Computationally designed liver-specific transcriptional modules and hyperactive factor IX improve hepatic gene therapy

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Key Points

• Liver-targeted gene therapy for hemophilia can be improved by using computational promoter design in conjunction with hyperfunctional FIX.
• Low and safe vector doses allow for stable supraphysiologic FIX that result in the induction of immune tolerance.

Introduction

Significant progress has recently been made toward the development of gene therapy for hemophilia B. Adenoassociated virus (AAV) vectors are among the most promising vectors for liver-directed gene therapy that are capable of achieving therapeutic factor IX (FIX) expression levels in patients suffering from severe hemophilia B.1,2 Nevertheless, there are still some issues related to the induction of AAV capsid-specific T-cell–mediated immune response against the AAV-transduced cells that need to be addressed.1-4 These inadvertent immune reactions curtailed long-term gene expression by eliminating the gene-modified cells and accounted for liver toxicity. Furthermore, the performance of these AAV vectors must be improved to achieve a bona fide cure.2 Consequently, there is a need to create the next-generation AAV vectors for liver-directed gene therapy that express higher FIX levels at lower vector doses, to the extent that stable physiologic levels of FIX can be attained, while preventing inadvertent AAV capsid-specific T-cell responses and liver toxicity. The availability of more potent vectors would also ease manufacturing needs. To increase the potency of AAV-FIX vectors, we explored the use of a bioinformatics algorithm that resulted in the identification of transcriptional cis-regulatory modules (CRMs) associated with robust hepatocyte-specific expression (M.K.C., M.Y.R., I. Petrus, P.D.B., S.S., D. Danso-Abieam, C. Le Guiner, G. Gernoux, O. Adjali, N.N., J. Willems, H.E., J. Matrai, M. Di Matteo, E.S.-K., B. Yan, A. Acosta-Sanchez, A. Meliani, G. Cherel, V. Blouin, O. Christophe, P. Moullier, F. Minogozzo, and T.V., manuscript submitted March 2013).5 These CRMs contained evolutionary conserved clusters of transcription factor binding-site motifs that confer high tissue-specific gene expression. We thus combined these hepatocyte-specific CRMs (HS-CRMs) with a synthetic codon-optimized hyperfunctional FIX transgene (ie, Padua R338L) that conferred 15-fold higher expression and activity levels than its wild-type counterpart.6,7 This novel combination approach substantially reduced the dose requirement for reaching therapeutic efficacy and thus facilitates future scale-up and clinical translation.
Study design

Additional information can be found in supplemental Methods, available at the Blood Web site. Identification of the HS-CRM relied on computational design based on a modified distance difference matrix multidimensional scaling approach, as described elsewhere.5 Generation and initial characterization of the codon-optimized FIX (coFIX) with the hyperactivating Padua mutation (ie, coFIX-R338L) were described previously.7 The most robust HS-CRM (designated as HS-CRM8) was cloned upstream of a strong liver-specific TTR promoter. This 72-bp HS-CRM8 element is derived from the human SERPINA1 gene and contains several putative transcription factor binding sites, including FOXA1, CEBP, HNF1, MyoD, LEF-1, and LEF-1/TCF. Some of these TFBSs are partially overlapping. (C) Chromatin immunoprecipitation assay confirming the binding of FOXA1 and CEBP on HS-CRM8. Antibodies specific to FOXA1 and CEBP and polymerase chain reaction (PCR) primers specific for the corresponding TFBS were used. In particular, PCR primers were designed to amplify a region within the vector corresponding to HS-CRM8 (that binds FOXA1 and CEBP), an untranscribed region on chromosome 6 was used as negative control (–). Binding events per 10^3 cells (mean ± standard deviation) were determined for each of the corresponding primer pairs. Significant differences compared with the negative control were indicated (Student t-test, *P < .05). (D) Confocal microscopy of different organs of mice injected with AAV9-HS-CRM8-TTR-GFP (5 × 10^11 vg/mouse; n = 4) with 4′,6-diamidino-2-phenylindole nuclear staining (top panels). A representative confocal scan is shown. Non-injected mice were used as controls (bottom panels). Pictures were taken at ×20 magnification.

