

Application of *in-vitro*-cultured primary hepatocytes to evaluate species translatability and AAV transduction mechanisms of action

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Recombinant adeno-associated virus (AAV) is an effective platform for therapeutic gene transfer; however, tissue-tropism differences between species are a challenge for successful translation of preclinical results to humans. We evaluated the use of *in vitro* primary hepatocyte cultures to predict *in vivo* liver-directed AAV expression in different species. We assessed whether *in vitro* AAV transduction assays in cultured primary hepatocytes from mice, nonhuman primates (NHPs), and humans could model *in vivo* liver-directed AAV expression of valoctocogene roxaparvovec (AAV5-hFVIII-SQ), an experimental gene therapy for hemophilia A with a hepatocyte-selective promoter. Relative levels of DNA and RNA in hepatocytes grown *in vitro* correlated with *in vivo* liver transduction across species. Expression in NHP hepatocytes more closely reflected expression in human hepatocytes than in mouse hepatocytes. We used this hepatocyte culture model to assess transduction efficacy of a novel liver-directed AAV capsid across species and identified which of 3 different canine factor VIII vectors produced the most transgene expression. Results were confirmed *in vivo*. Further, we determined mechanisms mediating inhibition of AAV5-hFVIII-SQ expression by concomitant isotretinoin using primary human hepatocytes. These studies support using *in vitro* primary hepatocyte models to predict species translatability of liver-directed AAV gene therapy and improve mechanistic understanding of drug-drug interactions.

INTRODUCTION

Recombinant adeno-associated virus (AAV) vectors are often used in gene therapy to deliver therapeutic transgenes and transduce numerous tissue types across multiple species. Advantages of AAV vectors for gene transduction include lack of pathogenicity and low genome integration frequencies.^{1–4} Studies of AAV vectors of multiple serotypes demonstrate differences in AAV transduction efficiency between mice, nonhuman primates (NHPs), dogs, and humans.^{2,5–10} Thus, using animal data to estimate appropriate dosing in humans can result in an incorrect estimation of doses needed to reach therapeutic levels of transgene expression.¹¹

AAV hepatic transduction for monogenic diseases, such as hemophilia A and B, is being studied in several ongoing clinical trials, as these diseases allow the use of clinically validated biomarkers.^{12–14} Among serotypes studied, AAV5 shows promise for translatability and species scaling when used as a vector for B-domain-deleted (BDD) coagulation factor VIII (FVIII) protein.⁹ The investigational gene therapy valoctocogene roxaparvovec (AAV5-hFVIII-SQ) is a replication-incompetent AAV5 vector containing a >4.9-kb genome encoding a codon-optimized SQ variant of BDD human FVIII (hFVIII-SQ).^{9,13,15} In a phase 1/2 trial, AAV5-hFVIII-SQ showed multiyear FVIII expression and effective bleeding control in 13 patients with severe hemophilia A who received either a 4×10^{13} or 6×10^{13} vg/kg dose.^{13,16} In a phase 3 trial, participants who received 6×10^{13} vg/kg experienced a significant increase from baseline in FVIII activity at 2 years post-infusion, as well as significantly reduced FVIII utilization and bleeding compared with their baseline values while receiving FVIII prophylaxis pre-infusion.^{17,18}

Translatability of AAV transduction and expression across species continues to be a challenge for development of efficacious gene therapy. Because of the extensive differences in AAV tropism across species,² we sought to determine if *in vivo* liver-directed AAV vector expression could be predicted using an *in vitro* primary hepatocyte culture model across different species. Specifically, we evaluated whether *in vitro* primary hepatocyte expression results across species would correlate with *in vivo* findings for AAV5-hFVIII-SQ. In addition, we assessed and compared transduction efficiency of a novel liver-directed capsid between mouse and NHPs *in vitro* and *in vivo* and evaluated different BDD-FVIII vector constructs. We then demonstrated the utility of the primary human hepatocyte model to study drug-AAV5-hFVIII-SQ interaction by investigating the

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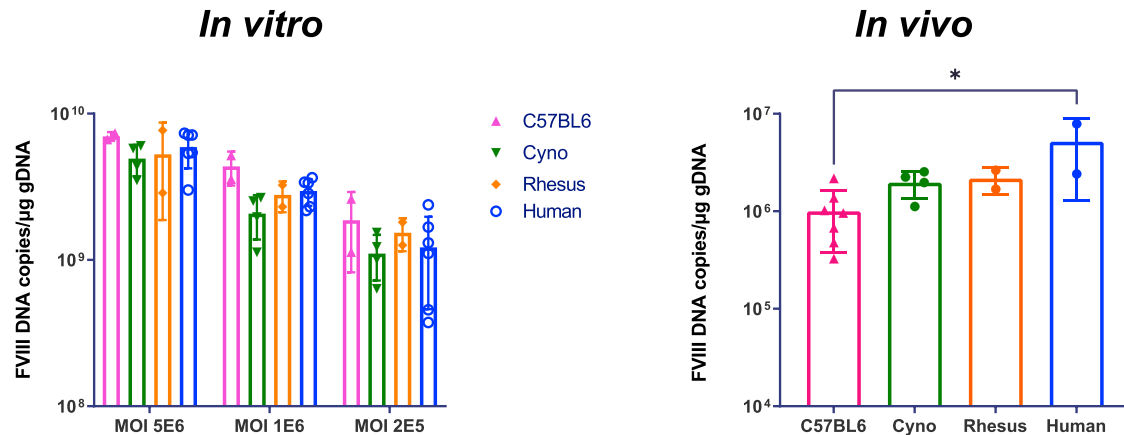


Figure 1. Comparison of hFVIII-SQ vector DNA levels in transduced hepatocytes *in vitro* and *in vivo*

In vitro data are mean \pm SD, where each point represents an individual donor or a pool of donors. *In vitro* data were analyzed by two-way analysis of variance with a subsequent Student's *t* test; within MOIs, there were no significant differences between species. *In vivo* data are mean \pm SD. *In vivo* data were analyzed by two-way analysis of variance, and there were no significant differences between species except for mouse versus human. Cyno, cynomolgus monkey; FVIII, factor VIII; MOI, multiplicity of infection; SD, standard deviation. **p* < 0.05.

mechanisms of action (MOAs) mediating the effects of isotretinoin on FVIII expression.

RESULTS

Characterization and validation of *in vitro* primary hepatocyte models by comparing with *in vivo* data of AAV5-hFVIII-SQ

Cross-species transduction-efficiency variation *in vitro* could be attributed to differing methods of cell culture or transduction. To eliminate this possibility and determine if the same cell culture protocol could be used to transduce primary hepatocytes from humans, cynomolgus monkeys, rhesus macaques, and mice (purchased from Lonza [Basel, Switzerland]), we assessed levels of green fluorescence protein (GFP) expression after transduction with AAV5-CMV-GFP by using 3 different ratios of multiplicity of infection (MOI). MOI ratios of 2×10^5 , 1×10^6 , and 5×10^6 were chosen; with these 3 MOIs, the virus concentrations in cell culture medium were close to theoretical blood vector maximum concentrations of 1.2×10^{13} , 6×10^{13} , and 2×10^{14} vg/kg per dose, respectively, bracketing the doses used in AAV5-hFVIII-SQ clinical trials. At each MOI tested, primary hepatocytes from different species expressed GFP, with human primary hepatocytes having the lowest percentage of cells scoring GFP positive; this was also demonstrated by GFP enzyme-linked immunosorbent assay (ELISA) quantification (Figures S1A and S1B). Overall, cynomolgus monkey and rhesus monkey hepatocytes produced 2.5- to 4-fold less protein, and human hepatocytes produced 24- to 41-fold less protein, than mouse hepatocytes (Figure S1B). Despite the differences in GFP protein expression between species, primary hepatocytes from all species studied were successfully transduced using the same cell culture protocol. Additionally, we tested an AAV8 construct in the *in vitro* system (Figure S1C) and found that AAV8 transduced mouse hepatocytes better than NHP and human hepatocytes, similar to trends previously observed *in vivo*.^{14,19}

To validate the *in vitro* hepatocyte model, we evaluated *in vivo* AAV5-hFVIII-SQ vector transduction using the same cell culture protocol and MOIs. Overall, vector DNA levels demonstrated a dose response in primary hepatocytes across species transduced with various MOIs (Figure 1). At each MOI, the same small relative differences in FVIII DNA copy number were observed across species in transduced primary hepatocytes, with cynomolgus monkey hepatocytes having the lowest copy number. These differences in DNA copy number across species correlated with *in vivo* liver-transduction data.

