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Philip Zakas_____ _Jan 30, 2016_____

Date

Evolutionary approaches to coagulation factor VIII biopharmaceutical engineering

By

Philip M. Zakas

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Molecular and Systems Pharmacology

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Evolutionary approaches to coagulation factor VIII biopharmaceutical engineering

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Bachelor of Science, Elon University 2009

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An abstract of

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Abstract

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Deficiencies of coagulation factor VIII (FVIII) result in the bleeding disorder hemophilia A. Current treatments are limited to protein replacement through intravenous infusions of recombinant or plasma-derived FVIII. Despite adequate management of the disease in several countries, FVIII replacement therapy remains unavailable to 75% of the global population. Gene therapy through adeno-associated or lentiviral vector delivery offers the potential for a long-term treatment or cure; however, *in vivo* biosynthesis of FVIII has not achieved therapeutic levels at clinically tolerable viral doses. This biosynthetic limitation is the largest obstacle in the development of improved protein therapeutics and the establishment of gene therapy protocols. Characterization of orthologous FVIII molecules from mammalian species has led to translational discoveries regarding FVIII biosynthesis and biochemistry. Mouse FVIII demonstrates a six-fold increase in stability following thrombin activation. Canine FVIII demonstrates a two-fold increase in coagulant activity per molecule. Porcine FVIII demonstrates 10-100 fold enhanced biosynthesis compared to human FVIII in heterologous expression systems. Incorporation of porcine domains into human FVIII resulted in a hybrid molecule that retains high biosynthesis, demonstrating the ability to bioengineer a FVIII molecule with enhanced therapeutic properties. To expand this ortholog-based bioengineering approach, we characterize a novel FVIII ortholog derived from sheep for unique biochemical characteristics. Traditional bioengineering efforts for FVIII through rational design or directed-evolution are not feasible. Structural data regarding FVIII is limited. Directed-evolution approaches require large quantities of recombinant protein and are likely to result in an inactive molecule; 1437 unique missense mutations within the 2332 FVIII residues have been documented in hemophilia A patients. A novel approach to bioengineering is critical for the development of improved FVIII therapies. In pursuit of this, we investigate the molecular evolution of extant and predicted FVIII sequences through ancestral sequence reconstruction and establish this methodology as a platform for bioengineering. We constructed and characterized predicted ancestral sequences to the most studied extant FVIII molecules and found incremental changes in amino acid sequence that result in significant changes in biochemical properties. Using this platform, we engineered novel FVIII molecules with enhanced biochemical properties through minimal amino acid substitutions.

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List of Abbreviations

AAV: adeno-associated viral

AHA: acquired hemophilia A

APC: activated protein C

AQ: activation quotient

ASR: Ancestral sequence reconstruction

BDD: B-domain deleted

BHK: baby hamster kidney

BiP: immunoglobulin-binding protein

BSA: bovine serum albumin

CDC: Centers for Disease Control and Prevention

cFVIII: canine factor VIII

CHAMP: CDC Hemophilia A Mutation Project

CHO: Chinese hamster ovary

CHOP: CCAAT/-enhancer-binding protein homologous protein

CNX: calnexin

COPII: coat protein II vesicles

COS-1: simian kidney fibroblast

CRT: calreticulin

EDEM1: ER degradation-enhancing alpha-mannosidase-like protein 1

ER: endoplasmic reticulum

ERAD: ER-associated degredation

ERGIC: ER-Golgi intermediate compartment

ET3: human/porcine high expression hybrid

FACT: normal pooled human plasma

FcRn: neonatal Fc receptor

FDA: Food and Drug Administration

FEIBA: factor eight inhibitor bypassing activity

FII: factor II; prothrombin

FV: factor V

FVII: factor VII

FVIII: factor VIII

FIX: factor IX

FX: factor X

FXI: factor XI

FXIII: factor XIII

FIIa: activated FII; thrombin

FVIIa: activated factor VIII

FVIIIa: activated factor VIII

FIXa: activated factor IX

FXa: activated factor X

FXIIIa: activated factor XIII

GRP78: glucose-regulated protein MW 78.0

GTI: glucosidase I

GTII: glucosidase II

HAMSTeRS: Hemophilia A Mutation, Structure, Test and Resource Site

HBST: HEPES buffered saline with Tween 80

HCV: hepatits C virus

HDX: hydrogen-deuterium exchange

HEK293T-17: human embryonic kidney cell line

HepG2: hepatocellular carcinoma cell line

hFVIII: human FVIII

HIV: human immunodeficiency virus

HAS: human serum albumin

HSC: Hematopoietic stem cells

HSV: herpes-simplex virus

IACUC: Institutional Animal Care and Use Committee

Ig: immunoglobulin

ITI: immune tolerance induction

ITR: inverted terminal repeat

kb: kilobases

kDa: kilodalton

LMAN1: lectin, mannose-binding 1; ERGIC-53

LRP1: low-density lipoprotein receptor-related protein

LSEC: liver sinusoidal endothelial cell

LV: lentiviral

MAb: monoclonal antibody

MCFD2: multiple coagulation factor deficiency protein 2

MFGE8: milk fat globule-EGF factor 8

mFVIII: murine factor VIII

MSC: mesenchymal stem cells

oFVIII: ovine factor VIII

PACE: paired basic amino acid cleavage enzyme

PBS: phosphate-buffered saline

pdFVIII: plasma-derived factor VIII

PDI: protein disulphide isomerase

PEI: Polyethyleneimine

PEG: polyethylene glycol

pFVIII: porcine factor VIII

PUP: previously untreated patient

SAXS: small angle x-ray scattering

SCID: severe combined immunodeficiency

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIN: self-inactivating

SIPPET: Survey of Inhibitors in Plasma-Products Exposed Toddlers

TPO: thrombopoeitin

UDP-GlcNAc: uridine diphosphate N-acetylglucosamine

UGT: UDP-glucose: glycoprotein glucosyltransferase

UTR: untranslated region

vWF: von Willebrand factor

WAS: Wiskott-Aldrich syndrome

Introduction

Hemophilia

Hemophilia is a rare X-linked bleeding disorder affecting approximately 400,000 people worldwide. It is characterized by spontaneous joint bleeds, hemarthroses, and potentially life-threatening bleeding episodes. There are two forms of hemophilia, A and B, each resulting from the deficiency of individual coagulation factors in the plasma and each occurring at different frequencies in the population. Hemophilia A is the absence of functional coagulation factor VIII (FVIII), a procofactor for factor IX (FIX) in the intrinsic pathway of blood coagulation. Hemophilia B is a bleeding disorder caused by the absence of FIX, a serine protease which proteolytically activates FX downstream in the coagulation cascade. Because both FVIII and FIX are required for coagulation, individual deficiencies of either factor results in an identical bleeding phenotype. Hemophilia A or B occurs due to mutations within either the F8 or F9 gene, respectively, on the X chromosome or through mutations that affect proper mRNA transcript splicing or polypeptide assembly and secretion. Both diseases present a range of clinical severities correlating with the circulating levels of functional coagulation factor. Typically, mild phenotypes are observed when plasma FVIII levels are within 5 - 45% normal concentrations and severe, life-threatening symptoms occur at levels less than 1%. Globally, hemophilia A is more common, afflicting approximately 1:5,000 male births while hemophilia B is approximately four times less common (World Federation of Hemophilia). Treatment of hemophilia by protein replacement infusion successfully prolongs the average lifespan of the

afflicted, however, due to manufacturing and cost limitations, it is predicted that 75% of the global population has no access to FVIII replacement products.

Genetic Causes of Hemophilia A

Hemophilia was first observed as a familial trait and recorded in the Jewish Talmud in the 2nd century AD, providing exception to circumcision if two brothers had died from the procedure [1, 2]. In 1803, physician John Conrad Otto described a disease affecting mostly males and occurring with greater frequency within certain families. It was not until 1947 that two forms of hemophilia were identified resulting from separate protein deficiencies. Both *F9* and *F8* genes are located on the long arm (q) of the X chromosome at positions Xq27.1-27.2 and 28, respectively. *F8* is one of the furthest coding gene sequences distal to the centromere and may therefore be prone to a greater frequency of recombination events generating increased genetic diversity as well as defects. The 186 kb *F8* gene was cloned and characterized in 1984 [3-5]. *F8* has 26 exons ranging from 69 to 262 nucleotides; two of these exons, exon 14 and exon 26, are greater in size containing 3106 and 1958 nucleotides, respectively. There are six introns that exceed 14 kb, including intron 22 whose 32 kb sequence contains sequence motifs with a high frequency of intra-chromosomal recombination. This intron 22 inversion accounts for almost 50% of all severe hemophilia A cases [6] and another inversion at intron 1 accounts for another 5%. According to the Factor VIII Variant Database (formerly HAMSTeRS: Hemophilia A Mutation, Structure, Test and Resource Site) and CHAMP (Centers for Disease Control and prevention Hemophilia A Mutation Project) databases, there are a total of 2931 unique mutations resulting in hemophilia A. These mutations occur throughout the FVIII coding sequence as well

as within the endogenous promoter and untranslated regions (UTRs). The majority of disease-causing mutations are missense substitutions followed by frameshift deletions. Because FVIII has multiple complex interactions with cellular and plasma proteins, single point mutations that affect structure and/or binding affinity can greatly reduce or abrogate coagulant function.

Role of FVIII in Hemostasis

The majority of coagulation factors are vitamin K dependent zymogen precursors of serine proteases. Factor VIII is a pro-cofactor for one such enzyme, activated (a) FIX, in the intrinsic pathway of blood coagulation (Figure i-1). During endothelial damage and tissue factor exposure, low levels of thrombin (FIIa) are generated from the inactive prothrombin form (FII) by activated factor VII (FVII). Thrombin will then activate factors XI, VIII, and V, in what is commonly termed the cascade, resulting in amplification of thrombin generation. The role of FVIII, following activation, is to recruit FIXa and FX to the platelet surface and form a complex referred to as the tenase complex. The interaction of FVIII with FIXa improves the catalytic efficiency of FIXa for FX. Activated FX will dissociate from the ternary complex and activate additional thrombin molecules. At high levels of thrombin, fibrinogen and factor XIII are cleaved into FXIIIa and insoluble fibrin, a fibrous molecule that cross-links platelets at the site of hemostatic demand. Without FVIII, the efficiency of FIXa to activate FX is 4 orders of magnitude lessened and thrombin amplification is not initiated. Therefore, mutations that affect the ability of FVIII to bind either FIXa, FX, or platelet phospholipid surfaces result in an inactive tenase complex and clinical phenotype. Similarly, mutations inhibiting the activation of FVIII by thrombin have also been shown to result in the disease phenotype.

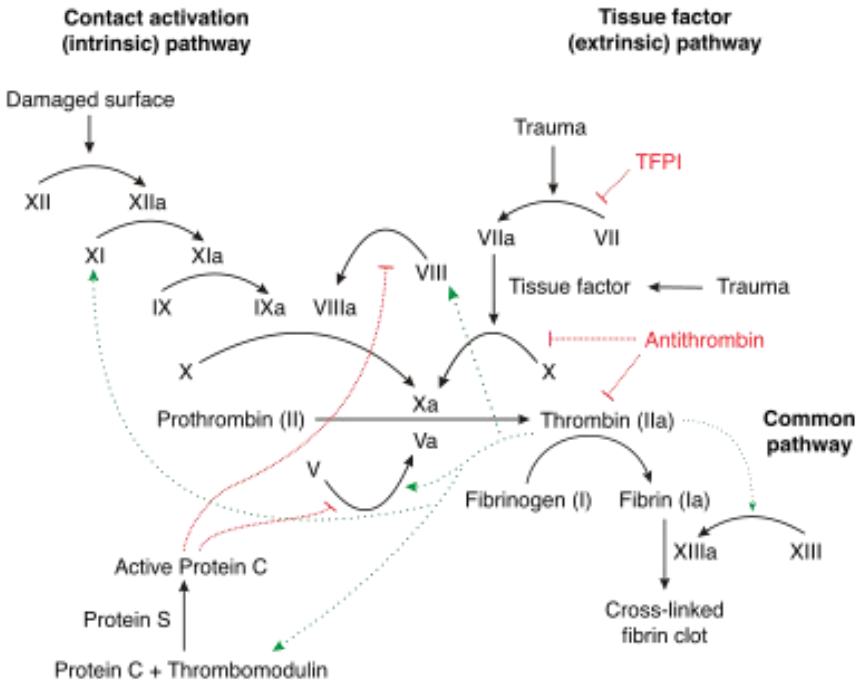


Figure i-1: The coagulation cascade. Intrinsic and extrinsic pathways converge at the formation of thrombin from proteolytic cleavage of prothrombin. The common pathway results in the generation of fibrin from fibrinogen. Image adapted from Knesek et al 2012 [7].

Treatment of Hemophilia A

Hemophilia A is a lethal disease without treatment, displaying a median life expectancy in the early teens. Initial treatment required whole blood transfusion from relatives and only during life-threatening events such as trauma or surgery. Prior to the discovery of the coagulation factors, remedies for hemophilia included lime, inhaled oxygen, the use of thyroid gland or bone marrow, hydrogen peroxide, and gelatin. When it was observed that particular snake venoms could cause blood to clot, dilutions of the venom were given as treatments for hemophilia. In 1937, it was discovered that the factor responsible for blood clotting could be isolated within the plasma [8]. Frozen plasma was being stored and used throughout the 50's and 60's; however, FVIII levels in the plasma were so low that large volumes were required for each patient. To meet the vast need of antihemophilic factors, plasma was collected from animal blood at abattoirs [9, 10]. Early treatment with animal plasma was successful in achieving hemostatic control, however, repeated exposures were complicated by allergic reactions and thrombocytopenia, particularly with bovine FVIII. A major technological advancement in clinical treatment came in 1964 with the ability to collect and store human plasma cryoprecipitate in freeze-dried concentrates [11]. This accomplishment reduced the need for non-human plasma and reduced storage limitations, infusion volumes, and increased shelf-life, allowing patients to treat at home. Cutter Laboratories in Berkeley, California began producing large volumes of the concentrate rapidly and throughout the 70's, the cryoprecipitate became the primary treatment. However, the mid-1980's brought the discovery that hepatitis C virus (HCV) and HIV could be transmitted through blood and blood products, such as cryoprecipitate. When screening for HCV began in 1992, approximately 44% of hemophilia A patients were positive

for the virus. More rigorous purification protocols, screening methods, and viral inactivation steps were implemented for plasma products and in 1992, the FDA approved the first recombinant FVIII product eliminating concerns of viral contamination.

Despite major technological advances, current treatment for hemophilia A patients is limited to protein replacement therapy through large volume infusions of either recombinant or plasma-derived FVIII. While protein replacement is successful in the management of an otherwise lethal disease, there are several limitations to current FVIII protein products. FVIII has a short circulating half-life of 12-14 hours, predominantly mediated by the clearance of carrier protein von Willebrand factor (vWF) to which FVIII remains bound until activation [12]. In order to achieve hemostatic protection prophylactically, multi-weekly IV infusions are required through either frequent venous access or a central port. Annual FVIII replacement costs can regularly exceed $250,000 per patient making FVIII products some of the most expensive drugs on the market . High cost is a result of low-level biosynthesis in recombinant heterologous expression systems coupled with rigorous purification requirements. The cellular biosynthetic efficiency of FVIII is 2 to 3 orders of magnitude lower than other plasma proteins. Plasma-derived FVIII also remains costly due to limited availability and measures required to remove harmful viral contaminants. Finally, approximately 30% of severe patients will develop neutralizing antibodies, termed inhibitors, against the exogenous protein. The formation of inhibitors remains the greatest obstacle in the management of hemophilia within patients with access to care.

Initially, patients with inhibitors comprised a refractory subset of patients with no treatment option. However, it was discovered in 1954 that porcine FVIII was able to restore hemostasis

even in the presence of anti-bovine FVIII inhibitors [13]. Similarly, porcine plasma could be used in anti-human FVIII inhibitor settings due to reduced antigen-antibody cross-reactivity. Currently, a recombinant porcine FVIII product (Obizur; Baxalta/Shire) is approved for acquired hemophilia, a severe auto-immune disorder in which a significant B-cell response is mounted against endogenous FVIII. Although several novel bypassing agents are marketed to obviate the need for FVIII (i.e. activated Xase, recombinant VIIa, bi-specific antibody ACE910), their ability to generate the required thrombin amplification for fibrin deposition is greatly reduced compared to FVIII.

FVIII Structure

Human FVIII is a large, heavily post-translationally modified polypeptide consisting of 2,332 amino acid residues. The structural domains are arranged A1-*a1*-A2-*a2*-B-*a3*-A3-C1-C2 with *a1*, *a2*, and *a3* representing small acidic subdomains at the borders of the A domains. These regions have been shown through mutagenesis to mediate thrombin binding and complete FVIII activation [14, 15]. Intracellular cleavage by PACE/Furin (paired basic amino acid cleavage enzyme) within the Golgi apparatus at the recognition motif prior to the *a3* subdomain results in a heterodimer of the A1-*a1*-A2-*a2*-B domain containing heavy chain and the *a3*-A3-C1-C2 light chain. Upon activation by thrombin in the circulation, FVIII is cleaved into a heterotrimeric A1-*a1*/A2-*a2*/A3-C1-C2 complex thereby releasing the *a3* subdomain and B domain.

The A domains share approximately 30% identity to ceruloplasmin, a copper binding plasma protein that plays a substantial role in iron metabolism [16, 17]. FVIII coordinates with 3 metal

ions that aid in protein folding and structural integrity through the A1 and A3 domains. Crystal structure analysis has shown that residues H267, C310, H315, H1954, C2000, and H2005 have direct contact with the copper ions [18]. Calcium ions are also required for coagulant function of FVIII by preventing the dissociation of the heavy and light chain dimer [19, 20]. Key residues for calcium interaction have been identified as D116, E122, D125, and D126 [21]. Furthermore, a range of bleeding phenotypes have been observed due to missense mutations at these residues that have >45% normal FVIII antigen levels but reduced activity [22-27], suggesting that hemostatic function, but not secretion, is dependent on ion binding. Within the FVIII A2 domain, residues 558-565 have been shown to mediate binding to FIXa and subsequent association with the tenase complex [28-30]. The A domains also share high sequence identity (~30%) to human factor V (FV), a pro-cofactor that is necessary for activation of FX and subsequent thrombin generation. As a result of this homology, several studies have replaced domains between FVIII and FV to investigate biosynthesis and secretion mechanisms [31].

The C1 and C2 domains of the light chain are homologous to the C1 and C2 domains of FV, the c-terminal domains of MFGE8 (milk fat globule-EGF factor 8) [32], and the discoidin family of proteins [33, 34]. These domains are most notably studied due to their affinity for vWF and acidic phospholipids including phosphatidylserine on cell membrane surfaces [35-37]. Similar to the A domains, numerous deletions in the C domains result in the bleeding phenotype due to perturbations in vWF and phospholipid binding [38, 39]. Due to these high level homologies with proteins of similar functions, it is thought that the diversity of the coagulation factors is the result of multiple gene and genome duplications.

The B-domain, however, does not share homology with the B-domain of FV, or any other protein. The role of the B-domain is predominantly thought to facilitate glycan-mediated intracellular transport due to its 16-19 N-glycosylation sites. The B-domain is removed during FVIII activation and does play any role in the coagulant function in the remaining FVIII heterotrimer [40]. As a result, numerous labs and biopharmaceutical companies have removed the B-domain from recombinant production without an observed loss in production, safety or efficacy [41-43].

Over 80% of the N-glycosylations occur within the B-domain (19/23). The remaining glycans are attached to asparagines N41, N239, N1810, and N2118. Interestingly, there have been no reports of disease causing mutations at any of these residues despite their suggested role in intracellular trafficking and protein folding. Factor VIII also has eight disulfide bonds: two within each A domain and one in each C domain. Only the A3 domain C1899 – C1903 bond is surface exposed, and mutagenesis of one or both cysteines improves secretion two-fold [44]. The remaining seven disulfides are indispensable for proper protein folding and structure.

FVIII Translation and Secretion

Until recently, the cellular origin of FVIII was not entirely known. It had been widely suggested that FVIII must be synthesized within cells that also generate vWF due to the short half-life of non-bound FVIII; however, vWF is generated within multiple cells including megakaryocytes, endothelium, and subendothelial connective tissue. While it was demonstrated that transplantation of a healthy liver can restore circulating FVIII levels [45, 46], the specific cell

type was debated due to the presence of FVIII mRNA found in multiple cell types [47], as well as other organs within various animal models [48]. Recent investigations into the liver as the primary source of FVIII biosynthesis have shown that liver sinusoidal endothelial cells (LSECs), and not hepatocytes, are the cellular origin [49, 50].

As the synthesized polypeptide elongates, a precursor oligosaccharide is attached to asparagine residues by uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) and glycosyltransferases within the cytoplasm allowing transport into the ER lumen. Translocation of the FVIII polypeptide to the ER lumen results in the processing of the 19 amino acid signal peptide, disulfide bond formation, and the next phases of N-glycosylation. The rate of biosynthesis is not thought be limited by these cellular processes, however, multiple cycles of glycan modification or disulfide isomerization may affect the duration that FVIII is retained within the ER. Following glycosylation and 'trimming' of the attached glucose molecules by glucosidase I and II (GTI and GTII), FVIII binds the ER chaperones calnexin (CNX) and calreticulin (CRT) which prevent the secretion of immature and improperly folded proteins [51]. When FVIII is correctly folded, it is translocated to the Golgi apparatus. However, mis-folded or mis-modified FVIII will remain in the ER and undergo multiple rounds of re-glucosylation by the UGT enzyme (UDP-glucose: glycoprotein glucosyltransferase) and 'trimming' in what is referred to as the calnexin cycle. Any FVIII molecule that remains in the calnexin cycle too long will be transported to proteasomes by ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1) for degradation through the ERAD (ER-associated degredation) pathway [52]. Similarly, failure of protein disulphide isomerase (PDI) to establish correct disulfide bonds and fold the internal hydrophobic regions of FVIII will result in ERAD due to aggregation of multimeric FVIII units

[53]. Proteasomal inactivation has resulted in an increase in intracellular FVIII without affecting levels of secreted and/or functional FVIII [51], suggesting a non-competitive role of FVIII degradation towards biosynthesis.

During the protein folding and modification process within the ER, FVIII is bound by GRP78 (glucose-regulated protein MW 78.0) also termed BiP (immunoglobulin-binding protein). Discovered as an ER chaperone during glucose starvation, BiP is also up-regulated during N-glycosylation inhibition, the accumulation of misfolded proteins within the ER, and FVIII overexpression [54, 55]. BiP is thought to be the central mediator of the unfolded protein response (UPR) because of its ability to induce cellular signaling to upregulate protein folding machinery such as PDI as well as ERAD proteins such as EDEM1. In times of persistent or overwhelming ER stress, BiP can also mediate apoptotic signals through CHOP (CCAAT/-enhancer-binding protein homologous protein) induction [56]. BiP has been predicted *in silico* to bind FVIII in the c-terminal region of the A1 domain, and mutagenesis of residue F309, the predicted interaction site, has resulted in modestly increased FVIII levels [57].

Following successful folding and disulfide formation, FVIII is released from BiP through ATP hydrolysis and transported to the ER-Golgi intermediate compartment (ERGIC) by the transmembrane protein LMAN1 (lectin, mannose-binding 1; also named ERGIC-53). LMAN1 binds and transports mannose-specific glycoproteins such as FVIII and FV through a calcium-dependent mechanism [58]. Mutations abrogating LMAN1 function or the interaction of LMAN1 with the transport complex MCFD2 (multiple coagulation factor deficiency protein 2) result in coagulopathy due to deficiencies in both FVIII and FV [59, 60]. These patients have 5-

30% normal circulating FVIII levels [61, 62], suggesting a secondary pathway, but this has not yet been elucidated. The LMAN1-MCFD2 complex recruits FVIII into COPII (coat protein II vesicles) for transport to the Golgi.

Within the Golgi apparatus, N- and O-glycans are further modified or added. Six confirmed tyrosine residues are sulfated at sites Y346, Y718, Y719, Y723, Y1664, and Y1680 [63] although heterogeneity has been observed [64]. These sulfations border thrombin cleavage sites and are critical for full coagulant activity as well as vWF association [65, 66]. The final stage in FVIII processing is the processing of the polypeptide into heavy and light chains by PACE/Furin cleavage at R1313 and R1648 although it has been widely observed that intact single chain FVIII is secreted without this processing. During secretion, FVIII associates with vWF intracellularly in Weibel-Palade bodies until granular release [67] although it is noted that mutations impairing vWF binding result in altered intracellular trafficking.

FVIII in the Circulation

Factor VIII circulates in the bloodstream at approximately 1 nM concentrations. Almost all the secreted FVIII remains in complex with a carrier protein, von Willebrand factor (vWF), a multimeric glycoprotein that protects FVIII from inactivation and delivers FVIII to the site of injury. Patients with vWF deficiency display, amongst multiple symptoms, hemophilia-like bleeding as a result of rapid FVIII clearance. The vWF binding regions have been mapped to the *a3* (including sulfated tyrosine Y1680), C1, and C2 domains on FVIII [68-71], and the D'D3

domains of vWF [72]. When bound to vWF, FVIII is oriented to allow thrombin activation [73, 74] while evading inactivation by FXa [75] and activated protein C (APC) cleavage [76].

Thrombin cleaves FVIII at three arginine residues, R372, R740, and R1689 resulting in the active heterotimer [77]. Once activated, the FVIIIa dissociates from vWF and is free to bind acidic phospholipids on the platelet surface. Factor Xa can also activate FVIII, however the tenase complex formed as a result of this activation is considerably less effective [78]. FVIIIa inactivation occurs by two methods. The predominant driving force of FVIIIa inactivation is through the spontaneous dissociation of the non-covalently bound A2 domain, often referred to as decay [78, 79]. Although this process is reversible, the low affinity and low concentration of non-bound A2 drives equilibrium towards the dissociated state, resulting in inactivation. FVIIIa can also be proteolytically cleaved by FXa or APC at K36 and R336 or R336 and R562, respectively [76-78]. The clearance of FVIII is largely mediated by vWF clearance through LRP1 (low-density lipoprotein receptor-related protein) and Siglec-5 (sialic acid-binding Ig-like lectin 5) [80, 81], however, LRP1 also binds directly to FVIII. The predominant sites of LRP1 recognition are the A2 (484-509), A3 (1804-1834), and C2 (2303-2332) domains [82-84]. While bound to vWF, the affinity of LRP1 for the C2 domain is reduced 90% [83].

Recombinant FVIII

The first generation of recombinant FVIII products were full length (B-domain containing) protein generated and purified from Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells in 1992 and 1993 by Baxter and Bayer, respectively [85, 86]. The Baxter product, Recombinate® or Bioclate®, was co-expressed with recombinant vWF in the presence of bovine

and human serum albumin (BSA, HSA) and thus required pasteurization and solvent/detergent treatment. Bayer's Kogenate® or Helixate® was made in the presence of only human plasma proteins to eliminate adverse reactions to *trans*-species proteins and eliminate bovine viral and prion contamination risks without pasteurization. In the second generation therapeutics, all albumin was removed from production but still contained human plasma components; the third generation excluded use of any animal compounds or human plasma proteins during the entire production and purification process.

In 2000, Pfizer received FDA approval for the first B-domain deleted FVIII product, ReFacto® derived from CHO cells and produced without animal compounds or human plasma proteins. Prior work had shown not only that the B-domain was dispensable for coagulant function [41], but also that mRNA and primary translation product levels were 17-fold increased over full-length FVIII [73]. These early reports in COS-1 (simian kidney fibroblast) and CHO cells observed a 10-fold increase in secreted activity as well as antigen compared to full-length FVIII.

The production of recombinant FVIII allowed for greater treatment of patients both in number and in infusion frequency. In 1995, prophylactic treatment of 2-3 infusions per week became the standard treatment and dramatically improved the quality of life for many patients. Despite these advances, the need for improved therapeutics remained. Production levels, even with the B-domain deleted products, were not sufficient to lower cost, nor was it possible to treat a greater global population of persons with hemophilia. Furthermore, the invasive nature and risks associated with venous access and central ports remain undesirable. The greatest limitation,

however, was, and remains, the development of neutralizing immunoglobulin (Ig) 'inhibitors' which completely abrogate the use of FVIII replacement therapy.

Clinical Immunogenicity of FVIII

FVIII is a highly immunogenic protein, causing robust T- and B-cell responses at only picomolar concentrations. Inhibitors typically form within the first 5 – 50 infusions of FVIII. Inhibitors form in approximately 30% of severe hemophilia A patients and 5 – 8% of mild or moderate patients [87]. There is no significant indicator of inhibitor formation risk. The most predictive factor is the specific mutation in the *F8* gene causing the bleeding phenotype, although environmental factors and treatment regimens also contribute [88-90]. The greatest risk lies within multi-exon deletions and early nonsense mutations, followed by inversions and finally missense substitutions. It is theorized that the mutations which generate complete or partial mRNA and translation products may play a role in central tolerance toward FVIII; however, no study has investigated the inhibitor prevalence in patients with circulating FVIII antigen but no activity (type II) compared to patients with no FVIII antigen (type I; Factor VIII Variant Database).

Additionally, there has long been debate over the immunogenicity of recombinant vs plasma derived FVIII products. Until recently, only retrospective analyses with insufficient statistical power were available. However, results of the multicenter prospective SIPPET (Survey of Inhibitors in Plasma-Products Exposed Toddlers) study utilizing previously untreated patients (PUPs) presented in December 2015, demonstrated that recombinant products, both full length

and B-domain deleted, are 1.9-times more likely to result in inhibitor formation than plasma derived products [91]. The importance of this study has led to protocol changes in the clinics for PUPs favoring plasma-derived products for the first several dozen exposures.

Treatment of inhibitor patients is limited to temporary use of bypassing agents such as factor eight inhibitor bypassing activity (FEIBA; FIIa and FXa), FVIIa or immune tolerance induction (ITI). Exogenous proteins aimed at bypassing the need for FVIII are less effective at thrombin generation and carry their own immunogenicity concerns. Because these bypassing agents are foreign antigens administered in the presence of endogenous coagulation factors, inhibitors formed against the exogenous protein may cross-react and eradicate endogenous proteins. Immune tolerance induction is the frequent infusion of bolus FVIII doses that overwhelm the immune system and generate tolerance through unknown mechanisms. ITI is extremely expensive; one retrospective study of 71 patients calculated the mean cost of approximately $65,000 per treatment month compared to $3,500 prior to ITI treatment over a median duration of 14.6 months [92]. This cost also carries considerable risk considering ITI is only successful in approximately 70% of cases [92-94].

Inhibitors to FVIII

Inhibitors are primarily IgG1 or IgG4 and predominantly form against the A2 and C2 domains [95]. However, the response is polyclonal and inhibitors form against all domains including the B domain and *a3* subdomain despite their absent role in hemostatic function. The term ‘inhibitor’ is in some ways misleading. Early antibodies against FVIII were discovered because of their

potential to neutralize FVIII activity in clinical settings, however, it is widely observed that IgG molecules frequently bind FVIII without inhibiting coagulant activity. It has been additionally observed that neutralizing inhibitors can interfere with FVIII through multiple mechanisms, potencies, and kinetics. Neutralizing inhibitors primarily act through steric hindrance, preventing the interaction of FVIII with FIXa [37, 71], FX [96], vWF and/or phospholipids [97-99], or thrombin [100]. Depending on the mechanism, inhibition of FVIII may be complete (Type I) or may result in continued residual activity despite saturating IgG concentrations (Type II). Because the population of inhibitors within a patient is polyclonal, the presence of type II inhibitors are masked by the presence of any type I inhibitors, making the evaluation of treatment options additionally complex.