Results and discussion

To improve the performance of AAV for liver-directed gene therapy, we explored a computational approach (Figure 1A) that led to the identification of an HS-CRM (designated as HS-CRM8) (Figure 1B) that was then cloned upstream of a strong liver-specific TTR promoter. This 72-bp HS-CRM8 element is derived from the human SERPINA1 gene and contains several putative transcription factor binding sites, including FOXA1, CEBP, HNF1, MyoD, LEF-1, LEF-1/TCF, that are strongly associated with robust liver-specific expression in vivo.
By using a chromatin immunoprecipitation assay, we subsequently confirmed robust binding of FOXA1 and CEBP on the HS-CRM8 element in livers from mice that were injected with AAV vectors containing HS-CRM8 (Figure 1C). By using GFP as reporter, we then assessed the tissue-specific expression pattern. We demonstrated by confocal microscopy that transgene expression was restricted to hepatocytes, whereas there was no detectable GFP expression in nonepithelial cells or in other tissue (Figure 1D). This is consistent with the liver-specific GFP messenger RNA expression (supplemental Figure 1A-B), despite AAV9 transduction in nonhepatic tissues (supplemental Figure 1C).

We subsequently determined the impact of the HS-CRM8 element on the expression of a synthetic hyperfunctional codon-optimized coFIX-R338L in an scAAV backbone (Figure 2B). Hydrodynamic transfection of the pAAV-HS-CRM8-TTR-co-hFIX-R338L plasmid (all plasmids are abbreviated as p) (Figure 2B) resulted in a significant (2 μg, P < .01; 5 μg, P < .001) 11- to 15-fold dose-dependent increase in circulating FIX expression levels (Figure 2D-E). The expression was assessed by using a validated hFIX-specific enzyme-linked immunosorbent assay (ELISA) (n = 4) on plasma samples collected at day 1 or 2 posttransfection. Similarly, to assess the impact of the Padua R338L mutation, hemophilic mice were hydrodynamically transfected with pAAV-HS-CRM8-TTR-co-hFIX-R338L (indicated as co-hFIX-R338L) and control pAAV-TTR-cohFIX-R338L (indicated as co-hFIX) (F). The clotting factor activity was measured by using a functional chromogenic FIX assay. Subsequently, we injected the cognate scAAV9-HS-CRM8-TTR-co-hFIX-R338L (designated as co-hFIX-R338L) (G) and scAAV9-HS-CRM8-TTR-co-hFIX (designated as hFIX) (J-L) in FIX-deficient hemophilic mice at a dose of 1 × 10^9 vg/mouse (5 × 10^9 vg/kg), 5 × 10^9 vg/mouse (2.5 × 10^10 vg/kg), and 2 × 10^10 vg/mouse (10^11 vg/kg) (n = 3 per group) (J-L). FIX activity and antigen levels were determined at the indicated times after AAV administration by using a chromogenic FIX activity assay and hFIX-specific ELISA, respectively. Results are presented as mean ± standard error of the mean.

*P < .05; **P < .01; ***P < .001 (Student t test); N.S., not significant (P > .1).

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We subsequently determined the impact of the HS-CRM8 element on the expression of a synthetic hyperfunctional codon-optimized coFIX-R338L in an scAAV backbone (Figure 2B). Hydrodynamic transfection of the pAAV-HS-CRM8-TTR-co-hFIX-R338L plasmid (all plasmids are abbreviated as p) (Figure 2B) resulted in a significant (2 μg, P < .01; 5 μg, P < .001) 11- to 15-fold dose-dependent increase in circulating FIX expression levels (Figure 2D-E) compared with the pAAV-TTR-co-hFIX-R338L (Figure 2A) control plasmid devoid of HS-CRM8. We then showed by hydrodynamic transfection of 2 microgram pAAV-HS-CRM8-TTR-co-hFIXR338L (Figure 2B) or pAAV-HS-CRM8-TTR-co-hFIX (Figure 2C) plasmids in FIX-deficient hemophilia B mice that the Padua R338L mutation resulted in a significant fivefold enhancement of FIX activity on both day 1 (P < .01) and day 2 (P < .001) (Figure 2F).

