was evaluated in one to two mice per AAV variant, and transduction efficiency was calculated by counting GFP positive cells in ten to twenty human clusters.

Human Ethics. Human liver samples were obtained under Royal Prince Alfred approval ethics proposals X16-0490 & HREC/16/RPAH/701 (“Pathogenesis of Human Liver Disease and Liver Cancer”) and X13-0436 & HREC/10/RPAH/130 (“Pathogenesis of human liver disease”), and Sydney Children’s Hospitals Network (SCHN) Human Research Ethics Committee proposal 18/SCHN/236 (“Establishment of a repository of liver cells to be used in researching gene-based approaches to the treatment of liver disorders”). The samples consisted of human liver donor tissues obtained at the time of liver transplantation.

AAV DNA isolation from primary tissues

DNA was extracted from human liver tissue, using a QIAamp Fast DNA Tissue Kit (Qiagen) according to the manufacturer’s instructions, with the following modifications: (i) Frozen liver chunks (~15 mg each) were homogenized in the digestion buffer using a plastic pestle and manual homogenization to minimize DNA shearing. (ii) Digestion at 56°C was carried out with intermittent agitation (5 sec/min) at 1,000 rpm for 30 minutes. (iii) Samples were triturated through a 22G needle to complete homogenization. (iv) Samples were incubated at 56°C for a further 90 minutes with intermittent agitation. PCR primers were designed to amplify full AAV2 capsid and 3’UTR sequences, by annealing a forward primer (Rep_mpx1_F) in the *rep* gene and a reverse primer (3UTR_mpx2_R) in the 3’ITR, spanning the A/D region junction (**table S1**). All amplification reactions were performed using Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs) as per the manufacturer’s instructions using 300 ng template DNA and the following thermal cycling conditions: 98°C for 30 sec; 40 cycles

of 98°C for 10 sec, 67°C for 30 sec, 72°C for 2 min 30 sec; 72°C for 10 min. Capsid amplicons were cloned using the StrataClone Blunt PCR Cloning Kit (Agilent) according to the manufacturer's instructions, and Sanger-sequenced at the Australian Genome Research Facility (Westmead, Australia) using standard M13 (-20) and M13 Reverse sequencing primers, as well as amplicon-specific internal sequencing primers. Samples were named similarly to the nomenclature used by Chen *et al.* (30), denoting the species and organ of origin, suffixed by ascending numbers in temporal order of their amplification from DNA and thus not linked to patient or sample numbers (AAV-hu.Lvr01).

Mouse studies

All animal care and experimental procedures were approved by the joint Children's Medical Research Institute and The Children's Hospital at Westmead Animal Care and Ethics Committee. CMRI's established *Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}* (FRG) mouse(25) colony was used to breed recipient animals. FRG mice were housed in individually ventilated cages with 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC)-supplemented in drinking water (8 mg / mL). FRG mice, 6 to 8 weeks old, were engrafted with human hepatocytes (Lonza Group Ltd., Basel, Switzerland) as described previously (25). Humanized FRG (hFRG) mice were placed on 10 % NTBC prior to transduction with vectors and were maintained on 10 % NTBC until harvest. Detailed information on all mice used in the study, including individual estimated repopulation, can be found in **table S2**.

The vector for injection was made up to a final volume of 150 µL using saline. Mice were randomly selected and transduced by intravenous injection (lateral tail vein) with the indicated vectors at a dose of 5×10^{10} vg/vector for NGS comparison, and at a dose of 2×10^{11} vg/vector for immunohistochemistry (with the exception of the *in vivo* adaptation, see "*in vivo* adaptation" subsection). Mice were euthanized by CO₂ inhalation 2 weeks after transduction for immunohistochemistry and 1 week after transduction for barcoded Next-Generation Sequencing analysis. Hepatocytes for flow cytometry analysis were obtained by collagenase perfusion of the liver (see below).

Isolation of human hepatocytes by collagenase perfusion

To perfuse mouse liver and obtain single-cell suspension, the inferior vena cava (IVC) was cannulated, and the solutions were pumped with an osmotic minipump (Gilson Minipuls 3) in the following order: 25 mL of Hank's balanced salt solution (HBSS) (-/-) (cat # H9394; Sigma), 25 mL of HBSS (-/-) supplemented with 0.5 mM EDTA, 25 mL HBSS (-/-), and 25 mL of HBSS (-/-) supplemented with 5 mM CaCl₂, 0.05 % wt/vol collagenase IV (Sigma) and 0.01 % wt/vol DNase I (Sigma).

Following perfusion, the liver was carefully removed and placed in a Petri dish containing 25 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). The blunt end of a scalpel blade was used to break the liver capsule to release the cells into the medium. After collection, the cells were spun down at $50 \times g$ for 3 min at 4 °C. The pellet was resuspended in 21 mL of DMEM and passed through a 100-µm nylon cell strainer. Isotonic Percoll (9 mL) (1 part of $10 \times$ PBS (-/-) with 9 parts of Percoll; GE Healthcare) was added to the cell suspension to separate live and dead cells. Live cells were pelleted at $650 \times g$ for 10 min at 4 °C and the pellet was resuspended in FACS buffer (PBS (-/-) with 5 % FBS and 5 mM EDTA). To delineate between mouse liver cells and human hepatocytes, cells were labelled with phycoerythrin (PE)-conjugated anti-human-

HLA-ABC (clone W6/32; Invitrogen 12-9983-42; 1:20), biotin-conjugated anti-mouse-H2Kb (clone AF6-88.5, BD Pharmingen 553568; 1:100) and allophycocyanin (APC)-conjugated streptavidin (eBioscience 17-4317-82; 1:500). GFP-positive labelled samples were sorted to a minimal 95 % purity using a BD Influx cell sorter. Flow cytometry was performed in the Flow Cytometry Facility, Westmead Institute for Medical Research, Westmead, NSW, Australia. The data were analyzed using FlowJo 7.6.1 (FlowJo, LLC).

Adeno-associated virus transgene constructs

AAV transgene constructs were cloned using standard molecular biological techniques. All of the vectors used in the study contain AAV2 ITR sequences. The AAV construct pLSP1-eGFP-WPRE-BGHpA, which encodes eGFP under the transcriptional control of a heterologous promoter containing one copy of the *SERPINA1* (hAAT) promoter and two copies of the *APOE* enhancer element, has been previously reported (47). Eighty four (n=84) versions of the pLSP1-eGFP-BC-WPRE-BGHpA construct were produced by cloning n=84 unique 6-mer barcodes downstream of eGFP.

AAV vector packaging and viral production

AAV constructs were packaged into AAV capsids using HEK-293 cells and a helper-virus-free system as previously described (48). Genomes were packaged in capsid serotypes AAV2, AAV8, LK03 and AAV13 using packaging plasmid constructs pAAV2, pAAV8, pAAV-LK03 and pAAV13, respectively. Replication-competent (RC) AAV variants were packaged by co-transfection of a corresponding p-AAV plasmid containing the full-length AAV genome (ITR2-*rep2-cap*-ITR2) and pAd5 into HEK-293T cells.