The majority of knowledge known regarding inhibitor mechanisms and epitopes comes from monoclonal antibodies purified from inhibitor patients [101-103], or generated within a murine hemophilia model [98, 100, 104, 105]. These monoclonal populations have allowed epitope mapping through homolog scanning mutagenesis [106-109], small angle x-ray scattering (SAXS) [110], hydrogen-deuterium exchange (HDX) [111, 112], phage display [113], and high resolution crystal structure analysis [114, 115]. These studies guide the engineering of novel FVIII molecules with reduced immunological profiles. One such example is the generation of a FVIII molecule with 3 alanine substitutions in the highly immunogenic 484-508 surface-exposed loop of the A2 domain [116]. This bioengineering effort was guided by the porcine FVIII sequence due to reduced cross-reactivity of inhibitors with epitopes within this region [106, 117, 118]. The engineered FVIII displayed indistinguishable activity and pharmacokinetics to human FVIII but generated statistically reduced inhibitor titers in hemophilia A mice. Although reduced

over 2-fold, the titers remained too elevated to suggest clinical utility, most likely due to the polyclonal response against the unmodified domains, however, it provides proof-of-principle for future engineering efforts.

Gene Therapy for Hemophilia A

Despite the advancements in the management of hemophilia A, all protein replacement therapies require repeated invasive administrations. The cloning and characterization of the FVIII gene in 1984 provided the foundation for genetic studies including the inception of a curative option through genetic manipulation. Hemophilia A is an ideal disease candidate for gene therapy trials due primarily to genetic errors affecting a single gene product. Although the plethora of disease-causing mutations precludes the use of a standardized gene correction technique, gene addition of an engineered cDNA could provide life-long endogenous production and mitigate the need for protein transfusions. Furthermore, a severe hemophilia A patient would only require modest (>1%) increases in circulating levels to dramatically improve quality of life and patient outcome. Finally, the development of coagulation assays and FVIII antigen quantification techniques provides the ability to accurately monitor the success of a gene therapy trial. Several hemophilia animal models, large and small, have been developed and well characterized. These models, including mice [119, 120], dogs [121], and sheep [122], have been extensively used for gene therapy development. Early gene therapy attempts for FVIII or FIX deficiency utilized retroviral, adenoviral, and adeno-associated viral (AAV) delivery systems as well as non-viral transplantation of stably expressing fibroblasts [123-126]. FVIII production levels following gene transfer were poor, only sporadically achieving 1% of normal or greater. With regard to

viral mediated transfer, increased virus dose was not clinically feasible due to the robust immune response against the viral components. This narrow therapeutic window has been the predominant obstacle in the improvement of gene therapy trials.

Developments of novel gene therapy products target both the delivery method as well as the FVIII transgene. While retroviruses were the most promising, uncontrolled replication and insertional mutagenesis resulting in leukemia forced the development of safer vehicles. With the characterization of lentiviruses [127] and development of self-inactivating (SIN) viral vectors [128], lentiviral mediated gene delivery has already successfully treated several diseases including severe combined immunodeficiency (X-linked SCID) [129], Wiskott-Aldrich syndrome (WAS) [130], β-thalassemia [131], and adenosine deaminase (ADA) [132]. Hematopoietic stem cells (HSCs) are a common target for *ex vivo* transduction protocols due to their pluripotency and cellular access to the bloodstream. HSCs differentiate into myeloid and lymphoid lineages with multiple cell types, each potentially expressing the transgene (for review, see chapter I). Another common gene therapy vehicle relevant to hemophilia is the use of adeno-associated viruses. There are several advantages to AAV over lentiviral approaches. First, AAV is a non-pathogenic virus that requires additional proteins from other viral infections, such as adenovirus or herpes-simplex virus (HSV), to replicate. AAV has a very low incidence of integration, thus the risk of insertional mutagenesis is almost minimal. A single intravenous administration of AAV is potentially sufficient to deliver the transgene, removing the invasiveness and risk associated with bone marrow transplantation with lentiviruses. Additionally, there are many capsid AAV serotypes that allow for greater tissue specificity. However, AAV has several limitations that support the use of lentiviral methods instead.

Because AAV does not integrate, any newly replicated cell will not contain the transgene and the population of transduced cells will dilute after each mitotic event. As a result, quiescent cells are often the targets for AAV therapies. AAV is also highly immunogenic; a robust B cell stimulation event neutralizes any repeat exposure to the capsid protein prior to the transduction event, eliminating the possibility of multiple administrations. It is estimated that up to 70% of the population has formed pre-existing antibodies to AAV peptides, eliminating their candidacy. One major limitation preventing hemophilia A gene therapy via AAV is the size and packaging capacity of the capsid protein. AAV is a small, 25nm diameter, virus with a DNA packaging capacity of roughly 4.9 kb. The smallest FVIII cDNA, without the promoter, polyA tail, or the necessary viral inverted terminal repeats (ITRs) is already almost 4.4 kb. Despite this, AAV studies using over-sized FVIII viral cassettes have achieved therapeutic levels of FVIII, most likely due to the recombination of FVIII fragments following incomplete packaging [133].

Current gene therapies have demonstrated far greater success in the treatment of hemophilia B. Liver derived FIX following AAV8 administration achieved 2-12% normal levels over 40 weeks following a single injection [134]. Currently, there are six ongoing or approved gene therapy trials for hemophilia B and only one for hemophilia A (BioMarin NCT02576795) and all seven trials are utilizing an AAV vehicle for delivery. There is still a great need for improved gene therapy research for hemophilia A. Efforts to enhance the FVIII transgene focus on size, and biosynthetic efficiency of the engineered FVIII. With low-level protein production driving high treatment cost, engineering of the FVIII cDNA is advantageous to both gene therapy and recombinant protein production. Furthermore, a FVIII sequence resulting in enhanced biosynthesis would theoretically improve the safety profile by reducing viral titer requirements.

Bioengineering FVIII

The development of a curative gene therapy product does not obviate the need for purified FVIII concentrates. Availability and exclusion criteria may prohibit a subset of patients from receiving gene therapy, requiring continued protein replacement. Several companies have launched new FVIII products designed to improve biosynthesis or circulating half-life through codon optimization or protein conjugation. The first FVIII engineering effort was the removal of the B domain which demonstrated 10-fold increased protein production [41]. Almost all new FVIII products for both recombinant production as well as gene therapy products incorporate this modification. It has been suggested that B domain plays a role in secretion due to glycan receptor mediated transport, and as a result, a FVIII molecule was produced with the first 269 amino acids of the B domain encompassing 6 N-glycans [135]. This molecule resulted in a 10-fold improvement in secreted FVIII activity compared to a full removal of the B-domain however, this finding was not reproduced in subsequent studies [136]. Additional engineering of this N-glycan rich region reduced the total size from 269 amino acids to 17 while maintaining all 6 N-glycan sites resulting in an additional 2-fold increase over the previous N6 molecule [137]. Codon optimization controls the DNA sequence based on codon usage bias within a host species or tissue and removes mRNA secondary structure. The amino acid sequence is unchanged, but the nucleic acid sequence is designed to utilize the most common tRNAs and thereby increase the rate of translation. Codon optimization of FVIII has demonstrated a 10-fold increase in protein levels in HepG2 cells following transduction [138], however, the effect of codon optimization is also cell specific [139, 140].

The past decade has seen the generation of several novel FVIII products aiming to extend the circulatory half-life of FVIII. This extension allows greater duration of protection and reduces the frequency of infusions needed to achieve adequate prophylaxis. Baxter and Bayer have each developed a PEGylated FVIII molecule with modest 1.3-1.5 fold enhancements in half-life [141, 142]. The addition of PEG is believed to reduce FVIII binding to clearance receptor LRP1. Biogen, however, has developed a FVIII molecule conjugated to the neonatal Fc protein (FVIII:Fc). This molecule is designed to escape clearance mechanisms by recycling to the bloodstream after uptake by endothelial cells and hepatocytes. The neonatal Fc receptor (FcRn) carries IgG molecules from breast milk across the gut epithelial border in weaning offspring, granting transferred immunity. In adults, it also recycles IgG through pinocytosis. The FVIII:Fc fusion protein prolongs the half-life 1.7 fold through FcRn mediated rescue from hepatocytes [143, 144]. The prolonged circulating half-lives of these molecules are quite modest, owing to the fact that vWF clearance remains the predominant driving force of FVIII elimination. Biogen is currently developing and testing a new FVIII conjugate, using XTEN technology to attach D'D3 fragments from vWF to the FVIII molecule. The addition of this vWF fragment should still protect FVIII from enzymatic inactivation without being cleared as rapidly due to the loss of the remaining vWF molecule [145].

A different approach to FVIII engineering has utilized the characterization of naturally occurring orthologs. The successful functionality of porcine FVIII-containing plasma fractions in the presence of anti-human and anti-bovine inhibitors warranted the development of the porcine cDNA and characterization of the recombinant protein [146]. It was shown in heterologous expression systems that recombinant porcine FVIII produced 10-100 fold more protein than

human FVIII despite similar transcript levels [147]. Subsequent studies demonstrated that the increased biosynthesis was due to higher secretion efficiency, and that the A1 and A3 domains were responsible for this enhanced biosynthesis [148]. The mechanism of increased secretion was found to be the reduced engagement of the UPR, and that secretion of human FVIII could be rescued with shRNA targeting BiP [133]. To date, this hybrid molecule, ET3, has achieved the highest levels of FVIII production following a gene therapy protocol and is actively being explored for both lentiviral and AAV mediated gene transfer [136, 149-152]. Despite the promising data from this state-of-the-art molecule, concerns remain regarding the immunological profile of the hybrid. The human/porcine hybrid, ET3 is 90.5% identical to human FVIII amino acid primary sequence, with 138 porcine residues. Both human and porcine FVIII result in the development of neutralizing inhibitors and similar IgG production in a mouse hemophilia A model [153]. In the phase II/III trial of OBI-1 (Obizur; BAX801) for the treatment of acquired hemophilia A (AHA), 5 of 28 patients developed de novo alloantibodies to the porcine product (10 patients displayed pre-existing cross reactive inhibitors against porcine FVIII) [154]. Despite the similar if not improved safety profile of porcine FVIII, the immunogenicity of ET3 is currently untested.

Orthologous FVIII

In the establishment of hemophilia animal models for gene therapy studies, other FVIII orthologs were characterized and utilized to maintain the colonies and has enhanced our understanding of FVIII biology. Administration of murine FVIII results in a reduced immunological response within a hemophilia A mouse model, allowing for the comparative immunogenicity of non-

murine FVIII [155]. The stability of the activated mouse FVIII molecule is also unique, demonstrating a prolonged half-life of 5-6 fold over human FVIIIa. It had been observed that mutations which increase the rate of the A2 domain dissociation result in mild hemophilia [156], and it has been demonstrated that increases in the FVIIIa stability would increase the potency of replacement products [157]. Investigations into the human residues responsible for A2 domain stabilization by alanine scanning mutagenesis identified several potential sites within the A1-A2 and A2-A3 interface and subsequently generated a FVIII with 4-fold enhanced FVIIIa stability [158, 159].

Porcine and canine FVIII also demonstrate a moderate 2-3 fold increase in FVIIIa stability, respectively [147, 160]. Canine, however, demonstrates a 2-fold higher specific activity, or activity per molecule, than human FVIII. Interestingly, canine FVIII is secreted predominantly as a single chain, unprocessed by PACE/Furin. Alignment of the canine amino acid sequence revealed that the R-X-X-R PACE/Furin recognition motif (RHQR in human FVIII) contains a single point mutation, HHQR. Incorporation of this sequence into human FVIII resulted in a 2-fold increase in FVIIIa stability, reduced time to occlusion in a ferric chloride injury model, and increased de novo biosynthesis of FVIII following an AAV gene therapy protocol [161]. The characterization of recombinant canine FVIII, like porcine and murine, has guided the engineering of potential therapeutics. Single-chain variants lacking the PACE/Furin motif have been explored; however, complications in *in vitro* measurements currently prevent commercialization efforts [162].

Large Animal Models of Hemophilia A

The development of mouse models of hemophilia made possible early investigations into the efficacy of novel proteins and gene therapies. The need for large animal models for safety and scale-up investigation are necessary prior to clinical trials and FDA approval. Currently, there are two locations with canine colonies. A colony of Irish setters is located at Chapel Hill, NC and miniature schnauzers and beagles are housed at Queen's University in Ontario. Both colonies of hemophilia A dogs possess a severe phenotype due to an intron 22 inversion, the same inversion that affects almost 50% of human patients, and display similar bleeding morphology and arthropathy [121, 163]. However, the Canadian dogs have been observed to develop inhibitors to canine FVIII with greater frequency [164]. These canine hemophilia models have been used for pre-clinical trials of novel recombinant FVIII [165], FVIIa [166], FXa, well as AAV mediated gene therapy [167, 168].

Recently, a hemophilia A line of alpine white ewe sheep was reestablished from cryopreserved semen. Genotyping revealed a severe phenotype caused by a frameshift and premature stop codon in exon 14, a naturally occurring mutation also found in humans [169]. The characterization of the disease closely mimics the disease pathology of humans, with spontaneous bleeds localized to joints, restricted locomotion, muscular hematomas, hematuria, and fatal hemorrhages. As an animal model, sheep have many characteristics that are advantageous for pre-clinical studies. First, sheep are similar in size to humans, weighing 8 pounds at birth and up to 160 lbs as adults, obviating the need for scale-up production after testing. Second, sheep have a similar immune system to humans which makes them ideal

candidates for inhibitor studies [170]. Because of similar developmental characteristics, sheep have been used extensively in the study of mammalian fetal physiology and are therefore ideal candidates for investigations into *in utero* gene therapy [171]. Furthermore, sheep typically give birth to one or two offspring per pregnancy, unlike dogs and mice. Finally, sheep, like humans, store vWF within platelets while canine vWF circulates in the plasma without significant intracellular storage [172]. While hemophilia A sheep were shown to develop inhibitors to human FVIII, homologous sheep FVIII was not available to determine the ability of sheep to generate inhibitors to FVIII of the same species. In chapters II and III we describe the production and characterization of recombinant ovine FVIII (roFVIII) for the maintenance of the sheep colony, as well as the exploitation of unique characteristics, including stability and antigenicity, for engineering purposes.

Ancestral Sequence Reconstruction as a Guide to Bioengineering

Current efforts to engineer proteins are guided by either rational design, in which detailed structure-function information is used to make specific substitutions, or directed evolution, in which natural selection is mimicked through multiple rounds of random mutation and selection. While rational design can be quick and inexpensive, it requires detailed information regarding the structure of the protein of interest. In the case of FVIII, there are only two crystal structures of the B-domain deleted variant at overall resolutions of 3.7 and 3.98 Å [18, 20], and no structural data regarding the thrombin activated FVIIIa which possesses all hemostatic function. Directed evolution approaches require no prior structural information. However, large quantities of recombinant protein are necessary for each round of mutation, selection, and screening. Given

the low biosynthesis of human FVIII, as well as the increased likelihood of mutations with undesirable effects, this approach is also not feasible for FVIII bioengineering.

Ancestral sequence reconstruction (ASR) is the prediction and generation of ancestral genes and proteins from existing sequences [173]. Using a large collection of nucleic acid and/or amino acid sequences, statistical models of evolution are incorporated into computational algorithms to 'recover' the ancestral sequences. With the advent of affordable de novo gene synthesis, ASR has become a feasible method to investigate the evolutionary path of single molecules across species. There are several advantages to ASR for protein engineering. First, unlike directed evolution, ASR provides high probability *a priori* that generated sequences will be functional. Second, the number of residue substitutions between ancestral nodes is greatly reduced. Therefore, observed changes in gene function between one ancestral node to the next can be investigated without structure information.

There are currently three methods of performing ASR: maximum parsimony, maximum likelihood, and Bayesian inference. Maximum parsimony minimizes the number of changes between all sequences to yield the shortest phylogenetic tree that minimally explains the provided data [174]. Because the parameters of maximum parsimony prioritize the simplicity of the tree, this method does not always generate a tree with the highest probability of 'correctness.' The Maximum likelihood method requires greater computing power because it allows parameters within the predictive algorithms, such as mutation rate, to change based on the resulting sequences of the previous prediction. As a result, the prediction parameters from one ancestral node to another 'evolve' with the actual sequences. Maximum likelihood incorporates

greater probability parameters, such as greater likelihood of a genetic transition (purine to purine or pyrimidine to pyrimidine) over a transversion (purine to pyrimidine or vice versa), while maximum parsimony does not. Finally, Bayesian inference is commonly accepted as the most accurate method. Unlike the other two methods, Bayesian inference produces a distribution of phylogenetic trees, each with calculated likelihood and variance.

The use of ASR has recently led to the development of several potential therapeutics. In the case of hyperuricemia and gout, the inability to break down and eliminate uric acid results in painful crystallizations within joints. Uricase, an enzyme which acts to degrade uric acid, is a pseudogene in humans and upper primates but is fully functional in other mammals. Through ASR, the small number of residue substitutions between modern human uricase and its common ancestors were identified [175]. As a result, the exact missense substitutions that inactivated uricase and persisted through evolution were identified. These substitutions could therefore be reversed through targeted mutagenesis and the resulting cDNA could be used for recombinant production and treatment.

Due to the unique biochemical characteristics that we have observed in numerous FVIII orthologs, we hypothesized that species-specific differentials were evolutionarily selected for to address the hemostatic need of each species. We employed ASR to generate the phylogenetic tree of mammalian FVIII and generate the predicted sequences of each common ancestral node. Our results demonstrate that ASR is an efficient and powerful method to identify genetic changes that result in observed phenotypic differences. Furthermore, this approach provides a novel platform for protein discovery and engineering that has not yet been employed.

Chapter I: Engineered Hematopoietic Stem Cells as Therapeutics for Hemophilia A

Introduction

Hematopoietic stem cells (HSCs) are the most routinely transplanted adult stem cell. Currently, they are utilized for the treatment of several genetic and acquired diseases including blood cancers, autoimmune disorders, and hematopoietic defects. HCSs are ideal candidates for gene therapy applications because they possess the capacity for self-replication and functionality to propagate the entire hematopoietic lineage, thus facilitating amplification of genetically-modified cells and expression of the transgene product from a multitude of hematopoietic cell types. An additional advantage is the tolerogenic effect HSCs have on host immunity, which in many contexts, is a barrier to successful gene therapy. Numerous HSC-targeted gene therapy studies have been conducted in a range of disease settings. Current pre-clinical research for HSC transplantation gene therapy of hemophilia A therapy is focused on i) identification of safe and efficient methods of nucleic acid transfer into HSCs, ii) optimization of the coagulation factor VIII transgene for high expression, iii) minimization of conditioning regimen-related toxicity with HSC engraftment and iv) overcoming complications due to pre-existing factor VIII immunity. Herein, we review the state of the art in HSC transplantation gene therapy of hemophilia A.

Clinical Gene Therapy of Hemophilia A

Loss of circulating FVIII activity due to mutations within the FVIII gene results in the X-linked, recessive bleeding disorder hemophilia A. The clinical presentation is a mild to severe bleeding phenotype that correlates with the patient's residual plasma FVIII activity level. Hemophilia A has been targeted by numerous academic and commercial entities as a prime candidate for gene transfer-based therapies for several reasons. First, modest increases in FVIII levels (to ≥ 1% of normal levels) can alleviate spontaneous bleeding episodes. Second, many different cell types are capable of synthesizing functional FVIII protein and virtually any tissue or cell type with access to the bloodstream can be targeted for gene transfer. Third, gene therapy should be more economical and less invasive than protein replacement therapy given that it would consist of limited (possibly only one) treatment events. There have been 3 phase 1 clinical trials of gene therapy for hemophilia A conducted to date and each employed a different gene-transfer strategy (for review see Doering and Spencer, 2010 [176]) . The first trial, sponsored by Transkaryotic Therapies, Inc., involved *ex vivo* gene modification of autologous dermal fibroblasts and transplantation into the greater or lesser omentum of twelve male patients [124]. Although no severe adverse events were observed in this trial, designed to assess safety, sustained FVIII levels above 1% of normal were not achieved. In a second study, sponsored by Chiron Corporation, retroviral particles containing a human B-domain deleted (BDD) FVIII transgene were introduced into thirteen male hemophilia A patients *via* peripheral vein infusion [126]. Again, FVIII levels above 1% of normal were not maintained and the trial was halted. The third trial, sponsored by GenStar Therapeutics, Inc., consisted of a single patient being infused with high-capacity adenoviral particles containing the full-length human FVIII cDNA. Following administration of viral vector, the patient developed transient chills, fever, back pain and headaches preceding the onset of thrombocytopenia and transaminitis. This patient did achieve

FVIII levels >1% of normal that were maintained for several months, but as predicted based on the non-integrating property of adenoviruses the FVIII activity eventually declined. The trial was halted due to the significant side effects observed. In summary, not only have there been no milestones of success in previous trials, to our knowledge, there are no approved or ongoing clinical trials utilizing gene transfer to treat hemophilia A.

Clinical Hematopoietic Stem Cell (HSC) Therapy

Hematopoietic stem cells (HSCs) first were discovered in the late 1940's as a result of the finding that spleen cells could protect mice from exposure to lethal doses of radiation [177, 178]. A comprehensive review of the history of HSC transplantation (HSCT) has been documented by E. D. Thomas, recipient of the Nobel Prize in Medicine 1990 for his pioneering work in this field [179]. Subsequently, HSCs have been implemented in the treatment of several genetic and acquired diseases including leukemia, non-Hodgkin's lymphoma, aplastic anemia, and sickle-cell disease. Annually, more than 20,000 clinical HCTs are performed. The ability of HSCs to reconstitute all cellular hematopoietic lineages, including myeloid, lymphoid, and erythroid populations through a combination of self-renewal and cellular differentiation endows them with unique clinical utility. Engrafted HSCs are capable of contributing to hematopoiesis for the duration of the patient's life. HSCs are harvested routinely from bone marrow aspirates or peripheral blood since they can be mobilized into the bloodstream using granulocyte-macrophage colony-stimulating factor. For the purpose of gene transfer-based therapies, HSCs

can be manipulated successfully *ex vivo*, allowing for the implementation of safety parameters prior to transplantation and subsequent engraftment.

In 1953, Medawar and colleagues made the fundamental discovery that mixed cellular chimerism and immune tolerance could be achieved following transplantation of a combination of T-cell depleted host and donor bone marrow cells into lethally-irradiated mice [180]. The result of a successful allotransplant is two genetically-distinct sources of hematopoietic cells referred to as a state of mixed cellular chimerism where immunotolerance to the foreign antigens has been established not only for hematopoietic cells, but for any other cell or tissue type derived from the host or donor. For example, acceptance of donor skin grafts often is used to demonstrate this phenomenon. However with any allogeneic transplantation, there exists significant risk that the donor cells, typically harboring HLA mismatches, will identify host tissue as foreign, triggering a cytotoxic immune response clinically described as graft versus host disease (GvHD). GvHD is the major side effect of HSC transplantation and carries with it significant morbidity and mortality risk. Therefore to avoid the risk of GvHD, the majority of HSCT gene therapy studies have utilized autologous cells. In this setting, the only antigen disparity is that of the transgene product, e.g. factor VIII (FVIII) in hemophilia A gene therapy. As is discussed herein, the immunotolerogenic nature of HSCT is a major benefit to gene therapy of hemophilia A, where the transgene product, FVIII, is a known potent immunogen (for review of FVIII immunogenicity, see [181]).

Strategies for genetic-modification of HSCs

Since the emergence of gene-transfer studies in the early 90's, HSCs have been targeted to restore therapeutic levels of certain proteins, the expression of which is affected by deleterious genetic mutations. In addition to the tolerogenic potential of HSCs, targeting this population allows for indefinite expression of a desired protein product from multiple terminally differentiated cell lineages. Several methods have been demonstrated to transfer functional genetic material or to correct existing genetic defects within HSCs, and are reviewed herein (Table I-1). Current methods for nucleic acid transfer include viral, chemical, and physical techniques. Viral vectors are the gold standard of genetic transfer into HSCs. Each viral vector system has different integration capabilities, as well as required components for nuclear uptake and expression. Choice of viral vector system is dependent on the transgene, transduction efficiency, and safety requirements. Common viral vector systems are based on retroviruses, lentiviruses, adenoviruses, adeno-associated viruses, and the herpes simplex virus. In addition to viral strategies, cationic lipids and other non-viral synthetic macromolecules have been used widely for cellular introduction of DNA [182]; however, to date, non-viral-based methods targeting HSCs show low potential as a therapeutic approach [183, 184]. Physical methods include particle-mediated transfection, electroporation, and hydrodynamics-based transfection. While very little research has been conducted on HSCs using particle-mediated transfections, electroporation has been used successfully to introduce genetic material into hematopoietic progenitors and their progeny [185-187].

Table I-1: Technologies for HSCT Gene Therapy

Technologies for HSCT Gene Therapy	Advantages	Disadvantages
γ-Retroviral Vectors	• Ability to target many cell types • Long-term expression due to integration • Increased safety due to SIN development	• Requirement for cellular division • Necessity of cytokine cocktails to stimulate HSC cycling • Insertional mutagenesis potential • Complex manufacturing
Lentiviral Vectors	• Wide range of cell targets • Long term expression due to integration • HIV-based human cell specificity	• Require multiple plasmids/elements provided *in trans* for production • Risk of insertional mutagenesis • Complex manufacturing
***Sleeping Beauty* Transposon Systems (SBTS)**	• Low complexity • Simple manufacturing (plasmid DNA only) • Potentially reduced immunogenic response	• Lower-level expression of the transgene product • Random insertion pattern • Potential for secondary or tertiary transposition events
Zinc Finger Nucleases (ZFNs)	• Targeted gene correction or addition • Potential to utilize endogenous genetic control elements • Long-term expression through chromosomal integration	• Safety remains undetermined • Risk of off-target mutagenesis • Require additional means of cellular entry • Limited sequence targeting potential
Peptide Nucleic Acids (PNAs)	• Site-specific modification • Useful in gene silencing • In vivo delivery and functionality possible	• Limited research to date • Risk of off-target sites of genetic modification • Low efficiency

The earliest proof of concept HSC gene-transfer studies utilized recombinant γ-retroviral vectors derived from murine leukemia viruses (MLV) and produced in recombinant form by transient transfection of NIH3T3 or HEK293T with viral packaging and expression plasmids [188, 189]. Packaging cells provide the accessory protein components required for the biosynthesis of non-replication competent retroviral vector. Pre-clinical studies using murine models demonstrated the versatility of this vector system to transfer nearly any transgene into a variety of cell types [190-192]. Soon after, however, it was shown that γ-retroviral vector systems could not transduce HSCs as efficiently as other rapidly dividing cell types. Subsequently, it was shown that γ-retroviral vectors require cellular division within the target cell to facilitate nuclear translocation of the pre-integration complex and integration of the transgene [193, 194]. Due to the requirement of cell division and the relatively infrequent replication rates of HSCs, protocols incorporating cytokine cocktails were developed and shown to bolster the transduction efficiency of murine HSCs [195-199].

To circumvent the need for cellular division, in 1996, Naldini and colleagues reported successful gene transfer in cell cycle arrested fibroblasts using a recombinant HIV-based lentiviral vector system [127]. Fibroblasts in G_1-S, G_2, and to a lesser degree, G_0 cell cycle stages showed increased transduction of firefly luciferase and β-galactosidase reporter genes compared to MLV-based vectors. Furthermore, this vector system was shown to transduce terminally differentiated neuronal cells *in vivo* following direct injection into the corpus striatum and hippocampus. This was the first study to demonstrate successful gene transfer into non-replicating cells using a retroviral vector. Although data support the ability of lentiviruses to

transduce non-dividing cells, it generally is recognized that dividing cells are transduced more efficiently. A comparative study of transduction efficiencies between MLV-based retroviral vectors and HIV-1 based lentiviral vectors in $CD34^+$ cells revealed the superiority of HIV vector integration into quiescent hematopoietic progenitors [200]. Subsequently, lentiviral vectors have been shown to transduce numerous non-dividing cell types including lung epithelial cells [201], neuronal cells [202, 203], and primary hepatocytes [204]. The ability of lentiviral vectors to infect quiescent cells is reliant upon two proteins: *gag* (matrix protein) and Vpr, although Vpr has been deleted in third and fourth generation lentiviral vectors without severe loss of infectivity. These proteins activate nuclear import machinery facilitating transport of the pre-integration complex through the nucleopore, decreasing the need for mitosis-induced nuclear permeability [205, 206]. With respect to the targeting of HSCs, recombinant retroviral vectors, and most recently lentiviral vectors, have been the system of choice for proof of concept studies in a variety of animal models.

In addition to recombinant retroviral vector, other non-viral technologies are being investigated for genetic modification of HSCs. In eukaryotes, transposon-transposase systems create duplications or transpositions of certain genetic sequences throughout the genome. In humans, transposons have been evolutionarily silenced, but one has been reconstructed from the salmonoid fish genome as a potential gene transfer and therapy tool [207]. *Sleeping Beauty* transposons systems, named for their evolutionarily dormant phase, employ an enzyme, termed transposase, that catalyzes transposition events. For gene therapy applications, a transgene cassette contained within a donor plasmid serves as the substrate for transposase-mediated

transposition into a host cell chromosome. Thus, the only two requirements for the *Sleeping Beauty* transposon system (SBTS) are i) a transposon containing the gene of interest, and ii) a source of transposase produced either in *cis* or in *trans*. The advantages lie in the simplicity of this design, as well as the reduced risk of immunogenic response [208]. SBTS have been validated in mouse models for the treatment of several genetic disorders, including hemophilia A where they have been shown to drive FVIII expression and phenotypic recovery for over 30 weeks [209-211]. However as is the case for most SBTS applications, the target cells were not HSCs and expression levels were lower than is routinely achieved using γ-retro- and lentiviral vectors.

Modification of the transposons, and specifically the transposase itself, has led to increased transposition and transgene expression [212]. Recently, hyperactive transposases have been identified through directed evolution-based screening and have been shown to improve the *ex vivo* modification of human cord blood $CD34^+$ cells following electroporation. In that study, genetically-modified cells retained the capacity for differentiation into all hematopoietic cell lineages. However, the efficiency of genetic modification did not exceed 27% [213, 214]. Despite significant progress, remaining hurdles for SBTS include i) low gene-transfer efficiency, which requires a method for getting the transposon and transposase into the target cell, and ii) safety concerns surrounding insertional mutagenesis. Of note, SBTS do demonstrate a more random integration profile than retroviral vectors, which predominantly target promoter regions within gene loci [215, 216]. Furthermore, the long-term stability of transposed sequences is not

known and it is possible that secondary, tertiary, etc. transposition events could further increase the risk of insertional mutagenesis.

Currently, the majority of gene therapy research consists of exploring and developing gene addition techniques where a functional transgene is introduced into a cell to restore expression. However, several groups now have identified methods for the correction of defective genes *in situ*. There currently are several mechanisms by which gene correction is being pursued. By virtue of its nature, gene correction allows for usage of the endogenous promoter and other regulatory elements and obviates the risk of insertional mutagenesis. Two prominent technologies for gene correction in HSCs include zinc-finger nucleases (ZFNs) and peptide nucleic acid (PNA) conjugates. Currently, both ZFNs and PNA-conjugates are in clinical trials for HIV and cancer treatment, respectively.

