High-efficiency Transduction and Correction of Murine Hemophilia B Using AAV2 Vectors Devoid of Multiple Surface-exposed Tyrosines

David M Markusic[1,2], Roland W Herzog[1–5], George V Aslanidi[1,2], Brad E Hoffman[1–3], Baozheng Li[1], Mengxin Li[6], Giridhara R. Jayandharan[1,2,7], Chen Ling[1,2], Irene Zolotukhin[1], Wenqin Ma[1], Sergei Zolotukhin[1–4], Arun Srivastava[1–5] and Li Zhong[6,8]

[1]Division of Cellular and Molecular Therapy, Cancer and Genetics Research Complex, Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida, USA; [2]Powell Gene Therapy Center, University of Florida College of Medicine, Gainesville, Florida, USA; [3]Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, Florida, USA; [4]Genetics Institute, University of Florida College of Medicine, Gainesville, Florida, USA; [5]Shands Cancer Center, University of Florida College of Medicine, Gainesville, Florida, USA; [6]Gene Therapy Center, University of Massachusetts Medical School, Worcester, Massachusetts, USA; [7]Department of Haematology, and Center for Stem Cell Research, Christian Medical College, Vellore, TN, India; [8]Division of Hematology/Oncology, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA

Elimination of specific surface-exposed single tyrosine (Y) residues substantially improves hepatic gene transfer with adeno-associated virus type 2 (AAV2) vectors. Here, combinations of mutations in the seven potentially relevant Y residues were evaluated for further augmentation of transduction efficiency. These mutant capsids packaged viral genomes to similar titers and retained infectivity. A triple-mutant (Y444+500+730F) vector consistently had the highest level of *in vivo* gene transfer to murine hepatocytes, approximately threefold more efficient than the best single-mutants, and ~30–80-fold higher compared with the wild-type (WT) AAV2 capsids. Improvement of gene transfer was similar for both single-stranded AAV (ssAAV) and self-complementary AAV (scAAV) vectors, indicating that these effects are independent of viral second-strand DNA synthesis. Furthermore, Y730F and triple-mutant vectors provided a long-term therapeutic and tolerogenic expression of human factor IX (hF.IX) in hemophilia B (HB) mice after administration of a vector dose that only results in subtherapeutic and transient expression with WT AAV2 encapsidated vectors. In summary, introduction of multiple tyrosine-mutations into the AAV2 capsid results in vectors that yield at least 30-fold improvement of transgene expression, thereby lowering the required therapeutic dose and potentially vector-related immunogenicity. Such vectors should be attractive for treatment of hemophilia and other genetic diseases.

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INTRODUCTION

The adeno-associated virus (AAV) is a small, single-stranded (ss) DNA-containing nonpathogenic human parvovirus, and recombinant AAV-based vectors are widely used for *in vivo* gene transfer applications.[1,2] It has been shown that recombinant AAV vectors can transduce a wide variety of cells and tissues,[2–5] and are currently in use in phase I/II clinical trials for gene therapy of a number of diseases such as hemophilia B (HB),[6,7] cystic fibrosis,[8] α-1 antitrypsin deficiency,[9] Parkinson's disease,[10] Batten's disease,[11] and muscular dystrophy,[12] and have shown clinical efficacy in Leber's congenital amaurosis.[13–16] However, in many cases, relatively large vector doses are needed to achieve therapeutic benefits. The requirement for large vector doses poses a challenge for not only vector production, but also increases the risk of immune responses. Thus, it is critical to develop novel AAV vectors with high-transduction efficiency at lower doses for human gene therapy.

Although AAV2 vectors efficiently target the liver, transgene expression is restricted to ~5% of the hepatocytes because of inhibition of viral DNA second strand synthesis due in part to phosphorylation of the cellular protein, FKBP52.[17] Self-complementary (sc) AAV vectors circumvent this step, but also further reduce the size of the expression cassette that can be packaged into viral capsids. Coexpression of T-cell protein tyrosine phosphatase and protein phosphatase 5 from scAAV vectors has been developed to prevent phosphorylation of FKBP52, resulting in improved transduction efficiency of murine hepatocytes by ssAAV vectors *in vivo*.[18,19] Interestingly, perturbations in epidermal growth factor receptor protein tyrosine kinase signaling to reduce phosphorylation of the FKBP52 protein augments transduction efficiency not only by increased viral second-strand DNA synthesis, but also by

Correspondence: *Roland W Herzog, Division of Cellular and Molecular Therapy, Cancer and Genetics Research Complex, 2033 Mowry Road, Room 203, University of Florida College of Medicine, Gainesville, Florida 32611, USA. E-mail: rherzog@ufl.edu; Arun Srivastava, Division of Cellular and Molecular Therapy, Cancer and Genetics Research Complex, 2033 Mowry Road, Room 492-A, University of Florida College of Medicine, Gainesville, Florida 32611, USA. E-mail: aruns@peds.ufl.edu; Li Zhong, Gene Therapy Center, 381 Plantation Street, Ste 250, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA. E-mail: Li.Zhong@umassmed.edu*

facilitating intracellular trafficking of viral particles from the cytoplasm to the nucleus.[20] Intact AAV2 capsids are phosphorylated at selected tyrosine residues by epidermal growth factor receptor protein tyrosine kinase. Tyrosine-phosphorylation of AAV2 capsids negatively affects viral intracellular trafficking by priming capsid ubiquitination and proteasomal degradation, which leads to reduced transgene expression and may render the transduced cells to become targets for capsid-specific cytotoxic T-lymphocytes.[21–23] These recent studies have yielded valuable insights into key steps in the intracellular trafficking of AAV2 vectors, allowing for the development of novel AAV vectors with further improvements in transduction efficiency.

Importantly, mutations in surface-exposed tyrosine residues on AAV2 capsids circumvent the ubiquitination step, thereby avoiding proteasome-mediated degradation, and resulting in high-efficiency transduction by these vectors in human cells *in vitro* and murine hepatocytes *in vivo*.[24] Additionally, improved human factor IX (hF.IX) expression has been obtained in several different strains of mice using the Y730F tyrosine-mutant AAV2 vector.[24] Mutagenesis of a surface-exposed tyrosine residue, Y733F, on AAV8 has also resulted in a substantial increase in gene transfer efficiency in retinal cells,[25] and it is expected that other serotypes will have improved gene transfer following tyrosine mutagenesis.

