Original article

Running Title: AAV gene transfer of factor IX fusion proteins

"Potential limits of AAV-based gene therapy with the use of new transgenes expressing Factor IX fusion proteins"

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Abstract

Introduction: The variety of treatment for Haemophilia B (HB) has recently improved with the emergence of both AAV-based gene therapy and bioengineered human factor IX (hFIX) molecules with prolonged half-life due to fusion to either albumin (Alb) or immunoglobulin Fc fragment (Fc).

Aim: Adeno-associated viral vectors (AAV) mediating expression of hFIX-Alb and hFIX-Fc fusion proteins was investigated for gene therapy of HB to explore if their extended half-life translates to higher plasma levels of FIX.

Methods: Single-stranded cross-packaged AAV2/8 vectors expressing hFIX-Alb, hFIX-Fc and hFIX were evaluated *in-vitro*, and in mice.

Results: Both hFIX-Alb and hFIX-Fc fusion proteins were synthesised and expressed as single chains of expected size following AAV mediated gene transfer *in-vitro* and *in-vivo*. The procoagulant properties of these hFIX-fusion proteins were comparable to wild-type hFIX. However, their expression levels were 3-fold lower than wild-type hFIX *in-vivo* most likely due to inefficient secretion.

Conclusion: This, the first, evaluation of hFIX-fusion proteins in the context of AAV gene transfer suggests that the hFIX-fusion proteins are secreted inefficiently from the liver, thus preventing their optimal use in gene therapy approaches.

Introduction

Two major advances have emerged recently for the treatment of haemophilia B (HB), an X-linked recessive bleeding disorder. The first is the development of extended half-life FIX molecules such as the human FIX-albumin (hFIX-Alb) and hFIX-immunoglobulin-Fc (hFIX-Fc) fusion proteins [1, 2]. These engineered proteins have enabled patients to reduce the frequency of prophylaxis without compromising clinical outcomes [3, 4]. The presence of the Fc domain or albumin allows interaction of the fusion protein with the neonatal Fc receptor (FcRn) [2, 5]. Interaction of fusion proteins with FcRn delays protein degradation in the lysosome and facilitates recycling back into the circulation. The second development is gene therapy, which leads to stable, long-term, dose dependent, expression of FIX at between 1-6% in patients with severe HB following a single administration of adeno-associated viral (AAV) vector encoding the wild-type hFIX gene [6]. This level of expression has altered the phenotype of the patients from severe to moderate or mild. Natural history studies show that patients with mild haemophilia who typically have FIX levels of 5-40% of normal have a much lower risk of spontaneous bleeding. However, epidemiology studies indicate that haemophilia patients with baseline factor level of >15% almost never have spontaneous haemorrhage [7]. Therefore, the ultimate goal of gene therapy should be to achieve stable expression at ≥15%. With the current technology, this will require higher dose of AAV vectors, which will be associated with increased liver toxicity [6, 8]. To address the challenge of mediating therapeutic expression of FIX using lower, potentially safer, vector doses, studies have employed a transgene expressing the hyperactive hFIX Padua variant [9], and recently showed clinical benefit [10]. However, the risk of such approach is the potential immunogenicity to the amino acid change to create hyperactive variant.

In this study, we investigated if liver-targeted gene transfer with AAV vectors encoding the hFIX-Fc and hFIX-Alb proteins would lead to higher steady-state plasma FIX levels than possible when using the wild-type FIX. Our study shows that hFIX-Alb and hFIX-Fc fusion proteins with procoagulant activity are synthesised following AAV mediated gene transfer *in-vitro* in human liver cells lines and *in-vivo* in murine liver. Expression of these fusion proteins is, however, lower than that of wild type FIX. The mechanisms limiting expression of the fusion proteins were explored to define the underlying obstacles.