All vector/virus were purified using iodixanol gradient ultracentrifugation as previously described (49). AAV preparations were titred using real-time quantitative PCR (qPCR) using *eGFP*-specific qPCR primers GFP-qPCR-For/Rev or AAV2-*rep*-specific qPCR primers Rep-qPCR-For/Rev (**table S1**). For *in vivo* testing of hu.Lvr capsids (study reported in **Fig. 1B-C**), culture adapted hu.Lvr07 (study reported in **Fig. 2G-H**) and AAV2 variants (study reported in **Fig4. A-B**) n=6 independent barcoded transgenes were packaged per capsid using two different concentrations (n=3 barcoded transgenes at high dose: 13 µg / transgene per preparation, and n=3 barcoded transgenes at low dose: 1.3 µg / transgene per preparation). The presence of the two distinct populations was confirmed by next-generation sequencing of the pre-injection mix. This approach effectively allows the simultaneous test of the same capsid at two doses in a single hFRG mouse. Following normalization to the pre-injection mix, no difference was found between results obtained with transgenes at high and low doses. Thus, data are presented normalized.

In vitro adaptation

The isolated AAV-hu.lvr07 was produced to package an ITR2-*rep2-cap*hu.lvr07-ITR2 replication-competent AAV2-based genome using the crude lysate production method previously described (50). The adaptation was performed following a previously published protocol for selection of a replication-competent library in the presence of wtAd5 (ATCC, Lot# 70010153) (50). Briefly, HuH-7 cells were infected in duplicates at five 10-fold serial dilutions. Cells were washed with 1× PBS 6 hours after infection and one of the duplicate series was co-infected with wtAd5, while the other dilution series served as a non-Ad5 replication control. No AAV genomes were detected in hAd5 only control (**fig. S12**).

Replication was measured 72 hours after wtAd5 co-infection by SYBR green qPCR quantification of AAV genomes in wtAd5 co-infected vs control wells, using *rep2* specific oligos (Rep-F1/Rep-F2). The sample with the highest AAV dilution that led to a 100-fold Cq increase over the respective non-Ad5 control was chosen for the subsequent round of selection. To monitor the progress of natural mutagenesis/culture adaptation, after each round of selection, n=24 random AAV genomes were recovered and sequenced with External_Seq F/R primers (table S1).

In vivo adaptation of RC-AAV2

Humanized FRG mice were injected with 1.2×10^{12} vg of replication-competent RC-AAV2 by i.v tail vein administration. 5×10^9 PFUs of wild-type human adenovirus-5 (ATCC, VR-5, Lot# 70010153) was administered intraperitoneally (i.p.) 24 h later. Xenograft livers were harvested 72 hrs after hAd5 administration, homogenized and snap frozen in liquid nitrogen. To extract AAV particles, approximately 0.3 g fragment of liver was subjected to three freeze-thaw cycles and mechanical homogenization in the presence of 2× w/v of PBS. Sample was subsequently centrifuged for 30 min at 4°C at top speed in a table-top centrifuge to separate the virus-containing supernatant from cellular debris. To inactivate wtAd5, the virus-containing supernatant was incubated at 65°C for 30 min. Following titration by qPCR, 200 µL of the virus-containing supernatant was administrated i.p. into hFRG mouse for subsequent round of selection. Non-engrafted naïve FRG mice were used as control. Consistent with previous findings(19), hAd5 did not support effective AAV replication in murine hepatocytes. A similarly engrafted hFRG (#445) was injected with hAd5 only and processed as described above. No detectable AAV genomes were found on hAd5 only control (fig. S26)

Barcode amplification, next-generation sequencing and distribution analysis

The 150 base pair region surrounding the 6-mer barcode was amplified with Q5 High-Fidelity DNA Polymerase (NEB, Cat# M0491L) using BC_F and BC_R primers (table S1). Next-generation sequencing library preparations and sequencing using a 2×150 paired-end configuration were performed by Genewiz (Suzhou, China) using an Illumina MiSeq instrument. A workflow was written in Snakemake (5.6)(51) to process reads and count barcodes. Paired reads were merged using BBMerge and then filtered for reads of the expected length in a second pass through BBDuk, both from BBTools 38.68 (<https://sourceforge.net/projects/bbmap/>). The merged, filtered fastq files were passed to a Perl (5.26)(52) script that identified barcodes corresponding to AAV variants. NGS reads from the DNA and cDNA populations were normalized to the reads from the pre-injection mix. The analyzed NGS data per sample can be found in tables S3-S5 and is detailed in Data File S1.

Heparin binding assay

The heparin affinity of various AAV vector variants was determined using a 1-mL HiTrap Heparin HP column (GE Healthcare, Cat #1704601, Lot #10276193) on an ÄKTA pure 25 M2 (GE Healthcare) fast protein liquid chromatography system. A detailed protocol can be found at the Supplementary Materials and Methods Section.

AAV structural visualizations

To visualize the location of the residues of interest on the AAV-hu.Lvr07 capsid, a 3D homology model of a VP3 monomer was created by supplying the isolated sequence to the online the SWISS-MODEL server (53). A 60mer of the VP3 was then generated using the oligomer generator subroutine of the online Viperdb server(54), and visualized with the COOT application (55). The surface maps were generated in Chimera(56) and the stereographic roadmap was created in the program RIVEM (57). The positions of the three AAV2 amino acid changes of interest were visualized on the AAV2 capsid surface using VP3 crystal structure coordinates, RCSB PDB # 1LP3, following icosahedral matrix multiplication as described for AAV-hu.Lvr07. The viral asymmetric unit (**Fig. 3E-F**) is shown as a black triangle bounded by 2-, 3-, and 5-fold axes, depicted by an oval, triangle, and pentagon respectively, in both the surface map and the stereographic roadmap.

Statistical analysis

Nonparametric statistical analyses were performed using the two-tailed Mann–Whitney test with the specified biological and technical replicates in each experimental group. (* P value $\leq$ 0.05; ** P value $\leq$ 0.01; **** P $\leq$ 0.0001, n.s. P value $>$ 0.05).

List of Supplemental Materials

Supplementary Materials and Methods

Fig. S1. Phylogenetic analysis of AAV Rep proteins isolated from human liver (AAV-hu.Lvr01, 02, 05 and 07).

Fig. S2. Murine transduction comparison of AAV-hu.Lvr capsids in the xenograft liver model.

Fig. S3. Multiple sequence alignment of prototypical AAV2 and AAV13 with related hu.Lvr capsids.

Fig. S4. HiTrap Heparin Column Binding assay of prototypical AAV2.

Fig. S5. HiTrap Heparin Column Binding assay of AAV-hu.Lvr01.

Fig. S6. HiTrap Heparin Column Binding assay of AAV-hu.Lvr02.

Fig. S7. HiTrap Heparin Column Binding assay of AAV-hu.Lvr03.

Fig. S8. HiTrap Heparin Column Binding assay of AAV-hu.Lvr04.

Fig. S9. HiTrap Heparin Column Binding assay of AAV-hu.Lvr05.

Fig. S10. HiTrap Heparin Column Binding assay of AAV-hu.Lvr06.