The introduction of selected single tyrosine-mutations into the AAV2 capsid has resulted in at least a tenfold increase in gene expression with the Y730F mutant providing the highest level of gene expression followed by Y444F and Y500F mutants.[24] We reasoned that a combination of multiple mutations in surface-exposed tyrosine residues would lead to a further improvement in gene transfer. In the present study, we identified an optimal triple-mutant AAV2 capsid. This improved vector enabled us to achieve long-term therapeutic expression of hF.IX in HB mice, which show poor hepatic gene transfer and lack tolerance to hF.IX when vector with wild-type (WT) AAV2 capsid is administered. The triple-mutant AAV2 vector may prove to be safe and efficacious in gene therapy trials in humans.

RESULTS

Multiple mutations in surface-exposed tyrosine residues further improve the transduction efficiency of AAV2 vectors in cells *in vitro*

In our previous studies, we modified each of the seven surface-exposed tyrosine residues (Y252, Y272, Y444, Y500, Y700, Y704, and Y730) on AAV2 capsids by site-directed mutagenesis with phenylalanine residues (tyrosine-phenylalanine, Y-F) and generated scAAV2-enhanced green fluorescent protein (EGFP) vectors.[24] Results showed approximately tenfold higher transduction efficiency of Y444F, Y500F, and Y730F vectors compared with the WT vectors in HeLa cells *in vitro*. To further examine whether multiple-mutations of surface-exposed tyrosine residues further enhanced transduction, we generated the following AAV2 capsid variants: seven double (Y252+730F; Y272+730F; Y444+730F; Y500+730F; Y700+730F; Y704+730F; Y444+500F), one triple (Y444+500+730F), one quadruple (Y272+444+500+730F), two pentuple (Y272+444+500+704+730F; Y272+444+500+700+730F), one sextuple (Y252+272+444+500+704+730F), and one septuple (Y252+272+444+500+700+704+730F). Each of the tyrosine-capsid mutant vectors was capable of encapsidating viral scAAV-EGFP genomes at similar titers except the septuple mutant, which was approximately tenfold lower (data not shown).

In side-by-side comparison using HeLa cells, all single and multiple Y-F mutants resulted in an improvement of transduction with the scAAV-EGFP vector (**Figure 1a,b**). Consistent with our previously published data,[24] Y730F was the most effective single-mutant, closely followed by Y444F and Y500F. Compared with Y730F, the best performing single tyrosine-mutant capsid, only the triple-mutant (Y444+500+730F, representing the combination of the three best single-mutants) and the septuple-mutant led to an additional significant increase in transgene expression (approximately four- to fivefold, **Figure 1a,b**). Interestingly, neither the double-mutant combinations of the 444, 500, and 730 residues nor quadruple-, pentuple-, or sextuple-mutants (that included these three residues) were effective.

The best performing multiple tyrosine-mutants on HeLa cells were then evaluated for transduction of a murine hepatocyte cell line, H2.35. At the moi chosen (2,000 vector genomes [vgs]/cell), Y730F, but not the WT vectors, was able to transduce H2.35 cells (**Figure 1c**). Similar to HeLa cells, the triple-mutant vector directed approximately fivefold higher levels of EGFP expression compared with Y730F (**Figure 1c,d**). However, the septuple-mutant was not superior to Y730F, and a minor increase in EGFP expression was observed with the Y444+730F double-mutant vector (**Figure 1d**).

Triple-mutant AAV2 vector is highly efficient in transducing murine hepatocytes *in vivo*

We have previously shown that AAV2 vectors containing specific single tyrosine-mutant capsids transduce murine hepatocytes *in vivo* approximately tenfold more efficiently than the WT capsids.[24] Based on initial *in vitro* results, several combinations of multiple mutations in surface-exposed tyrosine residues vectors were further evaluated *in vivo* for liver gene transfer. A dose of 1×10^9 vgs/mouse was injected via the tail vein of C57BL/6 mice. Four weeks postinjections, liver lobes were harvested and analyzed for *EGFP* gene expression using fluorescence microscopy. As can been seen in **Figure 2a**, the transduction efficiency of single tyrosine-mutant vectors was significantly higher (~5–18-fold) compared with the WT vector (**Figure 2b**), which is consistent with our published studies.[24] Again, Y730F was the best single-mutant, and the only capsid that yielded a robust additional increase in EGFP expression was Y444+500+730F triple-mutant (fivefold compared with Y730F, **Figure 2a,b**). Relatively minor increases, compared with Y730F, were observed with double-mutant Y444+730F, and the septuple-mutant (albeit these were statistically significant, **Figure 2b**). Consistent with *in vitro* data, the triple-mutant represents the most optimal tyrosine-mutant AAV2 vector for *in vivo* transduction of murine hepatocytes. Biodistribution studies of all the tyrosine-mutant vectors showed no evidence of *EGFP* gene expression in other tissues, such as heart, lung, kidney, spleen, pancreas, gastrointestinal tract (jejunum, colon), testis, skeletal muscle, and brain (data not shown). Thus, single- as well as the multiple-mutations in the surface-exposed tyrosine residues do not appear to alter the liver-tropism following tail vein injection of these vectors.