Zinc-finger nucleases combine a zinc-finger domain capable of site-specific DNA binding with a non-specific restriction nuclease domain. Upon dimerization at specified nuclear DNA recognition sequences, double-stranded DNA breaks are created that serve as substrates for homology-directed DNA repair (HDR). During HDR, the cleaved 3' ends invade the sister chromatid and replicate using the intact strand as a template. In the case of X-linked diseases such as hemophilia A, HDR is not possible unless a homologous DNA template is provided *in trans*. Without a template, the cleaved DNA is subject to random mutagenic events, i.e. insertions and deletions in an attempt to ligate the cleavage and avoid apoptosis. However when

a DNA template containing homologous sequences surrounding the corrected gene sequence or, alternatively, the entire transgene sequence 3' to the cleavage site, ZFN mediated cleavage and HDR results in replacement of the mutation with corrected sequence, or potentially, an entire transgene now under endogenous promoter and enhancer control. As ZFN-mediated gene correction has been subjected to limited testing in humans, the safety concerns are relatively unknown. Identification of off-target ZFN activity has, however, been demonstrated in pre-clinical studies including a recent study demonstrating the use of ZFNs for *in vivo* treatment of a murine model of hemophilia B [217]. This study utilized adeno-associated viral (AAV) vector to deliver episomally expressed ZFNs and a homologous template encoding the factor IX (FIX) cDNA. The AAV vector transduced primarily hepatocytes and the genetically-modified cells expressed FIX at 3-7% normal levels. However, the limited ability of AAV to target HSCs *in vivo* obviates the practicality of this strategy for HSC-directed gene therapy. Similar systems are being developed to target gene addition to specific sequences within the human genome. For example, it was recently shown that the CXCR5 and AAVs1 sites can be targeted for safe harbor gene addition using ZFNs that bind to and specifically cleave sites within these regions [218]. Introduction of donor DNA by an integrase defective lentiviral vector resulted in the introduction of genetic material at these specific and pre-determined sites.

Peptide nucleic acids (PNAs) are similar to ZFNs in that they also stimulate site-directed recombination using co-transfected donor DNA template. PNAs are polymers containing purine and pyrimidine bases covalently attached to a repeating N-(2-aminoethyl)-glycine backbone. The uncharged backbone diminishes electrostatic repulsion from DNA, allowing for higher affinity

binding to recognized sequences. As a result, PNAs can efficiently interrupt normal transcription processes and effectively knock out a gene product. There is ongoing research in exploring the use of PNAs as pharmacological transcription factor decoys to down regulate signaling and expression of oncogenic products [219]. In the case of genetic modification of HSCs, PNAs have only entered into investigation recently. Rogers and colleagues reported *in vivo* genomic modification of HSCs using PNA conjugates fused with a nuclear-localizing protein [220]. Current limitations to this method include the specificity of these PNA conjugates given that genomic modification of somatic tissues also was observed, as well as the low-level percentage of genetically-modified cells, which currently is less than 5%.

Clinical HSCT Gene Therapy

The first clinical gene therapy trials using HSCs revealed many of the safety concerns inherent with somatic cell genetic modification using recombinant retroviral vectors. Despite the pre-clinical and early clinical successes of retroviral based therapy for childhood X-linked severe combined immune deficiency (SCID), a 2002 report revealed the generation of T cell leukemia as a result of genetic-modification in one of the initial patients [221]. This trial was eventually suspended when 5 of 20 patients developed T cell acute lymphoblastic leukemia. As a result, a large effort was placed on understanding not only the mechanism of integration, but also the location and downstream effects of retroviral transduction. γ-retroviral vectors integrate into chromosomal sites that are actively transcribed, including potential proto-oncogenes, resulting in

aberrant transcription patterns and expression [222-226]. To date, insertion-site and clonality analysis remains a top safety parameter for HSC targeted gene therapy.

The creation and implementation of self-inactivating (SIN) viral vectors is predicted to alleviate some of the concern regarding integrating viral vectors. Deletions in the 3' LTR of retroviral vectors are transferred to the 5' proviral LTR resulting in transcriptionally inactive viral particles [227]. This is important because in lentiviruses, the LTRs are promoters with strong enhancer capabilities. As has been demonstrated, the strong enhancers can induce the transcription of genes near the integrated proviral sequence [228]. This safeguard, however, requires the insertion of a new promoter into the vector sequence, such as the elongation factor-1 alpha or cytomegalovirus (CMV) promoters. Promoter selection enables control over which cells express the transgene product and at what level. Current pre-clinical research and clinical trials are exploring the use of SIN lentiviral vectors because of their lower genotoxic profile and increased predicted ability to modify HSCs *ex vivo* compared to the original γ-retroviral vectors [229-233]. Although proof of concept studies clearly have shown the benefits of using SIN-lentiviral vectors, it is possible that these vectors may have adverse effects on viral titer, engraftment potential, and/or transgene expression. Current clinical trials are designed to answer these specific questions, and the gene therapy field has made safety of viral integration a primary concern.

Recent advancements in the safety profile of γ-retroviral vectors have led to two successful clinical trials of HSCT gene therapy for adenosine deaminase (ADA) deficiency and X-linked SCID. In August 2011, Gaspar et al. inserted the ADA cDNA into a γ-retroviral vector

pseudotyped with the gibbon-ape-leukemia-virus envelope [234, 235]. Six children ceased enzyme replacement therapy prior to treatment and were conditioned with either 140mg/m^2 melphalan or 4mg/kg busulfan intravenously prior to autologous HSCT gene therapy. Four of six subjects recovered immune function and three subjects no longer required ADA or immunoglobulin replacement therapy. All patients survived and no leukemia or other adverse events were observed within the 24 to 84 month follow-up. ADA expression was sustained in all hematopoietic lineages resulting in restored metabolic function, and functional T cell levels were elevated and sustained over 5 years post gene therapy. This trial is now one of two studies to present long-term, safe therapy using genetically-modified HSCs for the treatment of a monogenic disease [132]. In the treatment of X-linked SCID, Gasper et al. also used a similar γ – retroviral vector encoding the common subunit of the interleukin 2 receptor (IL2Rc) cDNA to transduce $CD34^+$ bone marrow cells stimulated with stem cell factor, thrombopoietin, interleukin-3, and Flt-3 ligand prior to transplantation [236]. Nonmyelosuppresive conditioning was used in this study and 10 of 10 patients showed elevated, functional polyclonal T cell populations over a 54-107 month follow-up. While most side effects were minimal and overcome with standardized course of action, one patient did develop T cell acute lymphoblastic leukemia due to up-regulation of the LMO-2 proto-oncogene. However, this patient maintained a polyclonal T cell population and currently is in remission. While the usage of γ-retroviral vectors has proven to be an effective and predominantly safe treatment option against ADA-deficiency SCID and X-linked SCID, the risk of oncogenic up-regulation is still a concern. ADA-deficiency SCID treatment has not, to date, resulted in the expansion of leukemic cells [237]. The emergence of T-ALL in X-linked SCID treatment but not ADA-SCID despite similar viral vector

preparation methods suggests that the discrepancies in safety are the result of transduction methods, biology of the corrected cells, and/or differences in the two disease states.

Immunological aspects of HSCT gene therapy

A major risk of allogeneic transplantation and even autologous transplants containing neo-antigens is that of immunological response and rejection. This issue is relevant to hemophilia A gene therapies because transplantation of HSCs expressing FVIII can induce an immune response to FVIII. Non-specific immunosupressants have shown success in reducing transplant rejection, however, they pose significant risk of subsequent infection. As a result, efforts to prevent specific inhibitor formation in gene therapy strategies have become high priority. Several studies have combined gene therapy approaches with immune tolerance strategies to prevent this inactivation response. HSCs remain ideal targets because the immune system is derived from these cells, and expression of transgenes within hematopoietic cells can induce immune tolerance.

Several labs have conducted HSCT gene therapy using mouse models to study immune tolerance through molecular chimerism. For example, it has been shown that retroviral gene transfer of the porcine glucosyltransferase UDP galactose gene, αGT, into naïve murine bone marrow cells induced tolerance to porcine xenografts containing the foreign epitope [238]. Furthermore, analysis of the B-cell population revealed that specific antibody-producing cells against the

xenograft were eliminated during immune reconstitution. In a subsequent study, retroviral gene transfer of the cDNA encoding H-2K^b, a murine major histocompatibility class I antigen, into HSCs resulted in long-term expression of the antigen in hematopoietic lineages. Furthermore, genetically-modified T cells facilitated tolerance to transplanted H-2K^b expressing targets, but retained the capacity to reject third-party grafts, suggesting that T cells are capable of induced tolerance via gene therapy [239]. Additionally, it was confirmed that this gene therapy approach induced negative selection of cells expressing the alloreactive T cell receptor in the thymus, and that thymic re-education is possible through genetic modification [240]. As described below, under certain conditions, expression of FVIII from genetically-modified HSCs induces long term tolerance in transplanted mice.

HSCT for the treatment of hemophilia A

Hemophilia meets several criteria for HSCT gene therapy. First, it is a monogenic X-linked disease caused by the deficiency of a single, essential blood coagulation factor. Second, FVIII functions in the circulation. Thus, hematopoietic cells are ideal vehicles for its delivery to the bloodstream. Third, mere picomolar concentrations of FVIII are sufficient to alleviate spontaneous bleeding episodes. Fourth, virtually all cell types tested have the capacity to biosynthesize FVIII, albeit at varying levels. Currently, there is no cure for hemophilia A and state of the art FVIII replacement therapy is cost prohibitive to the majority of people with the disease. Lastly, protein replacement therapy is plagued by complications arising from the

necessary intravenous route of administration. The risk/benefit ratio of gene therapy as a treatment for hemophilia A has resulted in the consensus that gene therapy is the most promising therapeutic advance on the horizon [241].

Evans and Morgan conducted the first preclinical study of HSCT-based gene therapy of hemophilia A [242]. Using a murine leukemia virus-based γ-retroviral vector, the human FVIII cDNA was transferred to murine bone marrow cells, which subsequently were transplanted into hemophilia A mice that were pretreated with a lethal dose of total body irradiation (TBI). In this study, correction of the FVIII deficiency was not achieved, but it provided an early indication of the low level biosynthesis of human FVIII as a major barrier to the development of hemophilia A gene therapy applications. In 2002, Tonn et al. showed that there are hematopoietic lineage specific differentials in FVIII biosynthesis [243]. It was observed that erythroid and megakaryocytic cells secreted higher levels of B-domain deleted (BDD) human FVIII compared to lymphoblastoid or T cell leukemia cell lines. The first demonstration of HSCT gene therapy based induction of therapeutic FVIII levels (generally accepted to be >5% or 0.05 U/ml FVIII activity) came in 2004 and 2005 by Hawley and colleagues [244, 245]. Using a bicistronic γ-retroviral vector encoding the BDD human FVIII transgene as well as an EGFP reporter transgene, genetically-modified cells were selected prior to transplantation leading to higher level engraftment of genetically-modified cells. Recognizing the technical expression limitations in these studies, a greater effort was placed in engineering the FVIII transgene for higher level expression from hematopoietic as well as other cell types. Point mutagenesis of endoplasmic reticulum chaperone immunoglobulin-binding protein (BiP) recognition site, specifically at residue 309, increased expression 2 – 3 fold [57]. Further characterization and engineering led to the generation of constructs containing 6 additional N-linked glycans in the B-domain that were

shown to further improve secretion [135]. Although it is predicted that these constructs with enhanced FVIII expression will benefit HSC-directed gene therapy, this has not been conclusively demonstrated in a head to head study against standard BDD human FVIII. In another study, Hawley and colleagues explored the use of a simian immunodeficiency vector system containing a B-cell specific enhancer/promoter to drive FVIII expression [246]. Therapeutic levels of FVIII were induced and intracellular FVIII was detected in $B220^+$ B cells and $CD138^+$ plasma cells, but not in HSCs. Transplantation under non-myeloablative conditioning induced a minor immunological response after subsequent challenge with FVIII. By targeting B-cells exclusively, tolerance to FVIII through molecular chimerism can be achieved. However, future studies are required to elucidate lineage-specific limitations in expression.

The benefits of using FVIII orthologs, however, have been well characterized. Characterization of porcine FVIII, has contributed greatly to overcoming the FVIII expression barrier. BDD porcine FVIII expresses at 10-100 fold greater levels than BDD human FVIII *in vitro* and *in vivo* [136, 147, 148]. The use of the porcine FVIII cDNA in gene therapy strategies was founded on the clinical history of plasma derived porcine FVIII in the treatment of acute bleeding episodes in persons with anti-human FVIII inhibitory antibodies. Of note, no species-based incompatibilities have been observed between porcine FVIII and the human blood coagulation components, including binding to von Willebrand factor. Currently, a recombinant BDD porcine FVIII product is undergoing late-stage clinical testing in inhibitor patients. Therefore, there is no technical obstacle to the utilization of the high expression property of porcine FVIII in gene therapy applications to overcome the FVIII expression barrier. Investigation into the mechanism

responsible for the expression differential revealed that the dominant characteristic in high level expression is improved post-translational secretory efficiency [148]. In support of this mechanism, it was demonstrated that recombinant porcine FVIII induces the unfolded protein response (UPR) to a lesser extent than human FVIII, thus explaining the previously observed differential in post-translational secretory transport [133]. Additionally, it was shown that pharmacogenetic knockdown of GRP78/BiP, a master regulator of UPR, using shRNA technology increased human FVIII production, and overexpression of X-box-binding protein 1 (XBP1), another UPR regulator, resulted in increased production of both human and porcine FVIII. Therefore, we are beginning to understand the basic mechanisms governing human and orthologous FVIII biosynthesis.

Several studies have demonstrated the utility of high expression porcine FVIII sequences in gene transfer-based applications for hemophilia A. For example, the BDD porcine FVIII transgene was transferred into HSCs using recombinant murine stem cell viral vector (a γ-retrovirus-derived vector) and the transduced cells were transplanted into hemophilia A mice subjected to a lethal dose of TBI [247]. All experimental mice expressed circulating FVIII activity levels near or exceeding 100% normal human levels that were sustained for over 18 months after transplantation. Subsequently, reduced-intensity conditioning regimens were explored including sublethal TBI (5.5 Gy), costimulation blockade (anti-CD40L and CTLA4-Ig), and a combination of busulfan and anti-thymocyte serum. Each of these regimens combined with HSCT gene therapy incorporating the BDD porcine FVIII transgene resulted in successful engraftment and sustained therapeutic FVIII expression in all hemophilia A mice [248]. Additionally, it was

shown that mice engrafted under the reduced-intensity conditioning regimens did not elicit an immune response following challenges with human FVIII. Furthermore, T cells from these animals were not activated upon stimulation with porcine FVIII suggesting that immunologic tolerance to FVIII was induced as a result of the HSCT gene therapy [249]. From this, it can be concluded that T cell suppression is critical to successful engraftment of genetically-modified HSCs encoding FVIII.

Investigations into the mechanisms underlying FVIII production and secretion differentials within orthologous and bioengineered transgenes have been pursued concomitantly with their application in gene therapy strategies. The use of porcine and high-expression human/porcine (HP) hybrid constructs have reduced the gene transfer requirements in terms of the genetically-modified cell dose and proviral copy number, thus increasing the safety profiles of proposed gene therapy protocols. Additionally through the use of HP constructs, it is possible to conserve the high expression characteristics of porcine FVIII within a predominantly human FVIII transgene. In one study, a HP construct containing a 9:1 ratio of human to porcine amino acid composition respectively, showed identical therapeutic performance to BDD porcine FVIII in the hemophilia A mouse HSCT gene therapy model [152]. These results are relevant and potentially critical to the design of future clinical gene therapy applications in light of the previous failures to achieve therapeutic FVIII expression levels in clinical trials.

Prior to approval for clinical testing in humans, a proposed gene therapy product must demonstrate pre-clinical safety and efficacy using multiple *in vivo* experimental systems. For hemophilia A, several model systems exist including the hemophilia A mouse model [120], canine model [250], and ovine model [169]. While characterization and experimentation with the ovine model is in the relatively early phases [251], the murine and canine models have been studied and utilized extensively in the pre-clinical development of novel hemophilia A therapeutics including gene-transfer based therapies (for review of the canine studies, see [252]). In the context of the broader application of HSCT gene therapy, most large animal studies have been performed using canine and non-human primate models (for review, see [253]). These models have been instrumental in the development of methods for stem cell harvest, *ex vivo* manipulation, and gene transfer as well as studying the biology of transplanted genetically-modified cells. Recently, Wilcox and colleagues demonstrated correction of canine Glanzmann thrombasthenia (GT), a rare platelet adhesion disorder, using HSCT gene therapy [254]. Affected dogs underwent autologous HSCT gene therapy incorporating a HIV-1-based lentiviral vector encoding a functional integrin αIIbβ3 gene. Post-transplantation analysis revealed approximately 5,000 αIIbβ3 receptors on 10% of platelets, resulting in improved bleeding times and reduced blood loss up to 5 years after treatment. However, as is the case for any model system, there are limitations to its ability to mimic human testing. For example, many reagents used in HSCT and HSCT gene therapy, including cytokines, stem cell markers, gene transfer vectors and conditioning agents, display species specificity. Therefore, reagents and dosing schemes may not be translatable from preclinical to clinical studies. Currently, the most relevant preclinical test of a HSCT gene therapy product is the ability to genetically-modify human $CD34^+$ cells without significantly diminishing their engraftment and hematopoiesis potential. To examine this, murine

(NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, referred to as NSG mice) xenotransplantation models are the gold standard. Therefore, there is no consensus on the best path toward regulatory approval for HSCT gene therapy and what role, if any, large animal models of hemophilia A will play in this process.

HSCT gene therapy for patients with inhibitors

The development of neutralizing antibodies against FVIII represents the most challenging and costly complication in the treatment of hemophilia A. Inhibitors emerge in 20-30% of patients with severe hemophilia and typically prevent future treatment with human FVIII replacement products. Instead, patients are routinely treated using FVIII bypassing agents such as recombinant activated factor VII or activated coagulation factor concentrates or, historically, porcine FVIII product. Currently, a recombinant BDD porcine FVIII product is in phase 3 clinical testing. Unfortunately, each of these treatments has significant limitations, e.g. subsequent immunity to porcine FVIII, and are best utilized on an acute basis and not for lifelong prophylaxis. Therefore, inhibitor patients represent the most at risk hemophilia A population in the developed world and therefore, can be considered prime candidates for novel experimental therapies such as HSCT gene therapy. However, due to the complexities of FVIII gene transfer and expression, historically, little attention has been devoted to this patient population.

Recently, it was demonstrated that the porcine FVIII transgene can be used to induce FVIII expression in mice with preexisting antibodies to human FVIII. To study the use of HSCT gene therapy for patients with inhibitors, humoral immunity to human FVIII is induced in mouse hemophilia A models by weekly intravenous administration of human FVIII over 4-6 weeks. Typically significant anti-human FVIII titers are observed in all animals, and in one study, it was shown that 10 – 14% of these antibodies have cross-reactivity to BDD porcine FVIII . Even in this unfavorable environment, using a gene transfer strategy incorporating porcine FVIII and myeloablative conditioning, complete correction of the FVIII deficiency (3.6 ± 1.3 U/ml) and eradication of the FVIII inhibitors ($t_{1/2}$ of 16 days) was observed [255]. This was the first successful report of HSCT gene therapy in an anti-FVIII inhibitor model where FVIII activity was restored and the inhibitors were eliminated. Despite the inherent difficulty of treating hemophilia A patients with pre-existing inhibitors, these results demonstrate the feasibility of HSCT gene therapy in this high-risk disease setting.

Summary

The field of HSCT gene therapy has advanced from proof of concept studies to the treatment of humans with acquired and genetic diseases. Several clinical trials have resulted in life-saving successes, and the curative potential of genetically-modified HSCs is now a reality with the number of disease applications growing rapidly, including chronic granulomatous disease, Wiscott-Aldrich disease, Fanconi anemia, β-thalassemia, and sickle cell disease. Hemophilia A

remains a prime candidate for HSCT gene therapy. Advancements in FVIII transgene design, viral vector engineering, and immunological conditioning have cured this disease in animal models and shown promise for upcoming clinical trials. Additional studies continue to elucidate methods and technologies to identify and isolate HSCs and improve transduction of this important gene therapy target. In addition, overcoming the current limitations of FVIII expression, as well as reducing the risks of the gene transfer procedure, such as insertional mutagenesis, is possible. Further advancements in the field of HSCT gene therapy have included novel mechanisms to target site-specific gene insertions or corrections using engineered nucleases or PNA-complexes. Through ongoing intensive research, the field of HSCT gene therapy is progressing towards safer, more efficient, and cost effective treatment options.

Chapter II: Development and Characterization of Recombinant Ovine Coagulation Factor VIII

Abstract

Animal models of the bleeding disorder, hemophilia A, have been an integral component of the biopharmaceutical development process and have facilitated the development of recombinant coagulation factor VIII (FVIII) products capable of restoring median survival of persons with hemophilia A to that of the general population. However, there remain several limitations to recombinant FVIII as a biotherapeutic, including invasiveness of intravenous infusion, short half-life, immunogenicity, and lack of availability to the majority of the world's population. The recently described ovine model of hemophilia A is the largest and most accurate phenocopy. Affected sheep die prematurely due to bleeding-related pathogenesis and display robust adaptive humoral immunity to non-ovine FVIII. Herein, we describe the development and characterization of recombinant ovine FVIII (oFVIII) to support further the utility of the ovine hemophilia A model. Full-length and B-domain deleted (BDD) oFVIII cDNAs were generated and demonstrated to facilitate greater biosynthetic rates than their human FVIII counterparts while both BDD constructs showed greater expression rates than the same-species full-length versions. A top recombinant BDD oFVIII producing baby hamster kidney clone was identified and used to biosynthesize raw material for purification and biochemical characterization. Highly purified recombinant BDD oFVIII preparations possess a specific activity nearly 2-fold higher than recombinant BDD human FVIII and display a differential glycosylation pattern. However, binding to the carrier protein, von Willebrand factor, which is critical for stability of FVIII in circulation, is indistinguishable. Decay of thrombin-activated oFVIIIa is 2-fold slower than

human FVIII indicating greater intrinsic stability. Furthermore, intravenous administration of oFVIII effectively reverses the bleeding phenotype in the murine model of hemophilia A. Recombinant oFVIII should facilitate the maintenance of the ovine hemophilia A herd and their utilization as a relevant large animal model for the research and development of novel nucleic acid and protein-based therapies for hemophilia A.

Introduction

Factor VIII (FVIII) is an essential glycoprotein procofactor within the intrinsic pathway of the blood coagulation cascade. In blood circulation, FVIII is non-covalently bound to von Willebrand factor (VWF) and is present at relatively low concentration (1 nM). Mutations in the *F8* gene often result in diminished or inactive plasma FVIII and are the molecular genetic cause of the monogenic, X-linked, bleeding disorder hemophilia A that affects approximately 1 in 7500 males worldwide. Current treatment is limited to intravenous infusion of plasma-derived or recombinant human FVIII (hFVIII) containing products. This therapy is only available to 30% of the world due to economic factors and requires multi-weekly injections to achieve prophylaxis, which must be maintained for the duration of the patients' life to avoid debilitating joint disease as well as life-threatening bleeding episodes. While gene therapy is being explored as a potential cure, additional research efforts are aimed at improving the therapeutic utility of recombinant FVIII.

Investigations into the biochemical properties of orthologous FVIII constructs have yielded insight into basic FVIII structure/function as well as translation into novel clinical agents. For example of the former, the characterization of recombinant murine factor VIII (mFVIII) revealed near complete stability at physiologic concentrations following thrombin activation [155]. Porcine FVIII (pFVIII) demonstrates 10 to 100-fold increased expression over hFVIII [147, 148], as well as decreased engagement of the endoplasmic reticulum-resident unfolded protein response [133]. Furthermore, Arruda and colleagues described the development and characterization of canine FVIII, which displays 3-fold higher specific activity than that of

hFVIII and currently is utilized to manage bleeding in canine hemophilia A colonies [160]. As for the development of novel clinical agents, plasma derived pFVIII has historically been used in the treatment of patients with pre-existing inhibitors to hFVIII and recombinant B-domain deleted (BDD) pFVIII currently is in clinical trials. Likewise, human/porcine (hp) hybrid transgenes with high expression properties are being developed for clinical gene therapy [152].

A line of sheep presenting with hemophilia A recently was re-established and the pathology, clinical profile, and molecular genetics were described [169]. Ovine FVIII (oFVIII) possesses 86% amino acid sequence homology to hFVIII outside of the B-domain and possesses a similar domain structure (A1-A2-B-ap-A3-C1-C2) defined by internal sequence homology. The causative mutation was identified as a single nucleotide insertion resulting in frameshift and a premature stop codon in exon 14 similar to a mutation documented in a human patient with severe hemophilia A [256]. In preliminary studies, administration of hFVIII or hpFVIII corrected the bleeding phenotype in this model transiently, but invariably induced the formation of high-titer anti-FVIII inhibitory antibodies eventually leading to premature mortality. Moreover, transplantation of genetically-modified mesenchymal stem cells expressing a BDD pFVIII transgene in this model corrected phenotypic hemarthroses and spontaneous bleeds for several months, thus establishing the potential of the model for the development of novel therapeutics. The utility of this model as a research and development resource hinges on the ability to maintain colonies of these clinically fragile animals. Toward this goal, the cloning, expression, purification and biochemical characterization of recombinant BDD oFVIII are described in the current study.

Materials and Methods

Materials

The cloning and characterization of full-length ovine FVIII in the pUC57 vector has been described previously [169]. Phusion High Fidelity PCR MasterMix, PNGase, and all restriction enzymes were purchased from New England Biolabs (Ipswich, MA). All cell culture materials were purchased from Corning Inc. (Corning, NY). AIM V and DMEM/F12 media was purchased from Invitrogen (Carlsbad, CA). Citrated FVIII-deficient plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical (Overland Park, KA). Activated partial thromboplastin reagent (aPTT) was purchased from Organon Teknika (Durham, NC). Monoclonal antibodies were provided by Dr. Pete Lollar (Aflac Cancer Center and Blood Disorders Service, Emory University, Atlanta, GA). Recombinant human thrombin was provided by Haematologic Technologies Inc. (Essex, VT). Desulfatohirudin was a generous gift from Dr. R. B. Wallis (Ciba-Geigy Pharmaceuticals) to our colleague Dr. Pete Lollar (Emory University, Atlanta, GA). SDS-PAGE precast gels were purchased from Bio-Rad (Hercules, CA). Polyethyleneimine was purchased from Polysciences, Inc. (Warrington, PA). A colony of exon 16-disrupted hemophilia A mice (E16 $^{-/-}$) was kept and maintained within the Emory Division of Animal Resources Pediatrics Facility.

Generation of BDD OFVIII

Replacement of the ovine B-domain with an SQ linker containing a PACE/furin recognition sequence was conducted by SOE mutagenesis as described previously [155]. Primers for heavy

chain (HC) and light chain (LC) were manufactured by Integrated DNA Technologies (Coralville, IA). HC forward: 5'- GAC CGG ATC GGA AAA CCT CTC GAG CCA CCA TGC ACA TCA AGC TCT GTA CCT GCC-3'; HC reverse: 5'-ATT CTG GGA GAA GCT CCT AGG TTC AAT GAC ATT GTT TTC ACT CAG CAG G-3'; LC forward: 5'-GTC ATT GAA CCT AGG AGC TTC TCC CAG AAT CCA CCA AGC TTG AAA CGC CAT CAA AGG-3'; LC reverse: 5'-AGT GGC AGG TGC TGC AGC GGC CGC CCT CAG TAC TGC TGC TGT GCC TCA C-3'. PCR amplification was conducted in the following cycles: 30 s at 98 °C, 35 cycles of 10 s at 98 °C and 30 s at 58 °C, and annealing at 78 °C for 13 minutes followed by 25 °C hold. Amplified products were digested utilizing NotI, AvrII and XhoI restriction nucleases and separated using SeaKem® GTG® Agarose (Lonza; Rockland ME) gel electrophoresis. Digested fragments were purified using a QIAquick Gel Extraction Kit (Qiagen) and ligated using a T4 DNA Quick Ligase (Promega). The final construct was cloned into ReNeo mammalian expression vector using NotI and XhoI restriction sites. BDD oFVIII ReNeo was sequenced by Beckman Coulter Genomics (Danvers, MA) using overlapping primers spanning the entire transgene.

Generation and Characterization of Stable OFVIII Expressing Clones

Naïve baby hamster kidney-derived (BHK-M) cells were transfected in 6-well plates with 1.5 µg/10^6 cells of ReNeo mammalian expression plasmid encoding the respective FVIII transgene. Polyethylenimine was administered at a final concentration of 6 ng/ml in DMEM containing 10% FBS. Media was replaced at 24 h and expanded at 48 h to 10 cm plates in media containing 500 µg/ml G418 (Gibco, Grand Island, NY) and cultured for 10-14 days. Fifty to sixty-eight

clones were selected and expanded. For determination of specific FVIII production rates, the clones were cultured for 24 h in serum free AIM V media and then counted by hemacytometer for normalization to units/10^6 cells/24 hr. FVIII activity measurements were made by one-stage coagulation assay and linear regression analysis of clotting times against a pooled human plasma standard (FACT) using a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France). Geneticin-resistant clones that expressed FVIII below the limit of assay detection (0.01 units/ml) in 24 h were not included in the statistical analysis. From 35 antibiotic resistant clones with BDD ovine FVIII activity above measurable detection, the highest expression clone was selected for further study. Peak expression from this clone was 6 units/10^6 cells/24 h.

Purification of Recombinant OFVIII

Recombinant oFVIII was purified using a two-step ion exchange chromatography procedure as cited previously [147]. Expressing clones were expanded into 500 cm^2 flasks in DMEM/F-12 growth media containing 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells at 60-70% confluency were washed 2x with 50 ml Dulbecco's phosphate-buffered saline (PBS) (Thermo Scientific) and re-fed with 125-150 ml AIM V serum free media. Media was collected every 24-48 h and replaced with equal volume of fresh AIM V. FVIII containing media was subjected to centrifugation at 2000 x *g* for 10 min and the supernatant was frozen at -80 °C in 0.05% sodium azide until time of purification. Media was thawed at 37 °C and loaded onto a 5 x 20 cm SP-Sepharose High Performance column equilibrated to 0.15 M NaCl, 20 mM HEPES, 5 mM $CaCl_2$, 0.01% Tween-80, pH 7.4 (HBST). The column was washed twice with equilibration buffer followed by 18% NaCl containing buffer prior to elution. Fractions were eluted over a

linear 0.18-0.65 M NaCl gradient in HBST. Fractions containing FVIII were assayed for FVIII activity and activation quotient (AQ) and those with AQ values greater than 20 were pooled. The AQ assay was conducted using both one-stage and two-stage coagulation assays as described previously [155]. The activation quotient is defined as the ratio of FVIII activity measured by two-stage coagulation assay divided by the FVIII activity measured by the one-stage coagulation assay. Pooled material was diluted to 0.15 M NaCl in the HBST, applied to a Source Q HR5/5 FPLC column and eluted with a linear 0.2-0.65 M NaCl gradient. Fractions were assayed by one-stage coagulation assay, absorbance at 280 nm, and SDS-PAGE. Final AQ and specific activity measurements of pooled material were recorded following a freeze/thaw cycle at -80 °C.

SDS-PAGE Analysis and Mass Spectrometry

Polypeptides were resolved by 4-15% gradient SDS-PAGE and fixed with 50% methanol/10% glacial acetic acid prior to staining with GelCode Blue (Pierce, Rockford, IL). Deglycosylation was conducted according to manufacturer's directions (NEB). Briefly, 2 µg purified protein was activated with 2 units recombinant human thrombin at 37°C for 10 min, denatured in denaturation buffer supplied by the manufacturer at 95°C for 10 minutes, and deglycosylated by incubation with 500 units PNGase at 37°C for 1 hour. Confirmation of N-linked glycan location was carried out by tandem mass spectrometry. OFVIII was treated with PNGase as described above, purified via SDS-PAGE, and stained with coomassie blue. Excised bands were subjected to in-gel digestion (12.5 µg/ml trypsin). Extracted peptides were loaded onto a C18 column (100-µm inner diameter, 20 cm long, ~300 nl/min flow rate, 1.9-µm resin from Dr. Maisch Gbmh, Ammerbuch-Entringen, Germany) and eluted during a 10–30% gradient (Buffer A: 0.1% formic

acid, 1% ACN; Buffer B: 0.1% formic acid, 99.9% ACN). The eluted peptides were detected by Orbitrap (300–1600 m/z; 1,000,000 automatic gain control target; 500-ms maximum ion time; resolution, 30,000 full-width at half-maximum) followed by ten data-dependent MS/MS scans in the linear ion trap quadrupole (2 m/z isolation width, 35% collision energy, 5,000 automatic gain control target, 150-ms maximum ion time) on a hybrid mass spectrometer, LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA). The acquired tandem mass spectrometer (MS/MS) spectra were searched against and decoy-concatenated Ovis aries database (807 target proteins and 1 customized coagulation factor VIII sequence) from the NCBI RefSeq protein database project (version 54) using the Sorcerer-SEQUEST Algorithm version 4.04 (Sage-N Research, San Jose, CA) with differential modification of + 0.984016 Da on Asn and +15.99492 on Met. Search results were filtered to 1% FDR and summarized by in-house programs, as described by Gozal et al [257]. Glycosylation sites were determined by differential analysis of peptide elution profiles and tandem mass spectrum between glycosylated and PNGase-F treated samples. In summary, ion chromatograms for peptides containing a matching asparagine modification mass shift were extracted from the PNGase-F treated sample. Only ions that have a noise-level chromatogram in the glycosylated sample were considered to be confidently matched to a glycopeptide.