Fig. S11. HiTrap Heparin Column Binding assay of AAV-hu.Lvr07.

Fig. S12. Representative controls for the in vitro adaptation.

Fig. S13. Population shift of hu.Lvr02 N469 and E555 during in vitro adaptation.

Fig. S14. HiTrap Heparin Column Binding assay of caAAV-hu.Lvr07^{E530K}.

Fig. S15. HiTrap Heparin Column Binding assay of AAV-hu.Lvr07^{G593R}.

Fig. S16. HiTrap Heparin Column Binding assay of AAV-hu.Lvr02^{D469N+E555K}.

Fig. S17. In vitro heparin competition assay of hu.Lvr02 and culture adapted AAV-hu.Lvr02^{D469N+E555K}.

Fig. S18. HiTrap Heparin Column Binding assay of AAV13.

Fig. S19. HiTrap Heparin Column Binding assay of AAV3B.

Fig. S20. HiTrap Heparin Column Binding assay of AAV2-R588I.

Fig. S21. HiTrap Heparin Column Binding assay of AAV2-R588T.

Fig. S22. HiTrap Heparin Column Binding assay of AAV2-R487Q.

Fig. S23. Murine transduction comparison of AAV2 capsid variants in the xenograft liver model.

Fig. S24. AAV yield comparison of RC-AAV2 and RC-AAV2-R588I.

Fig. S25. Immunohistochemical staining of engrafted human hepatocytes with human GAPDH and human fumarlyacetoacetase hydrolase (FAH).

Fig. S26. ddPCR analysis of chimeric liver lysate of hFRG #445.

Fig. S27. Murine transduction comparison of AAV-hu.Lvr07 and its adapted (E530K) variants in the xenograft liver model (FRG #164).

Table S1. Primer sequences

Table S2. hFRG individual mouse data

Table S3. In vivo comparison of hu.Lvr variants – NGS Read Summary

Table S4. In vivo comparison of culture adapted hu.Lvr07 variant – NGS Read Summary

Table S5. In vivo comparison of AAV2 variants – NGS Read Summary

Table S6. Summary of HiTrap Heparin Column Binding Assay

Table S7. Clonal Analysis of AAV2 in vivo adapted variants Round 1.

Table S8. Clonal Analysis of in vivo adapted variants Round 2.

Table S9. Clonal Analysis of *in vivo* adapted variants Round 3.

Data File S1 – Statistical analyses

Data File S2 – NGS data in tabular format for Figures 1B-C, 2G-H, 4A-B.

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Author contributions:

M.C.-C., C.V.H., A.W., I.E.A. and L.L. designed the experiments. M.C.-C., S.H.Y.L, E.Z. and R.G.N. performed the in vivo and in vitro vector comparisons and the in vivo replicative adaptations of RC-AAV2. C.V.H. isolated the AAV-hu.Lvr variants from human liver tissue. A.W. performed the tissue culture adaptation of AAV-hu.Lvr07 and M.C.-C and G.B. performed the tissue culture adaptation of AAV-hu.Lvr02. B.H.N., G.B. and M.C.-C generated the chromatography data. A.B. and M.A.M generated the structural visualizations. S.S. generated the script to identify barcodes corresponding to AAV variants. M.C.-C., C.V.H., A.W., B.H.N, S.H.Y.L, E.Z., R.G.N., G.B., S.S., A.B., generated reagents, protocols, performed and analyzed data. M.C.-C., C.V.H., M.D., I.E.A. and L.L. wrote the article and generated the figures. All authors reviewed, edited, and commented on the article.

Competing interests:

M.C.-C., C.V.H., S.L.G., I.E.A. and L.L. are inventors on patent applications filed by Children’s Medical Research Institute related to AAV capsid sequences and in vivo function of novel AAV variants (PCT/AU2020/050703, Title: Methods and AAV vectors for in vivo transduction). L.L. is a founder and scientific advisor of LogicBio Therapeutics and is the scientific founder of Reformer Therapeutics. L.L. and I.A.E. are co-founders of Exigen Biotherapeutics. A.J.T. is a co-founder and scientific consultant for Orchard Therapeutics, consultant for Rocket Pharmaceuticals, Generation bio, bluebird bio, 4Bio Capital Partners, and Sana Biotechnology. G.J.L. consults for Gyroscope Therapeutics. L.L. and I.A.E. have consulted on technologies addressed in this paper. L.L. has stocks and/or equity in LogicBio Therapeutics, Reformer Therapeutics, Orphic Scientific and Exigen Biotherapeutics. I.A.E.

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Data and materials availability:

All data associated with this study are in the paper or supplementary materials. Computer code has been archived at Code Ocean [DOI: 10.24433/CO.7176285.v1](58) DNA sequence data for AAV variants AAV-hu.Lvr01 to AAV-hu.Lvr07) has been deposited in GenBank (Accession numbers MT709008-MT709014).

Figure Legends

Figure 1. AAV capsids isolated from human liver transduced human hepatocytes in vivo with high efficiency (A) Phylogram depicting the deduced evolutionary relationships among human liver-derived (AAV-hu.Lvr01 to 07) and related AAV capsid sequences (AAV1, 2, 6, 3B, 8 and 13). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. (B-C) In vivo comparison of physical (B) and functional (C) transduction of AAV-hu.Lvr capsids in the xenograft liver of the hFRG mouse model. Each AAV variant was used to package six unique barcoded ssAAV-LSP1-GFP-BC⁽¹⁻⁶⁾-WPRE-BGHpA cassettes and an equimolar mix of all eleven variants was used. Percentage of NGS reads mapped to each capsid in human hepatocytes at the DNA (cell entry, physical transduction, average of 877 vg / diploid human genome) (B) and cDNA (expression, functional transduction) level (C), normalized to the pre-injection mix, is shown. Statistical significance was calculated by comparing the performance of each AAV-hu.Lvr variant with its closest parental variant (i.e. hu.Lvr02 with AAV2). (D) Representative immunohistochemical analysis of hFRG mouse liver transduced with indicated variants. Purple: human GAPDH; green: vector-expressed GFP; blue: DAPI (nuclei). Scale=100 μ m. (E) Quantification of the percentage of transduced human hepatocytes per human cluster, n=10 human clusters / mouse, N=2 mice / vector for AAV2, hu.Lvr01-02, hu.Lvr06-7 and N=1 mouse / vector for the rest. (F) Results of in vitro heparin competition assay, multiplicity of infection (MOI) = 100,000 vector genomes / cell.

AAV2 served as a control. Data are mean $\pm$ SD. Statistical significance was calculated using the two-tailed Mann-Whitney test (* P value $\leq$ 0.05; ** P value $\leq$ 0.01; **** P $\leq$ 0.0001, n.s. P value $>$ 0.05).