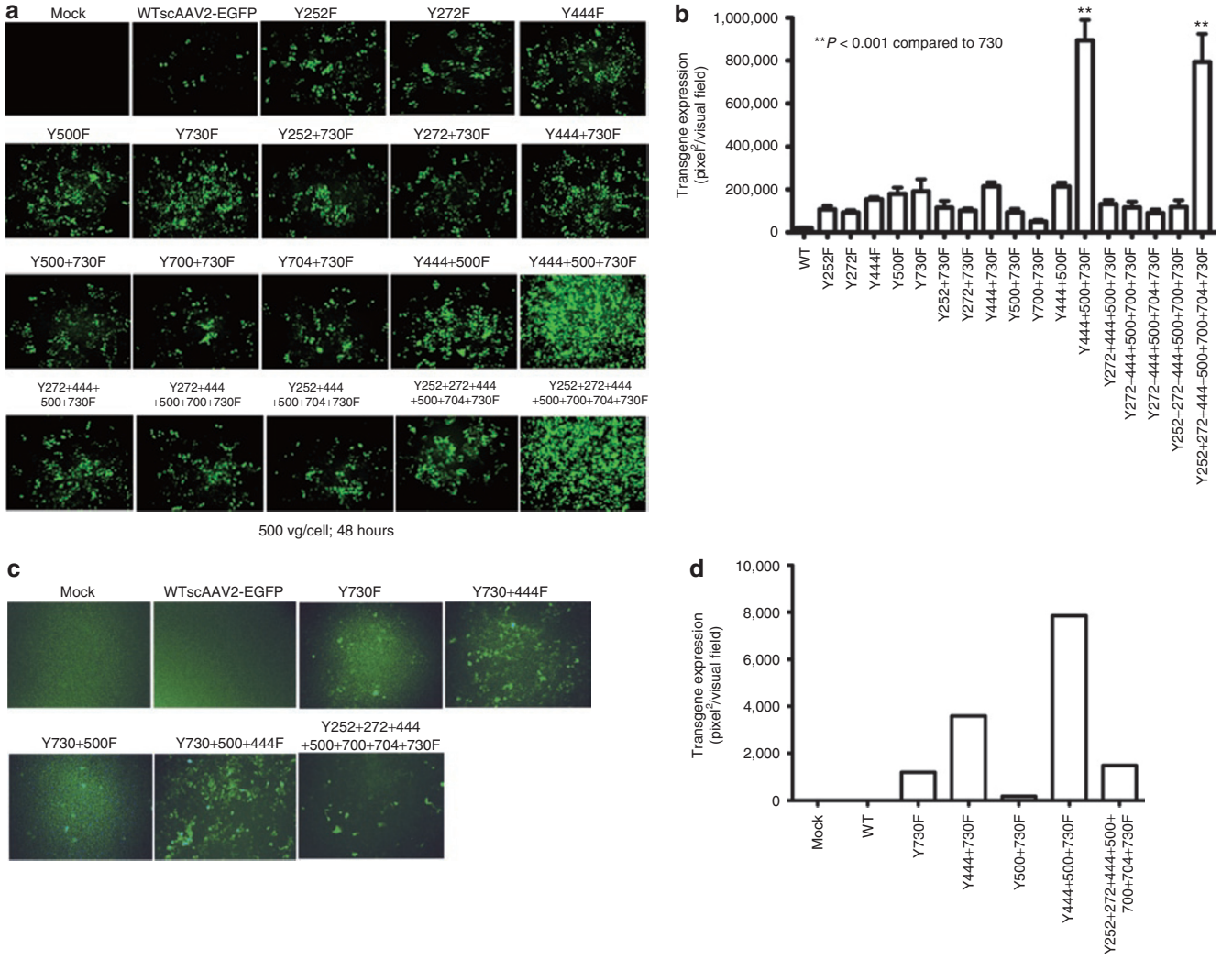

Figure 1 AAV2-mediated transgene expression in (a,b) HeLa cells and in (c,d) H2.35 murine hepatocytes following transduction with single and multiple surface-exposed tyrosine-mutant capsid scAAV2-EGFP vectors. (**a**,**c**) Transgene expression was detected by fluorescence microscopy 48 hours postinfection with (**a**) 500 or (**c**) 2,000 vgs/cell. Original magnification ×100. (**b**) Quantitative analyses of AAV2 transduction efficiency in HeLa cells. (**d**) Quantitative analyses of AAV2 transduction efficiency in H2.35 cells. Images from five visual fields were analyzed quantitatively by ImageJ analysis software. Transgene expression was assessed as total area of green fluorescence (pixel^2) per visual field (mean ± SD). One-way ANOVA and Dunnett's multiple comparison test was used to determine which multiple tyrosine-mutants provided significantly higher transgene expression ($P < 0.001$) compared to Y730F. ANOVA, analysis of variance; AAV2, adeno-associated virus type 2; EGFP, enhanced green fluorescent protein; scAAV2, self-complementary AAV2; vgs, vector genomes.

The most effective known serotype for *in vivo* transduction of murine hepatocytes is AAV8.[26] Therefore, a further comparison was performed between the triple-mutant AAV2 vector and an AAV8 vector for EGFP expression at a dose of 1×10^{10} vgs/mouse. AAV8-transduced livers expressed approximately fivefold higher level of EGFP as compared with the triple-mutant AAV2 vector (**Supplementary Figure S1**).

Next, long-term efficacy of hepatic gene transfer with WT, single (Y730F) and triple tyrosine-mutant (Y444+500+730F) scAAV2-EGFP vectors was determined. Liver lobes were harvested 2–26 weeks following tail vein injection of 1×10^9 vgs in C57BL/6 mice. As expected, gene transfer using the WT AAV2 capsid resulted in comparatively low EGFP expression in hepatocytes (**Figure 3a**). EGFP expression from single tyrosine-mutant vector (Y730F) and triple-mutant vector (Y444+500+730F) was significantly higher at all time points tested, ranging between 3- to 36-fold and an additional ~3 to 5-fold, respectively, compared with the WT vector (**Figure 3b**). Thus, tyrosine-mutant vectors directed a stable increase in transduction of hepatocytes *in vivo*.

In anticipation of extending these studies to include ssAAV2 vectors expressing a therapeutic gene, we also evaluated the effect of the tyrosine-capsid mutants on gene transfer efficiency of ssAAV2-EGFP vectors. C57BL/6 mice were injected via the tail vein with 1×10^{10} vgs of WT, single (Y730F), or triple tyrosine-mutant (Y444+500+730F) caspid ssAAV2-EGFP vectors. Liver lobes were harvested 2–17 weeks postinjections and analyzed for *EGFP* gene expression (**Figure 3c**). The transduction efficiency of the single tyrosine-mutant vector Y730F was again significantly higher (approximately fivefold at 2 and 17 weeks postinjection) than for WT vector (**Figure 3d**). Moreover, the triple-mutant

