Genetic modifications that increase factor VIII secretion *in vitro* and *in vivo*

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Expression of factor VIII (FVIII) in heterologous systems (e.g. recombinant FVIII manufacturing and gene therapy) is 2-3 orders of magnitude lower than that observed with other genes using similar expression strategies. We have used insights from investigating the regulation of FVIII secretion to bioengineer FVIII variants with improved secretion. Inefficient secretion of FVIII correlates with interaction with the protein chaperone BiP. We previously reported a BiP binding region within FVIII A1 domain residues 226-336. A point mutation, Phe309Ser, increased FVIIII secretion three-fold in transient transfection experiments in COS cells. A second strategy exploited insights into the role of the heavily glycosylated B domain. Combined deficiency of FV and FVIII results from null expression of the mannose-binding lectin LMAN1. LMAN1 shuttles between the endoplasmic reticulum (ER) and the Golgi compartment to facilitate glycoprotein trafficking through the secretory pathway. The FVIII B domain contains 18 asparagine (*N*)-linked glycosylation attachment sites, which may mediate interaction with LMAN1. Addition of several *N*-linked oligosaccharides within a short B domain spacer improves secretion of B domain-deleted (BDD)-FVIII 10-fold. When the Phe309Ser mutation is incorporated into constructs containing the short glycosylated B domain spacer, a further improvement in secretion is observed up to 25-fold compared to BDD-FVIII. These FVIII variants were expressed *in vivo* utilizing hydrodynamic tail vein injection of naked plasmid DNA into the $FVIII^{-/-}$ hemophilia A mouse model. Expression is peak from 24 to 48 h post-injection and continues for 72-96 h. Expression was analyzed from mouse plasma obtained from retro-orbital blood sampling and assayed for FVIII activity by chromogenic assay and for antigen by a human FVIII-specific ELISA. Average BDD-FVIII expression was 123 ng mL^{-1}. A variant with 226 amino acids (aa) of B domain with six consensus sites for N-linked glycosylation was expressed *in vivo* five-fold higher than BDD-FVIII and the hybrid FVIII variant which includes the Phe309Ser mutation, 309Ser/226aa/N6, was expressed 25-fold higher than BDD-FVIII. FVIII bioengineered for improved secretion will be an alternative for gene therapy strategies as well as recombinant FVIII production in manufacturing or transgenic strategies.

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