Material and methods

Construction and production of viral vectors

We designed a transgene expressing human FIX (hFIX) fused to mature albumin cDNA through FIXa- or FVIIa-tissue factor (TF) complex-cleavable linker previously described [1] under the control of our previously described small liver specific LP1 promoter [11]. A pUC57 cloning vector containing 201 bp of the 3' end of humanly codon-optimised hFIX (hFIXco) cDNA sequence followed by full linker and mature albumin cDNA sequences, flanked by EcoNI and NheI restriction sites were synthesized (GenScript Biotech Corp., Nanjing, China) and cloned using the Quick ligation™ kit (New England Biolabs.Inc, Hitchin, United-Kingdom). The control pAV-LP1-hFIXco-STOP-(Alb) vector expressing wild-type hFIX (hFIX-ctrl1) was created by introducing a single nucleotide substitution using Q5® site-directed mutagenesis kit (New England Biolabs.Inc) with specific primers, thus inducing a stop codon within the cleavable linker. To obtain pAV-LP1-hFIXco-Fc vector expressing hFIX covalently fused to the Fc fragment of human IgG1 (hinge, CH2, and CH3 domains) [2], the hFIX coding region was PCR amplified using Phusion® High fidelity polymerase (New England Biolabs.Inc) from our pAV-LP1-hFIXco with specific primers introducing an EcoRI restriction sites at the 5'-end of the hFIX cDNA, and allowing the removal of the hFIX stop codon and the introduction of BamHI restriction site. The Fc region of human IgG1 (hinge, CH2, CH3) was extracted from an in-house plasmid using BamHI and MluI. Using 3-way ligation the hFIXco (no stop codon) and Fc fragments were directionally cloned into our pAV-LP1-hFIXco to form pAV-LP1-hFIXco-Fc. The control vector pAV-LP1-hFIXco-STOP-(Fc) expressing wild-type hFIX (hFIX-ctrl2) was created by inserting the Fc fragment into pAV-LP1-hFIXco after the stop codon of hFIX using

restriction enzymes digestion with BamHI and MluI followed by ligation using Quick ligation kit[®]. All four cDNA sequences were sequenced.

Single-stranded cross-packaged AAV2/8 (ssAAV2/8) expressing hFIX fusion transgenes were made by the triple transient transfection of subclonfluent human embryonic kidney (293T) cells method described before [12-14]. Recombinant ssAAV2/8 vectors (ssAAV2/8-LP1-hFIXco-Alb, ssAAV2/8-LP1-hFIXco-STOP-(Alb), ssAAV2/8-LP1-hFIXco-Fc, and ssAAV2/8-LP1-hFIXco-STOP-(Fc) viruses) were isolated with affinity column chromatography as described previously [15]. The genome titres of rAAV-hFIX vectors were determined by mean of a validated quantitative polymerase-chain-reaction (QPCR) assay [16].

In-vitro assay

Huh-7 cells (ECACC 01042712) were maintained in conditioned media containing 5 µg/ml of vitamin K as described previously [17] and were transiently transfected with 1 µg of pAV-LP1-hFIXco-Alb, pAV-LP1-hFIXco-STOP-(Alb), pAV-LP1-hFIXco-Fc, and pAV-LP1-hFIXco-STOP-(Fc) vectors using Fugene[®]6 (Roche, Neuilly-sur-Seine, France). Huh-7 cells were also transduced *in-vitro* at an MOI of 10^5 vg/cell of all four corresponding ssAAV2/8 vectors, as previously reported [18]. Twenty-four hour conditioned media were collected as previously described [19] and assayed for FIX:Ag and FIX:C levels, and SDS-PAGE followed by immunoblotting. The intra cellular secretory traffic of hFIX-ctrl1, rFIX-Alb, rFIXFc proteins was analysed using various protease inhibitors and inhibitor of cellular protein transport diluted in 0.02% ethanol as described elsewhere [19]. Results were expressed as a percentage of values obtained with control culture medium containing 0.02% ethanol