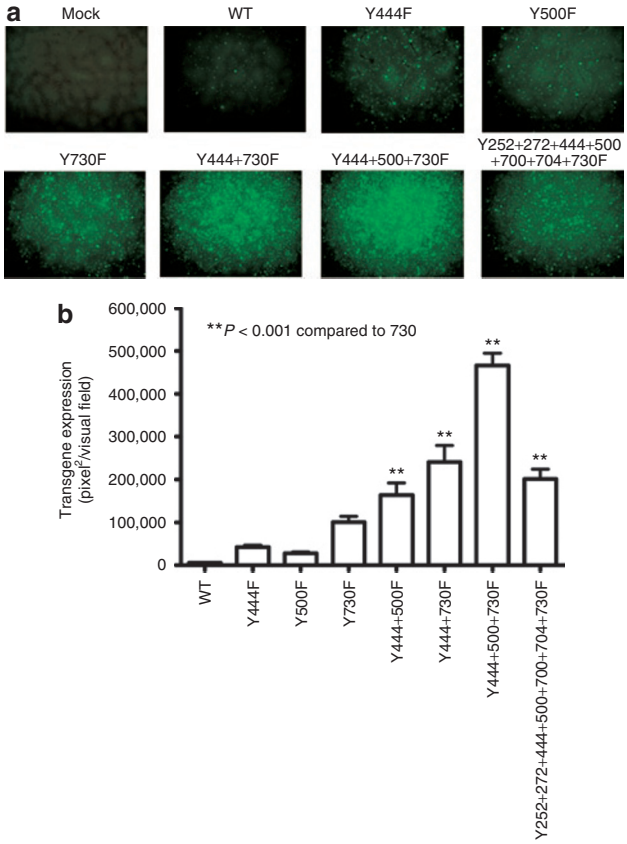

Figure 2 Hepatic gene transfer in C57BL/6 mice with single and multiple tyrosine-mutant capsid scAAV2-EGFP vectors. (**a**) Transgene expression was detected by fluorescence microscopy 4 weeks postinjection of 1 × 10^9 vgs/animal. Original magnification ×50. (**b**) Quantitative analyses of EGFP expression (see legend to Figure 1 for details). EGFP, enhanced green fluorescent protein; scAAV2, self-complementary adeno-associated virus type 2; vgs, vector genomes.

vector (Y444+500+730F) led to a further approximately fivefold increase in EGFP expression compared with Y730F (Figure 3d) at both time points. These data suggest that the observed increased transduction efficiency of tyrosine-mutant AAV2 vectors is independent of viral second-strand DNA synthesis.

Single (Y730F) and triple tyrosine-mutant vectors provide long-term therapeutic correction of murine HB

Recombinant ssAAV2 vectors were produced with WT single-mutant (Y730F), and triple tyrosine-mutant capsids expressing hF.IX from a liver-specific promoter (apolipoprotein E (ApoE)/human α_1-antitrypsin (hAAT); apolipropotein E enhancer and hepatocyte control region linked to the hAAT promoter).[7] In an initial experiment, these vectors were tested in C57BL/6 mice (2 × 10^{10} vg/mouse delivered via tail vein). Triple-mutant and Y730F vectors yielded ~20- and 50-fold higher systemic hF.IX levels compared with WT AAV2 capsid (data not shown).

To test the therapeutic efficiency of these two tyrosine-mutant capsid vectors, HB mice carrying a targeted deletion of the *F9* gene (strain C3H/HeJ/F9$^{-/-}$) were injected in the tail vein with 2 × 10^{11} vgs of either WT AAV2, Y730F, or the triple-mutant AAV2-ApoE/hAAT-hF.IX vectors (hepatic gene transfer in C3H strain is generally not as efficient as in C57BL/6, hence the higher vector dose). One month following gene transfer, HB mice injected with the triple-mutant vector expressed ~43-fold higher levels of hF.IX (n = 4, 627 ± 110 ng/ml; ~12% of normal levels in humans) than WT AAV2 (n = 3, 15 ± 4 ng/ml; <1% of normal) and approximately fourfold higher levels of hF.IX compared to Y730F vector (n = 4, 166 ± 69 ng/ml; 3–4% of normal) (Figure 4a). HB mice transduced with either Y730F or triple-mutant hF.IX AAV2 vectors showed sustained therapeutic hF.IX levels (Figure 4b) and improved coagulation (as measured by activated partial thromboplastin time) for the duration of the experiment (5–7 months, Figure 4c). The triple-mutant vector on average yielded approximately threefold higher hF.IX levels than Y730F during the course of the experiment, thus resulting in further correction of the activated partial thromboplastin time.

In several previous studies, we consistently found that C3H/HeJ/F9$^{-/-}$ mice injected with identical WT AAV2 vector via the portal vein at a similar dose expressed hF.IX only transiently in circulation and developed immune responses against hF.IX.[27–29] In the experiments presented here, average activated partial thromboplastin times of WT AAV2 vector-treated HB mice were, as reported previously, at the borderline between partially corrected and uncorrected mice at 1 month, but increased well into the uncorrected range by 2 months (Figure 4c). Subtherapeutic systemic hF.IX levels further decreased in two of three mice, similar to our previously published results for portal vein administration.[29] However, no evidence for antibody formation against hF.IX during this time was obtained (Figure 4d).

With the exception of one HB mouse, none of the Y730F- or the triple-mutant vector-injected HB mice showed evidence for antibody formation against hF.IX by immunoglobulin G (IgG) measurements or Bethesda assay (Figure 4d). One triple-mutant vector-transduced HB mouse had a transient IgG response at 3 months, which represented a transient and low-titer inhibitor of 2 Bethesda unit that spontaneously resolved (Figure 4d,e). Although the WT AAV2 vector-transduced mice had not formed antibodies against hF.IX, these animals, similar to nontransduced control mice, were not tolerant and formed IgG/inhibitors after challenge with recombinant hF.IX protein [1 (IU)] using either of two previously established challenge protocols (Figure 4d–f).[27,30] In contrast, neither subcutaneous injection of hF.IX in complete Freund's adjuvant nor four weekly administrations of hF.IX (one intraperitoneal followed by three intravenous, intravascular (i.v.) doses) broke tolerance to hF.IX (including in the animal with prior transient inhibitor formation) in Y730F- or the triple-mutant vector-transduced mice (Figure 4b–e). Challenge with repeated i.v. injections more closely mimics current treatment of patients with HB and, similar to treatment of humans with *F9* gene deletions, results in severe allergic and anaphylactic reactions in this strain of HB mice.[31] However, no such reactions were observed after challenge of Y730F- or the triple-mutant vector-transduced mice.