Immunoprecipitation

Purified polypeptide was activated with 1.4 μM thrombin, inactivated with 10 μM desulfatohirudin and incubated with mAb for 45 min at 37 °C in M-PER (Thermo Scientific, Rockford, IL) with 150 mM NaCl. Protein G Agarose (KPL, Gaithersburg MD) was added and

incubated at 37 °C for one hour. Following centrifugation at 14,000 x *g*, pellets were washed three times in 200 µl HBST followed by centrifugation at 14,000 x *g* for 5 minutes. Protein G complexes were resuspended in 25 µl HBST and heated for 5 minutes at 95° C. Supernatant was loaded into 4-15% SDS-PAGE gel for analysis.

Activated FVIII Decay Assay

Activated factor VIII (FVIIIa) was measured by chromogenic assay using purified human factor IXa, human factor X, and synthetic phospholipid vesicles as described previously [258]. Briefly, 20 nM oFVIII or hFVIII was activated with 100 nM human thrombin for 15 seconds at room temperature. Desulfatohirudin (150 nM) was added to stop the reaction and FVIII activity was measured at several time points.

von Willebrand Factor (VWF) Binding

Binding of oFVIII to human VWF was determined by ELISA. Thermo Scientific Immulon 1B plates were coated with 50 µl of 6 mg/ml human VWF in buffer A (20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, 0.05% Tween 20, 0.05% sodium azide) overnight at 4°C. Plates were washed twice with buffer A and blocked with 2% BSA in buffer A (blocking buffer). Plates were stored at 4 °C until use. Human and ovine FVIII were diluted in blocking buffer and applied to VWF coated wells following two washes with buffer A. Plates were incubated for 1 h at room temperature, washed twice with buffer A, and incubated with 1 µg/ml of biotinylated monoclonal antibody 4F4 1B (generously provided by Dr. Pete Lollar, Emory University) in blocking buffer

for 1 h at room temperature. Primary antibody incubation was followed by two washes with buffer A and the addition of streptavidin alkaline phosphatase (Jackson ImmunoResearch Labs, Inc., West Grove, PA) at 1:15,000 in buffer A for 1 h at room temperature. Substrate activation was preceded by two final washes in buffer A. Colorimetric transmission was initiated by the addition of 80 µl *para*-Nitrophenylphosphate substrate (Bio-Rad; Hercules, PA) and recorded in kinetic mode as the change in $A_{405}\,s^{-1}$. Rates were limited to a maximum optical density of 0.8 OD.

Efficacy of OFVIII In Vivo

Hemostatic challenge was conducted as previously described [151] with alterations. All animal studies were reviewed and approved by the Emory University Institutional Animal Care and Use Committee (IACUC). Briefly, oFVIII was diluted in saline to a concentration of 300 units/kg mouse body weight. Eight to twelve week old E16 $^{-/-}$ hemophilia A mice were infused with either saline or oFVIII via tail vein injection. Immediately following injection, mice were anesthetized with 3.5% isoflurane at a flow rate of 1,000 ml/min for 5 minutes. Tails were placed into a 15 ml conical tube with 13 ml sterile saline at 37 °C. Isoflurane was reduced to 2% at a flow rate of 500 ml/min and maintained for the duration of the experiment. At 15 min, tails were transected at 2 mm diameter as measured by wire gauge. In doing so, blood loss between mice is standardized to the diameter of tail vasculature rather than distal length. Blood was collected in a new, pre-weighed 15 ml conical containing 13 ml sterile saline at 37 °C for 40 minutes and measured by change in mass and recorded by mg blood loss per gram body weight. The mean evaporative loss of two vials of 13 ml sterile saline at 37 °C was used to correct for changes in

mass of the efficacy treatments. One mouse injected with oFVIII displayed blood loss of 7 mg/g body weight, however, this likely was attributed to a technical error in transection.

Results

Heterologous Expression of Recombinant OFVIII

Recombinant FVIII can be synthesized in two distinct, but functional forms, the endogenous full-length form and the BDD form that displays enhanced expression due to a 1/3 reduction in transcript and transgene product size. Therefore, a recombinant BDD ovine FVIII (oFVIII) expression plasmid was designed that contained a PACE/furin recognition site (RHQR) within a 14 amino acid linker between A2 and ap-A3 domains similar to those described previously for other orthologous FVIII constructs [42, 259]. Expression of full-length and BDD human and ovine FVIII in serum-free media was compared using stably transfected BHK-M cells as described previously [136, 147, 152, 155] (Figure II-1). Clones with measured activity production below 0.01 units/10^6 cells/24 h (1% normal FVIII levels) were not included in statistical analysis. Clones expressing full-length oFVIII (n = 37) displayed significantly higher FVIII production rates, 0.0472 and 0.793 units/10^6 cells/24 h (median and maximum, respectively), than clones expressing full length hFVIII (n = 15), 0.0142 and 0.1 units/10^6 cells/24 h (median and maximum, respectively) ($P < 0.001$, Mann-Whitney *U* test). Deletion of the B domain resulted in increased expression of both BBD oFVIII and BDD hFVIII over their full-length counterparts ($P < 0.001$ for both comparisons, Mann-Whitney *U* test) with median expression levels of 0.783 and 0.091 units/10^6 cells/24h respectively. Again, the BDD oFVIII production rate was significantly greater than the BDD hFVIII rate ($P = 0.005$, Mann-Whitney *U* test). A 2.3 fold difference in expression between the top producing clones was observed with maximum clonal expression of BDD oFVIII measured at 6.12 units/10^6 cells/24h and BDD hFVIII at 2.63 units/10^6 cells/24h. The top producing BDD oFVIII clone was selected for

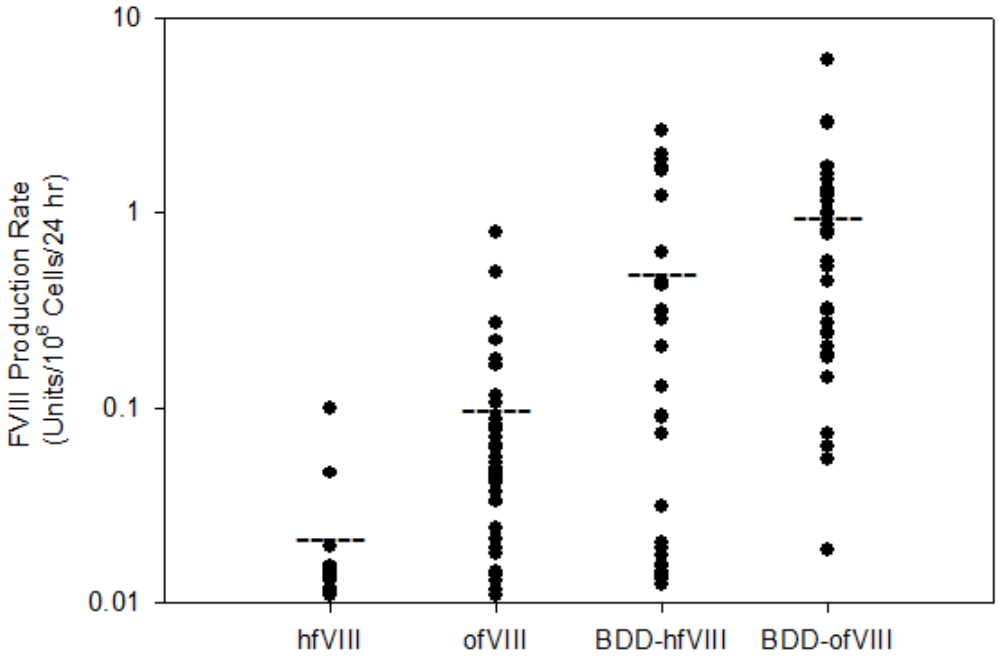


Figure II-1. Expression of Recombinant Ovine and Human FVIII. BHK-M cells were stably transfected with full-length and BDD FVIII constructs and selected with geneticin. Individual colonies were expanded in 6-well plates and FVIII activity was measured by one-stage coagulation assay in serum-free media after 24 hr culture. Cell numbers were determined at the time of activity measurement and data was normalized to 10^6 cells. The horizontal lines depict the mean values for each data set.

production, purification and biochemical characterization of the final product.

Purification and Biochemical Characterization of BDD OFVIII

Three independent expression and purification experiments were conducted although two were at smaller scale. The average specific activity determined from these independent preparations was 2,516 ± 503 U/nmol or 15,130 ± 3,030 units/mg (mean ± standard deviation) polypeptide. Specific activity was calculated by absorbance at 280 nm and an estimation of the molar extinction coefficient based on known tyrosine, tryptophan, and cysteine content [260]. In one experiment, approximately 20,000 units of BDD oFVIII was collected in 5.4 L of serum-free media and purified using a two-step ion-exchange chromatography procedure (Table II-1). The process yield was 25% and the final material had a specific activity of 3,050 units per nanomole (18,300 units/mg). One potential source of contamination that can affect dramatically the specific activity determination is the presence of activated FVIII (FVIIIa) in the final preparation. To address this issue, activation quotient (AQ) analysis of the peak fractions and final pooled material was performed. The activation quotient serves as a quality control metric for purified recombinant FVIII with an acceptable value being >20 and typically <80. A low AQ signifies the presence of FVIIIa in the material that can artificially inflate the activity measured in the one-stage coagulation assay and affect the accuracy of the specific activity determination. The AQ of the final BDD oFVIII preparation was 55. Furthermore, overall purity of > 95% (FVIII polypeptides) was confirmed by SDS-PAGE analysis (Figure II-2). The purified material from this preparation was utilized for all of the *in vitro* and *in vivo* characterization experiments

Table II-1. Purification of BDD OFVIII.

Sample	Vol. (ml)	A_{280}	Total A_{280}	Activity (U/ml)	Units	Units/ A_{280}	AQ	% Yield	Fold Pur.
Media	5,350	1.53	8,186	3.78	20,223	2.47	28	100	1
SP-Sepharose pool	35	0.415	14.5	353.4	12,370	853	49	61	345
Source Q pool	2.4	0.211	0.506	2,149	5,158	10,193	55	25.5	4,126

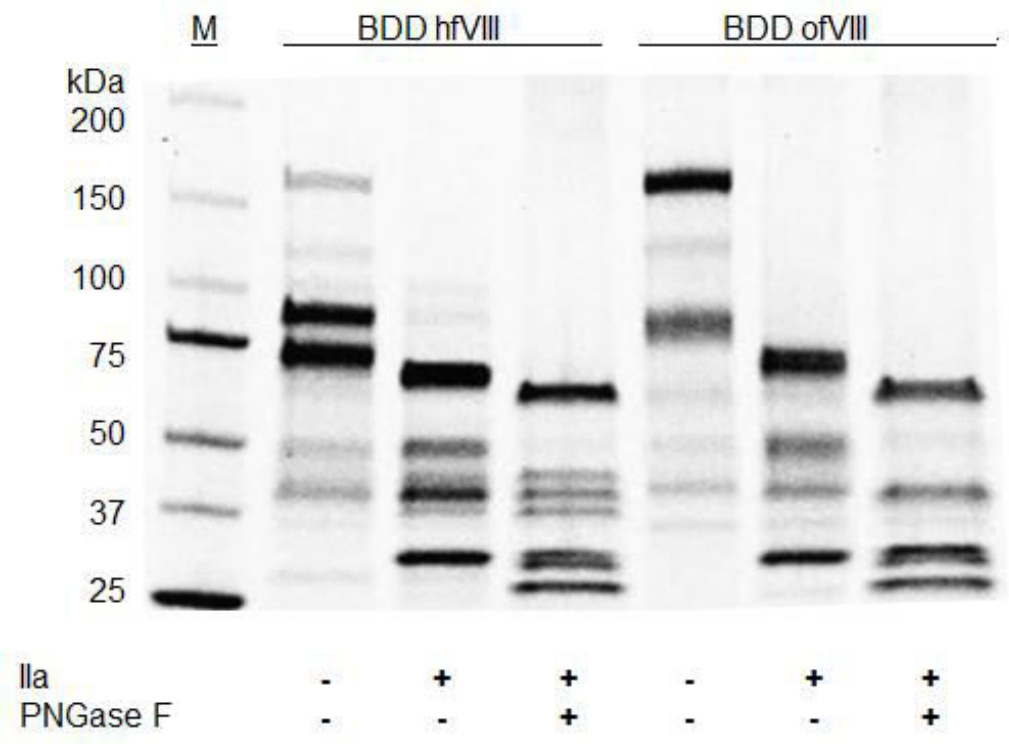


Figure II-2. Biochemical Analysis of BDD OFVIII. Recombinant oFVIII (2 µg) ± thrombin and PNGase treatment was resolved by SDS-PAGE and visualized by Coomassie blue staining. A molecular weight ladder was used to determine the relative mobility of the polypeptides.

presented herein.

Heterodimeric Structure, Glycan Analysis, and Thrombin Proteolysis of BDD OFVIII

FVIII circulates in plasma as a heterodimer of heavy and light chains associated in a metal ion facilitated, non-covalent manner. Typically, these two large polypeptides readily can be resolved upon visual inspection following SDS-PAGE. Unique to BDD oFVIII, the heavy and light chain polypeptides display similar relative mobility upon SDS-PAGE (Figures II-2 and II-3). When compared to the respective BDD hFVIII sequence, the ovine A1 and A2 domain sequences contain five and three amino acid residue deletions, respectively. This results in a predicted 1.1 kDa decrease in the overall size of the BDD oFVIII heavy chain. The overall size of both human and ovine light chains is identical based on *in silico* prediction as well as empirical SDS-PAGE analysis. Eight asparagine residues can be identified as potential sites of N-linked glycosylation based on *in silico* analysis: Asn-41; Asn-213; Asn-239; Asn-582; Asn-1720; Asn-1810; Asn-2118; Asn-2270. Of these sites, Asn-582 and Asn-2270 are not predicted to actually contain oligosaccharides. Consistent with these predictions, in many distinct human FVIII preparations, it has been demonstrated that Asn-582 is not glycosylated [261]. Furthermore, treatment of thrombin-activated oFVIII with PNGase F did not alter the mobility of the ovine A2 domain thus supporting the prediction that Asn-582 is not glycosylated in BDD oFVIII (Figure II-2). However, PNGase F treatment did affect a change in relative mobility of the oFVIII A1 and activated light chain fragments compared to BDD hFVIII, which is consistent with the prediction of additional

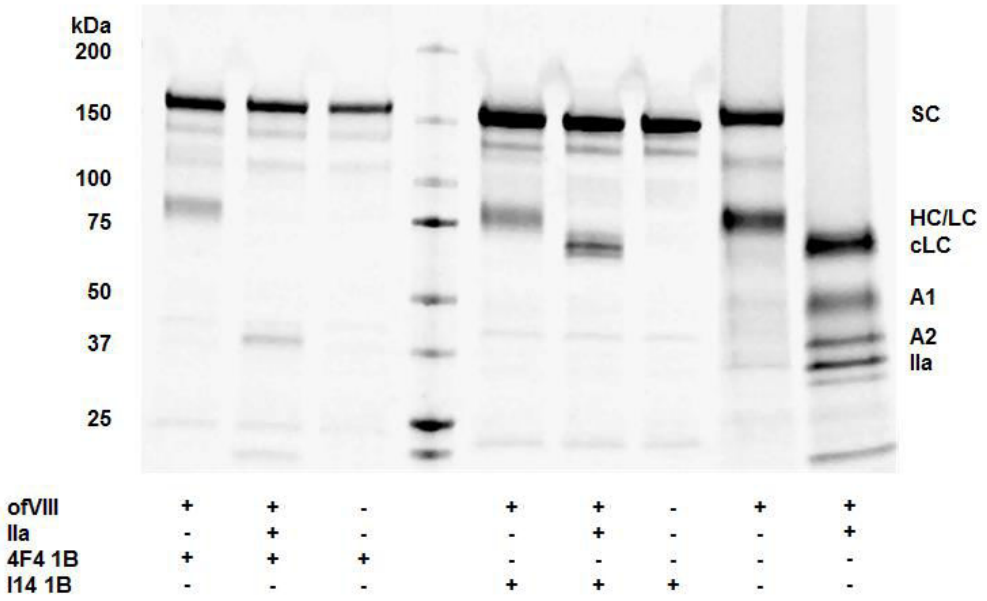


Figure II-3. Discrimination of the BDD OFVIII Heavy and Light Chains.

Immunoprecipitation using domain specific MAbs was performed by incubation with oFVIII in the presence and absence of thrombin. Heavy and light chains were dissociated using M-PER lysis buffer supplemented with 150 mM NaCl prior to MAb addition. FVIII heavy chain was precipitated with 4F4 1B, an A2 domain-specific mAb, and light chain was precipitated with I14 1B, a C2 domain-specific mAb. MAbs incubated with vehicle served as negative controls.

domain-specific N-linked glycans within both regions. Asn-213 is not present in the human sequence, but is conserved in canine and porcine while Asn-1720 is to-date a uniquely described potential glycosylation site, although this site is not supported by mass spectrometry. MS/MS analysis supports with high confidence the glycosylation of Asn-41, Asn-1810, and Asn-2118. There remains evidence of glycosylation of Asn-213 and Asn-239, however, limitations in the resolution require further investigation. A2 and C2 domain specific MAbs were used to elucidate mobility of heavy and light chains independently. Purified BDD oFVIII was incubated with either 4F4 1B or I14 1B MAbs in a solution known to dissociate the FVIII heavy and light chains of FVIII heterodimers. Consistent with prediction of identical relative mobility of the BDD oFVIII heavy and light chain polypeptides, independent precipitations of BDD oFVIII material with heavy and light chain-specific MAbs yielded polypeptide species of equal mobility (Figure II-3). Immunoprecipitations also were conducted in the presence of thrombin to verify domain-specific MAb interaction.

Decay of Activated OFVIII

When activated, FVIIIa serves as a cofactor for factor IXa, which executes the proteolytic cleavage of factor X into its activated form. Dissociation of the A2 domain from the A1/A3-C1-C2 heterodimer results in loss of pro-coagulant FVIII activity and can be measured indirectly by the generation of factor Xa in a purified system [258, 260, 262-264]. BDD oFVIII and BDD hFVIII were activated with thrombin and residual activity was measured over 30 minutes (Figure II-4). Similar to previous reports, hFVIIIa displayed a mean (± sample standard deviation) half-life of

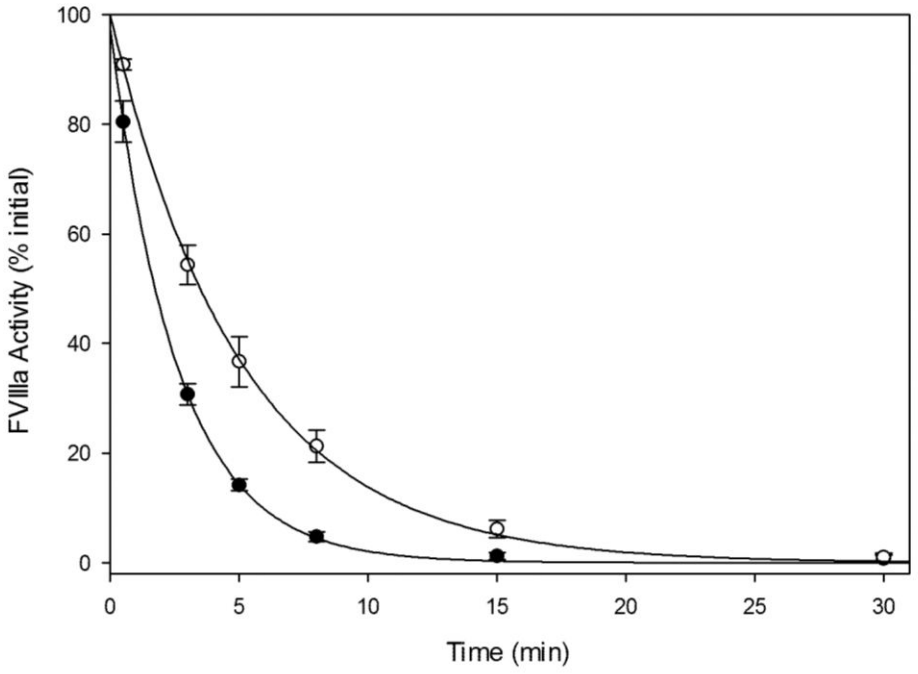


Figure II-4. Thrombin-Activated Decay Rate of OFVIIIa. Human (closed circle) and ovine (open circle) FVIIIa decay was measured by chromogenic Xase assay in which 20 nM FVIII was activated with thrombin and then stopped with desulfatohirudin. Activated FVIIIa in complex with phospholipid vesicles, activated factor IXa, and factor X was measured at 0.5, 3, 5, 8, 15, and 30 minutes to determine residual FVIIIa activity. Half-lives of 1.8 ± 0.09 and 3.5 ± 0.37 minutes were calculated for human and ovine FVIIIa, respectively. Data shown represents the percent of initial activity by semi-log extrapolation to time = 0. Regression analysis revealed Pearson correlation coefficients of 0.999 for both treatments.

1.8 ± 0.087 min [147, 155, 160] while the oFVIIIa half-life was prolonged significantly to 3.5 ± 0.37 min ($P = 0.001$, Student's *t* test).

VWF Binding

VWF is a plasma glycoprotein that performs many roles in the hemostatic system. One of which is to stabilize FVIII in circulation through non-covalent association. VWF circulates as non-uniformly sized multimers composed of individual 270-kDa monomers. Each VWF monomer is capable of 1:1 stoichiometric binding with FVIII. Upon proteolytic activation by thrombin or factor Xa, FVIII dissociates from VWF and is available to participate with factor IXa and Ca^{2+} in the formation of the tenase complex on a negatively charged phospholipid surface. In the absence of FVIII/VWF association, e.g. due to genetic deficiency of VWF or mutation of the FVIII/VWF binding sites, circulating FVIII levels are severely reduced and pathogenic bleeding often present phenotypically. The ability of oFVIII to bind human VWF, since a source of ovine VWF was unavailable, was assessed using an ELISA developed specifically for this study. Briefly, FVIII was captured by human VWF pre-adsorbed to a plate and detected using a MAb with an A2 domain epitope, which was demonstrated to possess equivalent affinity for human and ovine FVIII (Data not shown). Using this assay, BDD oFVIII and hFVIII displayed indistinguishable binding to VWF at physiologically relevant concentrations (Figure II-5).

In Vivo Efficacy of OFVIII

In order to demonstrate functionality of BDD oFVIII to restore the blood coagulation *in vivo*, hemophilia A mice were injected with either saline or oFVIII at a dose of 300 units/kg, which

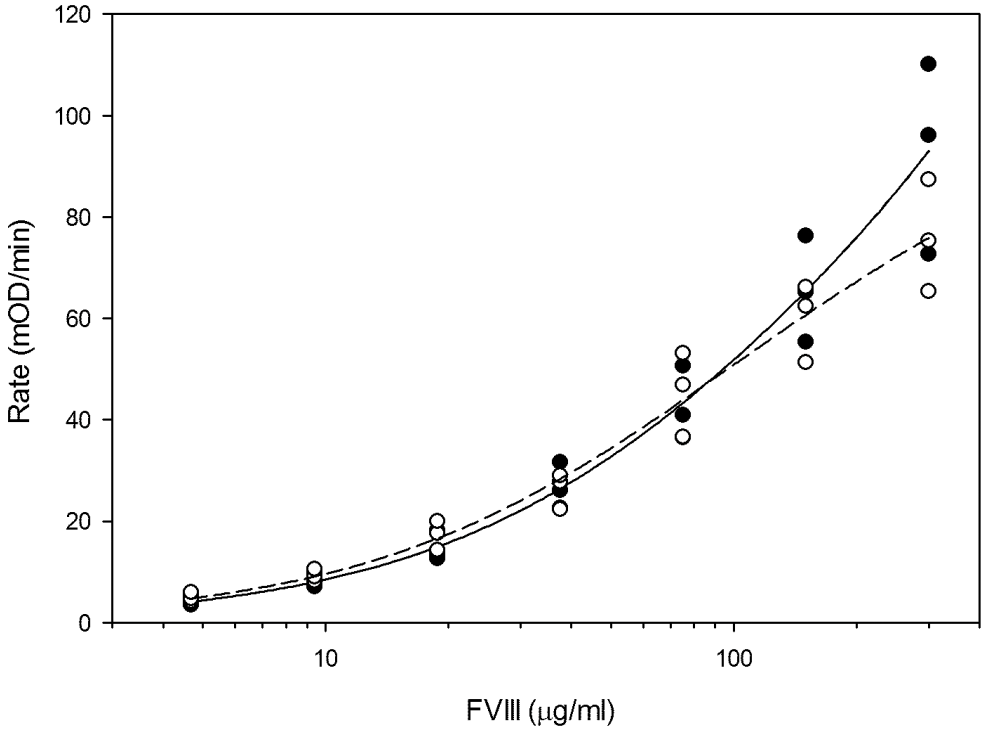


Figure II-5. BDD OFVIII Binding to VWF. Kinetic ELISA was conducted using human VWF to capture human (closed circles) or ovine (open circles) FVIII. Plates were coated with 50 µl of 6 mg/ml human VWF and blocked with 2% BSA. Monoclonal A2 domain FVIII antibody 4F4 1B was added to each well and colorimetric transmission was activated with *para*-nitrophenylphosphate substrate following streptavidin alkaline phosphatase. Data shown are the mean of three independent experiments ± sample standard deviation.

was determined previously to restore plasma FVIII activity to near endogenous murine levels (2.9 units/ml for C57Bl/6 mice in the colony at Emory University) [155]. Following FVIII or saline administration, a hemostatic challenge was induced via a tail transection at the 2 mm diameter position of the tail and blood loss was measured over a 40-minute period. Hemophilia A mice injected with saline alone demonstrated a mean (± sample standard deviation) blood loss of 32.2 ± 9.4 mg/g body weight (Figure II-6). In contrast, mice injected with oFVIII demonstrated a mean blood loss of 1.15 ± 2.57 mg/g body weight, which was significantly less than controls (P <0 .001; Mann-Whitney Rank Sum Test) and consistent with complete correction of the bleeding phenotype in this model.

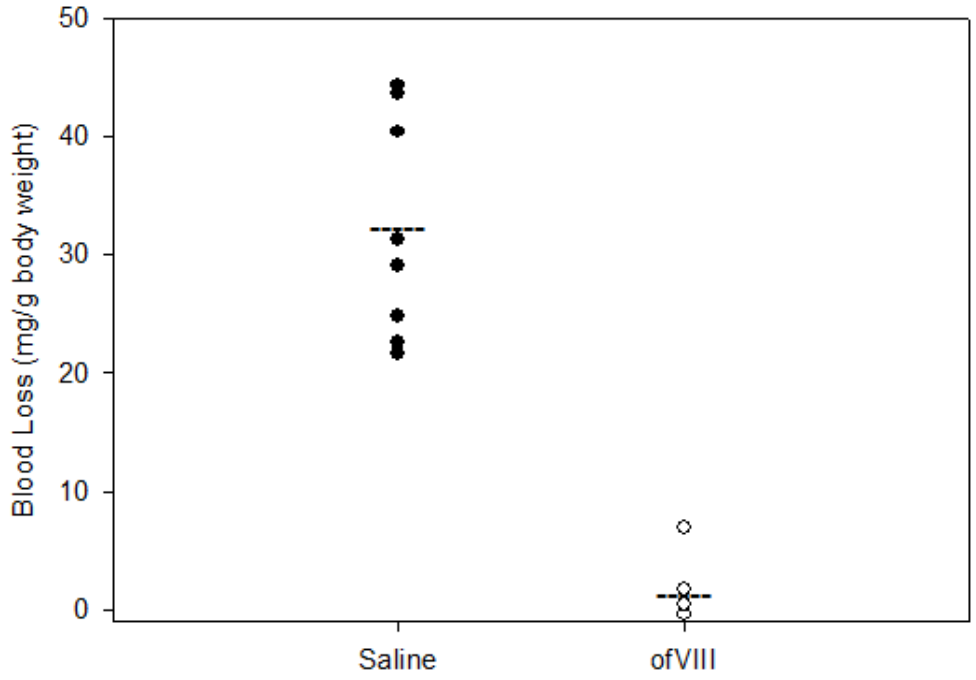


Figure II-6. In Vivo Efficacy of OFVIII in Hemophilia A Mice. Hemophilia A mice were injected with either 100 µl saline or 300 U/kg oFVIII in 100 µl sterile saline via tail vein injection (n = 8). After 15 min, bleeding challenge was induced via tail transaction at 2 mm diameter. Blood was collected in pre-weighed vials of 13 ml sterile saline at 37 °C. Blood loss was calculated and displayed as mg/g body weight. Mean blood loss for saline and oFVIII treatments were 32.2 ± 9.37 and 1.15 ± 2.57 mg/g body weight, respectively ($P < 0.001$; Mann-Whitney *U* test).

Discussion

Innovation in hemophilia A treatment has benefited significantly from the use of animal models of the disease and the study of orthologous FVIII molecules. For example, hemophilia A biopharmaceuticals continue to be hindered by low-level cellular production whether they be produced endogenously (plasma-derived FVIII), heterologously (recombinant FVIII), or following gene transfer into the patient (gene therapy). Secretion of FVIII is observed at 2-3 orders of magnitude lower than glycoproteins of similar size, including homologous factor V [265]. This is attributed to inefficient mRNA expression, protein mis-folding, and engagement of the unfolded protein response, and subsequent inefficient and rate-limiting transport from the endoplasmic reticulum to Golgi apparatus. Recently, we identified or generated orthologous and bioengineered FVIII constructs, respectively, that display enhanced secretion efficiency over BDD hFVIII. Specifically, certain pFVIII sequences were identified and shown to improve the efficiency of FVIII secretion by 10 – 100-fold. FVIII constructs containing these high expression porcine FVIII sequences now have been demonstrated to outperform BDD hFVIII in heterologous expression systems [44, 135, 140] and in preclinical gene therapy studies [136, 152, 249]. Therefore, a rational design approach for novel hemophilia A therapeutics has arisen out of the discovery of species-specific differentials in certain properties such as the rate of biosynthesis, half-life, antigenicity and immunogenicity [116, 147, 148, 152, 155, 258, 266].

Disregarding the B-domain, the majority of FVIII orthologs contain greater than 80% amino acid identity to human FVIII. Despite this primary sequence similarity, numerous unique properties have been characterized and utilized toward bioengineering improved FVIII constructs. During

re-establishment of a line of hemophilia A sheep, a new FVIII ortholog was cloned and characterized with the potential for discovering novel biochemical characteristics, while additionally providing a life-saving therapeutic for the ovine hemophilia A colony. In the current study, bioengineering, heterologous expression and biochemical characterization of oFVIII are described. Both recombinant full length and BDD oFVIII were shown to be expressed at greater levels than the equivalent hFVIII constructs in a BHK-M based heterologous expression system. These constructs were not modified beyond deletion of the B domain and the inclusion of a PACE/furin linker between the A2 and A3 domains as previously described [42, 259]. The biosynthesis levels observed for full length and hFVIII are comparable to previously published reports [136, 147, 152]. It was possible to purify significant quantities of BDD oFVIII using the same two-step ion exchange purification procedure that previously was described for recombinant BDD human, porcine and murine FVIII. OFVIII was purified to near homogeneity and shown to harbor specific activity higher than has been described previously for BDD human or murine FVIII [147, 155]. The purified product displayed AQ values indicative of very little to no contamination of the product with FVIIIa, which would artificially inflate the specific activity measurement. Therefore, it is concluded that the specific activity of BDD oFVIII is approximately 1.5 and 3 fold higher than recombinant BDD human and murine FVIII, respectively.