Subsequently, we produced the corresponding scAAV9-HS-CRM8-TTR-coFIX-R338L and assessed its performance in hemophilic FIX-deficient mice compared with the scAAV9-HS-CRM8-TTR-coFIX vectors at different vector doses corresponding to 1 × 10^9 vg/mouse (5 × 10^9 vg/kg), 5 × 10^9 vg/mouse (2.5 × 10^10 vg/kg), and 2 × 10^10 vg/mouse (10^11 vg/kg) (Figure 2G-L). AA9 was chosen because of its hepatotropic properties and reduced sero-prevalence compared with AAV2 in human patients. Although AAV9 can cross the blood-brain barrier, much higher vector doses are required. This is consistent with the low transduction in the brain after AAV9-HS-CRM8-TTR-GFP transduction (supplemental Figure 2C). Sustained supraphysiological FIX levels can be attained in a dose-dependent manner. Although the FIX antigen levels were comparable between both groups of mice, a significant increase in FIX activity was apparent in those mice treated with the co-hFIX-R338L transgene compared with the co-hFIX control (Figure 2G-L). Similarly, the
hyperfunctional co-hFIX-R338L resulted in a significant five- to sevenfold increased FIX activity over protein levels at all vector doses in those mice injected with scAAV9-HS-CRM8-TTR-co-hFIX-R338L (Figure 2G-I). In contrast, an activity:antigen ratio equal to 1 was observed for the co-hFIX control at all time points and vector doses (Figure 2J-L).

This is consistent with our previous results using integration-competent and integration-defective lentiviral vectors and with the increased FIX activity after muscle-directed AAV transduction in mouse and dog models. Most importantly, even at the lowest vector dose tested (5 × 10^10 vg/kg), it was possible to obtain supraphysiologic FIX levels that corrected the bleeding diathesis (supplemental Figure 2A). These FIX activity levels represent a robust improvement compared with the state-of-the-art AAV-FIX vectors, including those used in the most recent scAAV8-based clinical trial. hFIX messenger RNA expression was confined to the liver but not in other organs and tissues in mice injected with either scAAV9-HS-CRM8-TTR-co-hFIX-R338L or scAAV9-HS-CRM8-TTR-co-hFIX (supplemental Figure 2B), consistent with the GFP expression pattern (Figure 1). Transduction efficiency by quantitative polymerase chain reaction confirmed the predominant hepatotropic properties of AAV9 at the vector doses tested (10^10 vg scAAV9-HS-CRM8-TTR-co-hFIX-R338L: 9.2 × 10^4 copies per 100 ng genomic liver DNA; scAAV9-HS-CRM8-TTR-co-hFIX: 4.7 × 10^3 copies per 100 ng genomic liver DNA; P > .1; not significant).

To assess the immune consequences of expressing the hyperfunctional FIX Padua protein at high levels, we analyzed the anti-FIX antibody response before and after active immunization with wild-type FIX protein and Freund’s incomplete adjuvant. The results show that immune tolerance could be achieved because none of the mice treated with the scAAV9-HS-CRM8-TTR-coFIX-R338L vectors developed anti-FIX antibodies in contrast to the controls that were not treated with this vector (supplemental Figure 2C). This implies that hepatocyte-specific expression of the hyperfunctional FIX Padua does not increase the risk of antibody development but instead enables induction of FIX-specific immune tolerance. Previous studies have already shown that the success rates of immune tolerance induction to FIX antigen in hemophilia B mice correlates with the FIX transgene expression levels and that these higher expression levels may favor induction of regulatory T cells. Consequently, since higher FIX levels could be attained with the improved scAAV9-HS-CRM8-TTR-coFIX-R338L vector compared with the vectors containing the unmodified coFIX or the construct without CRM8, it can thus be inferred that the new gene construct is better suited for the induction of immune tolerance. To estimate the possible thrombotic risks associated with expression of hyperfunctional FIX, we determined dimer levels as a measure of fibrin degradation but detected no significant increase, even in those mice that expressed 3000% hyperfunctional FIX (supplemental Figure 3D), at least in the short term.

Collectively, our data indicate that the combination of computational vector design and the use of synthetic hyperactive coFIX-R338L represents a promising strategy for improving the efficacy of hemophilia B gene therapy by using AAV vectors, which will allow the use of lower and thus safer vector doses in patients suffering from hemophilia. A phase 1/2 clinical trial is currently underway to begin safety, efficacy, and optimal dose testing of an scAAV8 vector containing the Padua FIX gene, but preclinical and clinical data are not yet available.

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Authorship
Contribution: N.N. designed and performed experiments, analyzed data, and wrote the paper; M.Y.R., H.E., S.S., S.D., E.S.K., and O.G. performed experiments and analyzed data; P.D.B. conducted the bioinformatics analysis; B.T. and H.M.V. performed confirmatory experiments and analyzed data; and M.K.C. and T.V. designed experiments, coordinated the work, and wrote the paper.

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References