A dose-response pattern was also observed in FVIII RNA transcript levels in primary hepatocytes following transduction (Figure 2). Relative differences in RNA transcript levels between species were maintained at different MOIs in the hepatocyte models; similar differences were observed with *in vivo* results, even though the MOIs used *in vitro* bracketed the *in vivo* doses rather than corresponded to them exactly (because the dose concentration analysis was done post-dosing, *in vivo* doses represented actual doses administered). FVIII RNA levels observed in cynomolgus monkey and rhesus monkey hepatocytes were approximately 1- to 5-fold less than in murine primary hepatocytes (Figures 2A and 2B); *in vivo*, FVIII RNA levels in cynomolgus monkey and rhesus monkey livers were approximately 3- to 4-fold less than levels in mouse livers at 6–13 weeks post-dose. Compared with monkeys, greater relative differences in FVIII RNA levels were observed *in vitro* and *in vivo* between mice and humans: approximately 21- to 40-fold less in human hepatocytes and 10- to 105-fold less in human livers (Figure 2C). These data show that relative *in vitro* primary hepatocyte transduction efficiency corresponds with relative *in vivo* liver transduction with AAV5-hFVIII-SQ. Levels of FVIII protein in the culture media were below the limit of quantitation (data not shown), and thus we were not able to compare the *in vitro* data with *in vivo* data.

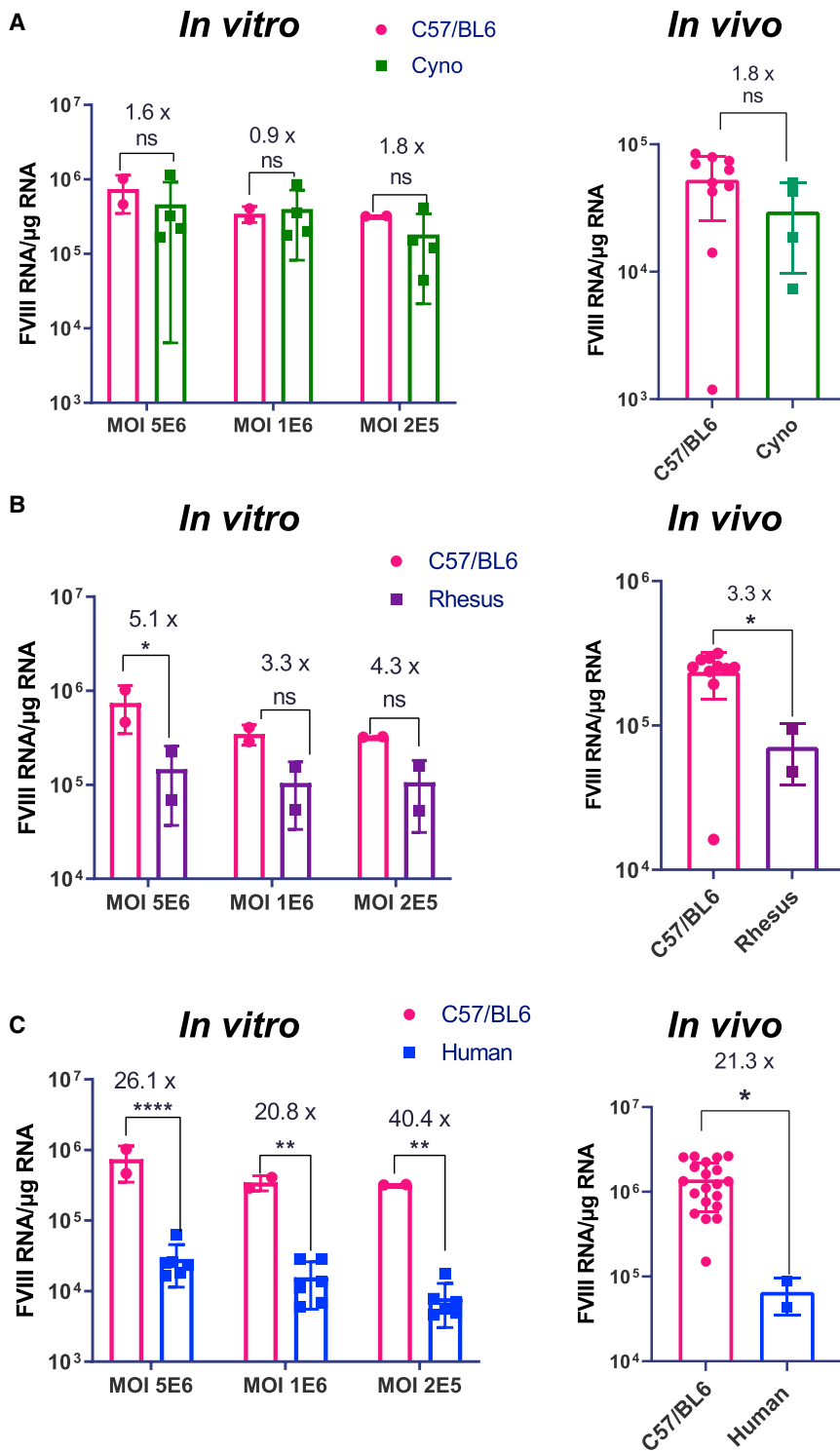


Figure 2. Comparison of hFVIII-SQ vector RNA levels in transduced hepatocytes *in vitro* and *in vivo*. (A–C) Comparison of hFVIII-SQ RNA levels in transduced mouse hepatocytes *in vitro* and *in vivo* to hepatocytes of (A) cynomolgus monkeys, (B) rhesus monkeys, and (C) humans. ns, not significant; * $p < 0.05$, ** $p < 0.005$, and **** $p < 0.0001$. *In vitro* data are mean \pm SD, where each point represents an individual donor or a pool of donors; data were analyzed by two-way analysis of variance with a subsequent Sidak’s multiple comparisons test. *In vivo* data are mean \pm SD; data were analyzed by Student’s t-test. Cyno, cynomolgus monkey; FVIII, factor VIII; MOI, multiplicity of infection; SD, standard deviation.

beta-chorionic gonadotropin (bCG) reporter gene were assessed: AAV-BMN.L3-hAAT-bCG, which uses a novel liver-targeting capsid, and AAV5-hAAT-bCG, which uses an AAV5 capsid. In murine hepatocytes at an MOI of 1×10^6 , AAV-BMN.L3-hAAT-bCG and AAV5-hAAT-bCG DNA levels were similar, while RNA levels were 5-fold less for AAV-BMN.L3-hAAT-bCG (Figure 3A). In contrast, *in vitro* transduction of cynomolgus monkey hepatocytes was considerably less efficient for AAV-BMN.L3-hAAT-bCG, as DNA levels were 8-fold less and RNA levels 90-fold less than for AAV5-hAAT-bCG. *In vivo*, transduction of either vector in mice at 2×10^{13} vg/kg resulted in similar bCG DNA and RNA levels in liver, but bCG DNA levels in cynomolgus liver transduced with AAV-BMN.L3-hAAT-bCG were 31-fold less than levels in livers transduced with AAV5-hAAT-bCG, resulting in a 98-fold difference in RNA levels (Figure 3B). Thus, the observed relative difference in transduction efficiency between the 2 vectors in mouse and cynomolgus monkey hepatocytes *in vitro* predicted transduction efficiency *in vivo* in these 2 species. These results suggest that primary hepatocyte cell cultures may serve as a beneficial *in vitro* tool to assess the transduction efficiency of liver-targeted AAV capsids across species.