BDD oFVIII shares 86% amino acid identity to BDD hFVIII with eight amino acid deletions in the heavy chain. Analysis of N-linked glycosylation patterns with the use of PNGase F endoglycosidase revealed a greater mobility shift in the activated light chain and A1 domain of

oFVIII than was observed for BDD hFVIII. Both the A1 and A3 domains of oFVIII have predicted N-linked glycosylation sites not present in hFVIII, the former also being present in canine, porcine, and murine FVIII. The results of mass spectrometry analysis of BDD oFVIII do not support the presence of N-linked glycosylation at Asn-1720, and an explanation for the differential mobility of the ovine FVIII light chain remains elusive. However, one possibility is that the processed glycans vary in size and composition thus accounting for the observed discrepancy. Similar to what was described for recombinant canine FVIII by Arruda and colleagues, the majority of secreted BDD oFVIII is maintained as single chain despite the presence of a consensus RHQR PACE/furin recognition site [160]. Entirely unique to oFVIII is the observation that there is not clear separation of the heterodimeric heavy and light chains observed upon SDS-PAGE. The chains are resolved upon immunoprecipitation of each polypeptide independently using domain-specific MAbs under conditions where the heterodimeric FVIII is dissociated. Following thrombin proteolysis, the A1 domain, A2 domain, and the activated light chain could be immunoprecipitated specifically. The biochemical basis of this observation is not yet understood. It seems likely that the amino acid deletions in the heavy chain coupled with potentially larger glycans bound to the light chain alter the relative mobilities in opposite, but converging directions. However, the predicted relative changes in molecular mass for these structural disparities (approximately 1 kDa for missing residues), may not be sufficient to account for this observation entirely. The differences in other post-translational modifications (e.g. O-linked glycosylation and tyrosine sulfation) may contribute to the diminished mobility of ovine light chain, but have not yet been characterized.

Following activation by thrombin, FVIII assumes a heterotrimeric structure with the A2 subunit being in weak association with the A1/A3-C1-C2 heterodimer, the latter of which is stabilized by coordination of a metal ion [263, 267]. Under physiologic concentrations of approximately 1 nM, the FVIII heterotrimer is thermodynamically unstable and the A2 subunit dissociates from the molecule with a half-life of 2 min for hFVIIIa. Dissociation of the A2 subunit results in the loss of FVIII coagulant function as demonstrated through identification of specific hemophilia A mutations that operate through this mechanism [156]. Although the physical factors directly attributing to A2 subunit dissociation are unclear, it has been shown that instability of the A2 domain association does not factor into one-stage coagulation assay FVIII activity measurements. As a result, measures of specific activity are independent of decay and must be due to factors other than A2 domain stability. However, in the presence of thrombin, mutations in the A1 or A2 domain resulting in diminished stability will show a reduced activity in the two-stage coagulation as compared to the one-stage assay and reduced ability to achieve hemostasis. Previously, we characterized the thrombin-activated decay of recombinant human, porcine, murine and hybrid FVIII molecules [147, 155]. Compared to those orthologs, oFVIII displays an intermediate half-life of 3.7 min, which is almost twice that of hFVIIIa but 0.5-fold that of pFVIIIa and <0.1-fold that of mFVIIIa. Increases in animal size and severity of potential thrombotic effects due to pressure differentials or vascular characteristics may provide an explanation for evolutionarily altered stability of FVIIIa within these species. Tight regulation of FVIII activity must be maintained to prevent unwarranted thrombotic events, as well as to allow cessation of bleeding events upon vessel injury.

In order to test the efficacy of BDD oFVIII, E16$^{-/-}$ hemophilia A mice were challenged with a tail transection after the intravenous administration of 300 units/kg oFVIII compared to saline. In an attempt to normalize physical hemodynamic-related properties due to vasculature size, transections were made at 2 mm diameter as opposed to a fixed distance from the distal end of the tail [268-270]. Furthermore, to prevent the false appearance of phenotypic recovery at low time points due to non-fibrinogenic platelet aggregation at the site of transection, mice were observed for 40 minutes post-challenge. Blood loss was reduced dramatically and phenotypic correction was observed through recombinant BDD oFVIII administration. Based on this result, we believe it practical to test the efficacy of BDD oFVIII in hemophilia A sheep and further assess the propensity for BDD oFVIII induced inhibitor formation (i.e., immunogenicity).

Assuming a typical weight of 75 kg, an adult hemophilia A sheep would require approximately 1.5 mg oFVIII per administration using an estimated therapeutic dose of 300 units/kg translated from the data obtained in the current study using hemophilia A mice. Due to the demonstrated bio-production characteristics of oFVIII shown herein, prophylactic treatment of hemophilia A sheep would be similar in product requirements to that of humans with severe hemophilia and may not be practical due to manufacturing and economic constraints, e.g. the typical cost of prophylactic severe hemophilia A care in the U.S. is $200,000 – 300,000 per patient per yr. However, a 5 kg neonatal lamb would require only 80 μg per administration. If effective, this post-natal treatment regimen may be complimented by gene therapy trials to measure the safety, efficacy, and immunogenicity of novel gene therapy strategies in neonatal sheep, which would greatly enhance the value of this model in biomedical research.

As has been observed in some canine hemophilia A lines [160, 271], hemophilia A sheep develop inhibitors to recombinant human FVIII following parenteral infusion [169]. Hemophilia A sheep possess a premature stop codon in exon 14 caused by a frameshift mutation and are believed to be an accurate phenocopy of severe hemophilia A in humans. If different in any demonstrable manner, the sheep model may display a more severe bleeding phenotype as well as higher inhibitor incidence. The physiology and clinical phenotype is mirrored in ovine and human, and the former model eliminates the requirement of scale up estimation of treatment dosages. Maintaining the ovine hemophilia A colony requires intensive effort and extensive resources. Thus, without adequate validation, this model likely will be lost as a testing ground for the efficacy and immunogenicity of novel hemophilia A biotherapeutics and gene therapy applications. The development and characterization of recombinant oFVIII should facilitate the validation which in turn will enhance the value and utility of this unique large animal disease model.

Chapter III: Expanding the Ortholog Approach for Hemophilia Treatment Complicated by Factor VIII Inhibitors

Summary

Background: The formation of neutralizing antibodies (inhibitors) directed against human coagulation factor VIII (hFVIII) is a life-threatening pathogenic response that occurs in 20–30% of severe congenital hemophilia A patients and 0.00015% of remaining population (i.e. acquired hemophilia A). Interspecies amino acid sequence disparity among FVIII orthologs represents a promising strategy to mask FVIII from existing inhibitors while retaining procoagulant function. Evidence for the effectiveness of this approach exists in clinical data obtained for porcine FVIII products, which have demonstrated efficacy in the setting of congenital and acquired hemophilia.

Objectives: In the current study, recombinant (r) ovine FVIII (oFVIII), was evaluated for antigenicity and procoagulant activity in the context of human patient-derived and murine model-generated FVIII inhibitors.

Methods: The antigenicity of roFVIII was assessed using i) inhibitor patient plasma samples, ii) murine anti-FVIII MAbs, iii) immunized murine hemophilia A plasmas, and iv) an in vivo model of acquired hemophilia A

Results: Overall, roFVIII demonstrated reduced reactivity to, and inhibition by, anti-hFVIII immunoglobulin in patient plasmas. Additionally, several hFVIII epitopes were predicted and empirically shown not to exist within roFVIII. In a murine hemophilia A model designed to mimic clinical inhibitor formation, it was demonstrated that inhibitor titers to roFVIII were significantly reduced compared to the orthologous immunogens, rhFVIII or rpFVIII.

Furthermore in a murine model of acquired hemophilia A, roFVIII administration conferred protection from bleeding following tail transection.

Conclusion: These data support the investigation of FVIII orthologs as treatment modalities in both the congenital and acquired FVIII inhibitor settings.

Introduction

Factor VIII (FVIII) is a procofactor in the intrinsic pathway of blood coagulation. Deficiency of FVIII activity resulting from genetic mutation of the X-chromosome-linked *F8* gene presents as a bleeding disorder, termed hemophilia A, that has a reported prevalence of 1 in 7,800 males [272]. Treatment consists of lifelong protein replacement via intravenous infusions of recombinant (r) or plasma-derived (pd) human (h) FVIII products. Upon repeated exposure, approximately 20-30% of severe hemophilia A patients develop inhibitory anti-hFVIII alloantibodies (inhibitors). In countries where replacement therapy is available, the immune response to hFVIII is the most significant complication affecting the management of patients with hemophilia A. Additionally, autoantibodies to hFVIII develop in non-hemophiliacs at a rate of 1.48/million/year producing an autoimmune condition termed acquired hemophilia A, which frequently results in life- or limb-threatening bleeding. [273-276]

On the molecular level, FVIII displays a domain structure A1-A2-B-*ap*-A3-C1-C2 where the A and C domains are defined by internal sequence homology and the heavy and light chains are separated by an activation peptide (*ap*) [3]. Although antibodies targeting each of the hFVIII domains can be found in patient plasmas, the A2 and C2 domains appear to contain the dominant immunogenic and inhibitory epitopes [106, 117, 277]. Epitopes, mechanisms of action, and kinetics have been defined for a large collection of anti-A2 and C2 domain murine monoclonal antibodies (MAbs) demonstrating that the murine hemophilia A model recapitulates many features of the anti-FVIII immune response observed in humans [98, 100, 278]. Recently, high resolution structural data of anti-hFVIII MAbs in complex with the FVIII C2 domain using small

angle x-ray scattering, x-ray crystallography, and hydrogen-deuterium exchange mass spectrometry was obtained and has brought the understanding of inhibitor mechanism of action to the atomic level [110, 111, 114].

Treatment options for hemophilia A patients with inhibitors are limited in terms of availability and efficacy. For example, in Immune Tolerance Induction (ITI) studies, the frequent administration of FVIII product at doses as high as 200 IU/kg/day, is effective at eradicating inhibitors in up to 70% of patients [279]. However, due to FVIII product supply constraints and expense, ITI is not an option for the majority of patients with hemophilia A. Aside from eradicating inhibitors, acute and frequently life-threatening bleeding can be treated in this setting using FVIII bypassing agents (e.g. activated prothrombin complex concentrate or activated recombinant factor VII). Plasma-derived porcine FVIII (pd-pFVIII) products also have been utilized although they no longer are available. However, a recombinant pFVIII (rpFVIII) product is under clinical development for acquired hemophilia A. The rationale for use of pd- or rpFVIII products stems from the presence of non-conserved amino acid sequence differences that confer reduced antigenicity and inhibition. Furthermore, it has been shown that there are several species-specific differentials in non-immunological properties between rh- and rp-FVIII [147, 148] and preclinical evidence exists to support the benefit of utilizing FVIII orthologs, i.e. proteins from different species that evolved from a common ancestral gene, or hybrid FVIII molecules engineered to possess sequences from multiple orthologs, in gene therapy applications [136, 152, 247, 248, 255].

A naturally occurring ovine model of severe hemophilia A has been identified and the responsible genetic lesion and disease phenotype was characterized [169]. Additionally, the ovine FVIII (oFVIII) ortholog was generated in recombinant form thereby facilitating biochemical characterization. B-domain-deleted (BDD) recombinant ovine FVIII (roFVIII) has been shown to display greater specific activity, prolonged half-life following activation by thrombin, functionality in a hFVIII-deficient plasma bioassay and efficacy in a murine hemophilia A tail-transection bleeding model [280]. As the rationale for clinical use of pFVIII is based on reduced antigenicity achieved through differential amino acid sequence, herein we sought to investigate the potential therapeutic utility of roFVIII which also contains a distinct repertoire of non-conserved amino acids, but still possesses procoagulant function in human plasma. Outside of the B-domain, ovine and porcine FVIII share 86 and 83% amino acid identity to human FVIII, respectively [146, 169]. We hypothesized that roFVIII would be less antigenic than hFVIII in plasma from human patients and hemophilia A mice with inhibitors and that there would be inter-patient differentials in the antigenicity to each FVIII ortholog. To test these ideas, the antigenicity of roFVIII was assessed using i) inhibitor patient plasma samples, ii) a collection of well-characterized murine anti-FVIII MAbs that mimic human inhibitors [98, 100, 278], iii) plasmas from hemophilia A mice immunized with either rhFVIII or rpFVIII, and iv) an in vivo bleeding challenge assay designed to model acquired hemophilia A.

Materials and Methods

Materials

Pooled citrated normal plasma (FACT) and FVIII-deficient plasma were purchased from George King Biomedical (Overland Park, KS). Automated APTT reagent was purchased from Trinity Biotech (Wicklow, Ireland). Inhibitor patient plasmas were drawn and banked in accordance with Emory University IRB protocol no. IRB00006290. Acquired patient samples were generously donated by Dr. David Green (Feinberg School of Medicine of Northwestern University, Chicago, IL). Patients were selected for inclusion if inhibitor titers against human exceeded 5 BU/ml and sufficient plasma was available. Streptavidin-alkaline phosphate conjugate was purchased from Jackson Immuno Research (West Grove, PA). Goat anti-mouse IgG-alkaline phosphatase conjugate and alkaline phosphatase substrate kit (AP pNPP) was purchased from Bio-Rad (Hercules, CA). Dimethyl pimelimidate was purchased from Thermo Scientific (Rockford, IL) and Protein A/G Plus was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Domain specific monoclonal antibodies were generated and purified as previously described [278]. MAb ESH-8 was purchased from American Diagnostica (Stamford CT). MAbs 413 and CLB-Cag 9 were gifts from American Red Cross (Rockville, MD) and Dr. Jan Voorberg (Sanquin-AMC Lamdsteiner Laboratory, Amsterdam, The Netherlands). BDD roFVIII, rpFVIII, and rhFVIII were generated and purified as described previously [147, 280]. Full-length rhFVIII was a generous gift from Hemophilia of Georgia. OTII C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and kept in accordance with Emory IACUC. Exon 16-disrupted hemophilia A mice have been previously described [120].

Inhibitor plasma and monoclonal competition ELISA

Competition ELISAs were performed as previously described [98-100]. Controls for each MAb against the A2 or C2 domain were replicated 11 and 16 times, respectively. Competition was defined as a reduction of signal greater than 2 standard deviations (SD) from the control mean. Only patient plasmas that displayed predominantly A2 and/or C2 specificity, as determined by homolog-scanning ELISA using human/porcine FVIII hybrids revealed (data not shown), were selected for competition ELISA analysis.

Cross-reactivity of inhibitor plasma and MAbs

An indirect ELISA was performed using plates containing adsorbed rhFVIII, rpFVIII, and roFVIII to which serial dilutions of patient plasma or MAbs were added followed by detection using goat anti-mouse AP-conjugated secondary antibody. ELISA titration curves were fitted to the 4-parameter logistic equation. The dilution of inhibitor plasma required to produce A_{405} of 0.3 was calculated by interpolation and compared across orthologs. The absorbance threshold was set as an arbitrary point in which colorimetric signal is approximately three times background while substrate remains in excess. MAb interactions that did not achieve an absorbance at 405 nm of 0.3 at the lowest dilution (10-fold molar excess) were designated as non-reactive or below the limit of detection.

FVIII inhibitor titer assays: patient plasma, mouse plasma, and MAbs

FVIII inhibitor titers against rhFVIII, rpFVIII, and roFVIII were measured using a modified Bethesda assay previously described [281]. For determination of the ortholog titers, pooled citrated FVIII-deficient plasma was combined with 0.8-1.2 units/ml of rpFVIII or roFVIII and buffered with 100 mM imidazole. Due to limited availability of plasma, not all patients could be fully screened. MAb inhibitor titer was calculated similarly using dilutions of MAbs at known concentrations and reported in BU/mg IgG.

Immunization of hemophilia A E16 F8$^{-/-}$ mice

Mice were immunized with BDD rhFVIII or rpFVIII as described previously [153]. Mice received six tail vein injections of 10 μg/kg FVIII at 7-day intervals followed by a final injection of 25 μg/kg FVIII two weeks after the sixth dose. Subsequently, terminal plasma collections were performed and these samples were used to determine inhibitor titer by modified Bethesda assay.

Murine acquired hemophilia A hemostatic challenge

In a blinded study, 8-12 week old C57BL/6 OTII mice received 10ug anti-A2 domain MAb 4A4 via intraperitoneal (IP) injection. After 15 minutes, the mice were administered 9 units of rFVIII or saline via intravenous tail vein injection. Hemostatic challenge was performed 2 hours after

rFVIII administration by tail transection at 2mm diameter and total blood loss over 40 minutes was recorded as previously described [151, 280].

Anti-oFVIII MAb generation, and domain specificity immunoprecipitations

Three 9-12 week old E16 $F8^{-/-}$ mice were injected with 1µg roFVIII each diluted in 100µl saline via tail vein each week for 7 weeks. Inhibitor titers were taken at week 8 via ELISA and Bethesda assay as described above. Two weeks after the last injection, mice were administered 1.25 µg roFVIII via tail vein. Three days after final immunization, the mouse with the highest ELISA and Bethesda titer was sacrificed and MAbs were generated and purified as previously described [278]. The 9 resulting MAbs were used to immunoprecipitate roFVIII following activation by thrombin and visualized by SDS-PAGE as previously described [280].

Results

RoFVIII displays reduced IgG binding and inhibition in inhibitor patient plasmas

As an expansion of the ortholog approach to hemophilia A treatment in the context of FVIII inhibitors, roFVIII was investigated for binding to IgG present in inhibitor patient plasmas (i.e. antigenicity) and resistance to inhibition conferred by the same. An indirect ELISA-based screen of 26 congenital and 10 acquired hemophilia A patient plasmas demonstrated reduced reactivity of plasma IgG to both roFVIII and rpFVIII compared to hFVIII with median values of 35.6 and 49.3%, respectively (Figure III-1A). No significant difference was observed between roFVIII and rpFVIII ($P = 0.097$; Mann-Whitney U test). Of the 36 plasmas tested, 32 displayed reduced reactivity to both roFVIII and rpFVIII and of these 22 demonstrated less reactivity to roFVIII compared to rpFVIII.

To measure inhibitor titers, a modified Bethesda assay utilizing the three FVIII orthologs was implemented. This analysis revealed that inhibitory titers against both roFVIII and rpFVIII were statistically reduced compared to hFVIII ($P < 0.05$) although they were not distinguishable from each other ($P > 0.05$; Kruskal-Wallis One Way ANOVA) with median titers of 7.25 (roFVIII), 4.4 (rpFVIII), and 34 BU/mL (rhFVIII) (Figure III-1B). Clinical experience shows that patients with inhibitor titers less than 5 often respond to high dose hFVIII replacement therapy while patients with inhibitor titers >10 BU/ml generally are not considered candidates for hFVIII infusion therapy [282]. Twenty-nine of the patient plasmas studied possessed inhibitor titers above 10 BU/mL against hFVIII and of those, 21 had <10 BU/mL titers against rpFVIII or

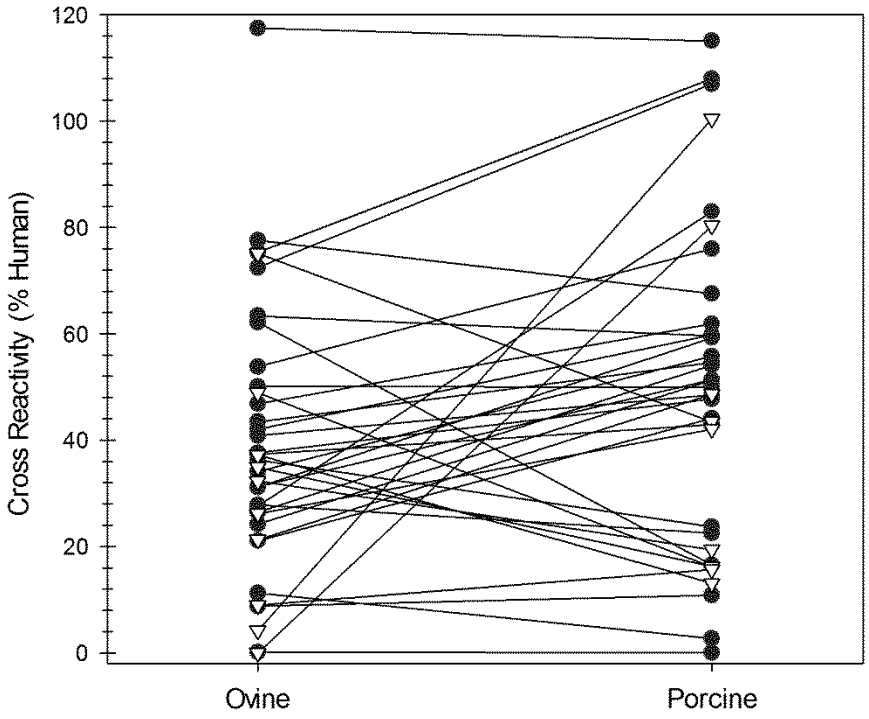


Figure III-1A. Antigenicity and inhibitor titers for inhibitor patient plasmas. (A) An ELISA was performed on 26 congenital (black circle) and 10 acquired (white triangle) hemophilia A inhibitor patient plasmas using rhFVIII, roFVIII, or rpFVIII as the capture antigen. Data are presented as the relative cross-reactivity to that observed with rhFVIII.

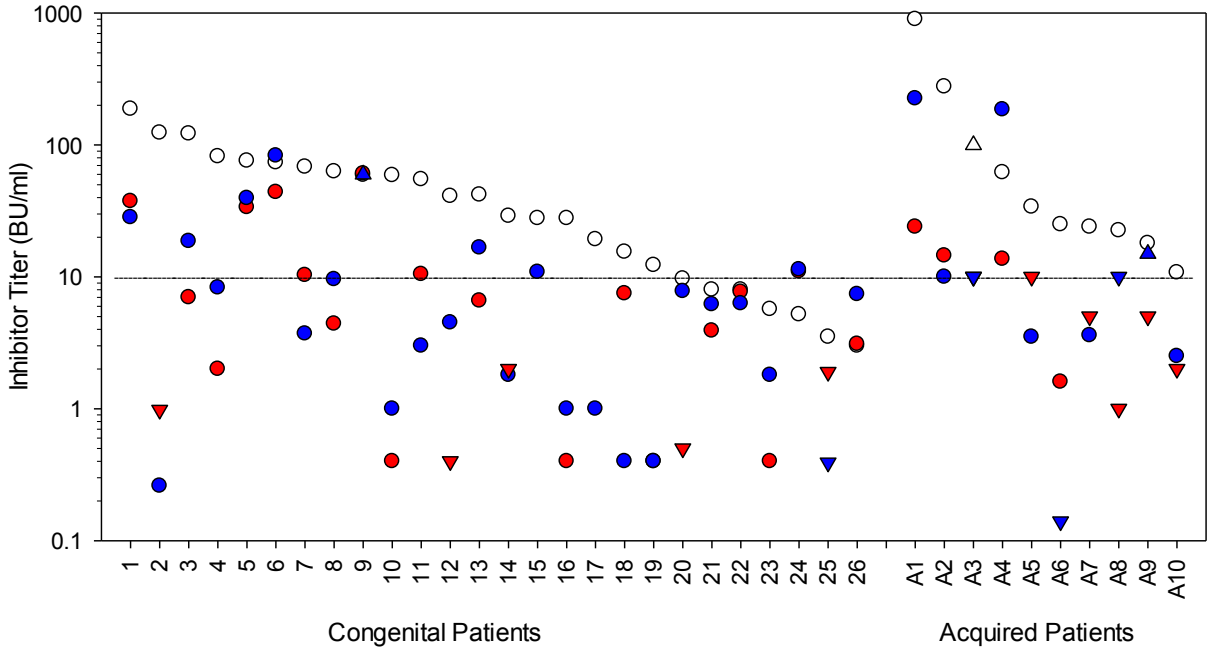


Figure III-1B. Antigenicity and inhibitor titers for inhibitor patient plasmas. (B) The inhibitor titer of each patient plasma against hFVIII (white circle), rpFVIII (red circle), and roFVIII (blue circle) was measured by modified Bethesda assay as described in Methods. Due to limited plasma availability, triangles depict maximum/minimum approximations corresponding with their orientation. For example, an inverted triangle represents a value less than the position of the triangle on the y-axis.

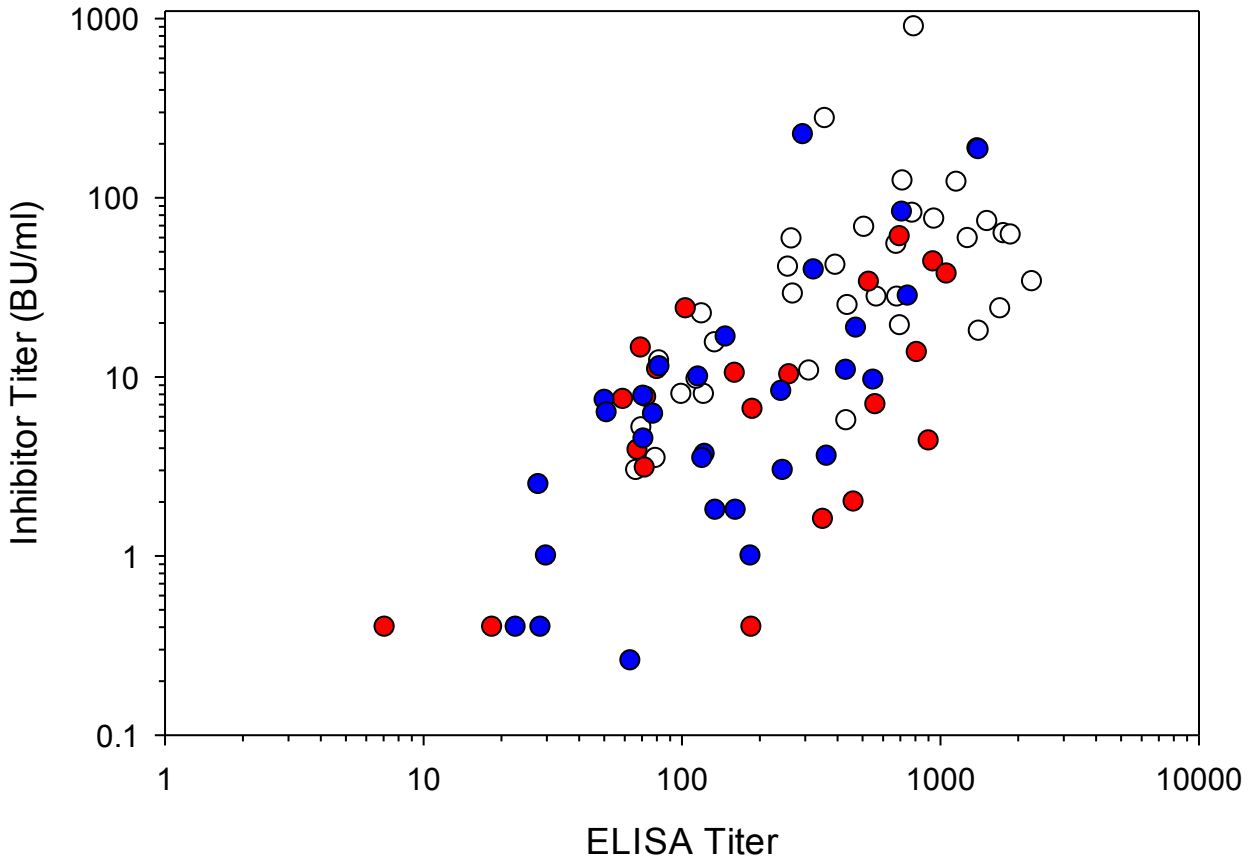


Figure III-1C. Antigenicity and inhibitor titers for inhibitor patient plasmas

(C) Patient plasma ELISA versus inhibitor titers against human (white), porcine (red), and ovine (blue) FVIII orthologs were plotted and analyzed for correlation. Significant non-zero correlations were observed with *P* values of 0.0028, and 0.0003 for p-, and o-FVIII while *P* = 0.4913 for hFVIII.

roFVIII. Furthermore, 5 of the plasma samples assayed harbored comparatively lower titers against roFVIII than rpFVIII and 2 of these plasmas had titers >10 BU/ml against both rhFVIII and rpFVIII suggesting that roFVIII exclusively might be effective in certain populations of inhibitor patients. Due to limited availability of certain patient plasmas, 2 patient plasmas could

not be tested for inhibitor titer and an additional sample (from patient 17) could not be tested for rpFVIII inhibitor titer. Significant correlations were observed between the ELISA and Bethesda titers determined for rpFVIII and roFVIII (P = 0.0028, and 0.0003, respectively, Student's two-tailed t distribution), but no significant correlation was observed for rhFVIII (*P* = 0.4913; Figure III-1C). Correlation coefficients for rhFVIII, rpFVIII, and roFVIII are 0.0145, 0.354, and 0.3827 respectively. These data demonstrate that hFVIII titers are not predictive of each other given that similar inhibitor titers spanned two orders of magnitude of ELISA titer. Inhibitor titers against rpFVIII or roFVIII were consistently refined within only one order of magnitude.

Distribution of A2 and C2 epitopes targeted by inhibitor patient plasmas

Inhibitor bank plasmas were screened for domain specificity by homolog-scanning ELISA incorporating single domain human/porcine hybrid molecules as described previously [278] (data not shown). Twenty patient plasmas of the initial 36 were shown to contain anti-hFVIII antibodies predominantly against the A2, C2, or both domains (Table III-1). For 18/20 patients, there was sufficient plasma available to interrogate the targeted A2 and C2 epitopes by competition with panels of MAbs known to recognize non-overlapping epitopes in these domains (Figure III-2) [98, 100]. Due to limited plasma availability, a single MAb was used to represent

each inhibitor group. Additional A2 – A and C2 – BC MAbs were added because of their clinical prevalence and inhibitor potency and efficacy. Successful competition with at least one of the A2 and/or C2 domain targeting MAbs was demonstrated for 15/18 patient plasmas. Furthermore, at least one patient plasma competed with each group of MAbs within the C2 domain but only 3/7 groups within the A2 domain. Within the C2 domain, 12/14 patient plasmas competed with group BC MAbs followed by 8/14 with groups A and C, 4/14 with group B, and 3/14 with group AB. In contrast, only 3/7 groups of A2 domain targeting MAbs were competed by the patient plasmas. Overall, the A2 epitopes targeted by the patient plasmas appeared to overlap primarily with those targeted by A2 – A (6/10 plasmas) followed by A2 – E (2/10 plasmas) and then A2 – B (1/10 plasmas) with no overlap/competition observed with A2 – BCD, – C, – D or – DE MAbs. Of note, MAb groups A2 – A and C2 – BC contain the most potent inhibitory MAbs and are the most prevalent groups with which patient plasma ELISA competition was observed.

