ELSEVIER

Blood 142 (2023) 21–22

The 65th ASH Annual Meeting Abstracts

ORAL ABSTRACTS

321.COAGULATION AND FIBRINOLYSIS: BASIC AND TRANSLATIONAL

Evolution Directed Gene Engineering (EDGE) Identifies Single Amino Acid Substitutions in FVIII with Six-Fold Increased *in Vitro* and *In Vivo* Activity

Lucas Van Gorder[1], *Reilly Peng*[1], *Julia Chau*[1], *Ben J Samelson-Jones, MD PhD*[2]

[1] The Children's Hospital of Philadelphia, Philadelphia, PA
[2] Division of Hematology, Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, PA

Background:Current AAV gene therapy for hemophilia A (HA) is limited by low sustained factor VIII (FVIII) levels. In contrast, AAV gene therapy for hemophilia B benefits from the widespread adoption of the single amino acid (AA) substituted high-specific-activity FIX variant R338L. We have previously shown that wild-type (WT) FIX has not evolved for maximal specific activity, but rather has evolutionary conserved AAs that limit procoagulant activity (Samelson-Jones et al. Blood Advances 2021). FVIII may have evolved with similar considerations. Based on this work, we developed a novel screening technique, termed Evolution Directed Gene Engineering (EDGE), to identify new hyperactive single AA substituted FVIII variant transgenes to address the current limitations of HA gene therapy.
Aims:To find and characterize single AA substituted FVIII variants that can enhance gene transfer.
Methods:EDGE evaluates AA positions fulfilling 2 criteria: 1) highly evolutionary conserved and 2) not associated with the disease phenotype. Here, EDGE compared 33 mammalian FVIII orthologues and then eliminated positions associated with known HA-causing missense mutations. FVIII variants were transiently expressed in Huh7 cells and cultured media was assayed for FVIII activity and antigen. Recombinant FVIII protein of lead candidates were purified to homogeneity and assayed for FVIII activity with one- and two-stage aPTT assays, thrombin generation assay (TGA), and enzyme kinetics studies. The *in vivo* efficacy of the variants was tested by tail clip assays of HA mice after retro-orbital delivery of the protein or liver-directed AAV gene therapy.
Results: Out of 52 AA positions identified by EDGE, we found 12 AA positions in FVIII where single AA substitutions increased specific activity ≥2-fold (Figure 1A). The largest increase in specific activity was 6-fold, which occurred for multiple AA substitutions at position 659. Purified recombinant protein of FVIII-K659M and -K659V both demonstrated 6-fold increased activity in one- and two-stage aPTT assays, but comparable thrombin activation and A2-stability to FVIII-WT. TGA show the FVIII-K659M/V variants have greater than 6-fold improvements in endogenous thrombin potential and peak thrombin generation. Compared to FVIII-WT, FVIII-K659M/V proteins have an increased k_{cat} and decreased *app*K_D which suggests that the mechanism of increased specific activity is due to enhanced interactions with FIXa. This is similar to the mechanism of hyperactivity of FIX-R338L.
FVIII-K659M/V also demonstrated enhanced procoagulant activity *in vivo*. In tail clip injuries of HA mice (n≥4) treated with purified protein, the FVIII-K659M/V proteins restored normal hemostasis at 5-fold lower concentrations than FVIII-WT. When mice were treated with equal doses of 2.5 μg/kg of protein, FVIII-K659M/V restored normal hemostasis while FVIII-WT had no significant change in blood loss (Figure 1B). As expected, 12.5 μg/kg of FVIII-WT protein was required to restore blood loss to WT mouse levels.
FVIII-K659 substituted variants also enhanced AAV based gene therapy. HA mice (n=6) treated with AAV8 vectors expressing FVIII-K659C had 6-fold higher plasma FVIII activity than mice (n=6) treated at the same dose with AAV8 vectors expressing FVIII-WT. HA mice that received 4×10^{12} vg/kg of AAV8 FVIII-K659C had less bleeding than HA mice that received the same dose of AAV8 FVIII-WT, but similar blood loss as WT mice after tail clip injury.
Conclusions:The success of EDGE in identifying multiple new high specific activity FVIII variants supports the hypothesis that FVIII-WT is not optimized for specific activity and thus is amenable to bioengineering approaches. The ≥5-fold enhanced *in vitro* and *in vivo* hemostatic activity of the FVIII-K659 substituted variants and initial murine gene therapy studies support their translational potential to address current limitations of HA gene therapy. These results also implicate K659 in modulating the FVIIIa/FIXa interaction. EDGE may also be applicable to other gene therapy targets as well.