Figure 2. In vitro adaptation of AAV-hu.Lvr07 attenuated in vivo transduction of human hepatocytes (A) Graphic representation of the in vitro evolution approach. (B-C) Population shift of hu.Lvr07 E530 and G593 during in vitro adaptation. Next-generation sequencing was used to determine the proportion of variants harboring mutations at hu.Lvr07 *cap* nucleotide position 1663 (leading to an E530K substitution) (B) and at nucleotide 1777 (leading to a G593R substitution) (C). PL, plasmid; RC, replication-competent; R1-R4, rounds 1 to 4. (D) In vitro heparin competition assay using HuH-7 cells transduced with culture-adapted variants of AAV-hu.Lvr07 expressing eGFP, with and without soluble heparin in the media. MOI = 40,000 vector genomes / cell. AAV2, AAV3B and AAV13 served as controls. Data are mean $\pm$ SD. Statistical significance was calculated using the two-tailed Mann-Whitney test, comparing the in vitro performance of AAV-hu.Lvr07 and the culture-isolated variants as indicated (* P value $\leq$ 0.05). (E) Surface map and (F) stereographic roadmap projection of a 3D model of AAV-hu.Lvr07 viewed down the icosahedral 2-fold axis. The viral asymmetric unit is shown as a black triangle and bounded by 2-, 3-, and 5-fold axes, depicted by an oval, triangle, and pentagon respectively, in both the surface map and the stereographic roadmap. The HSPG binding residues K532, R484 and R487 are colored blue, E530 is colored cyan and G593 is colored marine blue. (G-H) In vivo comparison of physical (DNA) and functional (RNA/cDNA) transduction of AAV-hu.Lvr07 and its culture adapted version (ca-hu.Lvr07-E530K) capsids in the xenograft liver model. AAV2, AAV8 and AAV13 were used as controls. Each AAV variant was used to package six unique barcoded cassettes as described before. Percentage of NGS reads mapped to each capsid in human hepatocytes at the DNA (cell

entry, physical transduction, average-of 611.97 vgs / diploid human genome) (**G**) and cDNA (expression, functional transduction) level (**H**), normalized to the pre-injection mix, is shown. Data are mean $\pm$ SD. Statistical significance was calculated using the two-tailed Mann-Whitney test by comparing the performance hu.Lvr07 with its culture adapted version (** P value $\leq$ 0.01).

Figure 3. HSPG-detargeting mutations accumulated upon in vivo replication of prototypical AAV2 (**A**) Schematic representation of the in vivo evolution approach. (**B-D**) Population shift of RC-AAV2 during in vivo adaptation experiments. The proportion of AAV2 with acquired mutations leading to amino acid changes at positions R487 (**B**), R588 (**C**) for adaptation experiment #1, and R588 (**D**) for experiment #2. RC: replication-competent; R1-R3: rounds 1 to 3. (**E**) Surface map and (**F**) stereographic roadmap projection of a 3D model of evolved AAV2 variants viewed down the icosahedral 2-fold axis. The HSPG binding residues R484, K532 and R585 (blue), R487 (light green) and R588 (yellow) are shown. (**G**) In vitro heparin competition assay. MOI = 100,000 vector genomes / cell. AAV2 served as a control. Data are represented as mean $\pm$ SD. Statistical significance was calculated using the two-tailed Mann-Whitney test by comparing vector performance with and without the presence of soluble heparin (* P value $\leq$ 0.05; n.s. P value $>$ 0.05).

Figure 4. AAV2 variants with mutations leading to HSPG-detargeting transduced human hepatocytes in vivo with higher efficiency than prototypical AAV2 (**A-B**) In vivo physical transduction (**A**) and functional transduction (**B**) comparison of AAV2 variants in the xenograft liver model. Each AAV variant was used to package six barcoded ssAAV-LSP1-GFP-BC⁽¹⁻⁶⁾-WPRE-BGHpA constructs and an equimolar mix of all seven variants was used in the study. AAV2, AAV8, AAV-LK03 and AAV13 served as controls. Percentage of NGS reads

mapped to each capsid in human cells at the DNA (average of 438 vgs / diploid human genome) (**A**) and RNA/cDNA levels (**B**), normalized to the pre-injection mix, is shown. (**C**) Representative immunohistochemical analysis of hFRG mouse liver transduced with indicated variants. Purple: human GAPDH; green: vector-expressed GFP; blue: DAPI (nuclei). Scale=100 μ m. (**D**) Quantification of the percentage of transduced human hepatocytes per human cluster. Data for control serotypes AAV2, AAV13, AAV8 and AAV-LK03 from **Fig. 1E** are shown again for reference. Data shown as mean $\pm$ SD, n=10 human clusters / mouse, n=1 mouse / vector for AAV2 variants, and N=2 mice for AAV2. Statistical significance was calculated using the two-tailed Mann-Whitney test (** P value $\leq$ 0.01; **** P $\leq$ 0.0001).

Table 1. Summary of HiTrap Heparin Column binding studies.

AAV Capsid	AAV predominantly detected in:	[NaCl] at elution peak maxima (mM)
hu.Lvr01-07	Flow Through	(-)
caLvr07 ^{E530K}	Elution Fraction	320.89
caLvr07 ^{G593R}	Elution Fraction	341.94
cahu.Lvr02 ^{D469N+E555K}	Elution Fraction	291.8
AAV2	Elution Fraction	454.34
AAV2-R588I	Elution Fraction	58.25
AAV2-R588T	Elution Fraction	98.09
AAV2-R487Q	Elution Fraction	175.97
AAV3B	Elution Fraction	278.69
AAV13	Elution Fraction	331.89

Table 2 Sequence alignment of AAV HSPG binding residues. Underlined are residues important for serotype-specific heparin binding. The numbers in parentheses indicate the sequence and structure-equivalent residue compared to AAV2.

Residue #	AAV2	AAV3b	AAV13	hu.Lvr07
484	<u>R</u>	R	R	R
487	<u>R</u>	R	R	R
530	E	E	<u>K(528)</u>	E
532	<u>K</u>	K	K(530)	K
585	<u>R</u>	S(586)	N(583)	N
588	<u>R</u>	T(589)	A(586)	T
593	A	<u>R(594)</u>	G(591)	G

Supplementary Materials and Methods

AAV sequence analyses

Deduced Rep and Cap amino acid sequences were aligned using the MAFFT version 7 online service (<https://mafft.cbrc.jp/alignment/server/>)(48), implementing default parameters and the E-INS-i strategy. Phylogenetic analyses were conducted in MEGA X(49) using the Maximum Likelihood method and JTT matrix-based model(50). The trees with the highest log likelihood (-4295.67 for Cap and -2909.84 for Rep) are displayed (**Fig. 1A**, and **fig. S1**, respectively). The percentage of trees in which the associated taxa clustered together is shown next to the branches, based on 1,000 bootstrap replicates. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.