To determine if the reduced reactivity and inhibition of rpFVIII and roFVIII was due to the absence of inhibitory epitopes, we tested the ability of anti-hFVIII A2 and C2 domain specific MAbs known to possess varying inhibitor titers and kinetics to bind and inhibit roFVIII via indirect ELISA. Within the A2 domain, cross-reactivity only was observed with two MAbs, 4F4 and G48, representing inhibitor groups A2 – B and A2 – C, respectively (Figure III-3). These antibodies previously were shown to have low human FVIII specific inhibitory activities of 330 and 5 BU/mg IgG respectively. To confirm that the diminished ELISA binding observed correlated with decreased inhibitor titers, specific inhibitory titers were measured for each MAb against roFVIII and rpFVIII (Table III-2). Both MAb 4F4 and G48 demonstrated no detectable

Table III-1. Inhibitor patient plasma Cross-reactivity and inhibitor titers of patient plasmas

		ELISA Cross Reactivity (%)		Bethesda Titer (BU/mL)		
Patient	**Domain**	**Porcine**	**Ovine**	**Human**	**Porcine**	**Ovine**
10	A2	3	11	59.0	0.4	1.0
14	A2	50	50	29.0	2.0	1.8
19	A2	23	28	12.3	0.4	0.4
20	A2	17	62	9.7	0.5	7.8
1	C2	76	54	187.6	37.5	28.3
3	C2	49	41	119.5	7.0	18.7
5	C2	56	34	76.0	33.8	39.6
7	C2	51	24	68.4	10.3	3.7
9	C2	55	43	59.3	60.7	60^
13	C2	48	38	42.0	6.6	16.7
21	C2	68	78	8.0	3.9	6.2
22	C2	60	42	8.0	7.7	6.3
23	C2	43	37	5.7	0.4	1.8
6	A2 + C2	62	47	73.8	43.9	83.1
2	A2 + C2	11	9	124.0	1.0	0.3
11	A2 + C2	24	36	55.0	10.5	3.0
12	A2 + C2	83	28	45.0	0.4	4.5
16	A2 + C2	0	0	27.9	0.4	1.0
17	A2 + C2	54	26	19.3	NT	1.0
A1	A2 + C2	13	37	900.0	24.0	225.0

NT: Not tested

^ : Value shown is a minimal approximation

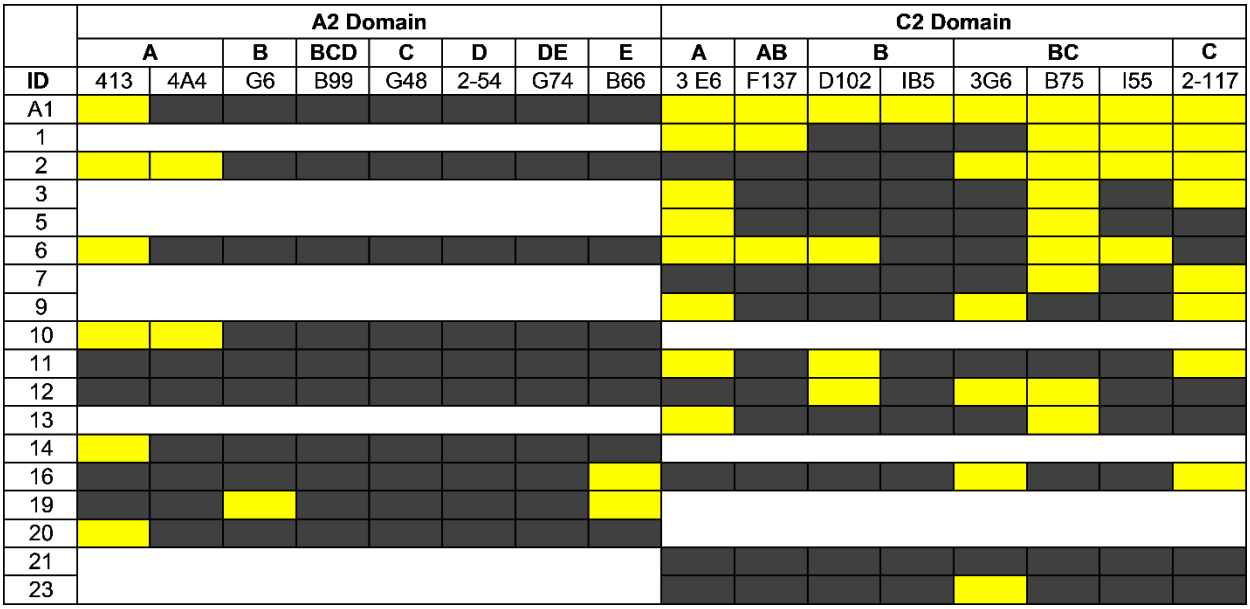


Figure III-2. Identification of A2 and C2 domain epitopes targeted by patient plasmas. A competition ELISA was performed with anti-A2 and -C2 domain MAbs competing against human inhibitor IgG for binding to hFVIII. HFVIII first was blocked with patient plasma and then incubated with individual biotinylated MAbs. Patient ID is listed along the y-axis in descending order of the Bethesda titers measured for each sample and individual MAbs ID/group are listed across the top. Absence of competition is represented by black shading, and white shading designates data not determined. Competition is defined as a reduction of kinetic signal outside 2 standard deviations of control kinetic rates and is represented as yellow shading.

titer against roFVIII or rpFVIII. None of the high specific inhibitory activity hFVIII A2 domain targeting MAbs demonstrated cross-reactivity to either FVIII ortholog.

Inhibitory MAbs display reduced reactivity to orthologous FVIII molecules

Cross-reactivity within the C2 domain, however, revealed moderate roFVIII-specific cross-reactivity with 3 inhibitors, I14, B75, and I55, at 30 – 50% of hFVIII reactivity. MAbs I14, B75, and I55 are characterized by specific inhibitory activities of 44,000, <1, and 10,000 BU/mg IgG against hFVIII, respectively. Of these, only I14 possessed an inhibitor titer to roFVIII with a specific inhibitory activity between 100 – 1,000 BU/mg IgG. An additional 3 MAbs, D102, G99, and 3G6, cross-reacted with roFVIII at 5 – 15% of the hFVIII level however, inhibitor titers against the orthologs were again nominal. Cross-reactivity against rpFVIII was observed only with two MAbs, 2-117 and D102, and the percent reactivity was between 5 – 10% that of the reactivity to hFVIII.

Due to the selection bias against identification of cross-reactive hFVIII C2 domain targeting MAbs, a similar pool of MAbs, this time generated against rpFVIII, were tested for cross-reactivity with rhFVIII and roFVIII. Ten MAbs with measurable inhibitor titers against rpFVIII were selected and tested by indirect ELISA. MAbs targeting the A1 or A2 domains of rpFVIII did not show any cross-reactivity to hFVIII or roFVIII, however all anti-rpFVIII light chain (A3 – C1 – C2) MAbs demonstrated cross-reactivity to roFVIII exceeding rpFVIII for all four C2

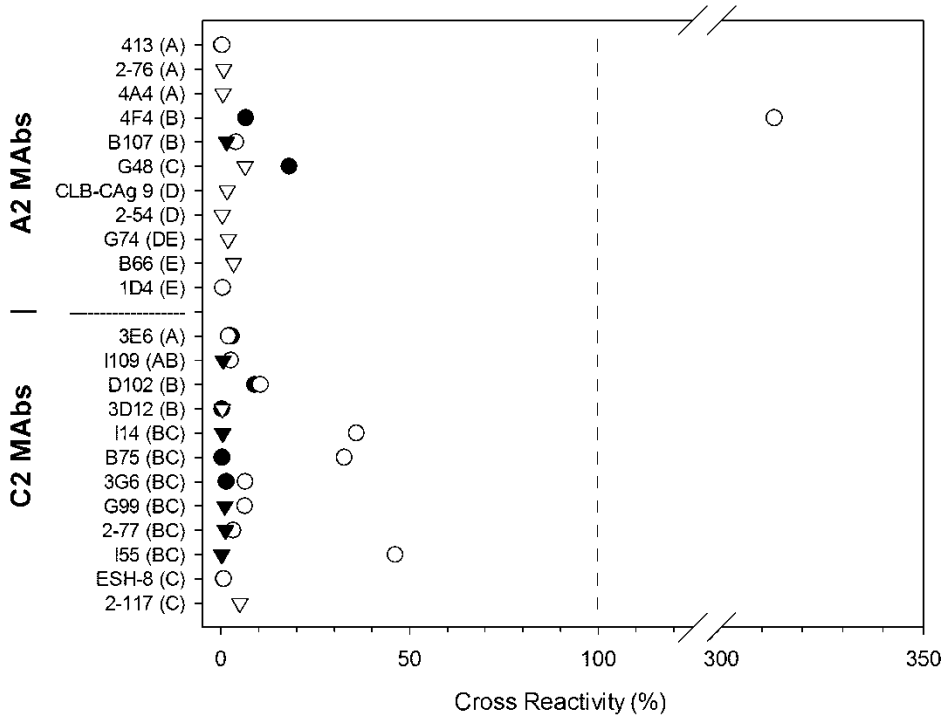


Figure III-3. Reactivity of anti-hFVIII A2 and C2 domain MAbs with FVIII orthologs. Panels of anti-A2 and -C2 domain targeting MAbs were assayed for cross-reactivity via indirect ELISA. Binding to roFVIII (white) and rpFVIII (black) is displayed as percent hFVIII binding as calculated by titration curve analysis. MAbs are listed on the y-axis by name with the inhibitor group classification in parenthesis. Triangles represent the maximal cross-reactivity percentages that could be determined experimentally with the available plasma.

Table III-2. Inhibitor titers of anti-human FVIII MAbs against ovine and porcine.

Inhibitor	Domain	Kinetics	Group	HumanTiter (BU/mg)	Porcine Titer (BU/mg)	Ovine Titer (BU/mg)
4A4	A2	I	A	25,500	< 8	< 8
413	A2	I	A	61,000	< 1	< 1
4F4	A2	I	B	330	< 1	< 1
B25	A2	I	C	18	< 4	< 1
1D4	A2	I	E	51,000	5	6
G48	A2	II	C	*	*	*
2-54	A2	II	D	****	*	*
3E6	C2	I	A	11	< 1	< 4
I109	C2	I	AB	3,100	< 1	< 1
D102	C2	I	B	8,600	10	5
3D12	C2	I	B	3,800	< 1	< 1
I14	C2	II	BC	****	*	**
B75	C2	II	BC	*	*	*
3G6	C2	II	BC	****	*	*
G99	C2	II	BC	****	*	*
2-77	C2	II	BC	****	*	*
I55	C2	II	BC	****	*	*
ESH-8	C2	II	C	****	*	*
2-117	C2	II	C	*	*	*

*: 0-100 BU/mg; **: 100-1,000 BU/mg; ***: 1,000-10,000 BU/mg; ****: > 10,000 BU/mg

domain targeting MAbs (Figure III-4). However, measurement of the specific inhibitory activities of these MAbs revealed near zero inhibition of either hFVIII or roFVIII (data not shown).

Pre-immunized hemophilia A mice display reduced inhibitor titer to roFVIII

To study the reactivity of anti-rFVIII immune plasma to roFVIII, samples from hemophilia A mice immunized with rhFVIII (n = 6) or rpFVIII (n = 10) were obtained from a previous study [153]. All mice displayed inhibitor titers to the specific immunogen of ≥ 10 BU/ml. However when tested for inhibition of roFVIII, all mice demonstrated reduced inhibitor titers against roFVIII as compared to the FVIII immunogen with mean reduction of 22 and 31 fold (P = 0.021 and 0.007, respectively for rhFVIII and rpFVIII; Paired t-test; Figure III-5).

RoFVIII restores procoagulant function in an in vivo acquired hemophilia A model

As each of the previous studies utilized in vitro surrogate assays for prediction of hemostatic function, an in vivo assay was developed to assess roFVIII functionality in vivo in a model of

acquired hemophilia A. Autoimmunity against endogenous FVIII develops unpredictably in individuals resulting in a transient but rapid development of inhibitors. Although the hyper-immune state can correct without intervention, affected individuals are high risk for loss of life or limb and in these cases immune tolerance induction is not recommended. The high potency, type I kinetics and prevalence of A2-group A inhibitors in patient plasma provided support for their use for modeling this condition. Because C2 domain inhibitors possess type II kinetics, the

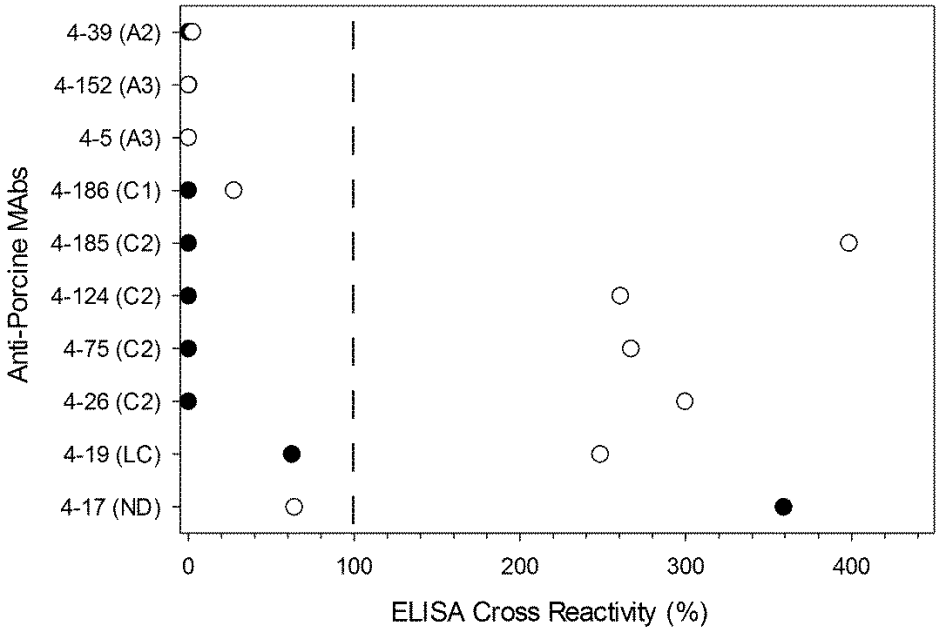


Figure III-4. Reactivity of anti-rpFVIII MAbs with roFVIII. Murine MAbs isolated from hemophilia A mice immunized with rpFVIII were screened for cross-reactivity to hFVIII (black) and roFVIII (white). The data presented are normalized to rpFVIII binding. MAbs are listed in the y-axis with the FVIII domain epitope specificity in parentheses. LC: Light chain; ND: not determined.

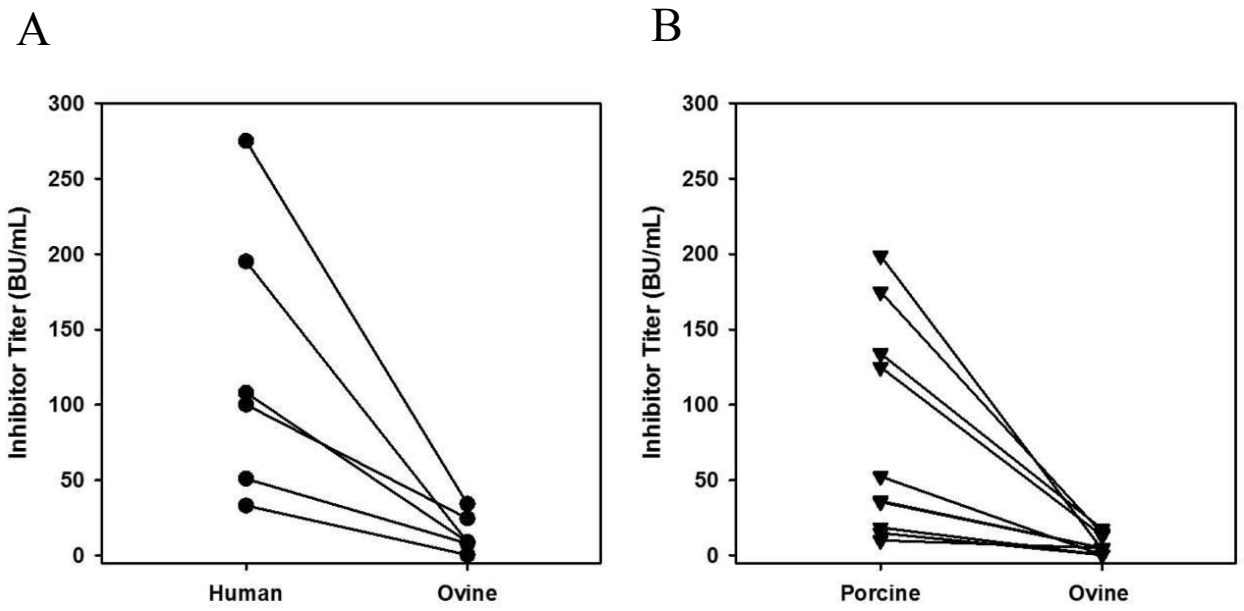


Figure III-5. Inhibitor titers in pre-immunized murine hemophilia A plasmas. Mice were immunized with either rhFVIII (A) or rpFVIII (B), respectively. Following the 7th injection, plasma was collected via terminal cardiac puncture and assayed for inhibitor titer to the original immunogen and roFVIII via modified Bethesda assay. Lines connect the intramouse inhibitor titers recorded for the two FVIII orthologs. N = 6 and 10 respectively.

residual activity even in saturating concentrations of inhibitor has empirically corrected a bleeding phenotype in mice. Therefore, *F8*$^{+/+}$ mice were administered MAb 4A4 at levels empirically shown to completely inhibit endogenous murine FVIII and predicted to neutralize infused rhFVIII. Subsequently, each animal received 9 units of one rFVIII ortholog, to achieve near 100% normal murine FVIII activity levels, or saline only and then were challenged by tail transection bleeding assay. Wild type mice were selected to more accurately recapitulate the acquired disorder due to continuous biosynthesis and secretion of endogenous FVIII into circulation. Mean blood loss of roFVIII treated mice was significantly reduced compared to saline and rhFVIII (10.0, 32.1, and 30.3 mg/g body weight, respectively; $P = 0.005$ and 0.007 respectively, Student's t-test) and no significant difference was observed between rpFVIII and roFVIII ($P = 0.421$, Mann Whitney *U*-test) (Figure III-6). Administration of rhFVIII did not significantly reduce bleeding over saline only ($P = 0.771$; Student's t-test).

RoFVIII produces high titer inhibitor titer mice with predominant A2 specificity

The previous findings of this study have all addressed the antigenicity of roFVIII with respect to existing anti-human or anti-porcine inhibitors. To address the issue of immunogenicity briefly, we sought to generate monoclonal antibodies against oFVIII. Following 8 injections of roFVIII, the paired ELISA/inhibitor titers of the 3 immunized *F8*$^{-/-}$ mice were 7,500/900, 2,500/71, and 700/35 (arbitrary units/BU per ml) respectively. Following purification of 9 anti-oFVIII MAbs, immunoprecipitation of activated roFVIII revealed a predominant A2 domain specificity with 6 of the 9 precipitating the A2 domain and 3 MAbs precipitating the cleaved light chain (cLC).

Although there is no data regarding the inhibitory status of these MAbs, these data suggest that immunogenic regions within the A2 are highly conserved in oFVIII.

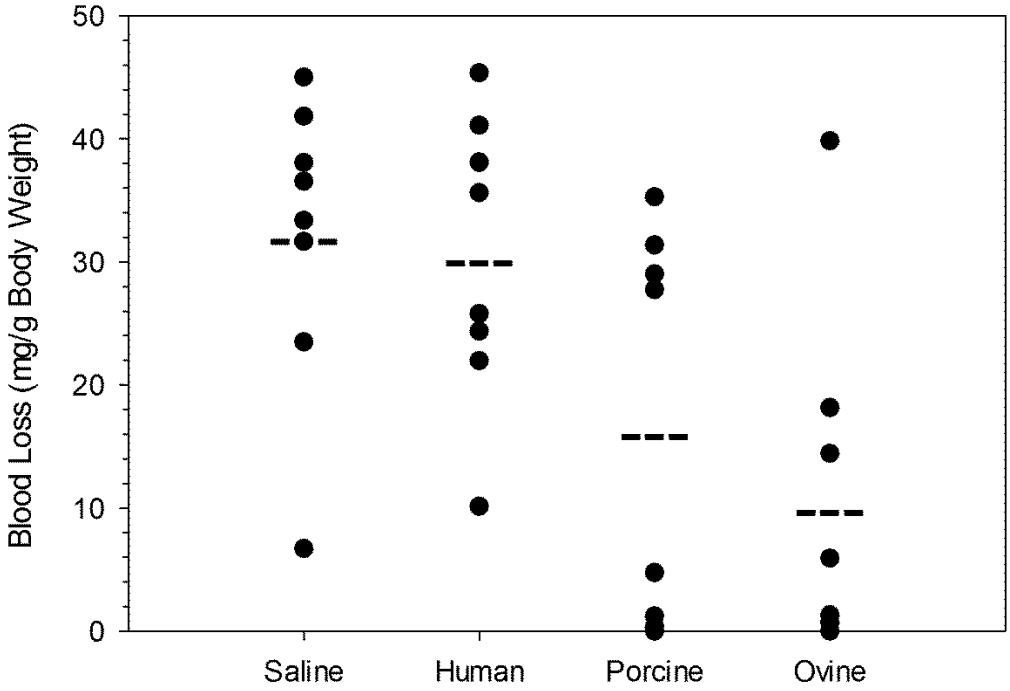


Figure III-6. In vivo testing of rFVIII orthologs in a murine model of acquired hemophilia A.

C57BL/6 mice were administered 10µg MAb 4A4 IP followed by 9 units rFVIII or saline only (n = 8). Blood loss over 40 minutes following tail transection was recorded. Dashes denote mean blood loss values for each experimental cohort.

Discussion

The development of anti-hFVIII inhibitors remains the most challenging complication of FVIII replacement therapy, which otherwise is effective at achieving and maintaining hemostasis in individuals with hemophilia A. While the development of humoral immunity to a protein replacement product is not unique, the doses (2 – 4 μg/kg) of FVIII needed to elicit this response are comparatively low [283]. Although knowledge regarding the antigen uptake, presentation, costimulatory signals, and predisposing genetic factors associated with FVIII inhibitor development is rather sparse, recent studies have described the epitopes, tertiary structures, mechanism of inhibition, and frequency of inhibitors in both human patients and FVIII-immunized murine models of hemophilia A [98-100, 114, 153, 284]. Well in advance of these high-resolution molecular studies, FVIII had been crudely isolated from animal plasmas (e.g. bovine and porcine) [285] and shown to control bleeding in hemophilia A patients with and without FVIII inhibitors [286, 287]. The clinical successes demonstrated using these animal FVIII preparations in inhibitor patients (both congenital and acquired) supported the commercial development of a highly-purified pd-pFVIII product (Hyate:C, formerly Speywood/Ipsen) in 1980 [288]. A decade later, Lollar and colleagues cloned the pFVIII cDNA [146] and began defining the major inhibitory epitopes present in hFVIII, but lacking in pFVIII, using hybrid human/porcine FVIII constructs and inhibitor patient plasmas [106, 109, 117, 118, 289]. This work also provided a scientific foundation and supported the development of a commercial rpFVIII product (OBI-1, Baxter International Inc.). However outside of the rpFVIII (OBI-1) and original bovine plasma FVIII studies, no further development of the interspecies antigenicity

differential has been pursued despite the obvious success of the approach, which is supported by the clinical utility of both pd- and r-pFVIII products.

Although the antigenicity and immunogenicity characteristics of FVIII orthologs largely have been ignored, their study has provided a platform for identifying structure/function relationships as well as interspecies differentials in biosynthetic, biochemical, and pharmacological properties that are thought to be exploitable for the rational design of improved rFVIII therapeutics and hemophilia A gene therapy applications. To date, FVIII orthologs from pigs, dogs, mice, monkeys (Dr. Pete Lollar, personal communication), and sheep have been generated and studied in recombinant form [147, 155, 160, 280]. Despite the interspecies differentials described above, each FVIII ortholog displays effective procoagulant activity in a human FVIII-deficient plasma bioassay and binds tightly to human von Willebrand factor, which is necessary for stabilization in plasma circulation. Furthermore, each of the FVIII orthologs displays unique biochemical properties in areas such as cellular secretion efficiency (rpFVIII > roFVIII > rhFVIII ≥ rmFVIII), decay rate following thrombin activation (rmFVIII > rpFVIII ≥ roFVIII > rhFVIII), and specific procoagulant activity (roFVIII > rpFVIII > rhFVIII > rmFVIII). The current study represents a continuation of this line of pursuit to identify FVIII sequences/molecules that can better address the clinical FVIII inhibitor problem.

Four key observations/findings were made in the current study. First, the inhibitor titers to roFVIII were significantly lower in most patient plasmas. Second, utilization of the ovine and porcine FVIII orthologs enabled further refinement of inhibitor epitopes within the A2 and C2 domains of hFVIII as well as determination of the inhibitor-epitope targeting frequencies within

an existing patient population. Third, murine anti- rh- and rp-FVIII inhibitor plasmas both demonstrate lower inhibitor titers against roFVIII suggesting its potential utility as a tertiary treatment for patients with inhibitors formed against pFVIII in addition to hFVIII. Fourth, roFVIII retains procoagulant activity and restores hemostatic protection *in vivo* in an acquired A2 domain specific hemophilia A murine model. It was reported in a previous study that A2 domain – group A inhibitors map to residues 484-508 [106]. Alignment of human, porcine, and ovine FVIII A2 domains within this region reveals that oFVIII and pFVIII share non-conserved residues R484S, Y487H, R489G, and F501M. RoFVIII also contains unique residues at L491F and I508V. In the current study, it was observed that group A inhibitors are present within several patient plasmas and A2–A MAbs do not cross-react with, or inhibit, either rpFVIII or roFVIII. In contrast to the A2 domain findings, for patient plasmas that displayed inhibitory titers above 10 BU against roFVIII and rpFVIII, there tended to be an abundance of polyclonal anti-C2 domain IgG that are predicted to bind conserved functional epitopes. Thus it can be concluded that in the absence of a significant C2 domain inhibitor population, shared non-conserved residues within the A2 domains of rpFVIII and roFVIII are responsible for retained activity.

The present study provides evidence for reduced antigenicity of roFVIII in the context of human inhibitor patients. While we acknowledge that roFVIII displayed no overall significant difference from rpFVIII, this study demonstrates response to exogenous FVIII orthologs is not universal. Due to inter-patient variation in inhibitor epitope specificity as well as inter-species differentials in epitope targeting efficiency, it seems reasonable to conclude that there would be distinct advantages to having multiple FVIII ortholog-based products in the clinical hemophilia A armamentarium. The current results also support the general investigation of orthologous

biomolecules not only as an approach to understanding structure/function, but also for the development of improved biotherapeutics. Given the ever-advancing push towards personalized medicine and the established clinical Bethesda assay for inhibitor detection, the case-by-case identification of the least antigenic and inhibited FVIII molecule may become the status quo.

Chapter IV: Bioengineering Coagulation Factor VIII through Ancestral Sequence Reconstruction

Materials and Methods

Materials

An-FVIII cDNAs were codon optimized with human codon bias and synthesized *de novo* by GenScript Biotech Corporation (Piscataway, NJ). SP-Sepharose, Source-Q chromatography resins, and Tricorn columns were purchased from GE Healthcare Life Sciences (Marlborough, MA). Lipofectamine 2000, Power SYBR® PCR Master Mix, RNAlater®, reverse transcriptase and RNase inhibitor were purchased from ThermoFisher (Grand Island, NY). HEK 293T-17 cells were purchased from ATCC (Manassas, VA), BHK-M cells have been previously reported [147, 155]. Serum-free AIM-V medium, Opti-mem®, and DMEM were purchased from Gibco®, ThermoFisher. Geneticin (G418) was purchased from Invitrogen, ThermoFisher. RNA and DNA isolation kits were purchased from Qiagen (Valencia, CA). Double stranded DNA fragments (gBlocks) were purchase from Integrated DNA Technologies (Coralville, IA). Polyethylenimine, Tween-80, and factor Xa chromogenic substrate were purchased from Sigma-Aldrich (St. Louis, MO). SDS-PAGE gels, protein standard, alkaline phosphatase substrate kit (AP pNPP), and Poly-Prep® columns were purchased from Bio-Rad (Hercules, CA). Pooled citrated normal plasma (FACT) and FVIII-deficient plasma were purchased from George King Biomedical (Overland Park, KS). Automated APTT reagent was purchased from Trinity Biotech (Wicklow, Ireland). Chromagenix FVIII Coatest was purchased from diaPharma (West Chester,

OH). Purified factor X and human alpha-thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). Factor IXa and phospholipid vesicles (PCPS) were generated and purified as previously described [266, 290, 291]. Cell culture flasks, Costar ELISA and EIA/RIA plates were purchased from Corning®, Sigma-Aldrich. Streptavidin-alkaline phosphate conjugate was purchased from Jackson Immuno Research (West Grove, PA). TransIT®-EE delivery solution was purchased from Mirus (Madison, WI). Domain specific monoclonal antibodies were generated and purified as previously described [98, 100, 278]. B-domain deleted recombinant oFVIII, pFVIII, and hFVIII were generated and purified as described previously [147, 280]. Exon 16-disrupted hemophilia A mice (E16) have been described previously [120].

An-FVIII sequence inference

An-FVIII sequence reconstruction was performed as described previously [292, 293]. Briefly, 47 available mammalian FVIII sequences were aligned using MUSCLE and an evolutionary tree was inferred using MrBayes (Figure 1) [294]. Ancestral sequences were inferred using both DNA and amino acid-based models in PAML VERSION 4.1 [295].

Biosynthetic efficiency of An-FVIII variants

HEK293T-17 and BHK-M cells were transfected with PEI or Lipofectamine 2000, respectively, in antibiotic-free media. Transiently transfected cells were washed with PBS and switched to serum-free media 48 hours after transfection and FVIII activity was measured by one-stage coagulation assay following an additional 24 h. Activity was normalized to human FVIII

activity. Stable BHK-M clones were generated as previously described [147, 155, 280] and 24 – 36 clones of each FVIII construct were isolated for screening. Clones resistant to G418 but lacking detectable FVIII activity were discarded. FVIII activity was measured in serum-free media by one-stage and normalized to cell counts taken at the time of FVIII activity determination. RNA for subsequent steady-state transcript levels was collected following a phosphate-buffered saline (PBS) wash and storage at -80 °C in RNAlater®. Transcript levels were determined using one-step RT-PCR compared to a plasmid standard curve for quantitation. Primers for transcript analysis are shown in Supplement 1 (S1).

Statistical Analysis

All calculations were performed using SigmaPlot 13 software (Systat Software Inc., San Jose, CA). FVIII expression comparisons made between An-FVIII constructs and hFVIII were performed using the Kruskal-Wallis one-way analysis of variance on ranks non-parametric test. Post hoc comparisons of the individual construct pairs were conducted using Dunn's test for multiple comparisons with P values < 0.05 being considered significant.

Purification of An-FVIII variants

The BHK-M clones producing the greatest concentration of FVIII were expanded into triple-flasks and FVIII was collected in serum-free media and stored at -20 °C with 0.05% sodium azide until purification. Cell debris was removed prior to storage by centrifugation at 1,000 rcf for 15 min. FVIII was purified by one- or two-stage ion exchange chromatography as previously

described [147, 280]. Elution fractions were analyzed for purity via SDS-PAGE and concentration was determined by one-stage coagulation. Specific activity of homogenous FVIII samples was determined by one-stage coagulation determination compared to λ280 following λ320 and buffer corrections. Purified FVIII was aliquoted and stored at -80 °C until use for biochemical characterization.

Non-proteolytic decay via A2 domain dissociation

Activated factor VIII (FVIIIa) was measured by chromogenic assay using purified human factor IXa, human factor X, and synthetic phospholipid vesicles as described previously [258, 280]. Briefly, 1 nM FVIII was activated with 100 nM human thrombin for 15 s at room temperature. Desulfatohirudin (150 nM final) was added to stop the reaction and FVIII activity was measured at several time points.

