Towards Improving the Treatment of Hemophilia A with Directed Evolution of the Factor VIII Transgene

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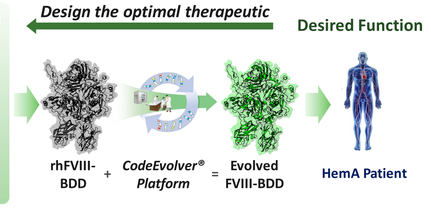
Abstract

Recombinant Factor VIII (FVIII) therapies were first approved by the FDA in 1992, yet the need for frequent infusions, appearance of neutralizing antibodies in patients, and limited efficacy has led to continued research into Hemophilia A (HemA) treatments, particularly gene therapy (1-3). The success of the Padua-FIX variant in Hemophilia B gene therapy raises the possibility that an improved version of the FVIII transgene might offer great promise for treating HemA. Specifically, a FVIII transgene with improved expression, secretion, stability, and cofactor potency as well as reduced immunogenicity could improve upon current gene therapy strategies by allowing lower doses and providing better patient outcomes. Rather than relying on the serendipitous identification of beneficial variants, we employed the CodeEvolver® directed evolution technology to engineer FVIII variants with better properties.

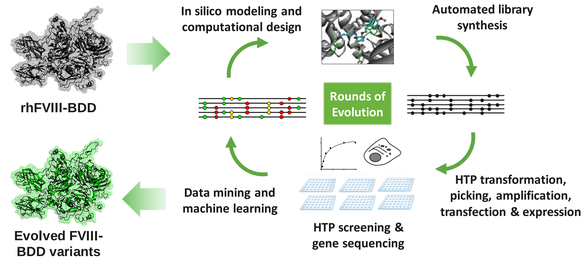
Using CodeEvolver®, which entails high-throughput protein expression, in vitro screening with patient-derived samples, next-generation sequencing, and bioinformatics, we screened >12,000 variants of a B-domain deleted FVIII (FVIII-BDD) over eight rounds of iterative evolution. Our screens identified mutant FVIII transgenes with superior properties as compared to wild-type FVIII-BDD. Whereas wild-type FVIII-BDD loses >50% of its activity in Hemophilia A patient plasma within 48 hours, engineered FVIII-BDD variants retain >80% activity after 4 days. Furthermore, the engineered FVIII-BDD variants show >30-fold increased expression from HepG2 liver cells and >20-fold improved potency in a chromogenic FXa generation assay. During our directed evolution program, to address concerns around the risk of patients generating FVIII neutralizing antibodies, we targeted in silico-predicted major histocompatibility complex (MHC) class II epitopes, reducing the number of predicted epitopes by 30%. Our results show the promise of protein evolution when applied to transgenes and gene therapy technologies, with the goal of improving outcomes for patients suffering from HemA and other genetic disorders.

Therapeutic Blueprint:
- Improve specific potency of WT protein
- Increase expression from patient liver
- Increase stability in circulation and decrease clearance
- Decrease immunogenicity



Codexis CodeEvolver® Technology

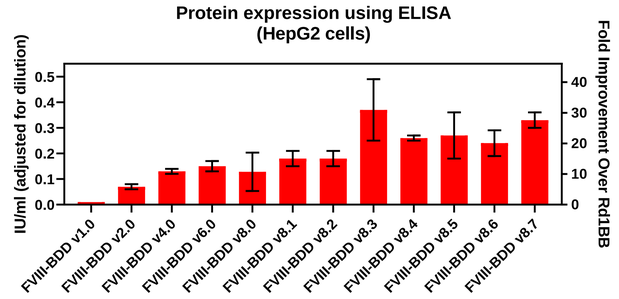
The CodeEvolver® directed evolution platform is a suite of technologies comprising protein library design, construction, high-throughput expression & screening, next-generation sequencing, and bioinformatics, used to identify protein variants with superior properties (4).



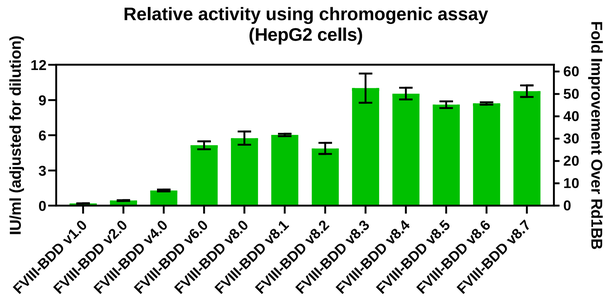
Results

FVIII-BDD variants display improved expression

HepG2 cultures were transfected with plasmid DNA of FVIII-BDD variants (recombinant human (rh) FVIII-BDD or variants FVIII-BDD v1.0 through v8.7). Cells expressed proteins for 3 days and protein accumulation was measuring using the total FVIII antigen ELISA kit (Abcam). Compared to rhFVIII-BDD, variants FVIII-BDD v8.3 and v8.7 display ~30-fold improved expression.



