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IN FOCUS

Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs. lentiviral vectors for hemophilia B gene therapy

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Summary. *Background:* Adeno-associated viral (AAV) and lentiviral vectors are promising vectors for gene therapy for hemophilia because they are devoid of viral genes and have the potential for long-term gene expression. *Objectives:* To compare the performance of different AAV serotypes (AAV8 and AAV9) vs. lentiviral vectors expressing factor (F) IX. *Methods and results:* AAV-based and lentiviral vectors were generated that express FIX from the same hepatocyte-specific expression cassette. AAV9 transduced the liver as efficiently as AAV8 and resulted in supra-physiological FIX levels (3000–6000% of normal) stably correcting the bleeding diathesis. Surprisingly, AAV9 resulted in unprecedented and widespread cardiac gene transfer, which was more efficient than with AAV8. AAV8 and AAV9 were not associated with any proinflammatory cytokine induction, in accordance with their minimal interactions with innate immune effectors. In contrast, lentiviral transduction resulted in modest and stable FIX levels near the therapeutic threshold (1%) and triggered a rapid self-limiting proinflammatory response (interleukin-6), which probably reflected their ability to efficiently interact with the innate immune system. *Conclusions:* AAV8 and 9 result in significantly higher FIX expression levels and have a reduced proinflammatory risk in comparison with lentiviral vectors. The unexpected cardiotropic properties of AAV9 have implications for gene therapy for heart disease.

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Introduction

Gene therapy could be an attractive alternative for the treatment of hemophilia B, because the therapeutic window is relatively broad and levels slightly above 1% of normal physiologic levels are therapeutic [1]. Adeno-associated viral (AAV) and lentiviral vectors are promising vectors for gene therapy for hemophilia B. However, side-by-side comparative studies are lacking. Both vector types do not express any potentially immunogenic viral proteins and can yield prolonged expression of therapeutically relevant proteins. AAV and lentiviral vectors can transduce non-dividing cells, including hepatocytes, which normally express factor (F) IX. Lentiviral vectors integrate stably into the target cell genome [2]. In contrast, only a fraction of the AAV genomes are capable of stable genomic integration and transgene expression is mainly determined by the non-integrated episomes [3].

AAV2-based vectors have been used in clinical trials, including gene therapy trials for hemophilia B [4,5]. Hepatic delivery of AAV-FIX vectors resulted in transient therapeutic FIX levels [5]. Though encouraging, further improvements of AAV vectors are required to attain higher and stable expression levels. One limitation of using AAV2 vectors is that pre-existing antibodies preclude efficient gene transfer, especially as 30–70% of the human population is seropositive for AAV2 [6]. In addition, transduction of hepatocytes with AAV2 is relatively inefficient [7].

The use of alternative AAV serotypes has been proposed as a means to overcome pre-existing immunity to AAV2 and to achieve higher clotting factor levels in patients [8]. It was therefore important to compare the relative efficacy, biodistribution and safety of the recently identified non-human primate serotype 8 vs. human serotype 9 for hemophilia B gene therapy

[9,10] and to assess the immune response and their ability to transduce antigen-presenting cells (APCs). Furthermore, we wanted to test the hypothesis of whether systemic gene delivery is hampered by vascular barriers and whether increased vascular permeability facilitated extra-vascular dissemination of AAV [11,12]. Finally, the liver-specific FIX expression cassette [13] was also incorporated into a lentiviral vector to compare the relative efficiencies and safety profiles of these two complementary gene transfer systems for hemophilia B gene therapy.

Materials and methods

AAV and lentiviral vectors

In the AAV-GFP vectors, the green fluorescent protein (*GFP*) gene was driven from the human cytomegalovirus promoter (*CMV*) (Fig. 1). In the AAV-CAG-FIX vectors, the human *FIX* cDNA was driven from a CMV/β-actin/β-globin (*CAG*) chimeric promoter [14] (Fig. 1). The CAG-FIX vector also contained an internal ribosome entry site (IRES)-GFP. In the AAV-Apo/AAT-FIX vector, the *FIX* cDNA (a kind gift from Dr Naldini, San Rafaelle Institute) was driven from the ApoE HCR/AAT promoter composed of the *apolipoprotein E enhancer/α1-antitrypsin promoter*, the *hepatocyte control region (HCR)* and the first *FIX* intron (kindly provided by Dr Miao, University of Washington) [13] (Fig. 1). GFP and FIX expression in the human immunodeficiency virus (HIV)-GFP and HIV-CMV-FIX vector was also driven by CMV [15], whereas in the HIV-Apo/AAT-FIX vector, FIX was driven from the ApoE HCR/AAT promoter identical to the one used in AAV-Apo/AAT-FIX (Fig. 1). This expression cassette was cloned in antisense orientation to preserve the FIX intron. Vector production and titration is outlined in the supplemental information.