Vectorization of AAV-hu.Lvr capsids

Capsid sequences were subcloned into a previously described packaging construct(47) as follows: To enable cloning, *cap* sequences were PCR-amplified using forward (Vec-Lvr-F, 5'-CCGATTAAATCAGGTATGGCTGCCGATGGTTATC-3', *Swa*I underlined) and reverse (Vec-Lvr-R, 5'-CACTAGGGATGTCATTAGAGTTAATGATTAACCC-3', *Nsi*I underlined) primer, which contained the *Swa*I/*Nsi*I restriction sites for subsequent cloning into the packaging construct. All amplification reactions were performed using Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs), 0.2 ng template DNA using the following thermal cycling conditions: 98°C for 30 sec; 40 cycles of 98°C for 8 sec, 65°C for 20 sec, 72°C for 1 min 15 sec; 72°C for 5 min. PCR products were subcloned into StrataClone Blunt Cloning plasmid (pSC-B-Kan) and validated by Sanger sequencing. Capsid sequences were then cloned directly from pSC-B-Kan downstream of *rep* into the AAV-helper plasmid(47) using *Swa*I/*Nsi*I restriction sites.

Heparin binding assay – Detailed

To prepare the sample for chromatography, 7×10^{11} vg of iodixanol gradient-purified rAAV vector encoding a LSP1-eGFP-BC-WPRE-BGHpA was diluted in dilution buffer (2.5 mM KCl, 1 mM MgCl, 10 mM phosphate, pH 7.4) to a final NaCl concentration of 40 mM and subsequently concentrated to a volume of 150 μ L using an Amicon® Ultra-15 Centrifugal Filter Unit (Merck, Cat #UFC910096) with a 100,000 kDa cut off.

The heparin column was equilibrated with 3 column volumes (CV) of buffer B (PBS + 1 M NaCl, pH 7.4), followed by 5 CV of buffer A (40 mM NaCl, 2.5 mM KCl, 1 mM MgCl, 10 mM phosphate, pH 7.4). 135 μ L of the concentrated rAAV was loaded into a 2-mL sample loop and applied to the heparin column with 4 CV of buffer A. Unbound particles were washed off with 20 CV of buffer A. For serotypes that bound to the column, the affinity of the serotype to heparin was determined by eluting it from the column with a linear gradient of 0-100 % buffer B (40-1137 mM NaCl) applied over 10 CV. The NaCl concentration at which the rAAV eluted from the column was measured at the maximum of the UV absorbance (A280) peak. After elution, 3 CV of 5 M NaCl followed by 5 CV of water was passed through the column to restore it for the next run. All chromatography steps were performed at a flow rate of 1 mL/min at room temperature. The flow-through and elution phases were collected as 0.25-mL fractions using the Fraction Collection F9-C (GE Healthcare).

The presence of rAAV in the A280 peaks observed in the flow-through of the sample application and elution phases was confirmed by running the corresponding fractions on SDS-PAGE and silver staining to detect AAV VP1, VP2 and VP3 proteins. The proportions of rAAV molecules in A280 peaks of the flow through and elution phases (**table S6**) were determined by SYBR® Green qPCR (Bio-Rad, Cat #172-5125) using GFP-qPCR-For and GFP-qPCR-Rev oligonucleotides (**table ST1**).

Human albumin ELISA

Human cell engraftment in chimeric mice were assessed by measuring presence of human albumin on peripheral blood, using the Human Albumin ELISA Quantitation Kit (Bethyl, cat # E80-129) as previously reported (25).

Vector DNA copy number per cell

Vector copy numbers were measured with primers GFP-qPCR-For/Rev using Droplet Digital (dd)PCR (Bio-Rad, Berkeley, US) with QX200 ddPCR EvaGreen Supermix (Bio-Rad, Cat# 1864034) and following manufacturer's instructions. Vector genomes were normalized to human albumin copy number using primers human_ALB_F/R_ddPCR (**table S1**) or mouse_ALB_F/R_ddPCR primers (**table S1**) for murine tissues.

Immunohistochemical analysis of mouse livers

Mouse livers were fixed with 4 % (w/v) paraformaldehyde, cryo-protected in 10-30 % (w/v) sucrose before freezing in O.C.T. (Tissue-Tek; Sakura Finetek USA, Torrance, California), as previously described(51). Frozen liver sections (5 µm) were permeabilized in -20°C methanol, then room temperature 0.1 % Triton X-100, and then reacted with anti-human GAPDH antibody (Abcam, Cat# ab215227, Clone EPR6256) Alexa Fluor® 647, and DAPI (Invitrogen, D1306) at 0.08 ng / mL. Hepatocellular origin of human cells was confirmed by co-staining of human GAPDH and human fumarylacetoacetase hydrolase (FAH) (**fig. S25**). For human Fah staining a rabbit polyclonal to human FAH (Sigma, #SAB210855) was used, followed by a secondary incubation with Alexa Fluor® 594 donkey anti-rabbit IgG (H+L) (Invitrogen, A-21207). After immunolabelling, the images were captured and analyzed on a Zeiss Axio Imager.M1 using ZEN 2 software. The percentage of transduced human hepatocytes per field of view was determined by counting total human GAPDH-positive cells and eGFP / human GAPDH double-positive cells.

Cell culture, vector transduction and heparin competition assay

HEK-293 cells were validated and provided by ATCC. HuH-7 cells were provided by Dr Jerome Laurence (The University of Sydney). All cells were cultured in *Dulbecco's* Modified Eagle Medium (DMEM) (Gibco, 11965-092) supplemented with 10 % FBS (Sigma Aldrich, F9423-500mL, Lot# 16K598), 100 Units/mL Penicillin, 100 µg/mL Streptomycin (Sigma Aldrich, P4458) and passaged using TrypLE Express Enzyme (Gibco, 12604-21). For HuH-7 cultures, media were supplemented also with non-essential amino acids (Gibco, 11140-050). All cells were tested for mycoplasma and were mycoplasma-free. For transduction studies, cells were plated into 24-well plates in complete DMEM at 2×10^5 cells per well and incubated overnight in a tissue-culture incubator at 37 °C / 5 % CO₂. 16 hrs later, the vector stock was diluted in 1 ml of complete DMEM and added to cells (at the indicated vector genome copies per cell (vgc/cell)). For the Heparin Competition Assay, cells were seeded at 10^5 per well into 24-well plates at day 0 and transduced at the indicated vgc/cell. When indicated, heparin sodium salt (Sigma, H3149-50KU, Lot SLBW2119) was supplemented from a 100× stock at 400 µg/mL (**Fig. 1F, 3G**) and at 800 µg/mL (**Fig. 2D**).

After a 72-h incubation, the cells were harvested using TrypLE Express) and analyzed for GFP using BD LSRFortessa cell analyser. The data were analysed using FlowJo 7.6.1.

DNA and RNA isolation

To extract DNA from sorted cells, the cells were resuspended in 200 µL lysis buffer (100 mM Tris-HCl pH 8.5 (Astral Scientific, BioSD8141-450ML), 5 mM EDTA (ThermoFisher), 0.2 % (w/v) sodium dodecyl sulphate (Sigma-Aldrich), 200 mM NaCl (Sigma-Aldrich) containing 50 µg/mL of proteinase K (Bioline). Samples were incubated overnight at 56°C degrees. DNA was treated with PureLink RNase A (Thermo Fisher Scientific, cat #12091021) and extracted using a standard phenol:chloroform protocol using phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich), followed by DNA ethanol precipitation(52).