Mice, tail-vein injection and blood collection

All animal work was carried out in the United Kingdom in accordance with the standards set in the care and use of laboratory animals by the National Institutes of Health. Vector was injected into the tail vein of 7- to 10-weeks old wild-type male C57BL/J6 immunocompetent mice (Charles River, Oxford, UK) and immunodeficient (NOD.CB17-*Prkdc*^{scid}/J; Charles River) mice as previously described [18] with 5 x 10^{10} vg per mouse of respective rAAV vectors. Blood samples were collected following tail-vein puncture regularly up to eight weeks after infusion. At termination, blood was collected from intra-cardiac puncture into 1/9 volume 3.2% citrate microtubes. Mouse platelet-poor-plasma (PPP) was immediately prepared for the measurement of FIX:Ag level.

Expression of hFIX constructs in mouse liver cells

Experimental mouse livers were harvested at the time of sacrifice for genomic DNA isolation using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Vector genome copy number in mouse liver was determined by QPCR as described previously [11]. To determine the level of mRNA transcripts, total RNA was extracted from mouse livers using RNeasy Blood & Tissue kit (Qiagen). RNA analysis was performed using reverse transcription (SuperScript™ IV VILO™ Master Mix, Invitrogen) followed QPCR on liver tissue samples. Integrity of the DNA and the RNA were determined by amplifying a 295-bp region of the murine GAPDH gene.

For analysis of hFIX.Fc in mouse liver lysates, ~0.05g of liver was homogenised in lysis buffer - 0.5% v/v Triton X-100, 0.01M Hepes, 1X cOmplete™ Protease Inhibitor Cocktail (Roche Diagnostics, Meylan, France) - , clarified by centrifugation before

protein concentration in lysate. Total proteins were quantified using Pierce™ BCA Protein Assay (ThermoFisher). 200µg of liver protein was incubated with biotinylated hFIX antibody (Affinity Biologicals) for 2 hours at 37°C. Positive control of coagulation reference plasma (Technoclone, Vienna, Austria) was treated in the same way. Streptavidin-coupled Dynabeads (ThermoFisher) were washed with PBS-0.05%Tween buffer before the liver lysate-antibody mix was incubated with beads for 30 min with rotation. Bead mix was washed 3 times in PBS-0.05% Tween before adding 10µL of reducing loading dye (2.5µL 4X NuPAGE™ LDS sample buffer (ThermoFisher) 6.5µL of water, 1µL of reducing agent), and was assessed for Western blot as described below.

Factor IX detection and measurement

FIX:Ag levels were determined using either human FIX specific ELISA as described previously [18, 20], or the ELISA ASSERACHROM:IX® Ag kit (Diagnostica Stago, Asnières, France). FIX:C levels were measured using the BIOPHEN chromogenic assay kit (Hyphen BioMed, Neuville-sur-Oise, France). Huh-7 cells and murine liver cell lysates samples were loaded onto a NuPAGE™ 4-12% Bis-Tris Protein Gel (Invitrogen™, Carlsbad, CA, USA)   for electrophoresis under reducing conditions (using Tris 25mM, glycine 190mM, SDS 4mM, pH 8.3 running buffer) and then transferred onto a nitrocellulose membrane (Amersham™ Hybond™-Cel, GE-Healthcare, Saclay, France). The nitrocellulose membrane was then blocked with 5% skimmed milk-PBS-0.05% Tween20® and exposed to HRP-conjugated affinity-purified goat anti-human FIX (1mg/ml) IgG (1:2000 dilution; Affinity biology, Ancaster, Ontario, Canada). Plasma samples from the mice were screened for the presence of Ab against hFIX using an ELISA method described previously [20].

Statistical analysis

Significant differences among groups were analysed via the non-parametric Mann-Whitney test Analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Results are expressed as mean±standard error to mean (SEM). A *P*-value of<0.05 was considered statistically significant.