At the end of the experiment, the WT AAV2-, Y730F-, and the triple-mutant vector-injected HB mice were sacrificed, and livers were sectioned and immunostained for hF.IX expression. Representative sections from WT AAV2, Y730F, and the triple-mutant vector-injected mice are displayed in Figure 5. The

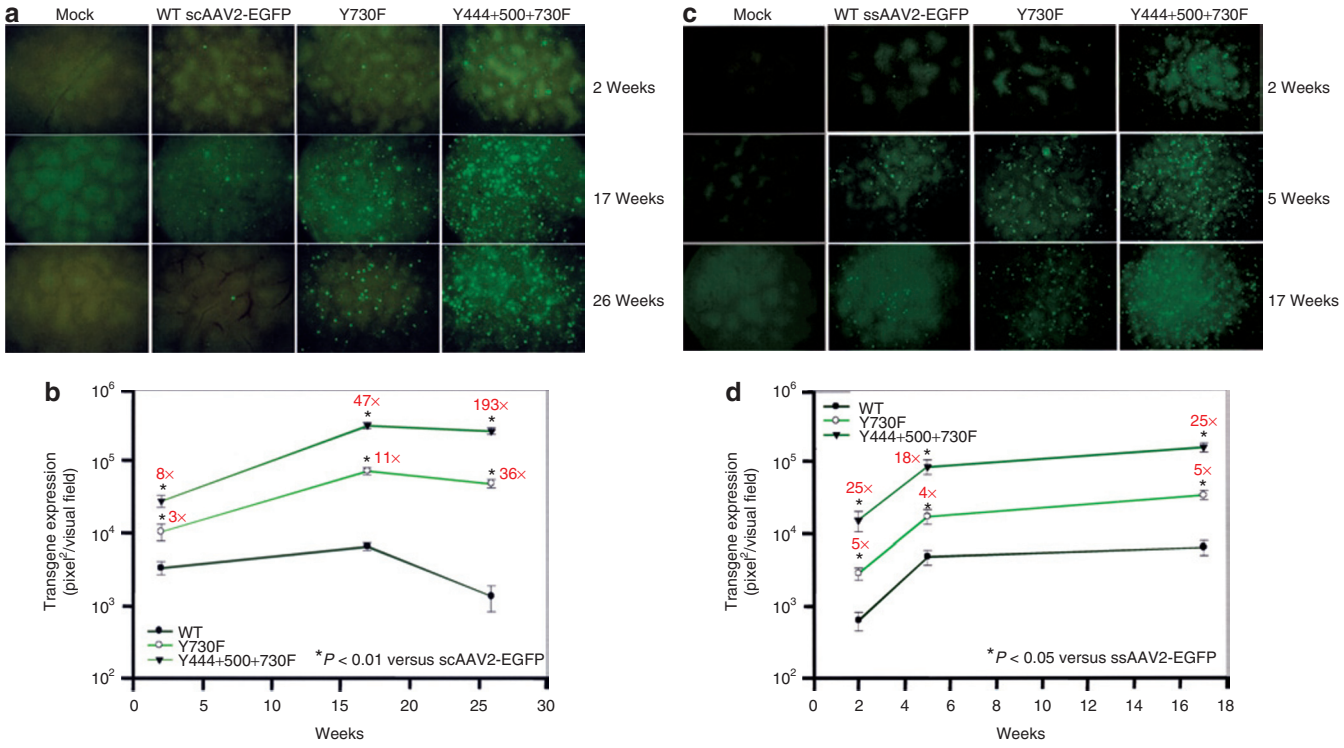

Figure 3 Time course of transgene expression in livers of C57BL/6 mice transduced with tyrosine-mutant capsid scAAV2-EGFP and ssAAV2-EGFP vectors. (**a**,**b**) Transgene expression was detected by fluorescence microscopy 2–26 weeks postinjection of 1 × 10^9 vgs/animal of scAAV2-EGFP vector (original magnification ×50) and quantitatively analyzed as described in the legend to **Figure 1**. *P < 0.01 versus WT scAAV2-EGFP. (**c**,**d**) Transgene expression 2–17 weeks postinjection of 1 × 10^{10} vgs/animal of ssAAV2-EGFP vector. *P < 0.01 versus WT ssAAV2-EGFP. EGFP, enhanced green fluorescent protein; scAAV2, self-complementary adeno-associated virus type 2; vgs, vector genomes; WT, wild type.