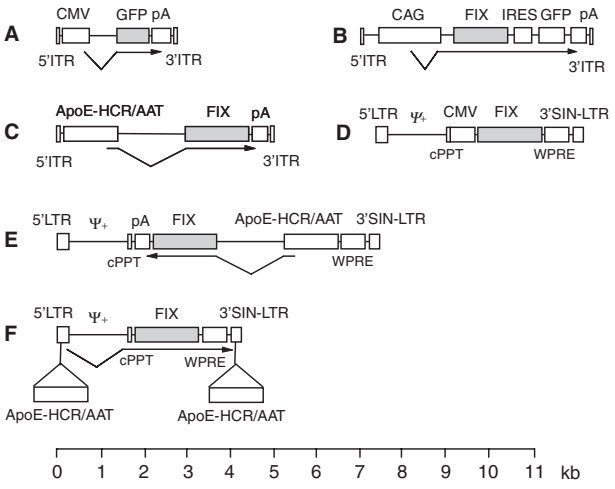

Fig. 1. Schematic diagram of adeno-associated viral (A–C) and lentiviral (D–F) vectors. (A) The human cytomegalovirus (CMV) promoter was used to express green fluorescent protein (GFP). Factor IX expression was driven from the CMV/β-actin/β-globin (CAG) (B), ApoE-HCR/AAT (C, E, F) or CMV (D) promoter. The central polypurine tract (cPPT), polyadenylation (pA) and internal ribosome entry site (IRES), packaging sequence (Ψ+), woodchuck post-transcriptional regulatory element (WPRE), long and inverted terminal repeats (LTR, ITR) are indicated (SIN, self-inactivating). The transcripts expressed in transduced cells are shown.

Animal studies

Animal procedures were approved by the institutional Animal Ethical Commission. One or 3×10^{11} AAV vector genomes (vg) were injected (i.v.) into the tail vein of adult 7-week-old male NMRI-Foxn1^{nu}/Foxn1^{nu} (nude) mice (Janvier, Le Genest Saint Isle, France) or FIX-deficient hemophilia B C57Bl/6 mice (kindly provided by Dr Verma, Salk Institute) [16] (2–5 mice/group). Adult hemophilia B mice were injected i.v. with 3×10^{11} vg of HIV-Apo/AAT-FIX vector, whereas adult SCID mice were injected i.v. with 10^9 transducing units of HIV-Apo/AAT-FIX or HIV-CMV-FIX vectors (3–5 mice/group). Blood was collected by retro-orbital bleeds and assayed for the presence of inflammatory cytokines by ELISA [Proteoplex murine cytokine array, Merck/EMD Biosciences, San Diego, CA, USA; interleukin-6 (IL-6) quantikine ELISA, R&D systems, Minneapolis, MN, USA]. Plasma measurements of aspartate aminotransferase levels were performed on a Modular System (Roche/Hitachi, Basel, Switzerland). GFP expression was monitored 1 month postinjection in intact organs under fluorescent light (488 nm) using a 515 nm long-pass filter (Montreal Biotech Inc., Dorval, Canada) or a Zeiss Stereo Lumar V12. GFP$^+$ area and mean fluorescent intensities were quantified using Zeiss-KS300 software (Zeiss, Zaventem, Belgium). Intact organs or 7 μm sections, stained with TOPRO-3, were analyzed by confocal microscopy (Axiovert 100M, LSM510, Zeiss). Human FIX expression was determined in citrated mouse plasma using a human FIX-specific ELISA (Asserachrome/Diagnostica Stago, Parsippany, NJ, USA). Phenotypic correction was analyzed by subjecting the mice (2–3 mice/group) to a 1-cm tail clip injury and by assessing survival rate. Mice were immobilized in a constrainer through which the cut tail was protruding, allowing continuous blood collection at room temperature. Total blood volume collected was then measured. Clotting times *in vitro* on citrated plasma were determined using an activated partial thromboplastin time (APTT) assay (Synth ASil; Instrument Laboratory, Lexington, MA, USA).

Quantitative polymerase chain reaction (qPCR) analysis was conducted on an ABI 7700 (Applied Biosystems, Foster City, CA, USA) using TaqMan® primers specific for the human GH poly A in the AAV vectors or using Lux® primers specific for the murine Gas6 gene (forward *GGACGAGTGCCAGCAGGAT*, reverse *CCCTCGCCCATCACAGG-JOE*). Animals were killed 1 or 2 months postinjection for AAV-GFP and AAV-CAG-FIX, respectively. Southern blot analysis was performed on genomic liver DNA restricted with BamHI and XhoI. Genomic DNA of non-injected mice that was spiked with a known amount of serially diluted pAAV-Apo/AAT-FIX vector plasmid was used as standard. Samples were

separated on a 1% agarose gel and hybridized overnight using a 3.1 kb ^{32}P-labeled probe corresponding to a BamHI–XhoI fragment of pAAV-Apo/AAT-FIX. Band intensities were quantified by densitometric analysis.

Results

Efficacy and safety of AAV8 vs. AAV9

Injection of AAV8-Apo/AAT-FIX or AAV9-Apo/AAT-FIX vectors (10^{11} vg) into hemophilia B mice resulted in stable supra-physiologic FIX levels (3000–6000% of normal) (Fig. 2A). These levels were thousandfold higher than what could be achieved with the AAV8-CAG-FIX or AAV9-CAG-FIX vectors (Fig. 2B–D). There was no significant difference in FIX or GFP expression between the mice cohorts injected with AAV8 or AAV9 (Figs 2 and 3) (GFP^+ hepatocytes: AAV9-GFP: 3.12 ± 0.25% vs. AAV8-GFP: 3.00 ± 0.45%; $P = 0.807$) (Fig. 3). To assess FIX clotting activity *in vivo*, a tail clip assay was performed. Hemophilia B mice injected with AAV9-ApoE/AAT-FIX or AAV8-ApoE/AAT-FIX survived the injury and exhibited a tenfold reduction in blood loss (only 90–135 μL of blood) and had normalized APTT values (36.2–38.5s), similar to wild-type. In contrast, most of the phosphate-buffered saline (PBS)-injected hemophilic control mice typically died within 2–5 h of the injury and had much more prolonged APTT values (> 90 s), consistent with the increased blood loss (1220 ± 180 μL). The extent of blood loss correlated with the FIX levels. Indeed, FIX-deficient mice that had been injected with a separate batch of AAV8-ApoE/AAT-FIX vector (with a lower infectious units/vector particle ratio), yielded lower circulating levels of FIX (1000 ng mL^{-1}, i.e. 20% of normal). Although these mice survived the injury, bleeding was more pronounced (620–1020 μL) than in those recipient mice that had supra-physiologic FIX expression levels.