Results

rAAV vector genomes direct low expression of hFIX-fusion proteins *in-vitro*

The albumin and human Fc fragments were cloned in-frame and downstream of the codon-optimised hFIX (hFIXco) gene, under the control of a liver-specific promoter to generate pAV-LP1-hFIXco-Alb (hFIX-Alb) and pAV-LP1-hFIXco-Fc (hFIX-Fc) AAV expression vectors, respectively. The Fc and albumin fusion sequences were downstream of the hFIX cDNA as described for the recombinant fusion proteins Alprolix® and Idelvion®. Control vectors were identical to these AAV expression cassettes with the exception of a stop codon introduced at the end of the FIX coding sequence to generate pAV-LP1-hFIXco-STOP-Alb (hFIX-ctrl1) pAV-LP1-hFIXco-STOP-Fc (hFIX-ctrl2) vectors (Fig. 1A). Equivalent amounts (1µg) of all four pAV constructs were transiently transfected into Huh-7 cells, to biochemically characterise the fusion proteins and assess procoagulant activity. Conditioned media collected after 24 hours was assayed for protein expression by hFIX specific Western blot analysis and ELISA. Under reducing conditions, the apparent molecular weight of hFIX-Alb, hFIX-Fc and hFIX-ctl1/2 were approximately 140 kDa, 100 kDa and 60 kDa, respectively (Fig. 1B). The FIX mean antigen levels ($\pm$ SEM) for hFIX-Alb 1.33 % ($\pm$ 0.07) and hFIX-Fc 1.14 % ($\pm$ 0.04) were 7 to 8-fold lower than hFIX-ctrl1 9.18 % ($\pm$ 0.48); $p<0.0001$). The hFIX-Alb recombinant protein, Idelvion®, was used as control in the same ELISA, and was detected at the expected antigen value, indicating that the lower FIX:Ag levels for the hFIX-fusion proteins on ELISA were not due to changes in conformation of the engineered proteins affecting detection [21]. The activity to antigen

ratio of the fusion proteins was ~1:1 suggesting that the fusion proteins were fully functional (Fig. 1C).

Next we produced and purified single-stranded AAV8 capsid pseudotyped vectors (ssAAV2/8) expressing the hFIX fusion proteins. The vector yields for the four vectors ranged from 1.74×10^{12} vg/ml $\pm$ 2.18×10^{11} with no significant differences in titres between vectors encoding hFIX-Alb and hFIX-Fc and their respective controls. *In-vitro* transduction of Huh-7 cells resulted in expression of hFIX-Alb, hFIX-Fc as single chain peptides as described above (data not shown).

Evaluation of AAV vectors encoding hFIX-fusion proteins *in-vivo*

To evaluate the hFIX fusion proteins *in-vivo*, adult male C57BL/6 mice were injected with 5×10^{10} vg per mouse of respective ssAAV2/8 vectors via the tail vein. Blood samples were collected at regular intervals over a period of eight weeks after gene transfer and assayed for hFIX:Ag levels. The kinetics of expression was similar for all four constructs with expression reaching steady state levels between 4-6 weeks after gene transfer. As with the *in-vitro* studies, both hFIX-Alb and hFIX-Fc proteins were detected at significantly lower levels than hFIX-ctrl1/2, with mean ($\pm$ SEM) steady state FIX:Ag levels of 38.86 % ($\pm$ 1.30) for hFIX-Alb and 227.40 % (14.57) for hFIX-ctrl1 ($p<0.0001$), and 60.30 % ($\pm$ 14.04) for hFIX-Fc and 167.90 % ($\pm$ 17.08) for hFIX-ctrl2 ($p=0.0006$; Fig.2A). No significant difference were seen between hFIX-Alb and hFIX-Fc levels of expression ($p=0.2773$). Antibodies to hFIX were not detected (data not shown) in any of the cohorts, thus ruling out a neutralising antibody response to the FIX-fusion proteins as a potential explanation for low plasma hFIX levels. Consistent with this the mean steady-state FIX:Ag level of the hFIX-Fc (24.45 % $\pm$ 4.70) was

significantly lower than hFIX-ctrl2 (72.68 % ± 18.05 ; p=0.0286) following AAV mediated gene transfer of the respective constructs in immunodeficient mice.