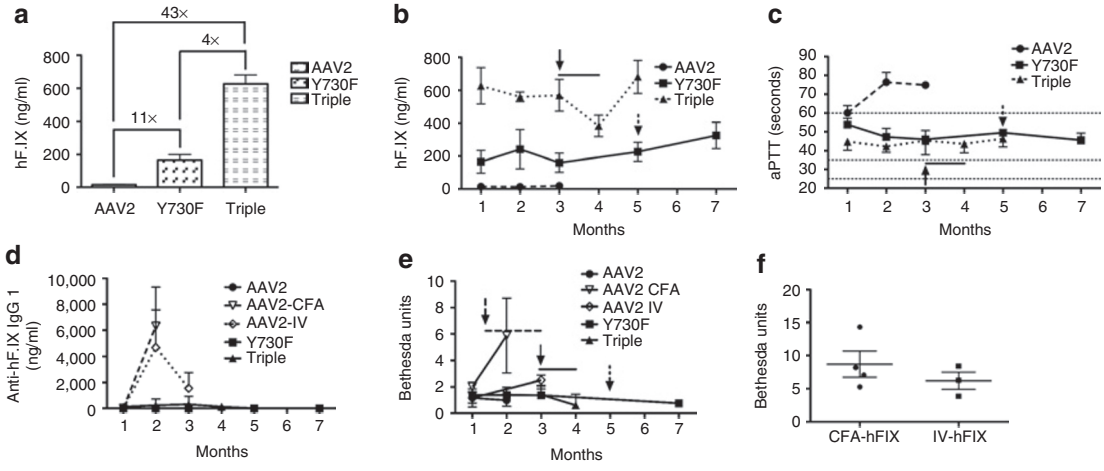

Figure 4 Systemic expression and humoral immune response to hF.IX following peripheral vein delivery of WT AAV2, Y730F, and triple-mutant ssAAV2-ApoE/hAAT-hF.IX vector. (**a**) hF.IX expression (mean ± SD) in HB mice 4 weeks following injection of 2 × 10^{11} vgs of WT (n = 3), Y730F (n = 4), or triple-mutant (n = 4) AAV2 vectors via the tail vein. (**b**) hF.IX expression (mean ± SD) in C3H/HeJ/F9$^{-/-}$ mice following injection of 2 × 10^{11} vgs/animal of WT, Y730F, or triple-mutant AAV2 vectors via the tail vein. (**c**) Coagulation times (aPTT in seconds, mean ± SD) (**b**,**c**) Y730F-injected mice were challenged at indicated time point (dotted arrow) with hF.IX in CFA. Triple-mutant transduced mice were challenged at indicated time point with repeated administration of hF.IX (solid arrow and line). (**d**) Antibody titers (IgG 1 mean ± SD) against hF.IX as a function of time after WT, Y730F, or triple-mutant AAV2 vector administration. Note that one mouse injected with the triple-mutant AAV2 vector formed a transient low-titer antibody. (**e**) Inhibitors to hF.IX as measured by Bethesda assay. AAV2 injected mice challenged at indicated time point with either hF.IX/CFA (n = 4, dashed arrow) or with repeated hF.IX (n = 3, dashed line). Y730F-injected mice were challenged at indicated time point dotted with hF.IX/CFA (dotted arrow). Triple-mutant transduced mice were challenged with repeated hF.IX (solid arrow and line). (**f**) Bethesda titers in naive (i.e., nontransduced) HB mice challenged with hF.IX/CFA or with repeated hF.IX administration (one i.p. injection followed by three weekly i.v. injections). AAV2, adeno-associated virus type 2; ApoE, apolipoprotein E; aPTT, activated partial thromboplastin times; BU, Bethesda unit; CFA, complete Freund's adjuvant; HB, hemophilia B; hF.IX, human factor IX; i.p., intraperitoneal; i.v., intravascular; scAAV2, self-complementary AAV2.

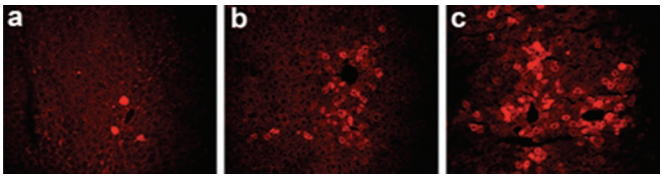

Figure 5 Representative immunofluorescence staining of hF.IX (red) in liver cross-sections after gene transfer to C3H/HeJ/F9$^{-/-}$ mice with (a) WT AAV2 3 months, (b) Y730F AAV2 7 months, or (c) triple-mutant AAV2 5 months following gene transfer. Original magnification: ×200. AAV2, adeno-associated virus type 2; hF.IX, human factor IX; WT, wild-type.

percentage of hF.IX expressing hepatoctyes were 0.8 ± 0.5, 5.3 ± 0.9, and 7.9 ± 0.9%, respectively (n = 3 animals per vector). Comparison of systemic expression and immunostaining suggests that the increased levels of hF.IX expression in the triple-mutant vector-injected mice were likely derived from both an increased number of transduced hepatocytes and a higher level of expression per cell. Indeed, quantitative PCR analyses performed to detect the presence of AAV genomes in liver tissue revealed the following: WT AAV2 (0.26 copy/diploid genome, n = 2), Y730F (0.32 copy/diploid genome, n = 2), and triple-mutant (2.61 copy/diploid genome, n = 3) in injected HB mice (data not shown). These studies indicate higher efficiency of gene transfer in murine hepatocytes with the triple-mutant capsid, which are consistent with our additional studies with murine fibroblasts.[32]

DISCUSSION

In recent years, recombinant AAV2 vectors have been widely used in human gene therapy clinical trials. For certain target tissues such as muscle or liver, high vector doses are needed in order to achieve clinical efficacy. Alternate serotype vectors have been developed with superior transduction efficiency in mice, which, however, have not consistently scaled up to large animals. Molecular shuffling and directed evolution technologies have been applied to develop libraries of chimeric capsids that can be screened for vectors with novel properties. Alternatively, one can take advantage of improved knowledge of AAV vector biology and vector-target cell interactions to develop more efficient and potentially less immunogenic vectors. Here, we describe AAV serotype 2 vectors with optimized tyrosine-mutant capsids, which further improve *in vivo* gene transfer to hepatocytes. Moreover, using a murine model of the X-linked bleeding disorder HB, we demonstrate the usefulness of such vectors for the treatment of a genetic disease.

The role of tyrosine-phosphorylation in capsid processing and intracellular trafficking of AAV vectors

A number of investigators have taken systematic approaches to elucidate some of the fundamental steps in the life cycle of AAV, which in turn have led to the development of novel viral vectors. The ubiquitin/proteasome pathway has been shown to play an essential role in AAV2 nuclear transport.[33–35] In the past years, we have shown that specific inhibition of epidermal growth factor receptor protein tyrosine kinase dramatically increases transduction efficiency with both ssAAV and scAAV vectors.[18,36–39] In a recent series of studies, we documented that during the process of navigation through the late endosome, epidermal growth factor receptor protein tyrosine kinase-mediated tyrosine phosphorylation of AAV2 capsid proteins promotes ubiquitination and degradation of AAV2, thus leading to impairment of viral nuclear transport and decrease in transduction efficiency in intact cells.[20,22] Based on these studies,[20] we mutated each of the seven surface-exposed tyrosine residues on viral capsids, which yield AAV2 vectors with significantly increased transduction efficiency *in vitro* as well as *in vivo*, compared with their WT counterpart.[24] Specifically, Y444F, Y500F, and Y730F mutant vectors are the most efficient, which suggests that these tyrosine residues constitute the primary signal for ubiquitination of the AAV2 capsid proteins. Indeed, these capsids largely bypass the ubiquitination step, which results in a significantly improved intracellular trafficking and delivery of the viral genome to the nucleus.[24]