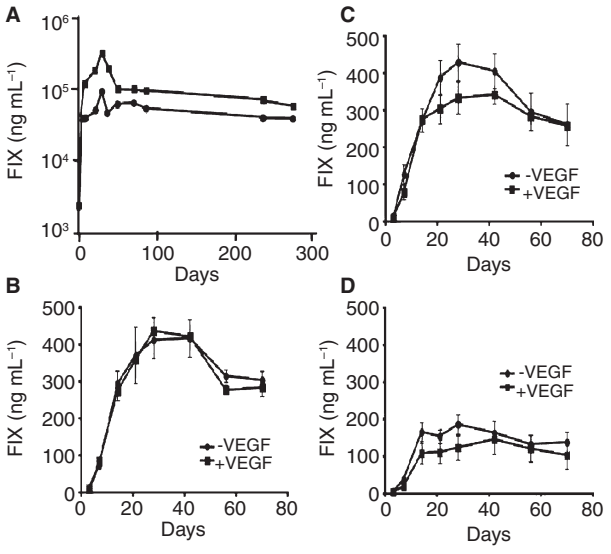

Fig. 2. (A) Factor (F) IX expression after i.v. injection of AAV8-Apo/AAT-FIX (circles) or AAV9-Apo/AAT-FIX (squares) vectors (10^{11} vector genomes) into hemophilia B mice. (B) FIX expression following injection of 3×10^{11} vector genomes AAV8-CAG-FIX, (C) 3×10^{11} vector genomes AAV9-CAG-FIX, or (D) 10^{11} AAV9-CAG-FIX with or without 10 μg vascular endothelial growth factor ($VEGF^{164}$) coinjection in nude mice.

qPCR analysis (Fig. 4) confirmed the GFP and FIX expression data, because AAV9 transduced the liver as efficiently as AAV8 (Figs 2 and 3). Gene transfer was more efficient in the liver, primarily into hepatocytes, than in any other organ (Fig. 4). Kupffer cells and sinusoidal endothelial cells were relatively refractory to transduction with either serotype (Fig. 3). Moreover, there were no GFP-positive splenic APCs (data not shown), consistent with the qPCR data (Fig. 4). AAV8 and AAV9 thus only minimally interact with the innate immune system, consistent with the lack of toxicity or proinflammatory immune responses (Table 1).

GFP expression data underestimate the actual percentage of transduced hepatocytes, because the CAG promoter is relatively weakly expressed in hepatocytes. Southern blot analysis was therefore performed on transduced liver from hemophilic mice injected with AAV9-Apo/AAT-FIX (Fig. 5). The actual average transduction efficiency corresponded to 42 copies/cell (or 4200%). These results confirm that the AAV9-Apo/AAT-FIX vectors transduced the liver very efficiently, even when a relatively low vector dose was employed (10^{11} vg/i.v.), consistent with the PCR data (Fig. 4).

Analysis of vascular endothelial growth factor ($VEGF)^{164}$ effect on vector performance

Previous studies had shown that VEGF enhanced vascular permeability and AAV6-mediated gene delivery in large areas of skeletal muscle [11]. This justified using VEGF in an attempt to enhance skeletal and/or hepatic transduction with AAV8 or AAV9 and to increase FIX expression from the ubiquitous CAG promoter. It was not known, however, whether vector penetration into the liver would also be enhanced by VEGF. AAV8-CAG-FIX or AAV9-CAG-FIX vectors were injected into nude mice with a safe dose of $VEGF^{164}$. Although a vector dose-dependent increase in FIX levels was apparent (Fig. 2), $VEGF^{164}$ coadministration did not enhance the performance of the AAV8 or AAV9 vectors because circulating FIX levels remained essentially unchanged (Fig. 2B–D).

Cardiotropism of AAV9

AAV9-GFP transduction resulted in efficient transduction into the heart, as revealed by widespread stable GFP expression in the myocardium (Figs 6 and 7), whereas AAV8-GFP resulted in more limited gene transfer in the adult heart (Fig. 6) [AAV9-GFP: 70.4 ± 2.3 % vs. AAV8-GFP: 6.4 ± 0.6 % and $P < 0.0001$ (two-tailed Student's *t*-test)]. This is consistent with the qPCR showing a significant enhancement (up to fifteenfold) of cardiac gene transfer with AAV9 than AAV8 (Fig. 4). An independent experiment was conducted to assess whether cardiac gene transfer is vector dose-dependent. Injec-