Vector genome copy number in mouse liver did not show a significant difference in proviral DNA in the hFIX-Fc cohort when compared to hFIX-ctrl2 (13.9 ± 5.2 vg/cell and 24 ± 2.7 vg/cell, respectively; p=0.100; Fig. 2B). In contrast, the copy number for hFIX-Alb proviral DNA in the livers of hFIX-Alb cohort was significantly higher when compared to hFIX-ctrl1 (61.7 ± 7.9 vg/cell vs 16 ± 5.7 vg/cell; p=0.0286; Fig. 2B). This most likely reflected an underestimation of the hFIX-Alb vector titre, leading to administration of a higher vector dose than planned.

To determine if the reduced expression from AAV2/8-hFIX-Alb or -hFIX-Fc was due to inefficient transcription, we performed RNA analysis on liver tissue samples. The mRNA levels present in the hFIX-Alb cohort were lower compared to control samples, potentially suggesting altered transcription of the hFIX-Alb cDNA. There was a trend toward higher mRNA levels in the hFIX-Fc cohort compared to the control samples (Fig. 2C). This may indicate inefficient translation, protein misfolding and degradation, increased mRNA stability or inefficient secretion of the fusion construct.

Low expression of hFIX fusion proteins following gene transfer may be due to poor secretion from murine hepatocytes

Limited immunoprecipitation studies of murine liver cell-lysates showed that hFIX-Fc protein was present at relatively high levels within the hepatocytes when compared to controls (Fig. 3A). This result suggested an alteration of the hFIX-Fc fusion protein secretion. To further understand this observation, we next assessed cell-trafficking of hFIX variants in Huh-7 cells following transfection with plasmids

encoding hFIX-Alb and hFIX-Fc and hFIX-ctrl1/2 to understand the reasons for poor secretion of the hFIX-fusion proteins. Intracellular FIX concentrations were measured by ELISA. The secretion profiles of hFIX-Alb and hFIX-ctrl1 were similar, showing an increase in intracellular accumulation of hFIX-Alb and hFIX-ctrl1 following blockade of endoplasmic reticulum to Golgi transit by brefeldin-A but no change in intracellular FIX levels with either β-lactone, a proteasome inhibitor, or NH_4Cl an inhibitor of lysosomal activity. The trafficking profile for hFIX-Fc showed a degree of variation with a significant reduction of hFIX-Fc after inhibition of lysosomal activity (Fig. 3B).

Discussion

The treatment of HB is undergoing substantial changes, with the approval of extended half-life clotting factor concentrates and the parallel, ongoing validation of FIX gene therapy in several clinical trials. Covalent fusion of the Fc domain of human immunoglobulin, IgG1 or human albumin with recombinant hFIX protects the protein from intracellular lysosomal degradation and facilitates its recycling back into the circulation. This results in a 3-5 fold increase in the plasma half-life of FIX, which enables the reduction in the frequency of factor infusion in patients with severe HB. These products raise the possibility of consistently achieving higher trough levels leading to prevention of breakthrough bleeding and subsequent joint disease. Furthermore, animal studies have not suggested an increased inhibitor rate with extended half-life products [1, 2, 22]. Therefore, these hFIX-fusion proteins are highly attractive for the next generation of gene therapy vectors, primarily because of the possibility of increasing steady-state plasma FIX levels due to their extended half-life, thus improving the potency of gene transfer without having to increase the vector dose and associated toxicity [6, 8].