Comparison of AAV5-canine FVIII vectors to identify the optimal expression constructs

Next, we evaluated the utility of the primary hepatocyte cell culture system to select the optimal expression constructs among 3 different AAV5-canine (c) FVIII vector constructs in hemophilia A model mice and dogs: HLP-nco-cFVIII-SQ (construct 1), HLP-co-cFVIII-SQ (construct 2), and HLP-co-cFVIII-V3 (construct 3). Construct 1 uses a non-codon-optimized cFVIII,

Comparison of novel liver-directed AAV capsid and AAV5

Following successful *in vitro* transduction of primary hepatocytes with AAV5-hFVIII-SQ, the applicability of this model to screen novel capsids was assessed. Two vectors delivering a cynomolgus monkey

different AAV5-canine (c) FVIII vector constructs in hemophilia A model mice and dogs: HLP-nco-cFVIII-SQ (construct 1), HLP-co-cFVIII-SQ (construct 2), and HLP-co-cFVIII-V3 (construct 3). Construct 1 uses a non-codon-optimized cFVIII,

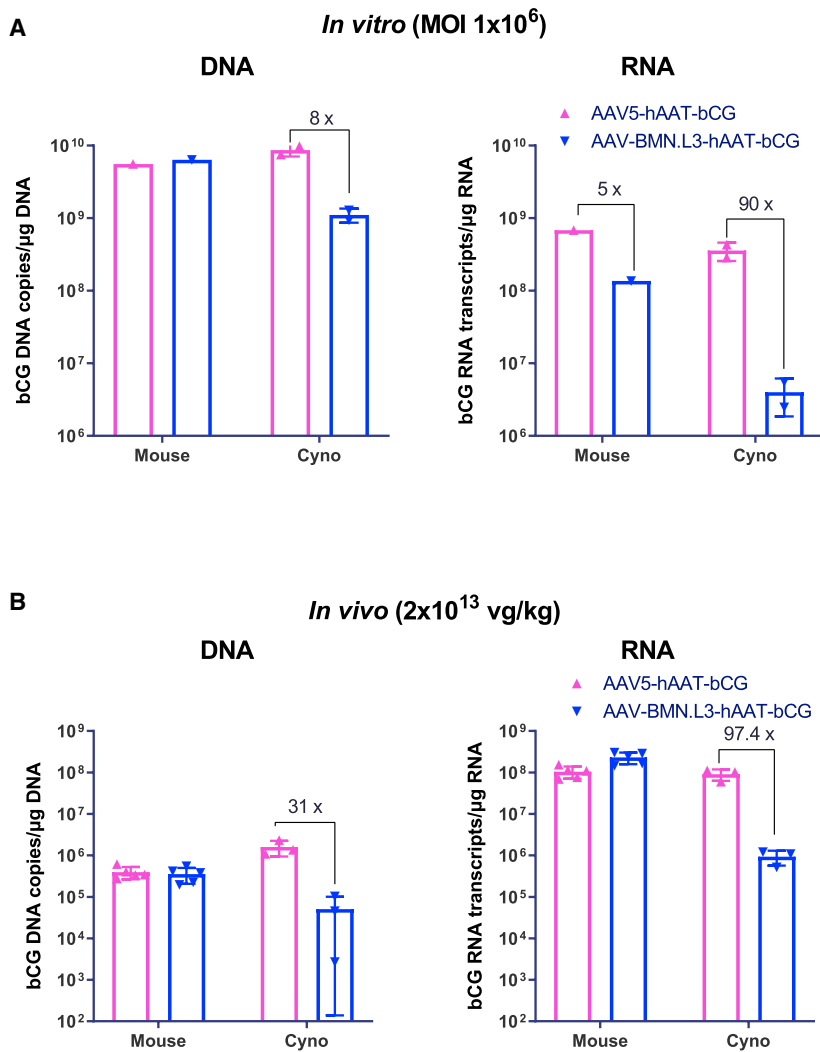


Figure 3. Transduction of a novel AAV capsid in hepatocytes *in vitro* and *in vivo*

(A and B) Comparison of transduction efficiency between a novel AAV capsid (AAV-BMN.L3-hAAT-bCG) with AAV5-hAAT-bCG using bCG as a reporter gene in mouse and cynomolgus monkey showing (A) DNA copy numbers and RNA transcripts in *in vitro* primary hepatocytes and (B) liver DNA copy numbers and RNA transcripts *in vivo*. Data are mean \pm SD. AAV, adeno-associated virus; bCG, beta-chorionic gonadotropin; Cyno, cynomolgus monkey; SD, standard deviation.

constructs 2 and 3 had significantly higher RNA transcript levels than construct 1 ($p < 0.01$; Figure 4D). At week 26, FVIII activity in plasma in hemophilia A dogs dosed with AAV5-cFVIII was greater with constructs 2 and 3 compared with construct 1 (Figure 4E), mirroring the *in vitro* RNA results. In the primary *in vitro* system, cFVIII-SQ protein was not detected, likely because amounts were below the assay detection threshold. Thus, the RNA results from the *in vitro* hepatocyte model predicted the *in vivo* outcomes for 3 different constructs in mice and dogs.

Effect of isotretinoin on FVIII expression

In the phase 3 trial of AAV5-hFVIII-SQ (NCT 03370913), concomitant isotretinoin, a synthetic retinoid used to treat severe acne, temporarily decreased FVIII activity without alanine aminotransferase (ALT) elevation in 1 participant. Specifically, this participant's FVIII activity, as measured by chromogenic substrate assay, declined from 75 IU/dL at week 62 to <3 IU/dL at week 64 after initiating isotretinoin. Following discontinuation of isotretinoin at week 72, his FVIII activity partially recovered to 46

IU/dL at week 122 (Figure 5A). We hypothesized that an interaction between isotretinoin and AAV5-hFVIII-SQ may contribute to the observed change in FVIII activity, although no such interaction has been reported before.

We therefore used the primary human hepatocyte *in vitro* model to understand the potential mechanisms mediating the temporal effects of isotretinoin on FVIII transgene expression to explain these results observed during the clinical trial. The effect of isotretinoin could be through modulation at the vector genome level, on transcription from AAV5-hFVIII-SQ, over FVIII-SQ protein production and/or secretion, through direct inhibition of circulating FVIII activity, or through interference with the chromogenic assay. Incubation using different doses of all-*trans* retinoic acid (ATRA), the most active metabolite of isotretinoin, with recombinant FVIII-SQ protein spiked in human FVIII-deficient plasma demonstrated that ATRA did not directly suppress FVIII activity (Figure S2A).

while constructs 2 and 3 use codon-optimized cFVIII; construct 3 also contains an additional V3 linker sequence that facilitates transit through the endoplasmic reticulum.²⁰ Using an MOI of 1×10^6 for *in vitro* transduction, all 3 vector constructs demonstrated similar FVIII DNA copy numbers in mouse hepatocytes. However, when examining RNA levels in mouse hepatocytes across constructs, construct 1 produced the lowest level of RNA transcripts. In particular, both constructs 2 and 3 produced approximately 8-fold greater *in vitro* RNA levels compared with construct 1 ($p < 0.0001$; Figure 4A). These *in vitro* results correlated with *in vivo* data, with all 3 constructs showing comparable *in vivo* DNA copy numbers in mice, but constructs 2 and 3 had significantly greater RNA transcript numbers compared with construct 1 ($p < 0.05$ and $p < 0.01$, respectively; Figure 4B). In addition, FVIII plasma protein levels were significantly higher in mice dosed with constructs 2 and 3 versus construct 1 ($p < 0.01$; Figure 4C). Similarly, in dog primary hepatocytes, all 3 vector constructs demonstrated similar FVIII DNA copy numbers, while