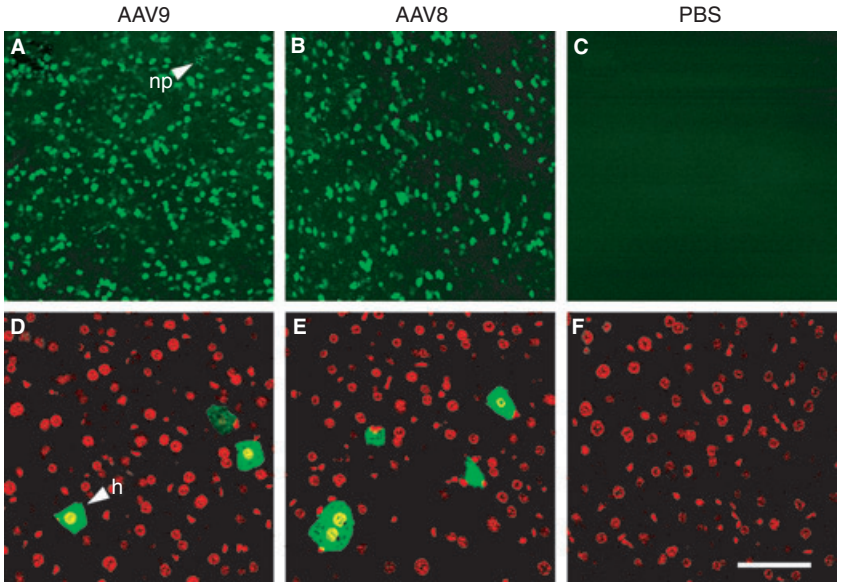

Fig. 3. AAV9 and AAV8 transduce liver with equal efficiency (3×10^{11} vector genomes). (A–C) Confocal microscopy on intact tissue or (D–F) on TOPRO-3 stained cryosections (red). A hepatocyte (H) and a non-parenchymal cell (np) are indicated. Scale bar corresponds to 500 μm (A–C) or 62.5 μm (D–F).

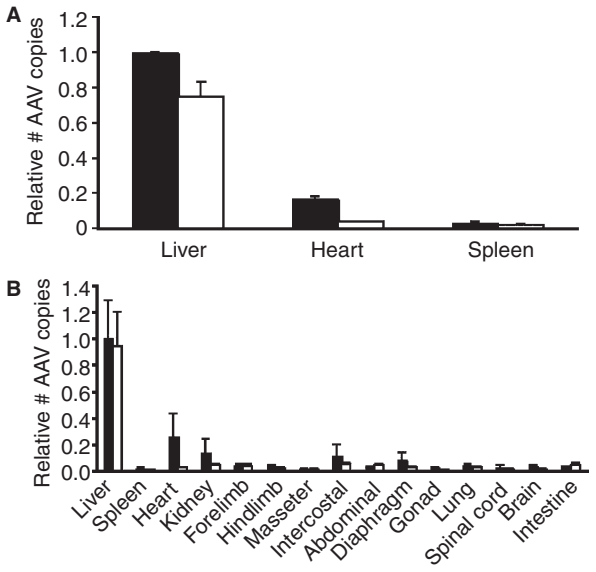

Fig. 4. Biodistribution and transduction efficiencies using AAV8 and AAV9 vectors (3×10^{11} vector genomes). (A) Relative AAV-green fluorescent protein (GFP) vector copies in heart, liver and spleen determined by quantitative polymerase chain reaction (qPCR) using vector-specific primers (white bars, AAV8; black bars, AAV9). AAV copy number is relative to AAV9 copies in liver. (B) Biodistribution of AAV8–CMV/β-actin/β-globin-factor IX (AAV8-CAG-FIX) and AAV9-CAG-FIX was determined by qPCR (white bars, AAV8; black bars, AAV9).

tion of 3×10^{11} vg AAV9-GFP resulted in widespread cardiac gene transfer (88 ± 2 % GFP^+ area), whereas a 3-fold lower vector dose resulted in a concomitant decrease (two-tailed Student's *t*-test: $P < 0.05$) in cardiac transduction efficiency (38 ± 14 % GFP^+ area) (Fig. 7). Similarly, the mean GFP fluorescent intensity was vector dose-dependent [3×10^{11} vg: 45 ± 3 arbitrary units (AU); 10^{11} vg: 20 ± 3 AU; two-tailed Student's *t*-test: $P < 0.005$]. Biodistribution analysis indicated that, with the exception of various skeletal muscle groups, including diaphragm, other tissues typically had much lower vector copies (Fig. 4).

GFP expression was apparent in the testis following AAV9 transduction and to a much lesser extent by AAV8 (Fig. 8), but was restricted to the peritubular and intertubular tissue and the intertubular blood vessels (Fig. 8). In contrast, GFP-positive cells could not be detected within the seminiferous tubules and there was no evidence of transduction into spermatogonia or sperm cells. This implies that the risk of inadvertent germline gene transfer is limited, consistent with previous reports using AAV2 [5,17].

Efficacy and safety of lentiviral vectors

VSV-G pseudotyped lentiviral vectors (HIV-Apo/AAT-FIX) were injected into hemophilia B or SCID mice, yielding stable FIX levels near the 1% therapeutic threshold, similar to what could be achieved with HIV-CMV-FIX (Fig. 9). Another construct was generated in which the ApoE HCR AAT promoter was cloned into the 3′ long terminal repeats (LTR) of the lentiviral vector, which is copied into the 5′ LTR in the transduced cells (Fig. 1F). However, this vector failed to yield any detectable FIX expression (data not shown), which underscores the importance of FIX intron A, as opposed to the native lentiviral intron, in obtaining higher FIX expression levels [13]. Although transaminases were not significantly different from PBS-injected controls, IL-6 levels had increased transiently (HIV-Apo/AAT-FIX: 389 ± 37 pg mL^{-1} vs. PBS: 49 ± 22 pg mL^{-1}, $P = 0.0013$), which probably reflects an acute, self-limiting proinflammatory immune response.