RNA from sorted cells was extracted using the Direct-Zol kit (Zymogen Cat# R2062) and treated with TURBO DNase (ThermoFisher, Cat# AM2238). cDNA was synthesized using the SuperScript IV First-Strand Synthesis System, following manufacturer's instructions (ThermoFisher, Cat# 18091050).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis Kit (NEB, Cat#E0554S), following manufacturer's instructions, using AAV2_R588T_F_SDM, AAV2_R588T_R_SDM, AAV2_R487Q_F_SDM and AAV2_R487_R_SDM primers (table S1).

Sanger sequencing

When specified, clones were Sanger-sequenced at the Garvan Molecular Genetics facility of the Garvan Institute of Medical Research (Darlinghurst, NSW, Australia) with External_Seq_F, External_Seq_R, and internal_cap_Seq primers (table S1).

Figure S1. Phylogenetic analysis of AAV Rep proteins isolated from human liver (AAV-hu.Lvr01, 02, 05 and 07). AAV1, 2, 6, 3B, 8 and 13 were included as references. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure S2. Murine liver transduction comparison of AAV-hu.Lvr capsids in the xenograft liver model (hFRG #171). Each AAV variant was used to package six unique barcoded ssAAV-LSP1-GFP-BC(1-6)-WPRE-BGHpA cassettes and an equimolar mix of all eleven variants was injected intravenously into a humanized FRG mouse (total of 5.5×10^{11} vgs/mouse) ($n = 6$ BCs $\times$ 11 variants = 66 BCs total). **(A)** Percentage of NGS reads mapped to each capsid in mouse cells at the DNA (3.13 vgs / diploid murine genome) and **(B)** cDNA level, normalized to the injection mix, is shown.

A

	541		R585	R588	600
.AAV2	IFGKQGS	EKTNVDIEKVMITDEEEI	RTTNPVATEQYGSVSTNL	Q	RGNRQAATADVNTQGV
hu.Lvr02	IFGKQDS	GKTNVDIEKVMITDEEEI	RTTNPVATEQYGSVSTNL	Q	SGNTQAATSDVNTQGV
hu.Lvr06	IFGKQDS	GKTNVDIEKVMITDEEEI	RTTNPVATEQYGSVSTNL	Q	SGNTQAATSDVNTQGV
	*****	.	*	*****	*****

B

AAV13	PCYRQQRLSKQANDNNNSNFPWTGATKYHLNGRDSLVP	GPAMASHKDD	EEKFFPMHGTL	538
hu.Lvr04	PCYRQQRLSKQANDNNNSNFPWTAATKYHLNGRDSLVP	GPAMASHKDD	EEKFFPMHGTL	540
hu.Lvr07	PCYRQQRLSKQANDNNNSNFPWTAATKYHLNGRDSLVP	GPAMASHKDD	EEKFFPMHGTL	540
hu.Lvr01	PCYRQQRLSKQASDNNNSNFPWTAATKYHLNGRDSLVP	GPAMASHKDD	EEKFFPMHGTL	540
hu.Lvr03	PCYRQQRLSKQANENNNSNFPWTAATKYHLNGRDSLVP	GPMPAMASHKDD	EEKFFPMHGTL	540
hu.Lvr05	PCYRQQRLSKQANDNNNSNFPWTAATKYHLNGRDSLVP	GPAMASHKDD	EEKFFPMHGTL	539
	*****	.	*****	*****

Figure S3. Multiple sequence alignment of prototypical AAV2 and AAV13 with related hu.Lvr capsids. **(A)** Multiple sequence alignment of prototypical AAV2 with hu.Lvr02 and hu.Lvr06. Key arginine residues for HSPG binding on AAV2 (R585, R588) and corresponding positions in hu.Lvr02 and hu.Lvr06 variants (S585, T588) are marked with a blue rectangle. **(B)** Multiple sequence alignment of prototypical AAV13 with hu.Lvr01, 03, 04, 05 and 07. Key lysine residue for HSPG binding on AAV13 (K528) and structure-equivalent glutamate residues for the hu.Lvr variants are marked with a blue rectangle.

Figure S4. HiTrap Heparin Column Binding assay of prototypical AAV2. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

Figure S5. HiTrap Heparin Column Binding assay of AAV-hu.Lvr01. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

Figure S6. HiTrap Heparin Column Binding assay of AAV-hu.Lvr02. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

A AAV-hu.Lvr03

B

Figure S7. HiTrap Heparin Column Binding assay of AAV-hu.Lvr03. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

A AAV-hu.Lvr04

B

Figure S8. HiTrap Heparin Column Binding assay of AAV-hu.Lvr04. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

A AAV-hu.Lvr05

B

Figure S9. HiTrap Heparin Column Binding assay of AAV-hu.Lvr05. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

A AAV-hu.Lvr06

B

Figure S10. HiTrap Heparin Column Binding assay of AAV-hu.Lvr06. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

Figure S11. HiTrap Heparin Column Binding assay of AAV-hu.Lvr07. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

Figure S12. Representative controls for the in vitro adaptation. ITR2-*rep2*-*caphu.Lvr07*-ITR2 in the presence of hAd5. HuH-7 cells were infected ($n=6$) with AAV (AAV only), with AAV and hAd5 (AAV + hAd5) or with hAd5-only ($n=4$) (hAd5 only). 72 hours after hAd5 co-infection, quantification of AAV genomes and hAd5 genomes was performed using ddPCR with **(A)** *rep2* primers and **(B)** *E1A* primers, specific to AAV and hAd5, respectively (Table S1). n.d. – not detected.

Figure S13. Population shift of (A) AAV-hu.Lvr02 N469 and (B) AAV-hu.Lvr02 E555 during in vitro adaptation. Next-generation sequencing (NGS) was used to determine the proportion of variants harboring mutations at hu.Lvr02 *cap* nucleotide position 1405 (leading to a D469N substitution) (A) and at nucleotide 1663 (leading to a E555K substitution) (B). R1-R5, rounds 1 to 5. Sanger-sequencing of n=6 full-length capsids confirmed that the dominant variant harbored both mutations.

Figure S14. HiTrap Heparin Column Binding assay of caAAV-hu.Lvr07^{E530K}. (A) UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). (B) Representative SDS-PAGE/Silver Staining of the chromatography samples.

A caAAV-hu.Lvr07^{G593R} **B**

Figure S15. HiTrap Heparin Column Binding assay of caAAV-hu.Lvr07^{G593R}. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

caAAV-hu.Lvr02^{D469N+E555K}**A** **B**

Figure S16. HiTrap Heparin Column Binding assay of caAAV-hu.Lvr02^{D469N+E555K}. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

Figure S17. In vitro heparin competition assay of hu.Lvr02 and culture adapted AAV-hu.Lvr02D469N+E555K. Multiplicity of infection (MOI) = 40,000 vector genomes / cell. AAV2 served as a control. Data are mean $\pm$ SD. Statistical significance was calculated using the two-tailed Mann-Whitney test (n=4, * P value = 0.0286).