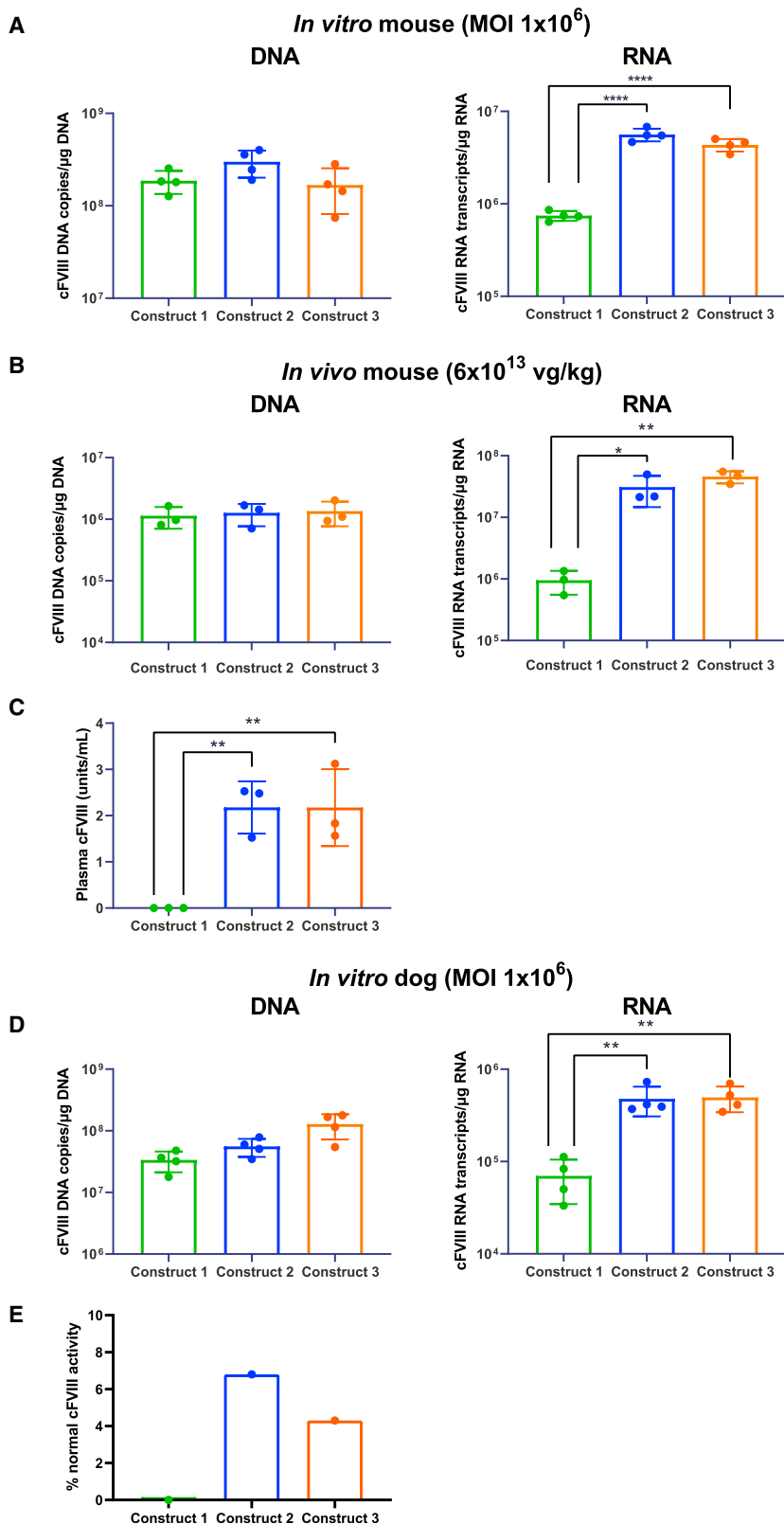


Figure 4. Assessment of transduction of cFVIII constructs in hepatocytes *in vitro* and *in vivo* (A–E) Comparison of cFVIII DNA and RNA for 3 vector constructs for (A) *in vitro* mouse hepatocytes and (B) *in vivo* mouse liver, (C) canine FVIII protein in mouse plasma, (D) cFVIII DNA and RNA dose response for *in vitro* canine hepatocytes, and (E) FVIII activity in dog plasma. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$. Data were analyzed with a one-way analysis of variance. Construct 1 is HLP-nco-cFVIII-SQ, construct 2 is HLP-co-cFVIII-SQ, and construct 3 is HLP-co-cFVIII-V3. Data are mean \pm SD. In (E), data are from single dogs; one dog per construct. Co, codon-optimized; cFVIII, canine FVIII; FVIII, factor VIII; HLP, hybrid liver promoter; MOI, multiplicity of infection; nco, non-codon-optimized; SD, standard deviation.

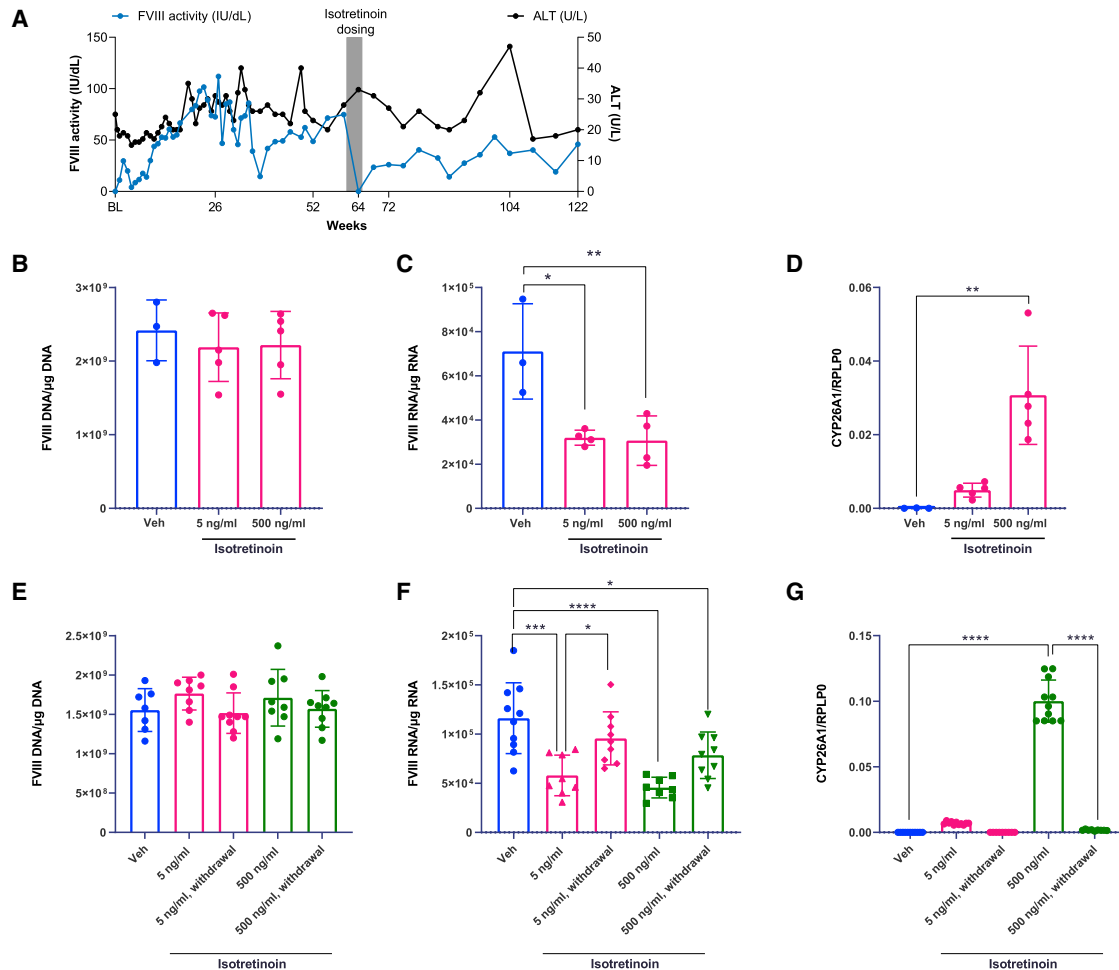


Figure 5. Investigation of the effect of isotretinoin on FVIII expression using the *in vitro* model.