Table 1 Blood parameters, serum transaminases and inflammatory cytokines at different time points following injection of 3×10^{11} vector genomes of AAV8-green fluorescent protein (GFP) or AAV9-GFP in nude mice

	AAV9			AAV8			Phosphate-buffered saline		
Time postinjection (h) pg mL^{-1}	2	6	24	2	6	24	2	6	24
Interleukin1 (IL-1) -α	20.3 ± 1.6	27.5 ± 8.8	22.3 ± 3.1	26.4 ± 7.6	16.9 ± 2.7	18.6 ± 5.2	18.7 ± 5.9	16 ± 4.4	16.1 ± 4.6
IL-1-β	23.6 ± 11.7	28.1 ± 15.6	21 ± 16.9	16.6 ± 9	6.5 ± 3.7	1.6 ± 1.6	31.7 ± 31.7	22.4 ± 22.4	27.6 ± 21.3
IL-2	13.7 ± 3.3	7.9 ± 5.3	3.5 ± 3.5	27.6 ± 5.8	6.7 ± 2.2	0.9 ± 0.9	23.3 ± 12.3	12.9 ± 7.5	14.8 ± 7.8
IL-4	6038 ± 639	3215 ± 326	3583 ± 350	6274 ± 208	2961 ± 250	3302 ± 506	5139 ± 262	2430 ± 45	3409 ± 673
IL-6	306 ± 7	456 ± 85	275 ± 14	308 ± 21	456 ± 105	260 ± 17	283 ± 35	367 ± 54	267 ± 8
IL-10	2435 ± 292	1708 ± 223	1569 ± 152	2503 ± 178	1602 ± 161	1385 ± 193	2141 ± 277	1276 ± 135	1444 ± 297
IL-12	1611 ± 264	1668 ± 351	1609 ± 363	1594 ± 82	1367 ± 84	1198 ± 90	1461 ± 364	1475 ± 403	1466 ± 313
GMCSF	12.1 ± 5.3	8.7 ± 5	8.8 ± 6.4	7.7 ± 1.5	3.6 ± 0.3	1.6 ± 0.8	25 ± 24.1	13.6 ± 11.5	19.6 ± 12.8
INF-γ	1700 ± 201	1466 ± 205	1281 ± 98	1832 ± 94	1378 ± 63	1170 ± 161	1613 ± 282	1226 ± 221	1243 ± 207
TNF-α	< 15	< 15	< 15	< 15	< 15	< 15	< 15	< 15	< 15
Time postinjection (d)	1	3	7	1	3	7	1	3	7
RBC (K μL^{-1})	6.3 ± 0.2	5.8 ± 0.2	6.4 ± 0.2	6.2 ± 0.1	6.0 ± 0.1	6.1 ± 0.2	5.9 ± 0.1	5.9 ± 0.2	6.1 ± 0.1
WBC (K μL^{-1})	3.5 ± 0.6	5.0 ± 0.7	6.8 ± 0.5	3.3 ± 0.2	4.6 ± 0.7	6.1 ± 0.6	2.4 ± 0.5	5.7 ± 0.9	5.7 ± 1.3
HGB (g dL^{-1})	12.5 ± 0.3	11.2 ± 0.3	12.0 ± 0.4	11.9 ± 0.3	11.6 ± 0.3	11.0 ± 0.3	11.2 ± 0.3	11.2 ± 0.4	11.2 ± 0.3
HCT (%)	32.4 ± 0.3	28.9 ± 0.8	33.5 ± 1.3	31.3 ± 0.8	29.9 ± 0.7	31.2 ± 0.9	29.5 ± 0.6	29.2 ± 0.8	31.6 ± 1.2
PLT (K μL^{-1})	857.3 ± 19.9	948.5 ± 40.0	> 1000	901.0 ± 37.0	> 1000	> 1000	824.0 ± 30.1	925.3 ± 16.1	> 1000
AST (U L^{-1})	20.0 ± 1.5	27.0 ± 1.1	11.0 ± 0.7	18.8 ± 0.8	28.8 ± 1.9	13.4 ± 1.0	20.2 ± 1.2	31.2 ± 2.5	14.0 ± 0.8

INF-γ, Interferon-γ; TNF-α, tumor necrosis factor-α RBC, red blood cell; WBC, white blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; AST, aspartate aminotransferase.

Although vector purification by chromatography reduced *in vitro* toxicity (supplemental Fig. 1), IL-6 induction was not abolished. The additional purification step did not compromise the functionality of the lentiviral vector, because the (functional titer/particle titer) ratio remained unaltered (1:500).

Discussion

The present study shows that gene therapy with AAV8 and AAV9-based vectors resulted in stable supra-physiologic human FIX levels in hemophilia B mice, which stably corrected the bleeding diathesis. Gene transfer with AAV8 or AAV9 was more efficient in the liver than in any other organ or tissue, which makes them particularly well-suited for liver-directed hemophilia gene therapy. Hepatic transduction with AAV9 vectors was at least as efficient as with AAV8 [18,19,9]. This is the first study on gene therapy for hemophilia B using AAV9, and it is consistent with recent results in hemophilia A models [20]. The stable FIX expression is in agreement with the long-term FIX expression following AAV8 delivery in non-human primates [21] and further supports the hypothesis that hepatic FIX expression results in FIX-specific immune tolerance, possibly involving regulatory T-cells [22,23].

One specific advantage of AAV9 over AAV8 is that it is more distantly related to AAV2, based on sequence homology and its reduced immune cross-reactivity with AAV2 [10]. This is important as even low titers of human anti-AAV2 antibodies can preclude *in vivo* gene transfer with AAV8 [5,6]. Nevertheless, an estimated 20% of the human population has detectable pre-existing antibodies to AAV8 and AAV9 (antibody titer > 1:20 resulting in 50% inhibition of transduction *in vitro*; J. Wilson, unpubl. obs.), implying that gene therapy subjects would still need to be prescreened for anti-AAV8/9 antibodies.

