Development of Coagulation Factor VIII Transgenes That Confer Greater Potency to Gene Therapies for Hemophilia A Through Ancestral Sequence Reconstruction

Christopher Coyle1, Jasmine Anickat1, Alex Condra2, Luke Blackmon2, Phillip M. Zakas2, H. Trent Spencer3, Eric A. Gaucher2, Christopher B. Doering5
1Graduate Program in Molecular and Systems Pharmacology, Laney Graduate School, Emory University, Atlanta, GA, 2Department of Biology, Emory University, Atlanta, GA, 3Gene Therapy Program, Children’s Healthcare of Atlanta, Emory University, Atlanta, GA, 4Infectious Disease Division, BIDMC, Clinical Adeno Virus Unit, Boston, MA, 5Affiliated Cancer and Blood Disorders Center, Emory University, Atlanta, GA, 6Department of Biology, Georgia State University, Atlanta, GA

Abstract

Clinical adeno-associated viral (AAV) vector gene therapy candidates for hemophilia A currently require vector doses >10X higher than is required for similar hemophilia B gene therapy despite the 25-fold lower requirements for coagulation factor VIII (FVIII) concentration. This highlights a ~250-fold difference in expression. Additionally, clinical hemophilia B gene therapy benefits from incorporating amino acid substitution(s) that increase factor IX (FIX) activity. It is anticipated that FVIII bioengineering would provide comparable advantages. Our group has pioneered efforts to capture the inherent advantageous biological properties of certain animal FVIII orthologs. More recently, we utilized ancestral sequence reconstruction (ASR) as a platform to study the evolutionary variation in coagulation factors V, VII, VIII, IX, X, and von Willebrand factor and discovered additional functional diversity that can be captured to engineer next-generation gene therapy and protein pharmaceuticals for bleeding disorders. For these studies, phylogenetic trees were constructed from extant sequences. Other than a single inconclusive variation within the interfered factor Vii phylogeny, all reconstructions were identical, further supporting the robustness of ASR. Here, C2Ms correspond to the distinct ancestral C domain decomposed (BDD) FVIII proteins were synthesized de novo. These inferred ancestral FVIII variants share 87-88% sequence identity with BDD human FVIII (hFVIII). Using transient expression from HEK293T/17 cells, the ancient FVIII variants showed 2-27 fold-higher FVIII activity than HSQ (n ≥ 3, p<0.0001). The top 3 ancestral (An) FVIII candidates, termed An63, An84 and An70, all had comparable FVIII activity at 27-, 24- and 22-fold greater than HSQ, respectively, as determined by one-stage coagulation assay (n ≥ 10, p<0.0001). Furthermore, codon optimized An63 and An70 in a liver-directed AAV cassette exhibited significantly greater FVIII production than HSQ when transduced into Huh7 cells (p<0.05, 0.01, respectively), suggesting high-expressing ancestral FVIII proteins can be efficiently expressed from liver cells. Upon purification of the candidate molecules, An63 was notably secreted in single chain, as opposed to the processed heavy-chain light-chain heterodimer. The specific activities of AnFVIII and An70 were 18,000 and 25,000 U/mg and 20,000 units/mg, respectively, roughly 2-4 fold higher than HSQ. In an effort to ‘humanize’ the lead candidate molecules, the C1 and C2 domains of HSQ were swapped with their cognate ancestral C1 and C2 domains. The resulting ancestral hybrids were non-inferior to their fully ancestral counterparts; however, the An70/HSQ hybrid had significantly diminished activity compared to An70 (n=15, p< 0.0001). These findings suggest that the C domains of An70 are required for high activity, while An63 and An84 C domains are not. This demonstrates the first observation of substitutions in the C domains of FVIII influencing the activity of a FVIII variant. Further studies are in progress to identify the mechanistic role for the C domain(s) in AnFVIII biosynthesis and/or activity. Collectively, these findings validate ASR approach as an enabling platform for the development of more potent gene therapy product candidates for hemophilia A, as has been achieved in hemophilia B with factor IX Padua.

Figure 1: Phylogenetic tree of coagulation Factor VIII, generated by ancestral sequence reconstruction using extant nucleic acid and amino acid sequences. The nodes of interest (circled in red) were liver codon optimized and synthesized de novo and further examined.

Figure 2: HEK293T/17 cells were transiently transfected with CDNA corresponding to the selected BDD AnFVIII molecules and examined with one stage aPPT-based coagulation assay. Data is normalized to the FVIII activity of HSQ (which was always within the standard range of detection) and analyzed with one-way ANOVA, multiple comparisons (to HSQ). Here, * p<0.03, ** p<0.002, *** p<0.0002, and **** p<0.0001.

Conclusions and Future Directions

- Ancestral sequence reconstruction is a valid method of protein engineering that can generate high-expression constructs with relatively low sequence identity to the human proteins.
- AnFVIII proteins are highly active in the human coagulation cascade and can be efficiently expressed my gene-therapy relevant cell types.
- For An63 and An84, the heavy chain confers most of the improvement in activity, however, domain swapping studies showed that epistasis between the heavy and light chains is critical to maintain the highest activity.
- Novel substitutions in the C domains of An70 likely account for the high expression, whereas they do not for An63, suggesting separate mechanisms of action for their high activities.
- Future studies will include AAV- and Lentiviral-mediated gene transfer in a murine hemophilia A model, identifying specific amino acids responsible for the high activity, and exploring the mechanisms responsible for AnFVIII’s high activity.

Figure 4: Table 2: Lead molecules, An63, An70 and An84 were stably expressed in 293F cells, propagated to 1L cultures in Freestyle293 medium, and subsequently purified by a 3-stage purification protocol. 1ug of FVIII was analyzed on a 4-15% SDS-PAGE and visualized with GelCode Blue. Some samples were activated with 4U of human thrombin for 3 and 37°C and quenched before loading. An63 was predominantly expressed in single chain form whereas An70 and An84 were a mix of single chain and heavy-chain-light-chain heterodimer. Purified samples were assessed for activity using one-stage coagulation assay and specific activity expressed in FVIII U/mg of protein.

Figure 5: AnFVIII's HSQ-hybrid molecules were generated according to the above (bi-scale) schematics. Constructs were tested identically to those in figure 2. Data is normalized to the FVIII activity of HSQ and analyzed with one-way ANOVA, multiple comparisons (to An63) where * p<0.0001.

Amino acid substitutions in the C domains of An63 were not required to maintain high FVIII activity, however substitutions in the A domains were.

Figure 6: AnFVIII's HSQ-hybrid molecules were generated according to the above (bi-scale) schematics. Constructs were tested identically to those in figure 2. Data is normalized to the FVIII activity of HSQ and analyzed with one-way ANOVA, multiple comparisons (to An70) where * p<0.03 and *** p<0.0001.

Substitutions in the C domains of An70 are required to maintain high FVIII activity, as evidenced by the 2-fold loss of activity of 70H-2 compared to An70. The poor activity of 70H-1 suggests epistasis between the C and A domains for superior FVIII activity.

Figure 7: AnFVIII's HSQ-hybrid molecules were generated according to the above (bi-scale) schematics. Constructs were tested identically to those in figure 2. Data is normalized to the FVIII activity of HSQ and analyzed with one-way ANOVA, multiple comparisons (to An84) where * p<0.03, ** p<0.002, *** p<0.0002, and **** p<0.0001.

All domains in An84 (except C1 where there are no substitutions) were required to maintain high FVIII activity, however the A2 domain confers the largest activity increase. Domains on their own could not restore high FVIII levels suggesting epistatic effects between domains.