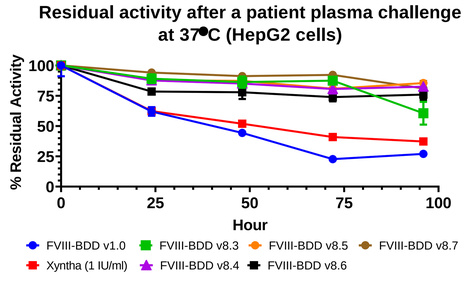
FVIII-BDD variants display improved cofactor potency



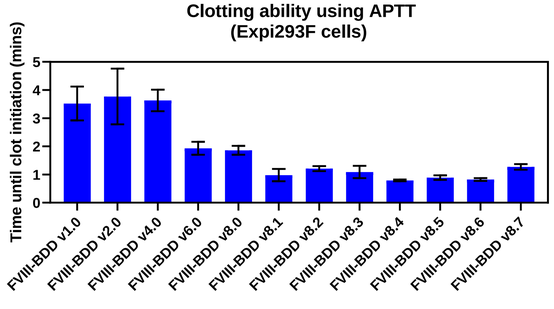
HepG2 cultures were transfected with plasmid DNA of FVIII-BDD variants (recombinant human (rh) FVIII-BDD or variants FVIII-BDD v1.0 through v8.7). Cells expressed proteins for 3 days and FVIII cofactor potency was measured via Factor Xa generation using the chromogenic BIOPHEN™ FVIII:C assay (HYPHEN BioMed). Compared to rhFVIII-BDD, variants FVIII-BDD v8.3 through v8.7 display ~50-fold improved cofactor potency.

FVIII-BDD variants display improved stability in patient plasma

HepG2 cultures were transfected with plasmid DNA of FVIII-BDD variants (recombinant human (rh) FVIII-BDD or variants FVIII-BDD v1.0 through v8.7). Cells expressed proteins for 3 days and the supernatants containing FVIII-BDD variants were challenged with incubation with HemA patient plasma at 37 °C for 100 hr. At selected time points, samples were taken and FVIII cofactor potency was measured. rhFVIII-BDD retained only ~25% of its activity after 100 hr, while variants FVIII-BDD v8.2 through v8.5 retained >75%.

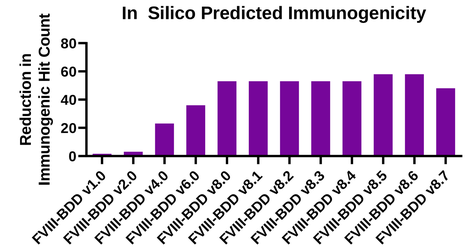


FVIII-BDD variants display improved clotting times



Expi239F cultures were transfected with plasmid DNA of FVIII-BDD variants (wild-type recombinant human (rh) FVIII-BDD or variants FVIII-BDD v1.0 through v8.7). Cells expressed proteins for 3 days and the supernatants containing FVIII-BDD variants were collected and diluted. Clotting times were measured using the Activated Partial Thromboplastin Time (APTT) assay. The diluted rhFVIII-BDD required ~3.5 min to induce clot formation, while variants FVIII-BDD v8.1 through v8.6 induced clot formation within 1 min.

FVIII-BDD variants are predicted to have less immunogenic regions



The immunogenic hit count (IHC) is a predicted value that reflects the number of regions across a protein sequence that are at risk for being particularly immunogenic due to their predicted ability to bind to a large number of MHC class II alleles. If a peptide is predicted to bind to at least 50% of these alleles, it is counted toward the IHC score. As observed in the graph, variants throughout the optimization process have reduced IHC scores, indicating that they have a fewer predicted number of high-risk immunogenic regions.

Conclusions

Factor VIII likely has evolved in nature to be transiently expressed as needed in response to bleeds. However, the protein suffers from low yield, poor stability, and fast clearance from the blood, causing challenges for the treatment of HemA using protein replacement and gene therapy.

Using directed evolution, we discovered variants of FVIII-BDD, containing up to 31 amino acid modifications, that overcome the fundamental biophysical limitations of the native rhFVIII-BDD sequence and have:
- Improved expression & secretion
- Improved stability in HemA patient plasma at physiological conditions
- Improved cofactor potency (i.e., greater enhancement of Factor IXa activity)

In addition to the biophysical improvements, FVIII-BDD was also evolved to improve the immunogenicity risk profile. FVIII-BDD v8.1 through v8.7 are predicted to have 30% less MHC class II epitopes compared to FVIII-BDD, potentially reducing the risk of patients generating neutralizing antibodies against our variants in clinic.

We believe that these evolved FVIII-BDD variants may be useful for a new generation of gene therapies capable of significantly improving outcomes in patients with Hemophilia A.

References

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