(A) Clinical outcomes and isotretinoin use in a clinical trial participant who received a single 6×10^{13} vg/kg dose of valoctocogene roxaparvec. (B–G) Effects of 2-day isotretinoin treatment on (B) FVIII DNA copies, (C) FVIII RNA transcripts, and (D) CYP26A1 gene expression, and 4 days post-isotretinoin removal on (E) FVIII DNA copies, (F) FVIII RNA transcripts, and (G) CYP26A1 gene expression in transduced human hepatocytes. * $p < 0.05$, *** $p < 0.005$, and **** $p < 0.0001$. Data are mean \pm SD. Data were analyzed with a one-way ANOVA. FVIII activity was measured per CS assay (LLOQ, <3 IU/dL). In the 5- and 500-ng/mL groups, cells were collected for analysis after 2 days of treatment with isotretinoin. In the withdrawal groups, isotretinoin was applied for 2 days, then treatment was withdrawn for 4 days, and cells were collected for analysis. ALT, alanine aminotransferase; BL, baseline; CS, chromogenic substrate; FVIII, factor VIII; LLOQ, lower limit of quantitation; SD, standard deviation; Veh, vehicle.

In human primary hepatocytes transduced with AAV5-FVIII-SQ at an MOI of 1×10^6 , isotretinoin at doses of 5 and 500 ng/mL (doses correspond to $0.01 \times$ and $1 \times C_{max}$ per suggested dosing, respectively²¹) had no effect on FVIII vector DNA levels (Figure 5B). However, isotretinoin significantly decreased FVIII RNA levels by approximately 50% ($p < 0.05$ and $p < 0.01$ at 5 and 500 ng/mL, respectively; Figure 5C). Interestingly, the suppression of FVIII RNA levels by isotretinoin did not occur in a dose-dependent manner, unlike the typical canonical response shown by CYP26A1, a direct target gene of retinoic acid receptors (Figure 5D).²² Following removal of isotretinoin, FVIII DNA copy numbers remained unchanged (Figure 5E). For high-dose isotretinoin treatment, FVIII RNA levels showed a partial recovery approximately 4 days following isotretinoin withdrawal, but for cells treated with low-dose isotretinoin that was subsequently

withdrawn, FVIII RNA levels showed a reversal to a level similar to vehicle-treated controls ($p > 0.05$; Figure 5F). The CYP26A1 mRNA level dropped to basal levels 4 days following isotretinoin withdrawal at either low- or high-dose treatment (Figure 5G). Additionally, no substantial changes in ALT levels were observed following treatment with both low- and high-dose isotretinoin, consistent with results in the clinical trial participant, suggesting no direct toxicity to hepatocytes and consistent with no change in the amount of vector DNA (Figure S2B).

In summary, results from the study assessing isotretinoin suppression of FVIII expression in human hepatocytes demonstrated that isotretinoin did not decrease hFVIII-SQ vector DNA but did decrease hFVIII-SQ RNA levels by approximately 50%. With high-dose

isotretinoin, this effect was partially reversed with the removal of isotretinoin, consistent with clinical observations. As the 500 ng/mL concentration that corresponds to $1 \times C_{\max}$, this higher dose likely mimics the clinical blood concentration resulting from daily isotretinoin dosing more accurately than the lower dose.

DISCUSSION

In this study, we demonstrated that the same cell culture conditions and protocol can be used to transduce primary hepatocytes derived from mice, cynomolgus monkeys, rhesus monkeys, dogs, and humans. While AAV-driven GFP protein expression levels varied across species, with mouse hepatocytes producing the most GFP and human the least, all were successfully transduced using the same methods. Moreover, *in vitro* cell cultures of primary hepatocytes from these species were successfully transduced with AAV5-FVIII-SQ, and in general, FVIII DNA and RNA copy numbers showed a dose response in primary hepatocytes transduced with different MOIs. Although FVIII protein was not detected here *in vitro*, liver FVIII RNA correlates with secreted FVIII protein.⁹ In all 4 species, the FVIII DNA and RNA results from the *in vitro* primary hepatocyte transduction experiment corresponded with *in vivo* liver transduction. We also demonstrated the utility of the *in vitro* hepatocyte model system for screening *in vivo* transduction efficiency of liver-directed novel capsids in mice and cynomolgus monkeys; again, *in vitro* results successfully modeled those observed *in vivo*. The *in vitro* system also successfully recapitulated *in vivo* outcomes of 3 different AAV5-c FVIII vector constructs in mice and dogs. Finally, we used the *in vitro* primary human hepatocyte model to investigate the effects of isotretinoin on FVIII expression following AAV5-FVIII-SQ transduction, illustrating the utility of this model in investigating potential drug-drug interactions following gene therapy and the mechanism underlying these. Overall, our results suggest that the *in vitro* primary hepatocyte model can be used to screen novel liver-targeted AAV capsids and will support future study of the MOA of liver-directed AAV gene therapy.

Current challenges with liver-directed AAV gene therapy include sustained expression, tropism translatability, limited efficiency in targeting of hepatocytes, and variability of response.^{2,5-10,13-15,23} The model system we present here provides an *in vitro* platform utilizing readily accessible primary hepatocytes. This system can be used to address the challenges of AAV gene therapy by more easily investigating transduction and transgene expression efficiency cross-species using novel AAV capsids and vectors, with rapid results that predict those seen *in vivo*. Our results suggest that *in vitro* RNA expression is a better predictor than DNA levels for *in vivo* results. This *in vitro* model can therefore be used to facilitate rapid screening, and promising constructs can be rapidly identified and prioritized for further experimentation. Furthermore, the human primary hepatocyte model offers an accessible platform for investigating molecular processes involved in transgene expression, such as drug-drug interactions and inter-subject variability by using multiple donors. We have recently applied this *in vitro* hepatocyte model to identify the mechanisms of interactions between the antiretroviral drug efavirenz and AAV5-hFVIII-SQ

that lead to loss of transgene expression in a clinical trial participant included the suppression of transcription.²⁴

A limitation of this study is that systemic effects, such as immunogenicity and tissue distribution, cannot be investigated. Other than enforcing multiple washing steps, no extra procedures were taken to remove untransduced AAV particles sticking outside of hepatocytes in our experiments, which might lead to an over-estimation of vector copy numbers in the system.²⁵ The short culture duration of this model also limited our ability to determine long-term transgene expression. This model was also not designed to predict or guide clinical dosing. Although we have some preliminary data suggesting the transduction of AAV8 in our system also displays a relative trend in potency (mouse > monkey > human) between different species corresponding to published *in vivo* results,^{14,19} the application of this system for other AAV serotypes needs further investigation. The relatively weak capacity of the *in vitro* model to synthesize, fold, or secrete large AAV-mediated proteins such as FVIII, as observed here, limits evaluation that can be done downstream of transduction and transcription for these transgenes. In particular, FVIII protein is known to be poorly secreted from the endoplasmic reticulum, and hepatocytes might not be equipped to produce and secrete FVIII protein efficiently, limiting the utility of this *in vitro* model to assess FVIII protein levels secreted in the media.²⁶ It should also be noted that use of codon-optimized sequences would have increased RNA expression. In the study examining cFVIII constructs, the primary hepatocytes used in the *in vitro* analyses did not come from hemophilia models; future establishment of *in vitro* hepatocyte cell culture lines from hemophilia A model mice and dogs may prove to be a useful tool. Finally, *in vivo*, multiple liver cell types spatiotemporally cooperate with hepatocytes to maintain liver functions, including hepatic stellate cells, Kupffer cells, and liver sinusoidal endothelial cells; this may impact AAV transduction, expression, secretion, and immune response *in vivo*.¹² The current *in vitro* primary hepatocyte model does not reflect this complicated *in vivo* environment, but efforts are being made to develop new culture models such as micro-organ systems that incorporate various cell types into primary hepatocyte cultures and mimic the liver microenvironment, which may further improve the predictive value of the *in vitro* hepatocyte transduction model.