Tyrosine-mutant AAV vectors for efficient hepatic gene transfer

In this new study, multiple Y-F mutants were screened for the formation of infectious viral particles and tested for a further augmentation of transduction efficiency. Importantly, all combinations of tyrosine mutants resulted in infectious viral particles, indicating that these tyrosine residues are not crucial for viral particle assembly. Interestingly, there were several combinations of tyrosine-mutants that resulted in similar or marginally better transgene expression when compared with the respective single tyrosine-mutants (**Figure 1b,d**). It is not unexpected that certain combinations of tyrosine-mutations may not have an additive effect on viral infectivity, as it is difficult to predict the effect on viral particle structure or interactions with cellular proteins by mutating multiple tyrosine residues. Although a link has been established between phosphorylated surfaced-exposed tyrosine residues and ubiquitination of capsid, it is unclear how this signal is relayed and which of these residues are predominately phosphorylated post entry. Out of all the tested tyrosine-mutant combinations, the triple-mutant (Y444+500+730F) vector resulted in the highest gene transfer efficiency in HeLa cells and in a murine hepatocyte cell line H2.35 *in vitro* and murine hepatocytes *in vivo*. Because the relative increase in transduction efficiency of triple-mutant ssAAV2 and scAAV2 vectors was not significantly different when compared with their WT capsid counterpart AAV2 vectors, these results suggest that the improved gene transfer efficiency gained from tyrosine-mutations in the capsids is independent of second-strand DNA synthesis. Our accumulating data support the hypothesis that phosphorylation of the Y444, Y500, and Y730 residues is critically involved in regulating AAV2 capsid ubiquitination, proteasome-mediated degradation, intracellular trafficking, and nuclear transport of viral particles.

Hepatic gene transfer of triple-mutant capsid AAV vectors for the potential treatment of HB

Hepatic administration of AAV2 vectors expressing F.IX has been successfully used to achieve long-term correction of HB in small and large animal models.[40] However, in a clinical trial on treatment of humans with severe HB (<1% hF.IX activity), an AAV2 vector dose of 2 × 10^{12} vgs/kg resulted in transient expression of hF.IX at >10% of normal coagulation activity for ~1 month, followed by transaminitis and return of hF.IX to baseline levels.[7]

Patients given a lower dose of 8×10^{10} or 4×10^{11} vgs/kg either did not experience an episode of transaminitis or experienced a mild low-grade transaminitis. Follow-up studies strongly suggested that the transaminitis was due to a cytotoxic T-lymphocyte response against AAV2 input capsid antigen displayed on transduced hepatocytes.[21,23] Data from the human trial also suggested that a reduction in the viral dose could avoid triggering an immune response against the viral capsid protein. However, such an outcome may not only depend on the vector being able to enter the target cell at lower doses but also on intracellular trafficking. Therefore, it is desirable to further develop viral capsids that are less prone to ubiquitination and proteasomal degradation. Indeed, our more recent *in vitro* studies with human and murine cells have revealed that the extent of ubiquitination and cytoplasmic accumulation of the triple-mutant vectors is further reduced, and as a consequence, these vectors gain more efficient entry into the nucleus.[32]

Here, we present data showing that hepatic transfer of a transgene cassette identical to the one used in the clinical trial resulted in much more robust expression in mice using single- or further optimized tyrosine–mutant capsids. Compared with Y730F, the triple-mutant capsid vector improved systemic hF.IX expression by an additional threefold, therefore representing an improvement of at least 30-fold compared with the WT AAV2 capsid vector. In addition, expression in the therapeutic range and partial correction of the coagulation defect was sustained in a strain of mice that exhibits stronger B and T-cell responses to hF.IX in gene or protein therapy compared with other strains.[29] For example, delivery of the AAV2 vector to the liver via the portal circulation results in induction of immune tolerance to hF.IX in C57BL/6, BALB/c, and other strains carrying the identical *F9* gene deletion.[27,41] In the C3H/HeJ/F9$^{-/-}$ strain used here, systemic expression of hF.IX from this combination of route and vector is limited by formation of inhibitory antibodies (inhibitors) against hF.IX and a CD8^{+} T-cell response targeting hF.IX-expressing hepatocytes (although these responses are weaker compared with other routes of administration).[29] Because we did not find inhibitors to hF.IX in WT AAV2 vector-treated mice here, but nonetheless observed loss of expression by 2 months, it is possible that, similar to portal vein administration, a CD8^{+} T-cell response to hF.IX eliminated a portion of transduced hepatocytes.[30] Another consideration is that peripheral vein administration of the WT AAV2 vector in the C3H/HeJ background results in less hepatocyte gene transfer compared with the portal vein route. The resulting low level of hF.IX expression may have been insufficient for stimulation of B cells. Nonetheless, in contrast to Y730F and triple–mutant injected HB mice, the WT AAV2 vector-injected mice developed inhibitors after being subsequently challenged with hF.IX protein, and therefore, lacked tolerance (**Figure 4d,e**).

Recently, we showed that AAV8 vector, which transduces murine hepatocytes much more efficiently than AAV2, directed stable expression and induced tolerance to hF.IX in C3H/HeJ/F9$^{-/-}$ mice over a dose range of 2×10^{9}–1×10^{11} vg resulting in ~9–20-fold increase in hF.IX protein expression per vector particle as compared with the results shown in **Figure 4d** for triple-mutant vector.[42] A similar (approximately fivefold) increase in EGFP transgene expression in hepatocytes of C57BL/6 mice was also observed using scAAV8 compared with triple-mutant scAAV2 vector (**Supplementary Figure S1**). These results are in contrast to the 500-fold dose advantage in hF.IX expression found for peripheral vein administration of AAV8 when compared with WT AAV2 capsid. It is encouraging that AAV2 vectors with mutations of surface-exposed tyrosines are approaching expression levels obtained with AAV8 vectors, directing both long-term expression and immune tolerance. We suggest that different approaches that improve the ratio of transgene expression to vector dose can aid in achieving tolerance to hepatocyte-derived antigen. Y-F vector-transduced HB mice not only remained unresponsive to hF.IX after challenge with antigen in adjuvant, but also did not show severe and deadly hypersensitive responses that we had observed after repeated i.v. injection of hF.IX protein in this strain.[30]

Because hepatic gene transfer is less efficient upon tail vein injection for AAV2 vectors, we have not been able before to achieve therapeutic levels of expression in the less efficiently transduced C3H/HeJ strain with AAV2 vectors using this route of administration, and instead had to use surgery for delivery into the portal circulation. With these improved AAV2-based vectors, it is now feasible to utilize a peripheral vein, thereby reducing invasiveness of the protocol. However, the effectiveness of such vectors using different routes of administration still remains to be evaluated in large animal models such as HB dogs before their clinical application. In addition, since tyrosine-mutant AAV2 vectors are not expected to circumvent neutralization by pre-existing neutralizing antibodies to AAV2 capsids in the human population, it would be interesting to test the effects of analogous tyrosine-mutations on hepatic gene transfer with other AAV serotypes or shuffled/hybrid capsids.