Although therapeutic FIX levels could be obtained in hemophilia B patients following AAV2 gene therapy, expression declined because of AAV2-specific cytotoxic T lymphocytes (CTL) that eliminated transduced hepatocytes [5]. In contrast, AAV8 does not activate T cells, which correlates with the lack of binding to heparan sulfate proteoglycan receptors on APCs [24]. This is consistent with the lack of APC transduction and IL-6 induction, as shown in the present study. The rapid onset of expression of AAV9 parallels that of AAV8, which most probably reflects the rapid uncoating vector particles [19,25]. The presumed rapid turnover of AAV8 and AAV9 capsids may further reduce this CTL risk.

Lentiviral transduction resulted in much lower FIX levels (~1%) and transiently induced IL-6, in contrast to AAV. This rapid proinflammatory immune response was due probably to their ability to efficiently interact with the innate immune system [2], but is limited compared with when adenoviral vectors are employed [26]. Despite this short-term innate immune response, FIX expression remained stable, suggesting that it did not increase the risk of an adaptive immune response against the transduced cells and/or the FIX protein.

Unexpectedly, AAV9 resulted in dose-dependent widespread and efficient cardiac gene delivery, which was more efficient than by any other vector, including AAV8 [27]. This may be due to improved vector transcytosis across the

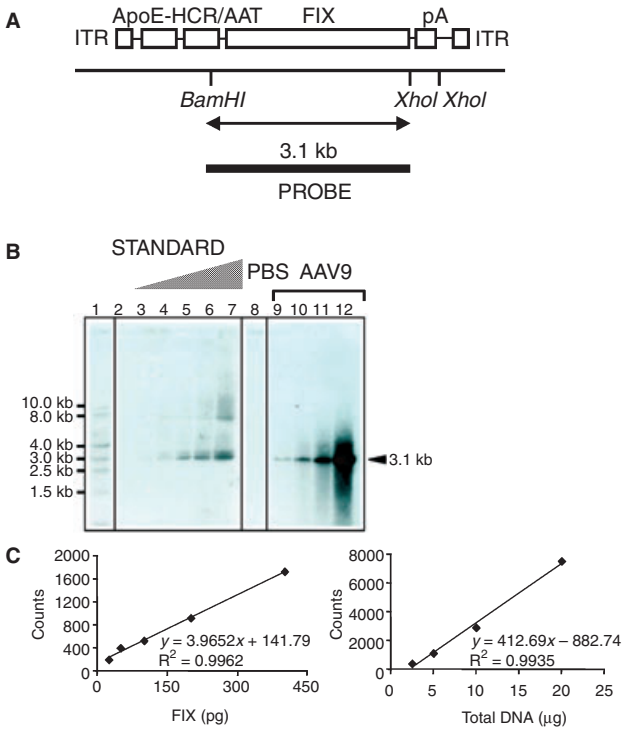

Fig. 5. Southern blot analysis of genomic liver DNA from hemophilia B mice injected with AAV9-Apo/AAT-factor (F) IX (10^{11} vector genomes). (A) Restriction map and vector-specific probe. (B) Genomic liver DNA from non-injected mice spiked with a known amount of serially diluted pAAV-Apo/AAT-FIX vector plasmid. (B and C, left panel) Linear correlation of the hybridization signal (arbitrary units) and the amount of spiked vector plasmid (range: 0.5–8 vector copies/cell, which corresponded to 24.5–393 pg FIX per 20 µg genomic DNA, 49.1 pg = 1 copy/cell). (B and C, right panel) Serial dilution of transduced liver DNA from mice injected with AAV9-Apo/AAT-FIX resulted in a hybridization signal that was linearly correlated with the amount of liver DNA (range 2.5–20 µg).

blood vessel barrier and obviated the need for pharmacological enhancement of vector penetration, neonatal recipients or catheter-based delivery. Previously, AAV6 resulted in cardiac gene transfer in adult mice, upon coinjection with VEGF [11]. Until now, AAV8 was considered the most efficient vector for cardiac gene delivery compared with AAV1, 2, 5, 6, and 7 [12]. However, in that study, higher vector doses were employed and a self-complementary vector design was used to enhance vector performance.

Whereas VEGF coadministration enhanced muscular transduction with AAV6 [11], other studies showed that it had no effect on cardiac or muscular transduction with AAV1, AAV6 or AAV8 [12]. We have shown that VEGF did not enhance the performance of AAV8 or AAV9 vectors. Because the liver is more efficiently transduced by AAV8 and AAV9 compared with skeletal muscle, the lack of any VEGF-mediated enhancement of FIX expression suggests that vascular barriers do not seem to hamper vector penetration into the liver parenchyma. This may be due to the inherent small size of the AAV vector particles (20 nm) relative to the liver fenestrae (150 nm).

Preclinical studies in larger animal models are warranted to further validate the use of AAV8 vs. AAV9 for hemophilia B gene therapy and to further confirm the superiority of AAV9 for cardiac gene delivery in non-murine species. It is encouraging that the superior transduction of AAV1 in skeletal muscle compared with AAV2 holds true for both mice and dogs [8,28]. Although some studies in large animal models suggest that AAV8 may be superior to AAV2 for liver-directed gene transfer [29], other studies showed that their performance is comparable in dogs and primates [18,30].