In the knowledge that hFIX-Fc and hFIX-Alb recombinant proteins were amenable to production at scale, we set out to evaluate these long-acting hFIX-fusion proteins in the context of AAV gene therapy. Whilst these fusion proteins, when expressed following gene transfer, had the expected procoagulant activity with an antigen:activity ratio of 1:1, their level of expression was significantly lower levels than wild-type hFIX following AAV-mediated gene transfer into the liver of mice or following transient transfection of the AAV expression cassette plasmid into human liver cell lines. The observed reduced expression from both plasmid and AAV vectors

suggests a potentially cross-platform issue with expression from liver cells that is likely to be observed with other gene transfer vectors including integrating vectors such as those based on lentivirus.

The mechanism behind the reduced plasma expression of hFIX-Fc and hFIX-Alb following gene transfer observed in this study remains unclear. Preliminary analysis suggests that the reasons for the poor expression of the two hFIX-fusion proteins may be different. Notably, the mRNA levels present in the hFIX-Alb cohort were not different compared to control samples despite higher *in-vivo* transduction rate. This suggests inefficient transcription of the hFIX-Alb proviral vector. The trend for higher mRNA levels after hFIX-Fc delivery compared to controls may indicate increased mRNA stability or a subsequent issue with protein secretion. Numerous examples in the literature exist of increased mRNA stability mediated by 3' regions of genes including the globin genes [23]. The possibility that improved mRNA stability mediated by the Fc moiety, which is downstream of the hFIX cDNA, exists. The limited immunoprecipitation studies of murine liver cell-lysates showed that hFIX-Fc protein was present at relatively high levels within the hepatocytes when compared to controls, suggesting an alteration of the hFIX-Fc fusion protein secretion. In addition, intracellular trafficking studies indicated that the secretion profile of hFIX-Fc fusion proteins was different than the ones of hFIX-Alb and hFIX-ctrl1. Studies with NH_4Cl, which inhibits the autophagic flux into lysosomes, led to hFIX-Fc proteins be redirected from the lysosomal compartment to organelles such as endosome with a quicker degradation rate [24], or increased the level of misfolded hFIX-Fc protein within the cell resulting in elevated endoplasmic-reticulum stress and a higher apoptotic rate [25]. These data suggest that the autophagy-lysosome pathway, involved in the intracellular

protein quality control [26] by removing large misfolded proteins and proteins aggregates [27], is activated in liver cells expressing hFIX-Fc fusion proteins [28]. This hypothesis is in accordance with the propensity of Fc fusion proteins to form protein aggregates [28], which is mostly induced by hydrophobic interactions [29]. Indeed, it has been shown that the coupling of the Fc region to the hFIX resulted in a difference in hydrogen atom exposition of residues 408-414 [30], which potentially favoured hydrophobic interactions and subsequent formation of hFIX-Fc fusion protein aggregates.

Our hypothesis, therefore, is that the lower plasma circulating levels of the hFIX-fusion proteins is likely due to inefficient secretion of these proteins from hepatocytes when compared to wild-type hFIX protein. There are reports of mutations within FIX or factor VIII (FVIII) genes leading to changes in protein configuration and poor secretion from the producer cells [19, 31]. The role of PACE/furin expression could also be considered as a potential limiting underlying mechanism for low hFIX fusion protein secretion [32, 33]. It is intriguing to speculate whether similar observations are made during the large-scale manufacture of these proteins as recombinant concentrates, though it is possible that changes in secretion pattern of hFIX-fusion protein may not be detected if the producer cells are lysed prior to purification of the hFIX-fusion protein.

One limitation of our study is that we did not inject HB mice, which would have allowed evaluating the hFIX activity level and the resulting correction of the bleeding phenotype after gene transfer of AAV vectors expressing hFIX-fusion proteins. However, both hFIX-fusion proteins have a lower specific activity compared to wild-type hFIX [1, 2]. This suggested that the lower hFIX:Ag level for both hFIX-fusion

In light of these results we believe the way forward for improving the efficiency of AAV mediated gene transfer is to use a gain-of-function, hyperactive form of FIX. Indeed results from a recent phase I/II clinical trial [10] showed that the use of the hFIX-Padua variant, a naturally occurring variant with approximately 8-fold higher specific activity compared to wild-type hFIX [9], in an AAV vector, could result in sustained mean steady-state FIX:C of about 34%. Interestingly, the FIX:Ag expression levels, in patients who had a negative status for cross-reactive material, were similar to that observed in the previous clinical trial sponsored by UCL/St Jude [6, 10].