AAV13

A

B

Figure S18. HiTrap Heparin Column Binding assay of AAV13. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

AAV3b

Figure S19. HiTrap Heparin Column Binding assay of AAV3b. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

AAV2-R588I

Figure S20. HiTrap Heparin Column Binding assay of AAV2-R588I. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

AAV2-R588T

Figure S21. HiTrap Heparin Column Binding assay of AAV2-R588T. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

AAV2-R487Q

Figure S22. HiTrap Heparin Column Binding assay of AAV2-R487Q. **(A)** UV280 chromatography profile with the pooled fractions in the flow through (cyan) and elution (magenta) that were quantified by qPCR. mAU, milli-absorbance units. Measured conductivity (mS/cm, millisiemens per centimeter) is shown in red (right y-axis). **(B)** Representative SDS-PAGE/Silver Staining of the chromatography samples.

Figure S23. Murine liver transduction comparison of AAV2 capsid variants in the xenograft liver model (hFRG #201). Each AAV variant was used to package six unique barcoded ssAAV-LSP1-GFP-BC(1-6)-WPRE-BGHpA cassettes and equimolar mix of all eleven variants was injected intravenously into a humanized FRG mouse (total of 3.5×10^{11} vgs/mouse) ($n = 6$ BCs $\times$ 7 variants = 42 BCs total). Percentage of NGS reads mapped to each capsid in mouse cells at the (A) DNA (6.75 vgs / diploid murine genome) and (B) cDNA level, normalized to the injection mix, is shown.

Figure S24. AAV yield comparison of RC-AAV2 and RC-AAV2-R588I. $n=5$ crude productions using plasmids harbouring ITR2-rep2-AAV2-ITR2 / ITR2-rep2-AAV2-R588I-ITR2.

Figure S25. Immunohistochemical staining of engrafted human hepatocytes with human GAPDH and human fumarlyacetoacetase hydrolase (FAH). Hepatocellular origin of human cells was confirmed by co-staining of human GAPDH and human FAH. Red: human FAH; purple: hGAPDH; blue: DAPI (nuclei). Scale=100 μ m.

Figure S26. ddPCR analysis of chimeric liver lysate of hFRG #445. Humanized FRG mouse #445 was injected with 5×10^9 PFUs of wild-type human adenovirus-5 intraperitoneally and harvested 72 hrs after administration. No detectable AAV genomes were found.

Figure S27. Murine liver transduction comparison of AAV-hu.Lvr07 and its adapted version (E530K) variants in the xenograft liver model (hFRG #164). Each AAV variant was used to package six unique barcoded ssAAV-LSP1-GFP-BC(1-6)-WPRE-BGHpA cassettes and equimolar mix of all five variants was injected intravenously into a humanized FRG mouse (total of 2.5×10^{11} vgs/mouse) ($n = 6$ BCs $\times$ 5 variants = 30 BCs total). Percentage of NGS reads mapped to each capsid in mouse cells at the (A) DNA and (B) cDNA level, normalized to the injection mix, is shown.

Table S1 – Primer sequences

Primer Name	Sequence (5' – 3')
Rep_mpx1_F	CGCAGACAGGTACCAAAACAAA
3UTR_mpx2_R	ACTCCATCACTAGGGGTTCC
Vec-Lvr-F	CCGATTATAATCAGGTATGGCTGCCGATGGTTATC
Vec-Lvr-R	CACTAGGGATGCATTAGAGTTAATGATTAACCC
GFP-F1	TCAAGATCCGCCACAACATC
GFP-R1	TTCTCGTTGGGGTCTTTGCT
rep-F1	CTCAACCCGTTTCTGTCGTC
rep-R2	CACATTGACCAGATCGCAGG
BC_F	GCTGGAGTTCGTGACCGCCG
BC_R	CAACATAGTTAAGAATACCAGTCAATCTTTCACAAATTTGTAATCCAGAGG
AAV2_R588T_F_SDM	GAGGCAACACACAAGCAGCTACCGCAGATGTCAACAC
AAV2_R588T_R_SDM	TCTGGAGGTTGGTAGATACAGAACCATACTGC
AAV2_R487Q_F_SDM	ACCGCCAGCAGCAAGTATCAAAGACATC
AAV2_R487Q_R_SDM	AACAGGGTCCAGGAAGCCAG
External_Seq_F	TGTGGATTTGGATGACTGC
External_Seq_R	GACCAAAGTTCAACTGAAACG
internal_cap_Seq	GTTCTTGATGAGAATCTGTGGAGGAGG
human ALB_F ddPCR	TGCTGTCATCTCTTGTGGGCTG
human ALB_R ddPCR	AACTCATGGGAGCTGCTGGTTC
mouse ALB_F ddPCR	AACTGCTACTCCCCTCCTAC
mouse ALB_R ddPCR	TTTACCCCAAGTGACAGGAAAG
E1A-F	AACCAGTTGCCGTGAGAGTTG
E1A-R	CTCGTTAAGCAAGTCCTCGATACAT

Table S2 – hFRG individual mouse data

FRG ID	Gender	Age at injection (weeks)	[h Albumin] at injection (mg/mL)	Experiment	Figure
171	female	26	4.68	<i>In vivo</i> comparison AAV-hu.Lvr01/07 Variants	1B-C
166	female	19	1.78	<i>In vivo</i> comparison AAV-hu.Lvr01/07 Variants	1B-C
562	female	24	5.65	AAV2 IHC	1D-E
209	female	26	3.59	AAV-Lvr01 IHC	1D-E
140	female	31	3.91	AAV-Lvr02 IHC	1D-E
463	female	24	2.29	AAV-Lvr06 IHC	1D-E
413	female	26	2.39	AAV-Lvr07 IHC	1D-E
597	female	22	0.54	AAV2 IHC	1D-E
589	female	22	2.26	AAV8 IHC	1D-E
67	female	24	1.06	AAV13 IHC	1D-E
602	female	22	3.20	AAV-LK03 IHC	1D-E
61	female	24	2.04	AAV-Lvr01 IHC	1D-E
60	female	24	1.21	AAV-Lvr02 IHC	1D-E
162	female	19	3.94	AAV-Lvr03 IHC	1D-E
176	female	19	4.21	AAV-Lvr04 IHC	1D-E
579	female	22	3.73	AAV-Lvr05 IHC	1D-E
59	female	24	1.10	AAV-Lvr06 IHC	1D-E
58	female	24	1.64	AAV-Lvr07 IHC	1D-E
174	female	19	3.88	AAV2-R588T IHC	4C-D
118	female	19	3.19	AAV2-R487Q IHC	4C-D
65	female	24	1.54	AAV2-R588I IHC	4C-D
164	female	21	10.60	<i>In vivo</i> comparison AAV-hu.Lvr07 / culture adapted	2G-H
155	female	21	2.48	<i>In vivo</i> comparison AAV-hu.Lvr07 / culture adapted	2G-H
266	female	23	9.00	RC-AAV2 <i>In vivo</i> Adaptation #1 R1	3B-C
298	female	22	5.78	RC-AAV2 <i>In vivo</i> Adaptation #1 R2	3B-C
333	female	23	3.20	RC-AAV2 <i>In vivo</i> Adaptation #1 R3	3B-C
588	female	19	12.75	RC-AAV2 <i>In vivo</i> Adaptation #2 R1	3D
585	female	19	13.38	RC-AAV2 <i>In vivo</i> Adaptation #2 R2	3D
201	female	29	4.94	<i>In vivo</i> comparison AAV2 variants	4A-B
294	female	23	2.56	<i>In vivo</i> comparison AAV2 variants	4A-B
321	female	16	Non-engrafted	RC-AAV2 <i>In vivo</i> Control	/
473	female	16	Non-engrafted	RC-AAV2 <i>In vivo</i> Control	/
445	female	25	16.31	RC-AAV2 <i>In vivo</i> Control (Ad5 only)	S26