Conclusions

In vitro primary hepatocyte transduction results for AAV5-FVIII-SQ correlated with previous *in vivo* liver-transduction data in all species tested. Moreover, *in vitro* hepatocyte transduction results for a novel capsid and 3 different AAV5-c FVIII vectors correlated with *in vivo* data, supporting the use of primary hepatocyte models to effectively screen novel liver-targeting capsids and identify better expression constructs. In addition, in experiments applying this model to study drug-drug interactions, isotretinoin did not elicit hepatocyte toxicity and had no effect on FVIII DNA levels but suppressed FVIII RNA transcription in human primary hepatocytes, with FVIII mRNA expression restored approximately 4 days post-isotretinoin removal, consistent with clinical observations. Consequently, *in vitro* primary

hepatocyte models may be an important tool to screen novel liver-targeted AAV capsids and assess MOAs mediating interactions between drugs with AAV vectors.

MATERIALS AND METHODS

Vector genome structure

In this study, vectors utilized to transduce primary hepatocytes included AAV5-CMV-GFP, AAV5-FVIII-SQ (encoding BDD human or canine FVIII transgenes), AAV5-hAAT-bCG, and AAV-BMN.L3-hAAT-bCG (a novel liver-targeting capsid delivering a monkey hAAT-bCG reporter gene). As previously described, AAV5-hFVIII-SQ is a replication-incompetent AAV5 vector containing a >4.9-kb genome encoding a codon-optimized BDD human FVIII.^{9,15} The expression cassette is flanked by AAV2 inverted terminal repeats at the 5' and 3' ends.⁹ All constructs used a hybrid liver promoter.

Ethics statement

All *in vivo* mouse experimentation was performed in accordance with institutional guidelines under protocols approved by the Institutional Animal Care and Use Committee of the Buck Institute. All NHP procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Charles River Laboratory facility. All canine procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Queen's University facility. Human liver biopsy samples were obtained per protocol in the phase 1/2 trial; all participants provided written informed consent, and trial procedures aligned with the Declaration of Helsinki and local regulations.^{15,27}

In vitro study designs

This study was divided into several experiments. In the first set of experiments, the same cell culture condition and protocol were tested to determine their ability to transduce primary hepatocytes from humans, cynomolgus monkeys, rhesus monkeys, dogs, and mice and to determine if *in vitro* primary hepatocyte transduction correlated with *in vivo* liver-transduction data from previous studies for AAV5-hFVIII-SQ for all species included in this experiment.

The next group of experiments evaluated the applicability of the cell culture model to screen the novel capsid AAV-BMN.L3-hAAT-bCG, which delivered a cynomolgus monkey (cyno)-bCG reporter gene, and compared transduction *in vivo* versus *in vitro*. Transduction efficiency with the novel capsid was compared with AAV5-hAAT-bCG, which also delivers a cyno-bCG reporter using AAV5.

The last set of experiments studied transduction of AAV5-hFVIII-SQ into a human model to assess the effect of isotretinoin on FVIII activity and the MOAs mediating suppression of FVIII activity. AAV5-h(human)FVIII-SQ, AAV5-hAAT-bCG, and AAV-BMN.L3-hAAT-bCG were transduced into mouse, human, and NHP primary hepatocytes, while 3 different constructs of AAV5-cFVIII-SQ were transduced into mouse and dog primary hepatocytes.

To investigate the effect of isotretinoin on FVIII activity, *in vitro* transduction of AAV5-hFVIII-SQ at an MOI of 1×10^6 was performed using a human primary hepatocyte model. Six days following transduction, isotretinoin treatment (Sigma, St. Louis, MO, USA) was introduced at a dose of either 500 or 5 ng/mL, and treatment was continued daily, with cell medium collected every other day to measure ALT levels. These doses were chosen to correspond to $0.01 \times$ or $1 \times$ of C_{\max} per the suggested dosage of isotretinoin of 0.5–1.0 mg/kg/day with a mean C_{\max} ranging from 301 to 862 ng/mL and a serum half-life of approximately 21 h.²¹ On day 8, cells were collected for FVIII DNA- and RNA-level expression. For the remaining cells, isotretinoin treatment was halted in half of the cell cultures, while the remainder were treated until day 12 and were analyzed for FVIII DNA- and RNA-level expression.

In vivo liver samples

In the mouse and NHP *in vivo* studies, animals were administered a single vector dose, and liver samples were harvested for FVIII DNA and RNA analysis following euthanasia. Euthanasia was performed at week 8 in mice, week 6 in rhesus monkeys, and week 13 or 26 for cynomolgus monkeys. Human *in vivo* liver biopsies were obtained via transjugular ultrasound-guided percutaneous route at week 140 post-dose from a participant in the phase 1/2 clinical trial.¹⁵ A 1 mm by >2 cm long core tissue sample was obtained, and the sample was flash frozen in liquid nitrogen or an ice/ethanol bath. Samples were stored in a freezer set to maintain -80°C until nucleic-acid isolation.

In the study comparing 3 different constructs of AAV5-cFVIII-SQ, mouse and dog models of hemophilia A were used. Mice were RAG2/FVIII double-knockout mice, as previously described.⁹ Hemophilia A dog models that display genetic and phenotypic similarities to severe hemophilia A in humans, including a naturally occurring intron-22 inversion in the canine *F8* gene and a spontaneous bleeding phenotype, were obtained from Queen's University (Kingston, ON, Canada).^{28–31}

Hepatocyte culture

Primary hepatocytes were purchased from Lonza (Basel, Switzerland). Hepatocyte culture was performed in Bio-Coat collagen I 24-well plates (Corning, Corning, NY, USA), and thawing medium, plating medium, and maintenance medium (Lonza, Basel, Switzerland) were used for hepatocyte cell culture. Cryopreserved hepatocytes were thawed in a water bath set to 37°C for less than 2 min. Once hepatocytes were almost completely thawed, the hepatocyte vial was wiped with 70% alcohol in the biosafety cabinet, and the cells were placed into the thawing medium using a wide-bore pipette tip to transfer. The thawing medium with the hepatocytes was inverted by hand prior to centrifugation. Human hepatocytes were centrifuged at $100 \times g$ for 10 min, NHP cells at $100 \times g$ for 6 min, and mouse hepatocytes at $70 \times g$ for 5 min. Following centrifugation, the supernatant was aspirated, and cells were resuspended in 1 mL plating medium with a wide-bore pipette to achieve single-cell suspension. The suspension

was mixed with 5-mL plating medium, and hepatocytes were diluted for ideal concentration for seeding (target concentration was approximately 1 million \pm 20% cells/mL for humans and NHPs, 1 million \pm 10% cells/well in a 24-well plate for dogs, and 0.45 million \pm 10% cells/well in a 24-well plate for mice). Cells were evenly dispersed in the wells and then incubated.

In vitro AAV transduction

Twenty hours after seeding, plates were removed from the incubator, and medium was aspirated and re-plated with 600 μ L fresh hepatocyte basal medium (HBM). The concentration of AAV virus was calculated based on MOI, and the AAV virus was prepared in the HBM at 2 times the target concentration. Following this, 450 μ L HBM was removed, and 150 μ L AAV containing HBM was added to each well (volume was adjusted for mouse cells to achieve comparable MOIs). After 24 h, medium was removed, and cells were washed with HBM twice before 500 μ L HBM with supplements (Lonza, Basel, Switzerland) were added per well. Cell cultures were maintained by changing medium each day, and 6 days following transduction, cells were collected for measurements.