Conclusion

In conclusion, our study shows that hFIX-fusion proteins, though synthesized by hepatocytes appropriately, are not efficiently secreted, thus making them unsuitable for use in gene therapy approaches, despite their extended half-life as recombinant protein replacement therapies. Our preliminary data suggests that the intracellular trafficking of hFIX-Alb and hFIX-Fc may differ but further studies are required to elicit the mechanism behind these observations.

Authorship contributions

S Le Quellec and A Dane designed the study, performed experiments and wrote the manuscript. C Rosales performed animal experiments. J Mc Intosh, N Enjolras, C Négrier, and A Nathwani critically revised the manuscript and gave final approval.

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Figures caption list

Fig.1 Schematic diagram of hFIX vector genomes and expression of hFIX molecules *in-vitro*. A) AAV2-based expression cassettes containing hFIX fused to human albumin or human IgG1 Fc, flanked by SV40 small t antigen intron before the start codon of hFIX and a SV40 polyA sequences, and under the transcriptional control of liver specific promoter LP1. Equivalent control vector genomes (ctrl1 and ctrl2) were prepared by inserting a stop codon between the FIX sequence and the fused protein, ensuring the albumin or Fc sequence was not translated. The albumin fusion constructs contained a FXIa/FVIIa-tissue factor complex- cleavable linker. ITR, inverted terminal repeat; bp, base pair; hFIXco, codon-optimised human factor IX; Alb, human albumin; Fc, Fc region of human IgG1 (hinge, CH_2, CH_3); ctrl1/2, control vector 1/2; B) Anti-hFIX Western blot on culture medium of transiently transfected Huh-7 cells with pAV expression vectors. The hFIX-Alb fusion construct and its control (hFIX-ctrl1) are shown in the left side of the figure 1B, and hFIX-Fc fusion construct and its control (hFIX-ctrl2) are shown in the right side of the figure 1B. NT=not transfected. C) FIX:Ag levels and FIX:C levels on culture medium of transiently transfected Huh-7 cells. Idelvion® spiked at 4% was used as control. NT= not transfected. Mann-Whitney test was performed for comparison of hFIX-Alb or hFIX-Fc result and hFIX-ctrl1 groups.

Fig.2 Expression of hFIX fusion molecules *in-vivo*. A) Effect of Albumin and Fc fusion to FIX on level of FIX expression *in-vivo*. Wild-type male C57Bl/6 mice were injected with 5×10^{10} vg per mouse of AAV2/8.hFIX-Alb (n=4), AAV2/8.hFIX-Fc (n=3) or AAV2/8.hFIX ctrl1/2 (n=4; n=3) vectors. B) DNA was extracted from livers harvested from each treatment group and the number of vector genomes per diploid cell

was determined in triplicate using QPCR. C) Relative hFIX mRNA copies per vg per cell from liver tissue were determined in triplicate by RT-QPCR. Mann-Whitney test was performed for comparison between hFIX-Alb and hFIX-ctrl1 groups, and between hFIX-Fc and hFIX-ctrl2 groups. Error bars represent mean ± SEM.

Fig.3 hFIX fusion protein expression in mouse liver and *in-vitro* intracellular trafficking. A) hFIX immunoprecipitation (IP) of mouse liver lysates followed by Western with anti-hFIX antibody. Coagulation reference plasma was used as a positive control. B) Intracellular trafficking experiment. Mann-Whitney test was performed for comparison between FIX:Ag values obtained after incubation with inhibitors and those obtained from untreated Huh-7 cells for each vector group. Error bars represent SEM. Results were expressed as a percentage of values obtained with control culture medium containing 0.02% ethanol.