Antigenicity of An-FVIII variants

The cross-reactivity of monoclonal anti-human FVIII antibodies (MAbs) against ancestral FVIII was measured via direct ELISA as previously described [296]. Briefly, 1.5 µg FVIII was adsorbed to an ELISA plate in 20 mM Bicine, 2 mM $CaCl_2$ pH 9.0 for 2 h and blocked with 2% BSA in 20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$ at 4°C for at least 12 h. MAbs were selected due to A2 and C2 domain epitope recognition as well as anti-human inhibitor titers exceeding 1,000 Bethesda units (BU) /mg as previously determined [98, 100]. Biotinylated MAb at 4 µg/mL was added to the well for one h at room temperature, followed by 1:10,000 dilution

of strepavidin-conjugated alkaline phosphatase in blocking buffer. Absorbance at 405 nm was measured following addition of chromogenic substrate. Mean values of triplicates were recorded and normalized to anti-hFVIII signal as percent cross-reactivity.

Inhibition of E434V mutant by MAb 4A4

An *de novo* synthesized DNA fragment (gBlock) containing a single point mutation E434V was inserted into An-53 by enzymes NheI and BlpI and confirmed via sequencing. A polyclonal population of BHK-M cells producing the E434V mutant was generated and expanded for protein collection and subsequent inhibitor testing. Inhibitor titer of Mab 4A4 was conducted using a modified Bethesda assay [281]. Briefly, E434V FVIII or purified An-53 was diluted to 0.8 – 0.2 unit/ml in conditioned serum-free supernatant from naïve BHK-M cells and buffered with 0.1 M imidazole. FVIII and MAb 4A4 were combined and incubated at 37°C for 2 h, and residual FVIII activity was determined via one-stage coagulation assay.

ED_{50} up-down efficacy determination

All animal studies were performed under the guidelines set by the Emory University Institutional Animal Care and Use Committee. Hemostatic challenge was performed via tail transection as previously described [151, 280] and the ED_{50} was calculated by Dixon up-and-down method as previously described [297, 298]. Briefly, hemophilia A E16 mice were injected with saline, recombinant ancestral FVIII at varying doses, or bolus hFVIII diluted in 0.9% saline via tail vein. Mouse tails were incubated at 37 °C for 15 min prior to challenge. FVIII doses were

determined *a priori* and prepared immediately preceding injection. A bleeding event was defined as blood loss (g/kg) exceeding the standard deviation of wildtype mice in an identical challenge without infused FVIII (16 mg/g body weight). Following transection, blood was collected directly in conical tubes containing pre-warmed PBS and measured by change in mass after 40 min.

Hydrodynamic injection and de novo FVIII generation

Codon-optimized human, ET3, and An-53 FVIII were subcloned into an AAV expression cassette incorporating the liver-directed HLP promoter and a synthetic polyadenylation sequence. Plasmid DNA was linearized outside the inverted terminal repeat (ITR) sequences and the DNA quality and quantity was assessed via gel electrophoresis prior to injection. Hydrodynamic injections were conducted as previously described [150]. Briefly, mice were weighed prior to injection and varying doses of linear plasmid was diluted into Transit-EE hydrodynamic delivery solution totaling 10% body weight. Naked DNA was delivered to hemophilia A mice age 8 – 12 weeks by tail vein injection over the course of 5-8 seconds. Blood plasma was collected at several time points following injection and FVIII activity was measured by two-stage chromogenic assay according to the manufacturer's instructions using a standard curve generated from pooled citrated human plasma *via* ELISA using cross-reactive anti-hFVIII murine monoclonal antibodies (MAbs) and purified recombinant An-53 as a standard.

Abstract, Introduction, and Results

Bioengineering approaches to protein and nucleic acid-based therapeutics require either detailed structure-activity knowledge (*e.g.* rational design) or robust high-throughput recombinant DNA/protein capabilities (*e.g.* directed evolution), thus limiting successful implementation. Herein, we investigate ancestral sequence reconstruction (ASR) as a tertiary approach to biopharmaceutical engineering. ASR employs predictive models of molecular evolution with knowledge of extant protein diversity to identify, characterize, and bioengineer desired properties. Coagulation factor VIII (FVIII) represents an attractive target for ASR as current biopharmaceuticals, although efficacious, possess significant pharmacological limitations. To address these limitations, we resurrected ancestral (An) FVIII variants and identified molecules possessing superior biosynthetic efficiency, specific-activity, and stability. Furthermore, certain An-FVIII constructs displayed reduced overall binding and procoagulant inhibition by anti-FVIII antibodies. Furthermore, ASR facilitated the identification and elimination of a previously uncharacterized inhibitory epitope on human FVIII through single amino acid bioengineering. The current findings support the utility of ASR as a biopharmaceutical discovery/engineering approach.

As originally proposed by Pauling and Zuckerland in the 1960's, ASR entails the prediction of ancient DNA/protein sequences from extant sequences [299]. Recent technological advancements in *de novo* custom DNA synthesis and recombinant protein expression now facilitate the laboratory 'resurrection' and characterization of inferred ancestral proteins, thus rendering the approach significantly more robust (for review see [300]). Examples of scientific

advances made possible through ASR include the, i) deduction of environmental conditions during the Precambrian era [292], ii) examination of ancestral enzyme promiscuity [301], iii) study of ancient receptor-ligand interactions [302], iv) resurrection of a human pseudogene [175] and v) defining of an anti-cancer drug mechanism [303]. In the current study, ASR is applied as an upfront and direct approach to biopharmaceutical bioengineering that does not suffer the same limitations as existing methods; requiring only extant genome information, modest computing resources, and the recombinant production and analysis of a limited set of ancestral protein variants.

Factor VIII (FVIII) is an essential procofactor in the intrinsic pathway of blood coagulation. Deficiencies and/or defects in circulating FVIIII define the most common severe bleeding disorder, hemophilia A. Transient hemostatic correction in the setting of hemophilia A is achieved through intravenous infusion of FVIII-containing products. Although FVIII infusion therapy successfully converts this lethal disease into a clinically manageable condition, human (h) FVIII biotherapeutics possess several limitations. For example, cellular biosynthesis of hFVIII is 100-1000 fold less efficient than other recombinant proteins, resulting in high product costs that preclude 70% of the global population from receiving treatment. Furthermore, poor transgene productivity (i.e. FVIII biosynthesis/secretion) has proven to be the major barrier to investigational gene therapy applications for hemophilia A where gene transfer is constrained by dose-limiting vector toxicity and/or vector supply. A secondary limitation is short half-life, both as an inactive procofactor in circulation (~ 14 hours), and as an active cofactor following proteolytic activation by thrombin (~ 2 minutes). This labile nature necessitates frequent, 2 – 3 times per week, infusion of hFVIII products to achieve prophylaxis. A third major unresolved

limitation is the recognition of hFVIII products by the immune system through both immunogenic and antigenic mechanisms. This aspect remains the most significant clinical complication with approximately 30% of severe patients developing neutralizing anti-drug antibodies, termed ‘inhibitors’, that negate therapeutic effectiveness.

Interspecies differentials in molecular, cellular and immune recognition properties of coagulation factor VIII (FVIII) orthologs exist [147, 153, 155, 160, 280, 296]. Studies by colleagues and ourselves on porcine (p) FVIII are prime examples where somewhat unanticipated, but predictably beneficial, therapeutic properties of pFVIII have been discovered. First, pFVIII displays reduced cofactor inhibition in the presence of many hFVIII inhibitory antibodies [288]. This property is the basis for clinical use of recombinant pFVIII. A second property is efficient cellular secretion, which contributes to 10 – 100-fold greater overall biosynthesis than hFVIII [133, 147, 148]. This property currently is being exploited both for the development of a more economical FVIII infusion product as well as potentially curative lentiviral and adeno-associated viral vector-based gene therapy products [150, 152, 247]. Third, pFVIII displays a 2-3-fold extended half-life ($t_{1/2}$) following proteolytic activation by thrombin [147, 148, 266]. This property is predicted to provide increased hemostatic potency [157]. Individually, these attributes likely represent adaptive traits acquired through natural selection to promote hemostatic balance. Initially, we took a reductionist (i.e. hybrid ortholog FVIII testing) approach to the identification of ‘high expression’ (i.e. increased biosynthesis/secretion) and ‘slow decay’ (i.e. longer activated FVIII $t_{1/2}$) sequence determinants present in p- and murine (m)-FVIII, respectively [148]. This approach facilitated the mapping of functionally-determinant regions down to the domain (or subdomain) level but became intractable beyond that resolution due to the complexity of

epistatic effects [148, 266]. ASR provides a high-resolution mapping solution to ortholog sequence-activity relationships through comparisons among the branches on a phylogeny also taking advantage of the observation that ancient proteins often have unpredicted and/or expanded functionalities. In the current study, we apply ASR as an upfront FVIII discovery/bioengineering platform to develop superior FVIII infusion and gene therapy products with the expectation that similar approaches can be successfully employed to improve upon a diverse array of biopharmaceuticals, and in particular, other hemostatic coagulation factors.

An-FVIII sequence reconstruction was performed as described previously [292, 293] to infer a mammalian FVIII evolutionary tree (Figure IV-1A). Sufficient extant sequence data were available to accurately extend the phylogenetic tree and infer ancestral sequences beyond the class Mammalia. To assess the performance of An-FVIII sequence prediction, initially eight different ancestral cDNA were inferred, synthesized de novo, and then expressed in mammalian cell lines used for commercial recombinant FVIII manufacturing. Transfection of each An-FVIII cDNA encoding expression plasmid into both human embryonic kidney (HEK) 293T-17 and baby hamster kidney (BHK)-M cell lines resulted in secretion of functional FVIII into the conditioned culture media as determined by *in vitro* FVIII ortholog data, the early mammalian FVIII ancestors An-54, -53, and -52, demonstrated 11-, 9-, and 4-fold enhanced productivity (*i.e.* recombinant protein expression), respectively, compared to hFVIII. Of the remaining ancestors, the previously described bioengineered human/porcine hybrid FVIII, ET3, An-68 demonstrated

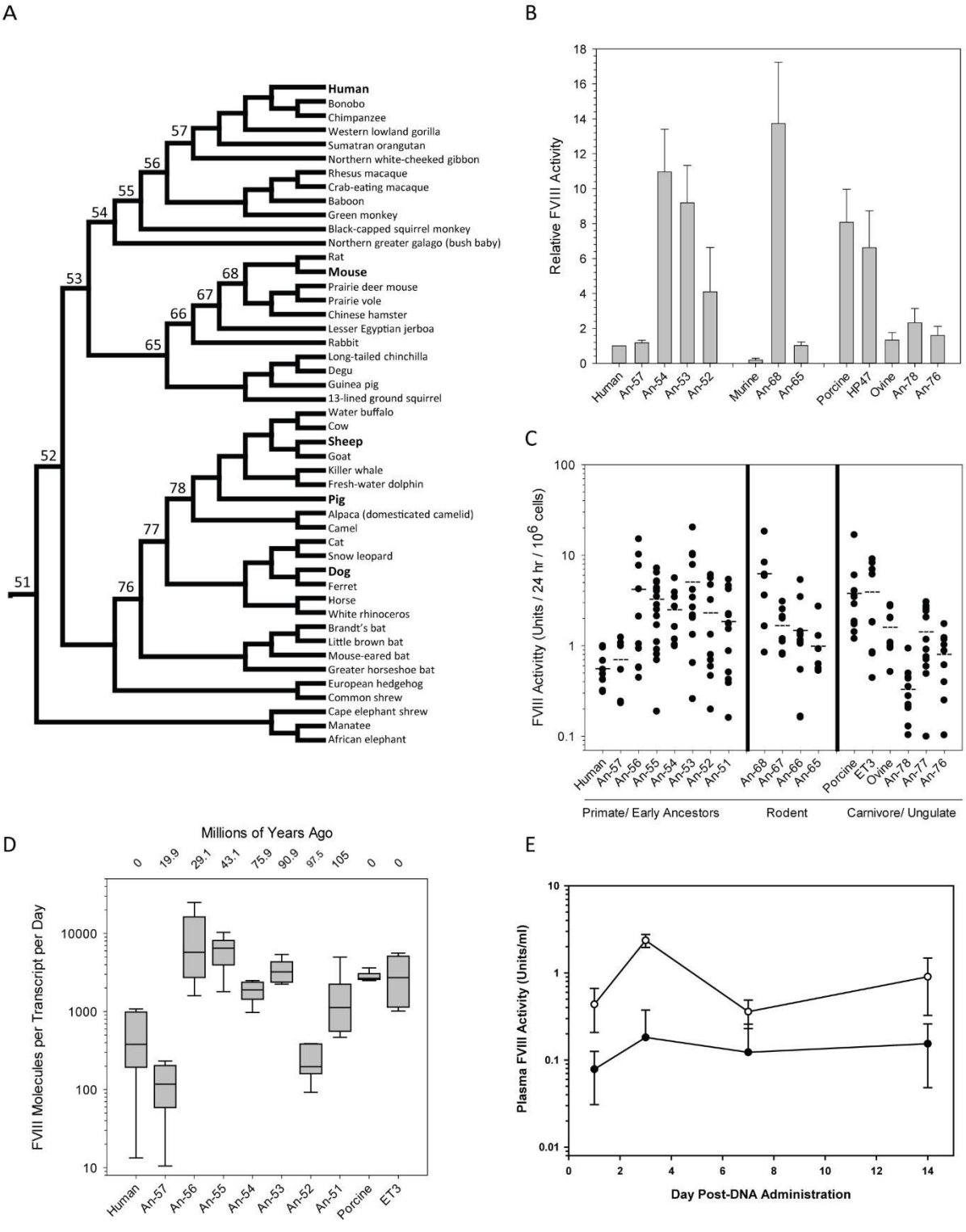
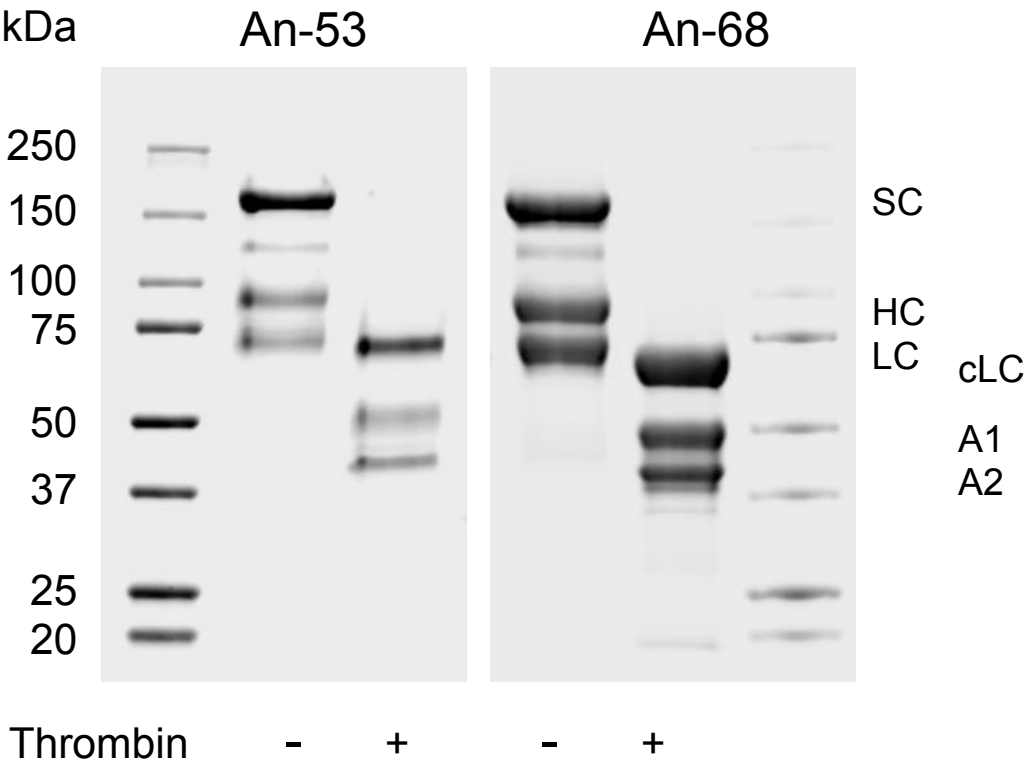

A
Human
Bonobo
Chimpanzee
Western lowland gorilla
Sumatran orangutan
Northern white-cheeked gibbon
Rhesus macaque
Crab-eating macaque
Baboon
Green monkey
Black-capped squirrel monkey
Northern greater galago (bush baby)
Rat
Mouse
Prairie deer mouse
Prairie vole
Chinese hamster
Lesser Egyptian jerboa
Rabbit
Long-tailed chinchilla
Degu
Guinea pig
13-lined ground squirrel
Water buffalo
Cow
Sheep
Goat
Killer whale
Fresh-water dolphin
Pig
Alpaca (domesticated camelid)
Camel
Cat
Snow leopard
Dog
Ferret
Horse
White rhinoceros
Brandt's bat
Little brown bat
Mouse-eared bat
Greater horseshoe bat
European hedgehog
Common shrew
Cape elephant shrew
Manatee
African elephant
B
Relative FVIII Activity
Human
An-57
An-54
An-53
An-52
Murine
An-68
An-65
Porcine
HP47
Ovine
An-78
An-76
C
FVIII Activity (Units / 24 hr / 10^6 cells)
Primate/ Early Ancestors
Rodent
Carnivore/ Ungulate
D
Millions of Years Ago
FVIII Molecules per Transcript per Day
E
Plasma FVIII Activity (Units/ml)
Day Post-DNA Administration

Figure IV-1: FVIII phylogenetic tree and expression analysis. A) Forty-seven available mammalian FVIII sequences were aligned using MUSCLE and an evolutionary tree was inferred using MrBayes [294]. Ancestral FVIII sequences were inferred using both DNA and amino acid-based models in PAML VERSION 4.1 [295]. From the inferred An-FVIII sequences, we initially selected 14 nodes (numbered) with corresponding B-domain deleted cDNAs for reconstruction. These sequences were human codon-optimized and synthesized *de novo*. Extant mammalian species for which recombinant FVIII characterization data exists are denoted by bold font on the cladogram (Supplemental Fig. 6 shows a phylogram). B) HEK 293T-17 cells were transiently transfected using PEI and FVIII production rate was measured by one-stage coagulation assay following 24 hours culture in serum-free media. Data are presented relative to hFVIII levels. An-53, An-54, and An-68 production rates were observed to be significantly higher than hFVIII ($P \leq 0.002$ for each comparison), but not significantly different from each other ($P = 0.465$; An-53 versus An-54). Error bars represent standard deviation from three independent experiments. C) Black circles depict expression levels from individual BHK-M clones stably expressing FVIII as determined by one-stage coagulation assay in serum-free media (n = 6 – 18). Dashed lines indicate the mean of each clonal set. Activity levels of An-53, An-55, An-68, pFVIII and ET3 are significantly different from hFVIII ($P < 0.05$). D) Biosynthetic efficiency was determined by comparison of the FVIII secretion rate to steady state mRNA transcript levels. Data are presented as FVIII molecules produced per transcript using a specific activity estimate of 1 unit/150 ng (n = 6). E) Eight- to twelve- week old hemophilia A mice were randomized and injected with 3 μg linearized ET3 (closed circles) or An-53 (open circles) encoding plasmid DNA. Mouse plasma was collected on the indicated days post hydrodynamic injection. Plasma FVIII levels were

measured using a chromogenic FVIII assay with pooled normal human plasma as a standard. Error bars indicate the standard deviation, n = 3 – 4 per cohort.

the greatest productivity at levels 14-fold above hFVIII extant. PFVIII and coagulation assay utilizing hFVIII-deficient plasma (Figures IV-1B and IV-1C). These data confirm the evolutionary compatibility of each An-FVIII procofactor with the necessary human hemostatic system components (e.g. coagulation factors IXa and X). Although not predicted based on demonstrated 8- and 7-fold increased productivity over hFVIII, respectively, consistent with previous reports [133, 136, 147, 148, 152]. Therefore, certain An-FVIII variants are produced at rates equivalent to, or possible greater than, the most efficient FVIII constructs described to date (*i.e.* pFVIII and ET3). Unexpectedly, two of these constructs, An-53 and -54, also harbor 55 – 75% less sequence dissimilarity than the existing high expression constructs, which should translate clinically to lower immunogenic risk (Supplemental Figure IV-S1).

Subsequently, another six An-FVIII variants were resurrected and all 14 sequences were used to generate stable production cell lines. Similar to the transient production results, An-53 and An-68 demonstrated the highest rates of stable FVIII production at levels 9- and 11-fold greater than hFVIII (Figure IV-1C). To test if the increased FVIII activity observed is due to pre-, post-, and/or cotranslational biosynthesis, steady state FVIII mRNA were measured and correlated to the rate of FVIII secretion (Figure IV-1D). An-56, An-55, and An-53 displayed significantly increased FVIII production per steady-state transcript compared to hFVIII, and were not significantly different from pFVIII or ET3. Therefore, efficient An-FVIII biosynthesis appears to result from cotranslational/posttranslational mechanisms consistent with data obtained previously for pFVIII and ET3 [133, 136, 147, 148, 152].



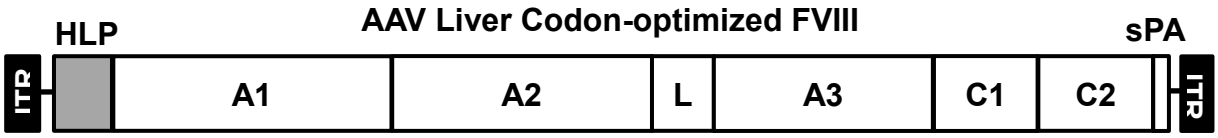
Supplemental Figure IV-S1: SDS-PAGE analysis of An-53 and An-68 following two-step ion-exchange. An-FVIII was treated with or without thrombin for 5 minutes at 37°C. Samples were reduced via DTT addition and denatured at 95°C for 5 minutes prior to loading into a 4-15% gradient SDS-PAGE gel. Protein was visualized by coomassie blue. SC: single chain; HC: heavy chain; LC: light chain; cLC: cleaved light chain; A1 and A2: FVIII A domains

As a test for i*n vivo* efficacy, ED_{50} values were obtained for two of the highest expressing An-FVIII constructs, An-53 and An-68. Briefly, each recombinant protein was purified to homogeneity and the purity of the resulting preparations was confirmed using SDS-PAGE (Supplemental Figure IV-S1), Specific activities of recombinant An-53 and An-68 were determined to be 18,500 and 8,770 units/mg, respectively. Hemophilia A mice were administered a single dose of FVIII and hemostatically challenged via tail transection. ED_{50} values of 89 and 47 units/kg were obtained for An-53 and An-68, respectively (Supplemental Table IV-S1). These data confirm the *in vivo* functionality of both An-FVIII constructs.

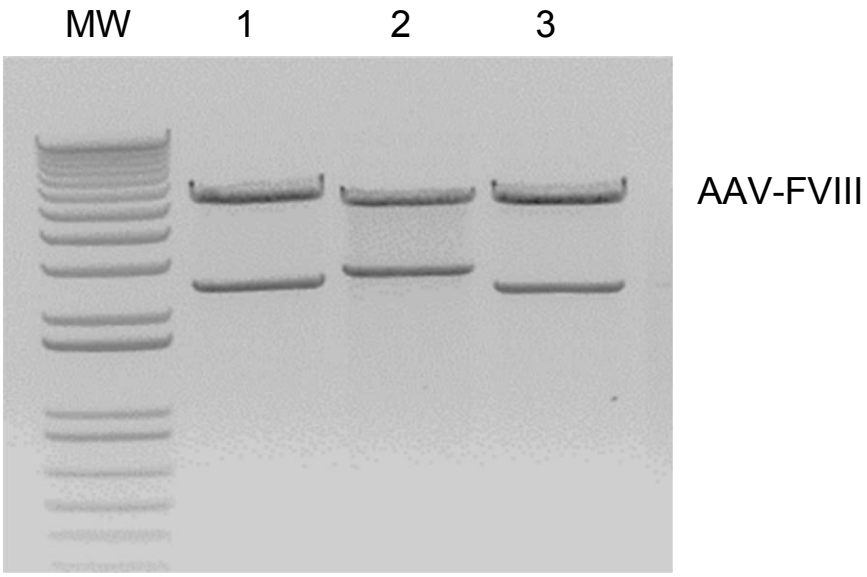
Liver-directed gene transfer is one of two leading platforms for clinical gene therapy of hemophilia A. As mentioned previously, the major limitation to all gene therapy protocols for hemophilia A, including both adeno-associated virus (AAV) and lentivirus (LV) vector-based platforms, continues to be the low-level biosynthesis of hFVIII at clinically achievable and/or safe vector doses. Given the enhanced *in vitro* biosynthesis and *in vivo* efficacy observed for An-53 as well as the high identity to hFVIII, we sought to test its performance following *in vivo* nucleic acid delivery to the liver of hemophilia A mice. An-53 and ET3 cDNAs were subcloned into an AAV expression cassette under the liver-directed HLP promoter (Supplemental Figures IV-S2 and IV-S3). Plasma FVIII levels were monitored over two weeks following injection. Compared to the bioengineered high-expression construct, ET3, An-53 demonstrated significantly increased and supraphysiologic peak FVIII levels of 2.37 units/mL (Figure IV-1E). At this dose of plasmid DNA, no detectable plasma FVIII activity was observed for animals receiving the hFVIII construct (Supplemental Figures IV-S4 and IV-S5). The specific activity of *in vivo* produced An-53 was measured via ELISA and shown to be similar to recombinant An-53

Supplemental Table IV-1. ED_{50} determination of An-53 and An-68 by Dixon up-and-down.

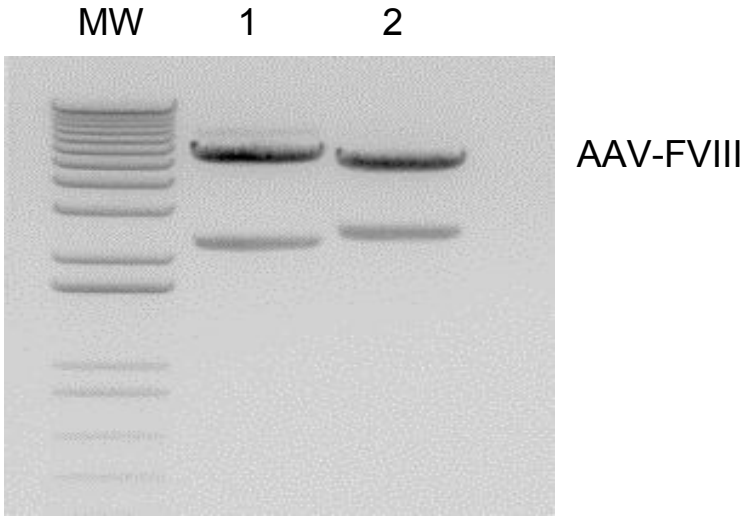
Dixon Up-and-Down Method for Small Samples								
Factor VIII	**Dose (U/mL)**	**Log (Dose)**	**Response: X = Bleed O = No Bleed**					
An-53	100	2		O				X
	63.1	1.8	X		O		X	
	39.8	1.6				X		
An-68	63.1	1.8	O		O			
	39.8	1.6		X		O		X
	25.1	1.4					X	
Saline	0	N/A	X	X				
hFVIII	200	2.3	O	O				



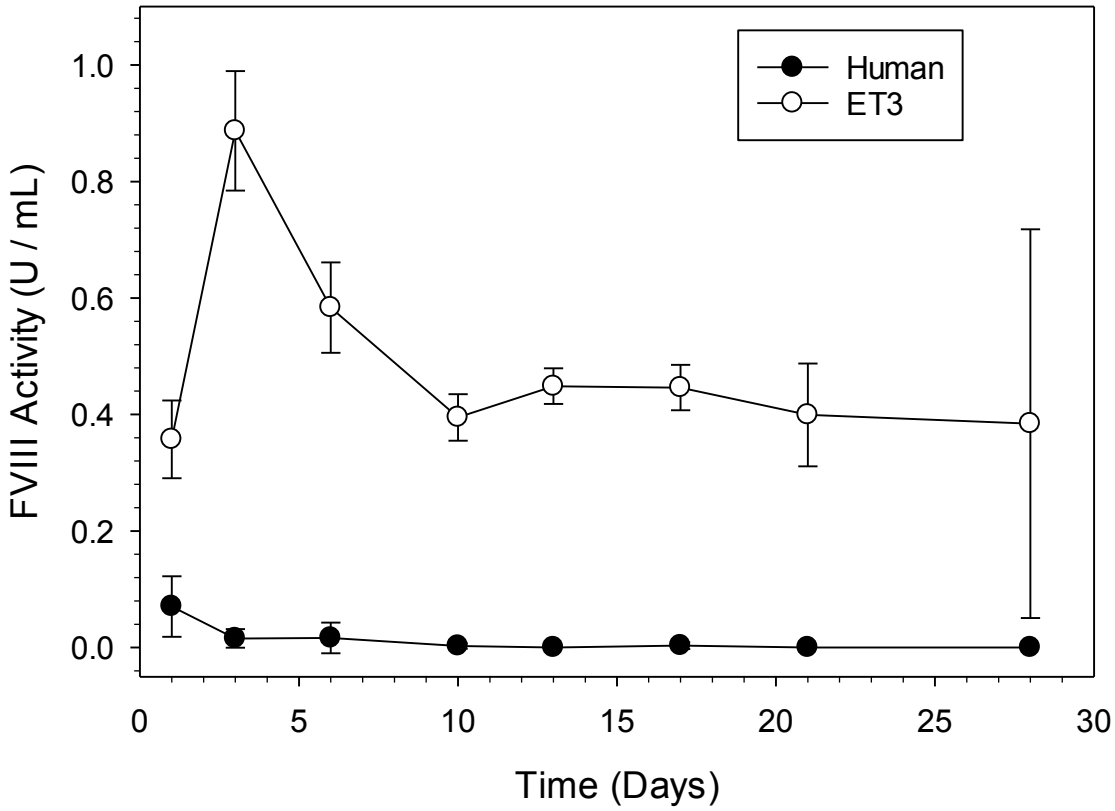
Supplemental Figure IV- S2: Schematic of linear AAV expression cassette for hydrodynamic injections. FVIII was liver codon optimized and subcloned under the liver-directed promoter HLP. The B-domain was removed and substituted with a small 14 amino acid linker (L). sPA: synthetic polyA tail.



Supplemental Figure IV-S3: DNA quality determination of ancestral FVIII cDNA. AAV-HLP-An-53 (1), AAV-HLP-An-56 (2) and AAV-HLP-ET3 (3) at one µg per well were assessed for quality, concentration, and linearity following enzymatic digestion. The FVIII containing AAV DNA fragment is labeled (right).



Supplemental Figure IV-S4: DNA quality determination of human and ET3 FVIII cDNA. AAV-HLP-Human FVIII (1) and AAV-HLP-ET3 (2) at two µg per well were assessed for quality, concentration, and linearity following enzymatic digestion. The FVIII containing AAV DNA fragment is labeled (right).



Supplemental Figure IV-S5: FVIII plasma levels following hydrodynamic injection. Human (closed circles) and human/porcine hybrid ET3 (open circles) FVIII levels were determined by two-stage chromagenic assay following hydrodynamic injection of 3 μg linear DNA. Plasma was collected and assayed for 4 weeks following injection.

at 13,000 ± 5100 units/mg (data not shown, n = 6).