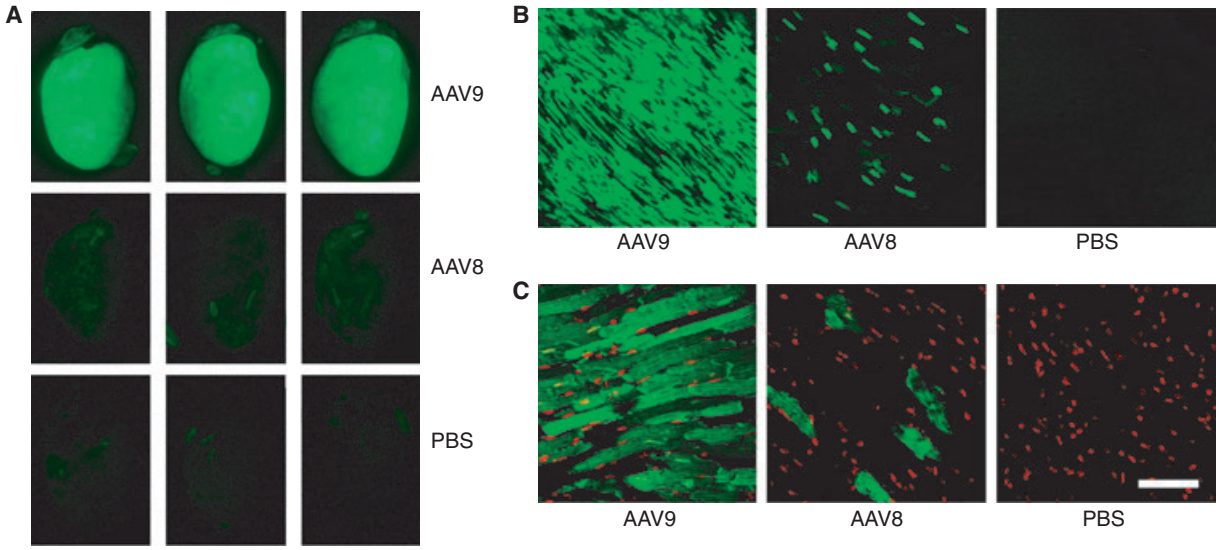

Fig. 6. Efficient and widespread cardiac gene transfer with AAV9 (3×10^{11} vector genomes). (A) Fluorescent imaging of intact hearts of mice injected with AAV9-green fluorescent protein (GFP), AAV8-GFP, or phosphate-buffered saline (PBS) as control (triplicates are shown). (B) Confocal microscopy on intact heart tissue and (C) on cryosections at higher magnification with TOPRO-3 nuclear staining. Scale bar corresponds to 500 µm.

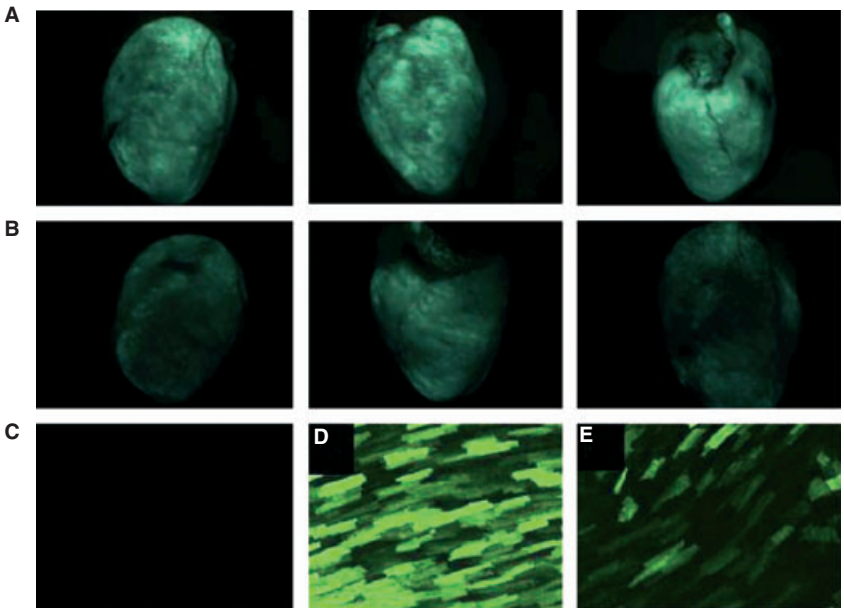

Fig. 7. Dose–response analysis of cardiac gene transfer with AAV9. Fluorescent imaging of intact hearts of recipient mice injected i.v. with AAV9- green fluorescent protein (GFP) at a (A) high (3×10^{11} vector genomes [vg]) or (B) low (10^{11} vector genomes) dose (triplicates are shown), or (C) with phosphate-buffered saline (PBS). Confocal microscopy of intact myocardium of (D) high- and (E) low-dose recipients. A representative confocal scan is shown. There was no background fluorescence in PBS-injected mice (data not shown).