Table S3 – In vivo comparison of hu.Lvr variants - NGS Read Summary

	Mapped NGS Reads
hFRG #171 - hu.Lvr variants	
Reads Pre-Injection Mix	1256264
Human Hepatocytes (DNA)	2615380
Human Hepatocytes (cDNA)	6045710
Murine Liver (DNA)	1793069
Murine Liver (cDNA)	2834706
hFRG #166 - hu.Lvr variants	
Reads Pre-Injection Mix	685272
Human Hepatocytes (DNA)	1257382
Human Hepatocytes (cDNA)	875967

Table S4 – In vivo comparison of culture adapted hu.Lvr07 variant – NGS Read Summary.

	Mapped NGS Reads
Reads Pre-Injection Mix	546967
hFRG #155- ca-hu.Lvr07 in vivo	
Human Hepatocytes (DNA)	583230
Human Hepatocytes (cDNA)	383150
hFRG #164- ca-hu.Lvr07 in vivo	
Human Hepatocytes (DNA)	551856
Human Hepatocytes (cDNA)	461820
Murine Liver (DNA)	732239
Murine Liver (cDNA)	769286

Table S5 – In vivo comparison of AAV2 variants – NGS Read Summary.

	Mapped NGS Reads
Reads Pre-Injection Mix	5280145
hFRG #201 - AAV2 variants	
Human Hepatocytes (DNA)	1112223
Human Hepatocytes (cDNA)	6784101
Murine Liver (DNA)	6658138
Murine Liver (cDNA)	650484
hFRG #294 - AAV2 variants	
Human Hepatocytes (DNA)	2544207
Human Hepatocytes (cDNA)	2295979

Table S6. Summary of HiTrap Heparin Column Binding Assay.

AAV Capsid	Flow Through (%)	Elution Peak (%)	[NaCl] at elution peak maxima (mM)
hu.Lvr01	59.97%	2.22%	-
hu.Lvr02	63.48%	3.24%	-
hu.Lvr03	42.37%	7.51%	-
hu.Lvr04	75.18%	3.22%	-
hu.Lvr05	68.76%	5.28%	-
hu.Lvr06	68.89%	5.80%	-
hu.Lvr07	46.26%	N/A	-
cahu.Lvr02 ^{D469N+E555K}	<0.01%	10.69%	291.8
caLvr07_E530K	0.65%	13.97%	320.89
caLvr07_G593R	0.29%	12.10%	341.94
AAV2	0.65%	39.77%	454.34
AAV2-R588I	0.62%	32.39%	58.25
AAV2-R588T	0.11%	13.18%	98.09
AAV2-R487Q	1.12%	16.48%	175.97
AAV3B	0.64%	14.05%	278.69
AAV13	5.38%	75.45%	331.89

Table S7. Clonal Analysis of AAV2 in vivo adapted variants Round 1. Full length *cap* sequences were aligned to prototypical AAV2. Table shows detected DNA changes and corresponding amino acid change.

Round 1			
Clone	Mutations	DNA Change #1	Amino acid change?
1	0	G1763T	R588I
2	0		
3	1		
4	0		
5	0		
6	0	G1763T	R588I
7	1		
8	0		
9	0		
10	0		
11	0	G1763T	R588I
12	0		
13	0		
14	0		
15	0		
16	0	G1763T	R588I
17	1		
18	0		
19	0		
20	1	C1991A	A664E
21	0	G1763T	R588I
22	1		
23	0		
24	0		
Clones Sequenced	24	Percentage	
Clones With R588I	4	16.67	
Clones With A664E	1	4.17	
AAV2 Clones	19	79.17	

Table S8. Clonal Analysis of in vivo adapted variants Round 2. Full length *cap* sequences were aligned to prototypical AAV2. Table shows detected DNA changes and corresponding amino acid change.

Round 2 EVO 1							
Clone	Mutations	DNA Change #1	Amino acid change?	DNA Change #2	Amino acid change?	DNA Change #3	Amino acid change?
1	0						
2	1	G1763T	R588I				
3	1	G1763T	R588I				
4	1	G1763T	R588I				
5	1	G1763T	R588I				
6	1	G1763T	R588I				
7	1	G1763T	R588I				
8	1	G1763T	R588I				
9	1	G1763T	R588I				
10	1	G1763T	R588I				
11	1	G1763T	R588I				
12	2	C496A	P166T	G1460A	R487Q		
13	1	G1763T	R588I				
14	1	G1763T	R588I				
15	1	G1763T	R588I				
16	3	C99A	P34C	C936A	Silent	G1763T	R588I
17	1	G1763T	R588I				
18	1	G1763T	R588I				
19	1	G1763T	R588I				
20	0						
21	1	G1763T	R588I				
22	1	G1763T	R588I				
23	1	G1763T	R588I				
24	1	G1460A	R487Q				
25	1	G1460A	R487Q				
Clones Sequenced		25	Percentage	NGS Data [Position R588I]			
Clones With R588I		20	80.00	88.62			
Clones With R487Q		3	12.00	/			
AAV2 Clones		2	8.00	9.95			

Table S9. Clonal Analysis of *in vivo* adapted variants Round 3. Full length *cap* sequences were aligned to prototypical AAV2. Table shows detected DNA changes and corresponding amino acid change.

Round 3					
Clone	Mutations	DNA Change #1	Amino acid change?	DNA Change #2	Amino acid change?
1	1	G1763T	R588I	G1763T	R588I
2	1	G1763T	R588I		
3	1	G1763T	R588I		
4	1	G1763T	R588I		
5	2	C252A	D84E		
6	1	G1763T	R588I		
7	1	G1763T	R588I		
8	1	G1763T	R588I		
9	1	G1763T	R588I		
10	1	G1763T	R588I		
11	1	G1763T	R588I		
12	1	G1763T	R588I		
13	1	G1763T	R588I		
14	1	G1763T	R588I		
15	1	G1763T	R588I		
16	1	G1763T	R588I		
17	2	G1763T	R588I	T2129C	V710A
18	1	G1763T	R588I		
19	1	G1763T	R588I		
20	1	G1763T	R588I		
21	1	G1763T	R588I	G1763T	R588I
22	1	G1763T	R588I		
23	1	G1763T	R588I		
24	2	G1326T	Silent		
Clones Sequenced	24	Percentage			
Clones With R588I	24	100.00			
Clones With R487Q		0.00			
AAV2 Clones		0.00			