Disclosures Samelson-Jones: *GeneVentiv:* Current holder of *stock options* in a privately-held company; *Biomarin:* Consultancy; *Genentech:* Consultancy; *Pfizer:* Consultancy, Honoraria; *Amarna:* Current holder of *stock options* in a privately-held company.

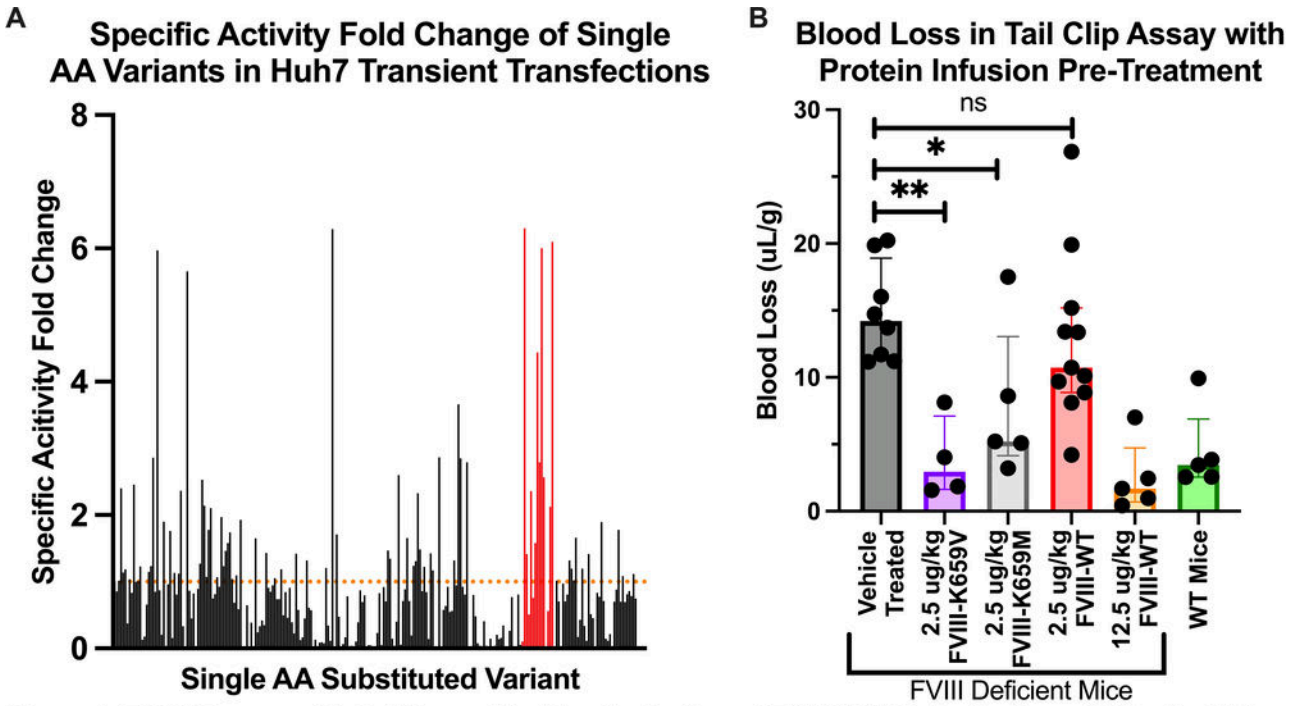

Figure 1: EDGE Screen with *In Vitro* and *In Vivo* Evaluation of FVIII K659 Substitutions. (A) *In vitro* fold change of specific activity. Single amino acid FVIII variants were transiently expressed in Huh7 cells and assayed for FVIII antigen and activity. Amino acid 659 variants are highlighted in red. Data is an average of n≥2 wells. (B) *In vivo* improvements in hemostatic function. Mice were pre-treated with a retro-orbital infusion of purified FVIII protein, then tails were clipped to a 3 mm diameter. Blood was collected for 12 minutes after injury and quantified. Data is displayed as median blood loss with individual points for each mouse treated with n≥4 in each group. (ns P > .05, * P < .05, ** P < .01)

Figure 1

https://doi.org/10.1182/blood-2023-178106