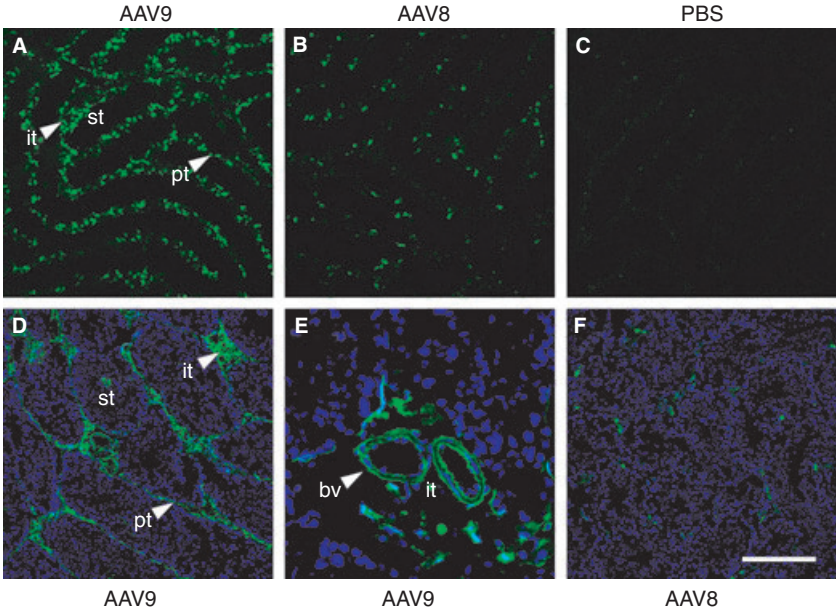

Fig. 8. Transduction of testis with AAV9 and AAV8. (A–C) Confocal microscopy on intact tissue and (D–F) on cryosections of the testis at higher magnification with TOPRO-3 nuclear staining (blue color). The seminiferous tubules (st), intertubular (it) and peritubular (pt) space and blood vessels (bv) are indicated. Vector dose: 3×10^{11} vector genomes/mouse (i.v.). Scale bar corresponds to 500 μm (A–C), 250 μm (D,F) and 62.5 μm (E).

The present study further strengthens the notion that alteration of the AAV capsid greatly influences the outcome of systemic gene delivery. These findings have implications for hemophilia gene therapy because cardiac expression of clotting factors may not be desirable because of an increased thrombotic risk. However, this could be overcome by using liver-specific promoters instead. Nevertheless, the identification of efficient cardiac gene delivery using AAV9 improves the prospect of gene therapy for heart diseases [27]. Moreover, AAV9 may facilitate the generation of animal models of heart disease and assist in validating new therapeutic leads by gene transfer.

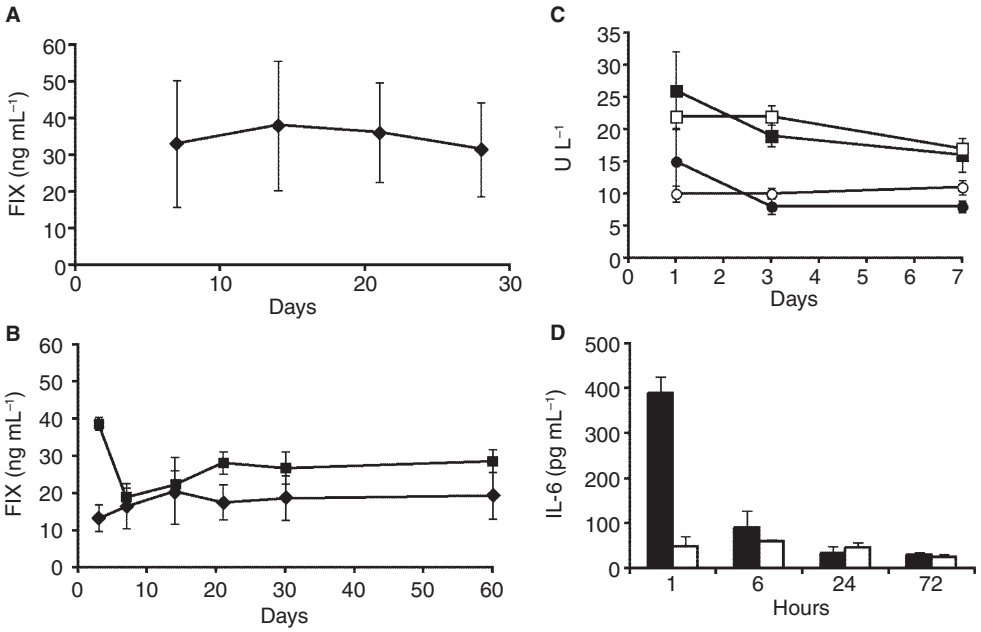

Fig. 9. Efficacy and safety of lentiviral vectors. (A) Factor (F) IX expression following lentiviral transduction. HIV-Apo/AAT-FIX (3×10^{11} vector genomes) vectors were injected i.v. into hemophilia B mice (circles); (B) HIV-cytomegalovirus-FIX (squares) or HIV-Apo/AAT-FIX (diamonds) vectors were injected i.v. into SCID mice (10^9 transducing units); (C) aspartate aminotransferase (squares) and alanine aminotransferase (circles) levels and (D) interleukin-6 levels following injection of 3×10^{11} vector genomes HIV-Apo/AAT-FIX (filled) or phosphate-buffered saline (empty).

Authors' contributions

T. VandenDriessche, L. Thorrez, A. Acosta-Sanchez, I. Petrus, L. De Waele, L. Ma, Y. Iwasaki, V. Gillijns and M. K. L. Chuah performed the experiments; T. VandenDriessche, L. Thorrez, A. Acosta-Sanchez, I. Petrus, L. De Waele, L. Wang and M. K. L. Chuah contributed to concept and design, analysis and/or interpretation of data; and T. VandenDriessche, L. Thorrez, L. Wang, D. Collen, J. M. Wilson and M. K. L. Chuah contributed to writing the manuscript. L. De Waele and J. M. Wilson contributed critical reagents.

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Disclosure of Conflict of Interests

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