In summary, our data strongly suggest that mutagenesis of critical tyrosine residues on AAV2 capsids is a key strategy by which highly efficient transduction of target cells can be achieved at reduced vector doses, which has important implications in the potentially safe and efficacious use of tyrosine-mutant vectors in human gene therapy.

MATERIALS AND METHODS

Construction of surface-exposed tyrosine residue mutant AAV2 capsid plasmids. A two-stage procedure, based on QuikChange II site-directed mutagenesis (Stratagene, La Jolla, CA) was performed using plasmid pACG-2 as described previously.[43] Briefly, in stage I, two PCR extension reactions were performed in separate tubes for each mutant. One tube contained the forward PCR primer and the other contained the reverse primer as described previously.[24] In stage II, the two reactions were mixed and a standard PCR mutagenesis assay was carried out as per the manufacturer's instructions. PCR primers were designed to introduce changes from tyrosine to phenylalanine residues as well as a silent change to create a new restriction endonuclease site for screening purposes.[24] All multiple-mutant AAV2 capsid plasmids were constructed using methods described above or standard subcloning strategies. All mutants were screened with the appropriate restriction enzyme and were sequenced before use.

Recombinant AAV2 vectors. Highly purified stocks of ssAAV2 and scAAV2 vectors with the WT, single-, and multiple-mutant capsids containing the enhanced green fluorescence protein (*EGFP*) gene driven by the chicken β-actin promoter (scAAV2-EGFP), and ssAAV2-ApoE/hAAT-hF.IX vectors containing the hepatocyte-specific expression cassette for hF.IX (hF.IX cDNA, a 1.4-kb portion of intron I of the *F.IX* gene),[7] an ApoE enhancer/hepatocyte control region, a hAAT promoter, and the bovine growth

hormone poly(A) signal were produced by triple transfection of HEK-293 cells, purified by CsCl gradient centrifugation, filter sterilized, and quantified by slot–blot hybridization as described.[44]

***Recombinant AAV2 vector transduction assays* in vitro.** Approximately 1×10^5 HeLa or H2.35 cells were used for transductions with recombinant AAV2 vectors as described previously.[23,24,26] The transduction efficiency was measured 48 hours post-transduction by EGFP imaging using fluorescence microscopy. Images from three to five visual fields were analyzed quantitatively by ImageJ analysis software (NIH, Bethesda, MD). Transgene expression was assessed as total area of green fluorescence (pixel^2) per visual field (mean ± SD). Statistical analysis was performed using a one-way analysis of variance and Dunnett's multiple comparison test to determine which multiple tyrosine-mutants provided significantly higher transgene expression ($P < 0.001$) compared with the Y730F single tyrosine-mutant vector.

Animal strains and experiments. All vectors were injected into the tail vein. C57BL/6 mice received ss and scAAV2-EGFP vectors at 1×10^9 or 1×10^{10} vg/animal. Liver sections from three hepatic lobes of the mock-injected and injected mice 2 weeks after injection were mounted on slides. Transgene expression was measured by EGFP imaging as described above. For hF.IX expression, ssAAV2-ApoE/hAAT-FI.X vectors were injected in C57BL/6 at 2×10^{10} or C3H/HeJ/F9$^{-/-}$ mice at 2×10^{11} vg/animal. Plasma samples from C57BL/6 mice were obtained by retro-orbital bleed or from C3H/HeJ/F9$^{-/-}$ mice in 0.38% sodium citrate collected from the tail as described previously.[27] Immunizations were done either by subcutaneous injection of 5 µg of recombinant hF.IX (BeneFix; Wyeth Pharmaceuticals, Philadelphia, PA) protein formulated in complete Freund's adjuvant CFA (Sigma 5881) or by a single intraperitoneal injection followed by three weekly i.v. injections of 5 µg of recombinant hF.IX. Naive HB mice were challenged as described above with the exception that following the second i.v. hF.IX injection, mice received an injection of antihistamine and a platelet-activating factor antagonist 10 minutes before hF.IX injection as previously described.[31] All animal experiments were conducted in accordance with the University of Florida Institutional Animal Care and Use Committee guidelines.

Analyses of plasma samples. Enzyme-linked immunosorbent assay-based measurements of hF.IX antigen and anti-hF.IX IgG levels in murine plasma samples were performed as described previously.[27] The enzyme-linked immunosorbent assay for anti-hF.IX IgG was sensitive to ~200 ng/ml. Measurements of the activated partial thromboplastin time was performed using a fibrometer also as described.[45] The Bethesda assay was used to measure titers of inhibitory antibodies against hF.IX as previously described.[29]

Immunohistochemistry. Liver tissue was frozen in optimal cutting temperature in a dry ice-isopentene bath. For hF.IX staining, cryosections of liver tissue were fixed/permeabilized with acetone for 10 minutes at room temperature. Sections were blocked with 5% donkey serum in phosphate-buffered saline for 30 minutes. Goat anti-hF.IX (1:200; Affinity Biologicals, Ancaster, Ontario, Canada) was applied in 5% donkey serum for 30 minutes. After washing, tissue sections were incubated with a secondary antibody Alex Fluor-568 donkey anti-goat IgG (1:200 dilution; Invitrogen, Oregon, WA). Fluorescence microscopy was performed with a Nikon E800 microscope (Nikon, Tokyo, Japan). Images were captured with a Cool Snap-Pro camera and analyzed with Image Pro-Plus software (Media Cybernetics, Silver Spring, MD). For EGFP staining, cryosections of liver tissue were fixed in 4% paraformaldehyde, permeabilized in 0.25% Triton X-100 in phosphate-buffered saline, and blocked in 5% normal goat serum for 1 hour. Rabbit anti-GFP Alexa Fluor-488 (1:400 dilution A21311; Invitrogen) was applied for 1 hour. Images were captured with a Cool Snap-Pro camera and analyzed with NIS-Elements Software (Nikon).

SUPPLEMENTARY MATERIAL

Figure S1. Representative immunofluorescent staining of EGFP (green) in liver cross-sections 2 weeks following gene transfer of 1×10^{10} vg to C57BL/6 mice with (**a**) triple-mutant scAAV2-EGFP ($n = 3$) or (**b**) scAAV8-EGFP ($n = 3$).

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