MOIs at 2×10^5 , 1×10^6 , and 5×10^6 correspond to a theoretical blood vector maximum concentration of 1.2×10^{13} , 6×10^{13} , and 3.5×10^{14} vg/kg, respectively, bracketing the doses used in AAV5-hFVIII-SQ clinical trials. The calculations are based on the following assumptions:

- With a 6×10^{13} vg/kg dose, assuming an average body weight of 70 kg, the participant receives a total of 4.2×10^{15} vg (6×10^{13} vg/kg \times 70 kg)
- Since AAV5-hFVIII-SQ is administered via intravenous (i.v.) injection, all 4.2×10^{15} vg circulate in the blood after dosing
- Assuming that blood is approximately 7%–8% of an adult human's weight, a 70-kg participant has a blood volume of 5.6 L (70 kg \times 8%; 1 kg water is equivalent to 1 L)
- The maximum theoretical C_{\max} of circulating AAV5-hFVIII-SQ in this participant is therefore 8×10^{11} vg/mL, given 4.2×10^{15} vg/5.6 L = 8×10^{14} vg/L = 8×10^{11} vg/mL

For hepatocytes seeded in 24-well plates with 0.4×10^6 cells/well and 0.4 mL medium volume at dosing, the MOI is therefore approximately 0.8×10^6 (8×10^{11} vg/mL \times 0.4 mL/ 0.4×10^6 cells).

Molecular analyses

DNA and RNA were extracted from primary hepatocytes and frozen liver samples using AllPrep DNA/RNA Micro Kit (Qiagen, Hilden, Germany) per the manufacturer's instructions and quantified by droplet digital polymerase chain reaction (ddPCR) and/or reverse transcription followed by quantitative PCR. Roughly 1 pg of DNA was used per ddPCR reaction, but methods were otherwise as previously described.³² Concentration of extracted RNA was measured using a Nanodrop 8000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and Quanti-iT RNA Assay kit (ThermoFisher Scientific) and then diluted to 10 ng/ μ L. For each sample, 160 ng

RNA was reverse transcribed to generate first-strand cDNA using SuperScript VILO Master Mix (Life Technologies, Carlsbad, CA, USA). No amplification (NA) controls were generated for each sample by omitting the reverse transcriptase. The cDNA and NA controls were diluted 1:10 with elution buffer containing 0.05% Pluronic F-68. Each ddPCR reaction used 5 μ L of diluted cDNA, 1 \times ddPCR Supermix for Probes (no dUTP) (Bio-Rad, Hercules, CA, USA), SQ primer sets (250 nM each), and SQ-FAM probe (900 nM) in a final volume of 25 μ L.

A Bio-Rad Auto Droplet Generator was used to generate droplets from reaction mix and QX200 Droplet Generation Oil for Probes (Bio-Rad), which were then transferred into a 96-well plate. PCR was performed in a C1000 Touch Thermal Cycler (Bio-Rad) as follows: 10 min at 95°C, 40 cycles of 30 s at 95°C and 1 min at 58°C, 10 min at 98°C, and hold at 4°C. Samples were read using a QX200 droplet reader (Bio-Rad), and the total concentration of target sequences and linked copies of target sequences were processed with QuantaSoft software (Bio-Rad). Primer and probe sequences are provided in Table S1. CYP26A1 ddPCR primers and probes were purchased from Bio-Rad.

ELISA

Plasma level of cFVIII protein or GFP protein level in cell lysates from AAV5-GFP transduced primary hepatocytes, and ALT level in primary human hepatocyte culture medium, were determined by ELISA according to the manufacturer's instructions. GFP and human ALT ELISA kits were obtained from Abcam (Cambridge, MA, USA), and a Canine FVIII Paired Antibody Set was purchased from Affinity Biologicals (Ancaster, ON, Canada).

FVIII activity assay

Canine FVIII activity was measured using the Biophen (HYPHEN BioMed, France) chromogenic FVIII substrate assay. Study samples were diluted 1:10 in assay buffer and run against a canine pooled (12–20 normal dogs) plasma standard curve generated by serial dilutions using canine FVIII-deficient plasma.

To test the effects of isotretinoin on FVIII activity in the plasma, different concentrations of Xyntha (Wyeth Pharmaceuticals, Madison, NJ, USA; 87.5, 43.75, 21.88, 10.94, 5.47, 2.71, and 1.37 ng/mL), a BDD recombinant FVIII protein, were incubated at 37°C with vehicle or different concentrations of ATRA (Sigma, St. Louis, MO, USA; 500, 50, 5, and 0.5 ng/mL), the most potent metabolite of isotretinoin, in plasma from human hemophilia A patients for 2 h. At the end of the 2-h incubation, the activity of vehicle- or ATRA-treated Xyntha was measured using COAMATIC Factor VIII Assay (Diapharma, West Chester, OH, USA).

Statistical analyses

Data for transgene DNA and RNA were analyzed by one-way analysis of variance or two-way analysis of variance with a subsequent multiple comparison post-test; a p value <0.05 was considered statistically significant. The statistical analysis is described in each figure. Data are

presented as mean \pm standard deviation. Statistical analyses were performed in Prism software (GraphPad, San Diego, CA, USA).

Data availability

Materials and protocols will be distributed to qualified scientific researchers for non-commercial, academic purposes. The AAV5-hFVIII-SQ vector and the AAV5-hFVIII-SQ vector sequence are part of an ongoing development program, and they will not be shared.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtm.2022.05.008>.

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AUTHOR CONTRIBUTIONS

S.F., B.K., S.B., D.L., and S.L. contributed to the study design. S.L., L.R., O.R., C.-R.S., B.H., and G.B. performed the molecular and biochemical analyses. L.Z. and P.B. performed the animal experiments. J.W., V.A., C.C., K.G., H.A., and P.C. contributed to vector design, production, and purification. All authors critically reviewed the manuscript and contributed to interpretation of the data.

DECLARATION OF INTERESTS

S.L., V.A., C.C., H.A., O.R., J.W., and B.K. are former employees of BioMarin Pharmaceutical, Inc., and may hold stock. L.R., C.-R.S., B.H., G.B., L.Z., K.G., P.C., S.B., and S.F. are employees and stockholders of BioMarin Pharmaceutical, Inc. P.B. has received research support from BioMarin Pharmaceutical, Inc.; Grifols; and Octapharma. D.L. has received research support from Bayer; BioMarin Pharmaceutical, Inc.; CSL; Octapharma; and Sanofi.

