Gene therapy may provide a cure for hemophilia and overcome the limitations of protein replacement therapy. Increasing the potency of gene transfer vectors may allow improvement of their therapeutic index, as lower doses can be administered to achieve therapeutic benefit, reducing toxicity of in vivo administration. Here we generated codon-usage optimized and hyperfunctional factor IX (FIX) transgenes carrying an R338L amino acid substitution (FIX Padua), previously associated with clotting hyperactivity and thrombophilia. We delivered these transgenes to hemophilia B mice by hepatocyte-targeted integration-competent and -defective lentiviral vectors. The hyperfunctional FIX transgenes increased FIX activity reconstituted in the plasma without detectable adverse effects, allowing correction of the disease phenotype at lower vector doses and resulting in improved hemostasis in vivo. The combined effect of codon optimization with the hyperactivating FIX-R338L mutation resulted in a robust 15-fold gain in potency and therefore provides a promising strategy to improve the efficacy, feasibility, and safety of hemophilia gene therapy. (Blood. 2012;120(23):4517-4520)

Methods

Additional information can be found in supplemental Methods (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). The codon usage optimization was carried out using proprietary algorithms (BaseClear). The R338L mutation was introduced using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Lentiviral vectors were produced and characterized as described. Adult hemophilia B mice were injected intravenously at the indicated
vector doses. D-dimer and FIX antigen levels were determined by ELISA and FIX activity with activated partial thromboplastin time or a chromogenic assay (Hyphen Biomed). Tail-clipping assay was performed as described. Tail-clipping assay was performed as described.12 All animal experiments were approved by the respective Animal Care and Use Committees.

Results and discussion

We cloned a canine codon-optimized FIX (co-cFIX) cDNA, or its wild-type (WT) counterpart (cFIX), into miR-142-regulated LVs under the transcriptional control of the hepatocyte-specific ET promoter (supplemental Figure 1). We compared the potency of the 2 transgenes in hemophilia B mice (5 or 10 × 10⁸ transducing units [TU]/mouse). FIX activity was 3.4- and 2.2-fold higher for the co-cFIX than its WT counterpart in both dose cohorts (Figure 1A-B). Vector genome content was similar in the liver of treated animals receiving matched doses of LVs. FIX inhibitors were undetectable by Bethesda assay in all treated mice (not shown).

These data show that a co-cFIX transgene can significantly increase expression, without any evident impairment of specific enzyme activity or increased immunogenicity.13

We next generated WT or co-cFIX transgenes bearing a point mutation corresponding to the previously described hyperfunctional FIX-R338L mutant that was shown to increase its specific
activity 5- to 10-fold. As shown in Figure 1C, hemophilia B mice treated with LV carrying the WT or hyperfunctional FIX-R338L (7 × 10⁹ TU/mouse) expressed ~8% of normal cFIX protein in the circulation, whereas mice treated with the hyperfunctional co-cFIX-R338L transgene reached ~20% (consistent with the aforementioned 2- to 3-fold increased expression of co-cFIX). However, both the hyperfunctional cFIX-R338L and co-cFIX-R338L transgenes exhibited >5-fold higher activity with respect to the protein levels, resulting in up to 125% of normal clotting activity for the co-cFIX-R338L transgene (Figure 1D). These data show that the hyperfunctional co-cFIX-R338L transgene provides a 15-fold gain in potency with respect to the WT sequence and is greater than what has been reported with other FIX mutants.

To evaluate whether the co-cFIX-R338L transgene allows lowering LV doses to reach therapeutic activity, hemophilia B mice were injected with 1.25 or 2.5 × 10⁸ TU/mouse. Treated mice expressed 0.7 and 3.4% of normal cFIX protein, respectively. However, their clotting activity was 6.4% and 19% of normal, with 6- to 9-fold hyperactivity with respect to protein level (Figure 1E-F). None of the mice treated with hyperfunctional transgenes developed inhibitors (not shown). In a tail-clipping assay, hemophilia B mice treated with low-dose co-cFIX-R338L LV lost significantly less blood than treated with a matched dose of co-cFIX LV and were indistinguishable from WT mice, indicating the superior performance of the R338L FIX in achieving hemostasis in vivo (Figure 1G).

We reproduced these findings using LVs encoding a human hyperfunctional co-hFIX-R338L, resulting in 5- to 7-fold increased activity over protein levels at 2 different LV doses and reconstituting supra-physiologic clotting activity in hemophilia B mice (up to >400% at the highest dose; Figure 1H-I). These mice did not develop anti-hFIX antibodies, even after challenge with hFIX protein, indicating that gene therapy induced immune tolerance to the WT protein (Figure 1J).

Although Integrase-Defective Lentiviral Vectors (IDLVs) exhibit a reduced genotoxic risk, hepatic transgene expression is less efficient compared with their integration-competent counterparts and tends to decline with time. We now show that using co-cFIX and hyperfunctional co-cFIX-R338L transgenes may offset some of the limitations of IDLVs, which now allow reconstituting FIX activity to fully therapeutic levels, exceeding 50% of normal FIX activity (Figure 2A-B). The extent of the increased expression and activity with these IDLVs was consistent with that observed with integrase-competent LVs.

To assess the possible risks associated with expression of hyperfunctional FIX, we determined D-dimer levels as a measure of fibrin degradation. D-dimers are not normally present in plasma, except when the coagulation system has been activated, as in the
case of thrombosis.17 We did not detect a significant increase in D-dimer levels in treated mice, including those that expressed the highest levels of hyperfunctional FIX (Figure 2C), suggesting that thrombotic risk was not increased after gene therapy in these hemophilic mice, at least in the short term (1-2 months after injection). We also performed histopathologic evaluation of liver, spleen, kidney, heart, lungs, and brain of mice long-term reconstituted (10 months after LV) to supra-physiologic levels by co-cFIX-R338L LV (up to 125% of normal FIX activity), and we found no difference between mice treated with WT and hyperfunctional transgene (not shown).

Thrombosis risk is expected to be low at the levels tested, and further ad hoc studies in permissive thrombosis models are required to establish the long-term safety of delivering hyperfunctional FIX transgenes.18 Our data, together with the known impact of the R338L mutation on substrate interaction rather than zymogen activation,10 suggest that expressing limited amounts of hyperfunctional FIX to reach a threshold therapeutic level represents a viable and promising strategy to improve the efficacy, feasibility, and safety of hemophilia gene therapy.

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References


Authorship

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Correspondence: Luigi Naldini, San Raffaele Telethon Institute for Gene Therapy and “Vita Salute San Raffaele” University, Via Olgettina 58, 20132 Milano, Italy; e-mail: naldini.luigi@hsr.it; and Thierry VandenDriessche, Free University of Brussels, Laarbeeklaan 103, Brussels, Belgium 1090; e-mail: thierry.vandendriessche@vub.ac.be.
Hyperfunctional coagulation factor IX improves the efficacy of gene therapy in hemophilic mice

Alessio Cantore, Nisha Nair, Patrizia Della Valle, Mario Di Matteo, Janka Mátrai, Francesca Sanvito, Chiara Brombin, Clelia Di Serio, Armando D'Angelo, Marinee Chuah, Luigi Naldini and Thierry VandenDriessche