REFERENCES

- Liu, X., Yan, Z., Luo, M., and Engelhardt, J.F. (2006). Species-specific differences in mouse and human airway epithelial biology of recombinant adeno-associated virus transduction. *Am. J. Respir. Cell Mol. Biol.* *34*, 56–64.
- Ellis, B.L., Hirsch, M.L., Barker, J.C., Connelly, J.P., Steininger, R.J., 3rd, and Porteus, M.H. (2013). A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with Nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype. *Virology* *457*, 10, 74.
- Wang, D., Tai, P.W.L., and Gao, G. (2019). Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* *18*, 358–378.
- Daya, S., and Berns, K.I. (2008). Gene therapy using adeno-associated virus vectors. *Clin. Microbiol. Rev.* *21*, 583–593.
- Watakabe, A., Ohtsuka, M., Kinoshita, M., Takaji, M., Isa, K., Mizukami, H., Ozawa, K., Isa, T., and Yamamori, T. (2015). Comparative analyses of adeno-associated viral vector serotypes 1, 2, 5, 8 and 9 in marmoset, mouse and macaque cerebral cortex. *Neurosci. Res.* *93*, 144–157.
- Gao, G., Lu, Y., Calcedo, R., Grant, R.L., Bell, P., Wang, L., Figueredo, J., Lock, M., and Wilson, J.M. (2006). Biology of AAV serotype vectors in liver-directed gene transfer to nonhuman primates. *Mol. Ther.* *13*, 77–87.
- Lisowski, L., Dane, A.P., Chu, K., Zhang, Y., Cunningham, S.C., Wilson, E.M., Nygaard, S., Grompe, M., Alexander, I.E., and Kay, M.A. (2014). Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. *Nature* *506*, 382–386.
- Nathwani, A.C., Gray, J.T., Ng, C.Y., Zhou, J., Spence, Y., Waddington, S.N., Tuddenham, E.G., Kemball-Cook, G., McIntosh, J., Boon-Spijker, M., et al. (2006). Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood* *107*, 2653–2661.
- Bunting, S., Zhang, L., Xie, L., Bullens, S., Mahimkar, R., Fong, S., Sandza, K., Harmon, D., Yates, B., Handyside, B., et al. (2018). Gene therapy with BMN 270 results in therapeutic levels of FVIII in mice and primates and normalization of bleeding in hemophilic mice. *Mol. Ther.* *26*, 496–509.
- Jiang, H., Lillcrap, D., Patarroyo-White, S., Liu, T., Qian, X., Scallan, C.D., Powell, S., Keller, T., McMurray, M., Labelle, A., et al. (2006). Multiyear therapeutic benefit of AAV serotypes 2, 6, and 8 delivering factor VIII to hemophilia A mice and dogs. *Blood* *108*, 107–115.
- Manno, C.S., Chew, A.J., Hutchison, S., Larson, P.J., Herzog, R.W., Arruda, V.R., Tai, S.J., Ragni, M.V., Thompson, A., Ozelo, M., et al. (2003). AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood* *101*, 2963–2972.
- Kattenhorn, L.M., Tipper, C.H., Stoica, L., Geraghty, D.S., Wright, T.L., Clark, K.R., and Wadsworth, S.C. (2016). Adeno-associated virus gene therapy for liver disease. *Hum. Gene Ther.* *27*, 947–961.
- Pasi, K.J., Rangarajan, S., Mitchell, N., Lester, W., Symington, E., Madan, B., Laffan, M., Russell, C.B., Li, M., Pierce, G.F., et al. (2020). Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. *N. Engl. J. Med.* *382*, 29–40.
- Nathwani, A.C., Reiss, U.M., Tuddenham, E.G., Rosales, C., Chowdhary, P., McIntosh, J., Della Peruta, M., Lheriteau, E., Patel, N., Raj, D., et al. (2014). Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N. Engl. J. Med.* *371*, 1994–2004.
- Rangarajan, S., Walsh, L., Lester, W., Perry, D., Madan, B., Laffan, M., Yu, H., Vettermann, C., Pierce, G.F., Wong, W.Y., et al. (2017). AAV5-Factor VIII gene transfer in severe hemophilia A. *N. Engl. J. Med.* *377*, 2519–2530.
- Pasi, K.J., Laffan, M., Rangarajan, S., Robinson, T.M., Mitchell, N., Lester, W., Symington, E., Madan, B., Yang, X., Kim, B., et al. (2021). Persistence of haemostatic response following gene therapy with valoctocogene roxaparvec in severe haemophilia A. *Haemophilia* *27*, 947–956.
- Ozelo, M.C., Mahlangu, J., Pasi, K.J., Giermasz, A., Leavitt, A.D., Laffan, M., Symington, E., Quon, D.V., Wang, J.D., Peerlinck, K., et al. (2022). Valoctocogene roxaparvec gene therapy for hemophilia A. *N. Engl. J. Med.* *386*, 1013–1025.
- Mahlangu, J., Ozelo, M.C., Peyvandi, F., Jayaram, K., Yu, H., Robinson, T.M., et al.; Pipe, SW, and on behalf of the GENER8-1 trial group (2022). Efficacy and safety of valoctocogene roxaparvec gene transfer for severe hemophilia A: Results from the GENER8-1 two-Year analysis. [abstract]. Presented at European Association for Haemophilia and Allied Disorders 2022 Virtual Congress.
- Nathwani, A.C., Rosales, C., McIntosh, J., Rastegarlar, G., Nathwani, D., Raj, D., Nawathe, S., Waddington, S.N., Bronson, R., Jackson, S., et al. (2011). Long-term safety and efficacy following systemic administration of a self-complementary AAV vector encoding human FIX pseudotyped with serotype 5 and 8 capsid proteins. *Mol. Ther.* *19*, 876–885.
- McIntosh, J., Lenting, P.J., Rosales, C., Lee, D., Rabbanian, S., Raj, D., Patel, N., Tuddenham, E.G., Christophe, O.D., McVey, J.H., et al. (2013). Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. *Blood* *121*, 3335–3344.
- Isotretinoin capsules (for oral use) (2020). Upsher-Smith Laboratories, LLC. Maple Grove, MN. <https://www.upsher-smith.com/products/isotretinoin-capsules/> Accessed March 2, 2022.
- Topletz, A.R., Tripathy, S., Foti, R.S., Shimshoni, J.A., Nelson, W.L., and Isoherranen, N. (2015). Induction of CYP26A1 by metabolites of retinoic acid: evidence that

- CYP26A1 is an important enzyme in the elimination of active retinoids. *Mol. Pharmacol.* 87, 430–441.
23. Nathwani, A.C., Reiss, U., Tuddenham, E., Chowdary, P., McIntosh, J., Riddell, A., Pie, J., Mahlangu, J.N., Recht, M., Shen, Y.-M., et al. (2018). Adeno-associated mediated gene transfer for hemophilia B: 8 year follow up and impact of removing "empty viral particles" on safety and efficacy of gene transfer. *Blood* 132.
 24. Ragni, M.V., Majerus, E., Giermasz, A., Fong, S., Yates, B., Scheeler, S., Razon, L., Liu, S., Yu, H., Reddy, D.B., et al. (2022). Valoctocogene roxaparvec gene transfer in participants with HIV [abstract]. *Haemophilia* 28, 101.
 25. Francois, A., Bouzelha, M., Lecomte, E., Broucque, F., Penaud-Budloo, M., Adjali, O., Moullier, P., Blouin, V., and Ayuso, E. (2018). Accurate titration of infectious AAV particles requires measurement of biologically active vector genomes and suitable controls. *Mol. Ther. Methods Clin. Dev.* 10, 223–236.
 26. Poothong, J., Pottakat, A., Siirin, M., Campos, A.R., Paton, A.W., Paton, J.C., Lagunas-Acosta, J., Chen, Z., Swift, M., Volkmann, N., et al. (2020). Factor VIII exhibits chaperone-dependent and glucose-regulated reversible amyloid formation in the endoplasmic reticulum. *Blood* 135, 1899–1911.
 27. Fong, S., Yates, B., Sihn, C.R., Mattis, A.N., Mitchell, N., Liu, S., Russell, C.B., Kim, B., Lawal, A., Rangarajan, S., et al. (2022). Interindividual variability in transgene mRNA and protein production following adeno-associated virus gene therapy for hemophilia A. *Nat. Med.* 28, 789–797.
 28. Hough, C., Kamisue, S., Cameron, C., Notley, C., Tinlin, S., Giles, A., and Lillicrap, D. (2002). Aberrant splicing and premature termination of transcription of the FVIII gene as a cause of severe canine hemophilia A: similarities with the intron 22 inversion mutation in human hemophilia. *Thromb. Haemost.* 87, 659–665.
 29. Giles, A.R., Tinlin, S., and Greenwood, R. (1982). A canine model of hemophilic (factor VIII:C deficiency) bleeding. *Blood* 60, 727–730.
 30. Tinlin, S., Webster, S., and Giles, A.R. (1993). The development of homologous (canine/anti-canine) antibodies in dogs with haemophilia A (factor VIII deficiency): a ten-year longitudinal study. *Thromb. Haemost.* 69, 21–24.
 31. Monahan, P.E. (2010). The expanding menagerie: animal models of hemophilia A. *J. Thromb. Haemost.* 8, 2469–2471.
 32. Sihn, C.R., Handyside, B., Liu, S., Zhang, L., Murphy, R., Yates, B., Xie, L., Torres, R., Russell, C.B., O'Neill, C.A., et al. (2022). Molecular analysis of AAV5-hFVIII-SQ vector-genome-processing kinetics in transduced mouse and nonhuman primate livers. *Mol. Ther. Methods Clin. Dev.* 24, 142–153.