FVIII is present in the circulation as a heterodimeric, A1-A2-B/ap-A3-C1-C2 domain structure, procofactor in non-covalent association with von Willebrand factor (VWF) until it is activated (a) by trace amounts of thrombin. Thrombin-mediated proteolysis at R372, R740, and R1689 releases FVIIIa from VWF to perform its procoagulant function as a necessary cofactor for the activation of factor X by the serine protease, activated factor IX. However following activation, the weakly-associated, but essential, A2 domain is subject to rapid, and nearly complete, equilibrium-governed dissociation (Figure IV-2A). Mutations that further decrease FVIIIa stability present as a mild bleeding phenotype confirming the physiological relevance FVIIIa A2 subunit decay [156]. We previously described 2 – 10-fold higher stability of pFVIIIa, ovine (o) FVIIIa and particularly mFVIIIa [147, 155, 266, 280] and speculated that stabilization of FVIIIa should result in a more potent FVIII product. This concept was recently substantiated in a preclinical hemophilia model [157]. Therefore, we initially sought to investigate the basis of differential FVIIIa stability through biochemical assessment of An-FVIIIa decay (Supplemental Table IV-2). The data suggest that hFVIIIa-like rapid decay existed with the last common mammalian ancestor, An-51, and persisted through the primate lineage and into extant humans and primates (Figure IV-2B). However, within the rodent lineage, An-66 and -67 display modestly prolonged decay rates with $t_{1/2}$ of 3.8 and 4.4 min respectively, progressing to the fully-extended mFVIIIa-like $t_{1/2}$ of 15.6 min observed for An-68 (Figure IV-2C). These data suggest that the previously described stability of mFVIIIa [155] is the result of incremental steps. Similarly, within the

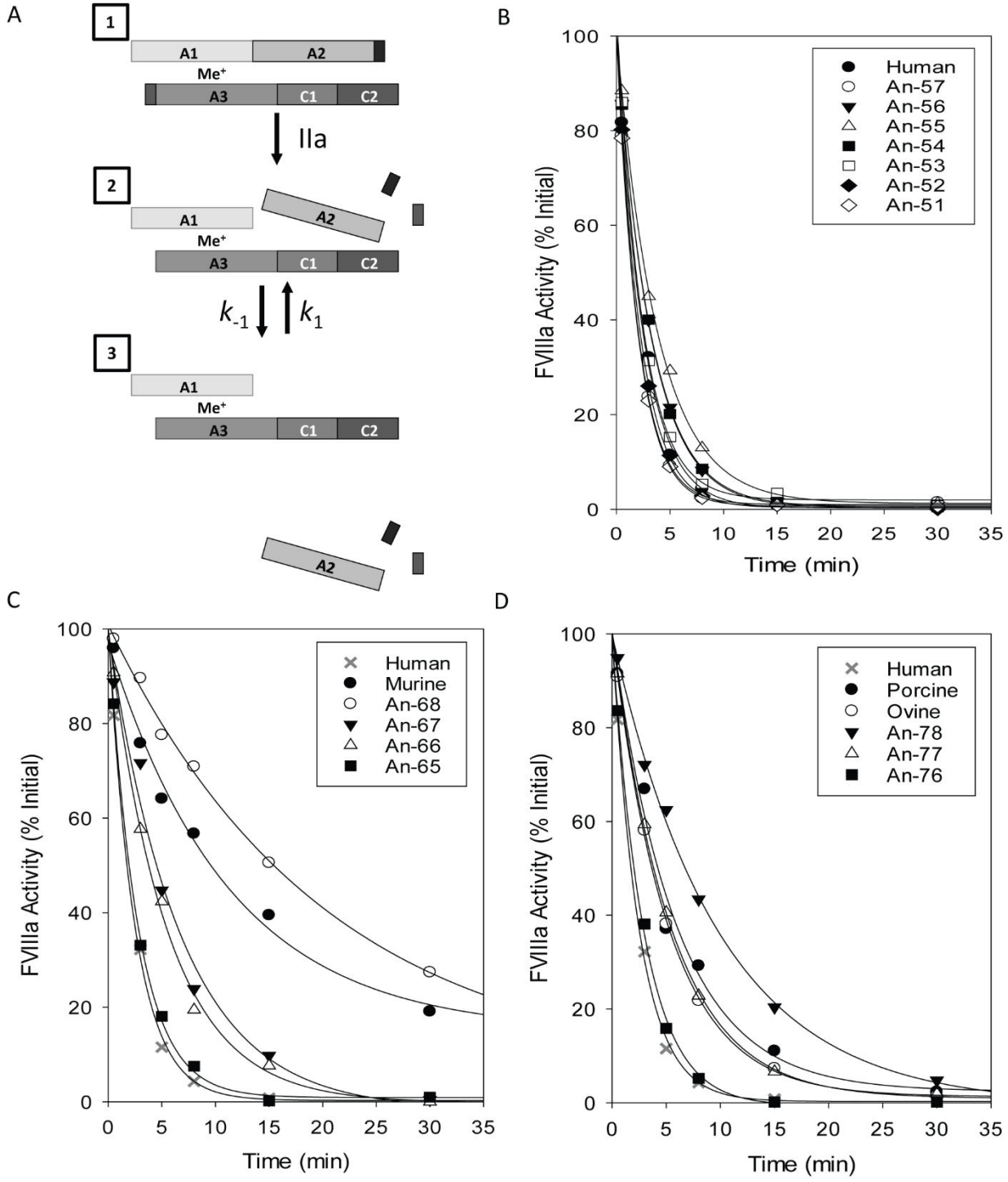

A
1
A1
A2
Me+
A3
C1
C2
IIa
2
A1
A2
Me+
A3
C1
C2
k-1
k1
3
A1
Me+
A3
C1
C2
A2
B
FVIIIa Activity (% Initial)
Time (min)
Human
An-57
An-56
An-55
An-54
An-53
An-52
An-51
C
FVIIIa Activity (% Initial)
Time (min)
Human
Murine
An-68
An-67
An-66
An-65
D
FVIIIa Activity (% Initial)
Time (min)
Human
Porcine
Ovine
An-78
An-77
An-76

Figure IV-2: An-FVIII activation and stability. A) 1. FVIII is secreted and circulates in blood in a heterodimeric A1-A2-B/ap-A3-C1-C2 procofactor form stabilized by a divalent metal (Me^{+}) ion. 2. Following proteolytic activation by thrombin (IIa), FVIII is converted to a heterotrimeric form (FVIIIa) wherein the A2 domain is no longer covalently attached to the Me^{+} ion stabilized A1/A3-C1-C2 heterodimer, but remains in weakly associated via noncovalent forces. 3. At physiologic concentrations, equilibrium for this reversible interaction lies far to the dissociated state and FVIIIa cofactor activity rapidly decays as the essential A2 domain subunit dissociates from the A1/A3-C1-C2 complex. B) The stabilities of thrombin-activated early ancestral and primate lineage An-FVIIIa molecules were assessed as described previously [266]. Briefly for all An-FVIIIa stability studies, purified recombinant FVIII samples were activated completely by incubation with a thrombin (t = 0). Subsequently, the thrombin was inhibited by addition of desulfatohirudin and FVIIIa activity decay was measured over 30 minutes at 23°C *via* indirect Xa formation following the addition of chromogenic substrate. C) and D) The decay rates of rodent lineage (C) and carnivore/ungulate lineage (D) An-FVIIIa were determined as described in B). Decay profiles for hFVIIIa serve as a reference in each experiment.

Supplemental Table IV-2: Comparison of FVIII sequences and FVIIIa stability

FVIII	Identity to hFVIII (%)	Amino Acid Replacements	FVIIIa $t_{1/2}$ (min)	k_{-1}^{*} (sec^{-1})
Human	100	0	1.75	0.396
An-57	99	14	1.5	0.483
An-56	98	19	2.2	0.307
An-55	98	26	2.7	0.255
An-54	96	57	2.2	0.312
An-53	95	69	1.9	0.380
An-52	94	82	1.6	0.445
An-51	93	92	1.4	0.487
Murine	85	216	11.5	0.0403
An-68	87	178	15.6	0.0284
An-67	91	117	4.4	0.1538
An-66	94	84	3.8	0.1840
An-65	94	80	2.0	0.356
Porcine	84	232	4.3	0.164
Ovine	85	209	3.7	0.189
An-78	91	124	6.7	0.103
An-77	92	103	3.8	0.180
An-76	93	101	2.0	0.344

* Dissociation rate constant for FVIIIa

carnivore/ungulate lineages, the development of a prolonged $t_{1/2}$ is the result of 4 or less amino acid replacements occurring between An-76 and An-77 (Figure IV-2D).

Other than cost, the greatest obstacle to treatment of hemophilia A is the development of anti-FVIII inhibitory antibodies and no clinical progress has been made towards reducing their incidence likely due to our lack of understanding of the immune response to FVIII. Using well-characterized anti-hFVIII murine monoclonal antibodies (MAbs) with high inhibitory titers and specificity for the dominant clinical epitope containing domains, *i.e.* A2 and C2 domains [98, 100], we initially examined the antigenicity of several An-FVIII variants (Figure IV-3A). Despite sharing 95% identity to modern hFVIII, An-53 displays markedly reduced cross-reactivity within the antigenically-dominant A2 and C2 domains. All group A anti-hFVIII MAbs bind a major epitope in the highly immunogenic 484-508 loop of hFVIII [100], and only MAbs 4A4 and G32 demonstrate cross-reactivity to An-53 with G32 possessing an inhibitory titer 45-fold less against An-53 compared to hFVIII (data not shown). In contrast, MAb 4A4 displayed an equivalent titer against An-53 and hFVIII.

Ignoring 4A4, An-53 has four amino acid replacements within the 484-508 loop that may account for the absence of cross-reactivity and/or inhibition by A2 inhibitors. Reconsidering 4A4, previous studies using hybrid h/p-FVIII showed that a functional epitope of 4A4 lies within residues 403-444 [100], but was not further defined. Alignment of several FVIII A2 domain sequences either possessing or lacking the 4A4 epitope allows for the identification of four residues that may be responsible for differential binding affinity. Three of these residues, Asp403, Gln410, and Asn414 are distal to the 484-508 surface loop and therefore appear

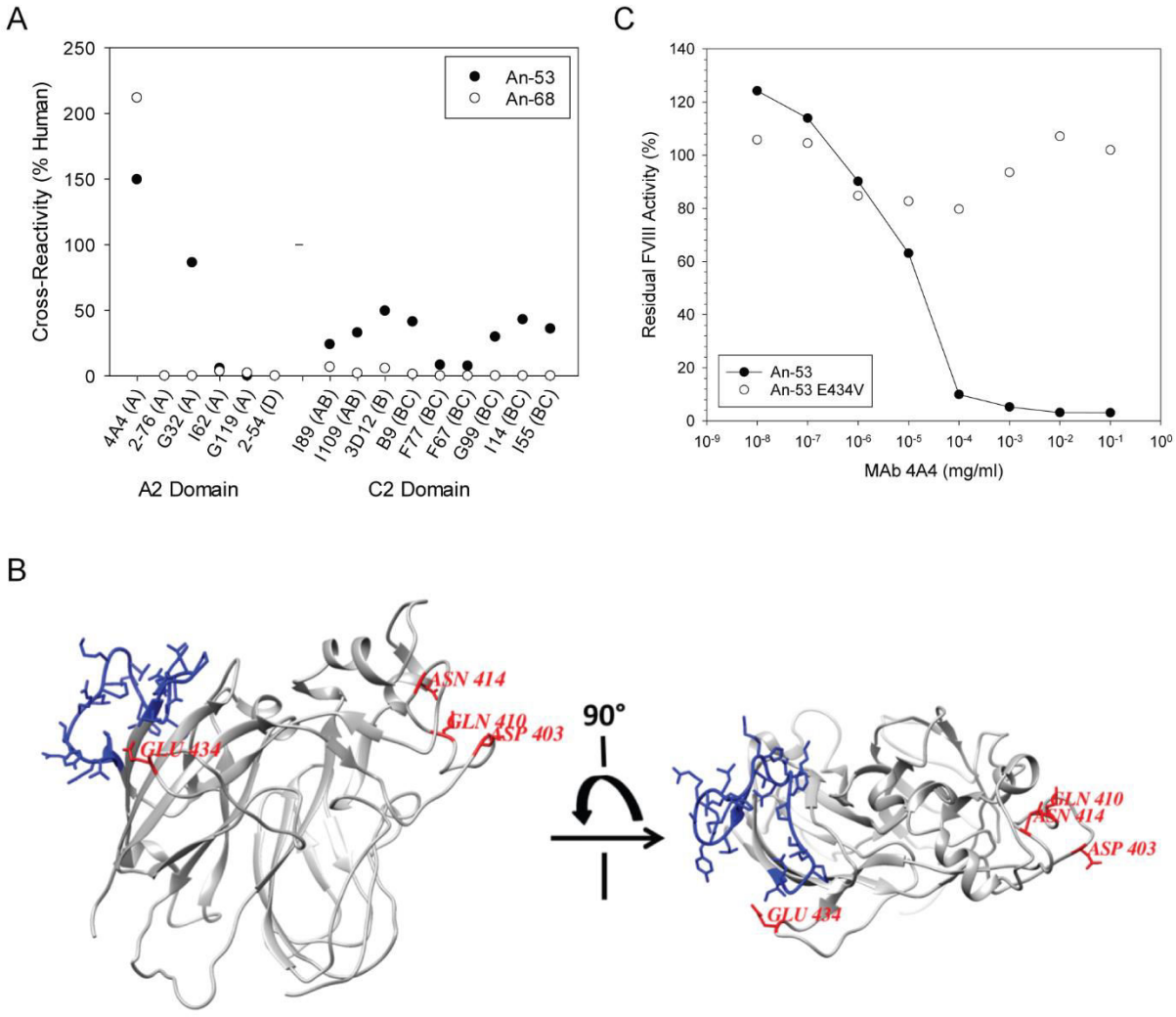


Figure IV-3: An-FVIII immune recognition and bioengineering. A) Cross-reactivity of anti-hFVIII inhibitory MAbs to An-53 (closed circles) and An-68 (open circles) was determined *via* direct ELISA. MAbs were selected for high potency and specificity to the A2 or C2 domain. Epitope groups within the domain are listed in parentheses (see [98, 100]). Cross-reactivity to An-FVIII is defined as the percent absorbance compared to hFVIII. B) The crystal structure of the hFVIII A2 domain [18] is shown with the highly immunogenic region and partial MAb 4A4 epitope, residues 484-508, depicted in blue. Through alignment of epitope containing or lacking

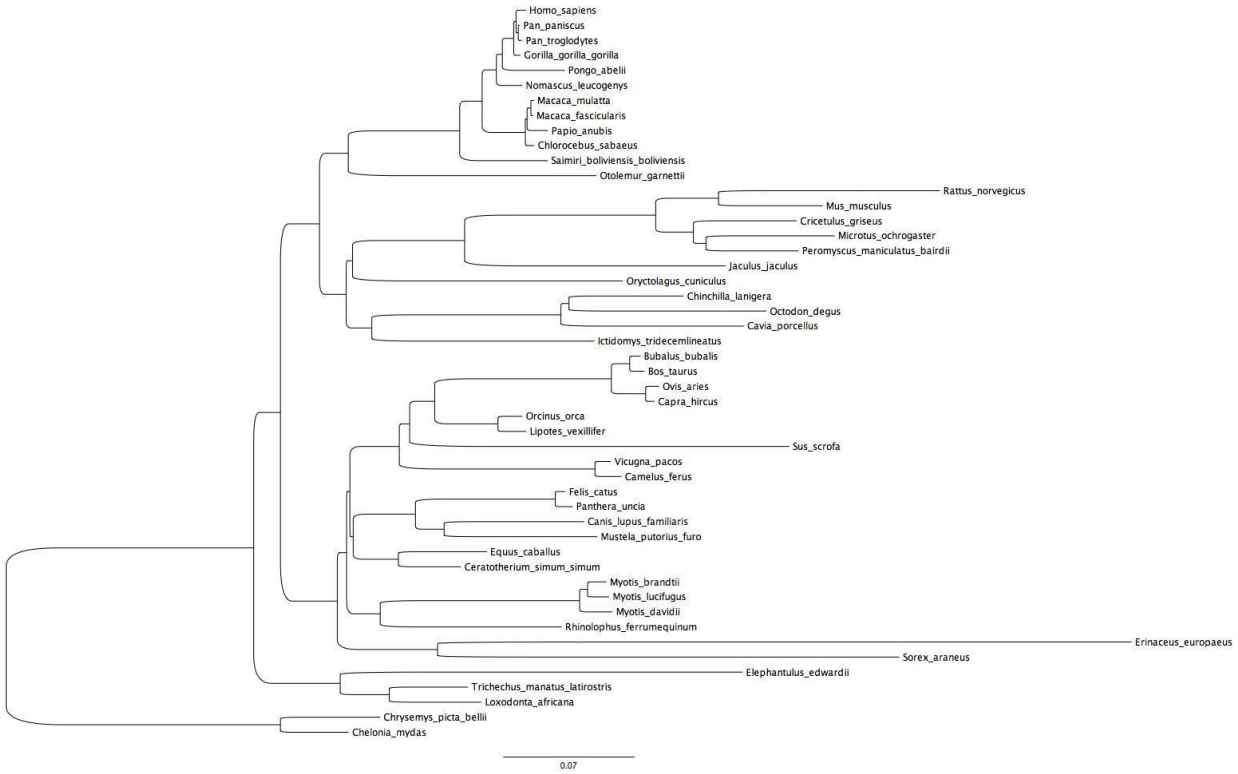
FVIII sequences, four residues are identified as potential contributors to binding energy of MAb 4A4 and labeled in red. C) Inhibitor titer of MAb 4A4 was measured by modified Bethesda assay. Dilutions of MAb 4A4 were incubated with 1 unit/mL An-53 or An-53 containing the residue substitution E434V, naturally occurring in pFVIII. Residual FVIII activity was measured by one stage coagulation assay. Inhibitor titer of approximately 40,000 Bethesda (inhibitory) units/mg is observed against An-53 while no inhibition of An-53 E434V is observed.

unlikely to contribute significant binding energy (Figure IV-3B). However, Glu434 is within close proximity to Ile508. We now demonstrate that replacement of this single amino acid, E434V, results in a >4,000-fold reduction in 4A4 inhibition (Figure IV-3C).

In the current study, we were able to identify An-FVIII molecules with predictably superior properties to current hFVIII products such as biosynthetic productivity, specific activity, cofactor stability and immune reactivity. These findings validate ASR as a tertiary approach to biopharmaceutical bioengineering through discovery of sequence-activity relationships that may have previously existed in nature. In the context of FVIII, it has been known for decades that distinct extant interspecies differences in several pharmaceutically-relevant properties exist. However until recently, the necessary protein engineering tools were not available to fully exploit this knowledge. ASR now provides a widely accessible strategy to support development of new classes of bioengineered protein replacement-based pharmaceuticals as well as probe fundamental aspects of hemostatic evolution.

Supplementary Table IV-3: qRT-PCR primers for steady-state transcript determination

An-FVIII	Forward Primer	Reverse Primer
Human	GCACAGCATCAATGGCTATGTTTT	GGGTGAGTGTGTCTTCATAGACC
Porcine	GCACAGCATCAATGGCTATGTTTT	GGGTGAGTGTGTCTTCATAGACC
ET3	GCACAGCATCAATGGCTATGTTTT	GGGTGAGTGTGTCTTCATAGACC
An-51	CAGTTCAAGAAAGTGGTCTTCCAGGAGTTTAC	CCGTGATGCCTGGTTCTTGAAGGTG
An-52	CAGTTCAAGAAAGTGGTCTTCCAGGAGTTTAC	TGATTCTTGAAGGTGACCATAATGTT
An-53	CAGTTCAAGAAAGTGGTCTTCCAGGAGTTTAC	TGATTCTTGAAGGTGACCATAATGTT
An-54	CAGTTCAAGAAAGTGGTCTTCCAGGAGTTTAC	TGATTCTTGAAGGTGACCATAATGTT
An-55	CAGTTCAAGAAAGTGGTCTTCCAGGAGTTTAC	TGATTCTTGAAGGTGACCATAATGTT
An-56	CAGTTCAAGAAAGTGGTCTTCCAGGAGTTTAC	TGATTCTTGAAGGTGACCATAATGTT
An-57	CAGTTCAAGAAAGTGGTCTTCCAGGAGTTTAC	TGATTCTTGAAGGTGACCATAATGTT



Supplemental Figure IV-S6: Phylogram of FVIII mammalian phylogeny. Scale bar represents 0.07 amino acid replacements per site per unit evolutionary time. Branch lengths leading to the two outgroup turtle species and the single rat species were shortened by 80% for scaling purposes.

Discussion

Hemophilia, if diagnosed and treated, is no longer a lethal disease. The majority of patients receiving treatment within developed countries live full and active lives. However, the dramatic limitations of existing therapeutics, including global availability, have resulted in large-scale, high-throughput research efforts by several major pharmaceutical companies. Bioengineering coagulation factors at the nucleic and amino acid level is now a major field in hemophilia research. The potential to cure hemophilia is apparent; the next decade will likely produce an FDA approved gene therapy product for both hemophilia A and B. Despite recent successes for hemophilia B patients receiving AAV gene therapy, long-lasting therapeutic FVIII treatments are not yet as advanced. Pre-clinical research for subsequent commercialization purposes hinges upon our understanding of FVIII biochemistry, adequate animal models that recapitulate the disease, and novel, rational approaches to gene/protein engineering. Our investigations into extant FVIII orthologs and ancestral FVIII contribute to and expand our knowledge of FVIII cellular biology, protein biochemistry, and molecular evolution.

The characterization of recombinant ovine FVIII for efficacy and immunogenicity is essential to preserve hemophilia A sheep as a large animal model for gene and cell therapy trials. The initial aim of Chapter II was to generate sufficient quantities of recombinant protein to maintain the hemophilia A sheep colony during pre-clinical testing absent the deleterious effects of inhibitor formation. The tolerance of hemophilia A animals to their host protein has been observed for

E16 mice [155], and the UNC Chapel Hill dog colony (although higher frequency of inhibitor formation is seen in the Queen's University dog colonies) [163, 164] and has allowed for hemostatic protection prior to a gene therapy trial [160]. These animal models are critical to the development of novel curative approaches due to their similar genetic disposition and clinical phenotype with human hemophilia A. Hemophilia A sheep display a severe bleeding phenotype with morbidity and mortality rates exceeding that of humans, predominantly due to umbilical cord bleeding upon birth and injury-prone activity [169]. Validation of this model for gene and cellular therapy has been demonstrated by mesenchymal stem cells (MSC) transplant following viral transduction [251]. Sustained levels of FVIII were achieved mitigating the need for exogenous protein infusion. Furthermore, the MSCs were shown to migrate to sites of injury within the joints and reverse the hemarthrosis. Sheep also provide a unique large animal model for novel *in utero* gene and cell based therapies [304, 305]. Sheep undergo naturally occurring changes in the primary sites of hematopoiesis, yolk sac/ fetal liver/ bone marrow, and fetal to adult hemoglobin switching, that mirror human development, establishing an excellent model for study. The potential benefits of *in utero* delivery include i) a reduced scale-up requirement due to the small size of the fetal sheep, ii) the curative potential prior to phenotypic development and bleeding complications during birth, iii) the ability to target multiple cell types prior to lineage commitment or quiescence, and iv) the potential to eliminate immune responses to FVIII due to central tolerance during immune development. Despite the potential utility of this animal model, human and porcine FVIII cannot prolong survival of the colony due to the high incidence of inhibitor formation [251]. Without the development of host oFVIII for immunogenicity testing, this key animal model may not persist.

Currently, investigations into the ability of ovine FVIII to correct the bleeding phenotype in hemophilia A sheep are in early stages, and thus no information regarding the immunogenicity of the host protein is available. However, it has been empirically observed during the formation of murine anti-ovine MAbs that immunization of hemophilia A mice required a greater number of exposures to oFVIII to generate mice with elevated ELISA and inhibitor titers compared to previous human and porcine immunizations. Furthermore, the genetic causation of the bleeding disorder in sheep is due to a premature stop codon in exon 14 which may allow the generation of partial translation products of the heavy chain. These partial peptides, if synthesized, may provide central tolerance to key epitopes within the A1 and A2 domains. Together this suggests that the immunogenicity of oFVIII is a potential area of investigation and may demonstrate success in the treatment of the hemophilia A sheep colony.

Similar to mouse, canine, and porcine FVIII, the characterization of the roFVIII protein has led to discovery of conserved biochemical properties not observed in rhFVIII. RoFVIII was shown to have several characteristics with potential therapeutic utility including increased biosynthesis, specific activity, and stability following thrombin. In a severe hemostatic challenge via tail transection, roFVIII was shown to be efficacious in hemophilia A mice at doses similar to rh- and rpFVIII. Similar to rpFVIII, oFVIII demonstrated reduced cross-reactivity to anti-human inhibitors, a characteristic pursued in Chapter III. This was observed with a panel of well characterized mouse anti-human monoclonal antibodies (MAbs) [98, 100], as well as patient plasma samples collected from the Emory IRB approved inhibitor bank. Amino acid identity between ro- and rpFVIII is 85% and thus it is not surprising to see similarly reduced inhibitor

titers. However, it is important to note that a non-trivial 24% of inhibitor patients displayed reduced inhibition of oFVIII compared to pFVIII in a clinical assay. This finding confirms that inhibitors to human FVIII are polyclonal and patient-specific. As a result, treatments must also be specifically tailored to the patients. Currently, several microarrays and ELISA-based assays are being developed for the clinical determination of inhibitor paratopes in order to identify the best course of action for each patient (Shannon Meeks, MD, Emory University, personal communication). As a proof-of-principle, we designed a competition based indirect-ELISA using patient plasma to block FVIII epitopes. We identified the existence of antibodies that directly compete with MAbs of high inhibitor titer and these inhibitors were both polyclonal and patient-specific despite similar diagnostic parameters. Therefore, it is highly considerable that future treatment strategies will identify the inhibitor types within a patient and administer FVIII treatments known to have reduced cross-reactivity. Due to the patient specific response towards ro- or rpFVIII, key epitope differences exist within the 15% non-identity. Because 18% of acquired hemophilia A patients receiving recombinant porcine FVIII (Obizur, Baxalta/Shire) developed de novo alloantibodies to the porcine protein, we investigated the ability of roFVIII to function in the presence of anti-porcine FVIII inhibitors. Mouse plasma immunized with rpFVIII displayed an average 31-fold reduction in inhibitor titer against roFVIII compared to rpFVIII. All 10 mice showed reduced inhibitor titers against roFVIII. Human plasma from the clinical trial using Obizur was not available for direct testing against roFVIII. Therefore, our data only suggest that roFVIII may be a potential tertiary treatment option, but this remains to be investigated.

While the characterization of orthologus FVIII has proven beneficial for the identification of biochemical properties, therapies for inhibitors, and the maintenance of the canine hemophilia colony, there is no assurance that this approach is useful for other proteins, or even other FVIII orthologs. Considering the effort and cost required to isolate genomic DNA and mRNA for the isolation of cDNA for each ortholog to characterize, this approach, without prior knowledge and rationale, may not be a worthwhile venture. Furthermore, the potentially therapeutic benefits that have been observed have proven difficult to translate into the human molecule. The enhanced stability of murine FVIIIa was observed in 2002, and no engineered molecule has identified the mechanism or replicated the stability to this degree. Similarly, the high biosynthetic efficiency of rpFVIII through diminished engagement of the UPR is due to a combination of amino acids within the A1 and A3 domains [133, 148] but further efforts to identify the responsible residues within each domain became an intractable effort and have not been solved since 2004. Difficulty in the identification of the key residues is due to the large number of substitutions despite high homology. Most FVIII molecules share amino acid identity with each other within the 80-90% range. Because of the large size of BDD FVIII, this results in 145-291 potential residues that may explain differential biochemistry.

FVIII is not an ideal candidate for rational design based engineering efforts due to the lack of structural information. There are only two crystal structures solved for human FVIII with resolutions of 3.7 and 3.98 Å, respectively [18, 20]. There are currently no crystal structures of FVIIIa due to its instability. Directed evolution approaches are not feasible for discovery due to the high probability of inactive mutants as well as the large quantity of recombinant protein

needed. To address this lapse in knowledge driven engineering approaches, we employed ancestral sequence reconstruction to identify the incremental changes that occurred throughout molecular evolution. Our findings answer key questions regarding the species-specific characteristics observed previously, as well as the potential mechanisms for each attribute.

The *F8* gene is located at the termini of the long arm of the X chromosome and therefore has an increased likelihood of recombination events. This is one possible explanation for the expansive diversity of mutations which result in deficient or inactive FVIII levels in circulation resulting in the disease phenotype. We hypothesized that natural selection has acted upon the hemostatic system to provide a balance between hemostasis and thrombotic risk. Because of the unique hemostatic requirements for each mammalian organism, i.e. size, posture, pedalism, metabolic rate, blood volume, etc. we further proposed that conserved properties of FVIII could be traced to a common ancestor. Through our investigation utilizing ASR, we have discovered that increased biosynthetic efficiency was commonly observed within early ancestral sequences and along the primate lineage until more recently (19 mya). The residues responsible for this have not yet been identified, nor has the mechanism. Because a distinct change in biosynthetic efficiency per transcript was observed between An-56 to An-57, it is hypothesized that the amino acid changes in An-57 reduced post-transcriptional efficiency in a manner similar to that observed with rpFVIII [133]. The low biosynthetic efficiency of FVIII sequences within the carnivore and ungulate lineages is consistent with current information regarding endogenous coagulation factor levels within current species. However, the rationale for the increased biosynthesis of An-68 remains unanswered. An-53, the common ancestor to both primates and rodents, displays high FVIII biosynthesis. Ancestral sequences 65, 66, and 67 display low biosynthesis, suggesting that mutations in the *F8* gene during the divergence of rodents and

primates resulted in reduced FVIII production, and this reduced production persisted through subsequent divergent events. Considering that An-67 and mouse FVIII are biosynthetically poor, and that An-68 is an intermediary node, the high biosynthesis of An-68 is likely to have persisted to a subsequent ancestor or modern species. Therefore, we may hypothesize that either the rat or Chinese hamster FVIII sequence is likely to display similar increased biosynthetic efficiencies for FVIII. Of note, several current recombinant products for the treatment of hemophilia, including Recombinate®, Advate®, ReFacto®, Turoctocog alfa®, and Xyntha®, are generated by Chinese hamster ovary (CHO) cells because of their observed increased FVIII production.

Following a similar rationale with regard to FVIIIa stability, the previously observed increases in FVIIIa half-life in mouse, sheep, dog, and pig could be explained by similar increased stability in their common ancestor, An-52. However, An-52 demonstrates a rapid half-life identical to human FVIII, and therefore our hypothesis ‘evolved’ to predict two independent selection events to account for the observed differences in modern species. Along the rodent lineage, early data from the first 8 sequences investigated showed that An-65 was also less stable following activation whereas An-68, however, demonstrated the longest observed FVIIIa half-life of almost 16 minutes. Therefore, the emergence of increased stability must have occurred in the transition from An-65 to An-66 or An-66 to An-67, either incrementally or en masse. Our data demonstrate that the transition from An-65 low stability to An-68 high stability was the result of two incremental changes, between An-65 to An-66 and more significantly, from An-67 to An-68. Given this information, it would be interesting to investigate the stability of rat and Chinese hamster FVIII to determine if this characteristic persisted. Looking to the carnivore and ungulate

lineage, differential stability was observed for An-76 and An-78. Based on the phylogenetic tree, the common ancestor to dog, sheep, and pig, all orthologs with similar intermediary stabilities, is An-77. Therefore, we hypothesized *a priori* that this sequence would demonstrate increased FVIIIa stability with the rationale that a single phenotypic emergence is more likely to explain the observed phenotype within descendants than multiple events giving rise to the same phenotype. Our hypothesis was supported, with An-77 presenting as the first sequence with enhanced stability along its phylogenetic branches.

As a platform for bioengineering, ASR reveals the incremental amino acid changes within branches of a phylogeny that result in a particular phenotype, dramatically reducing the number of residues to investigate. For example, the high expression human/porcine hybrid ET3 contains porcine A1 and A3 sequences resulting in 138 porcine residues (9.5%). The ability to further refine the minimal porcine residues responsible for high biosynthesis is not cost-effective by mutagenesis studies. However, An53-56, but not An-57 or human, display high FVIII biosynthetic efficiency. Strikingly, the number of residue changes between An-56 and An-57 is only 5. These 5 residues are being actively investigated for potential mechanism into enhanced biosynthesis. Similarly, with regard to FVIII stability, the number of residue changes giving rise to the increased stability of An-66 and An-77 from their preceding ancestor are only 4, and 5 respectively. Interestingly, these 9 mutations are non-overlapping and suggest different mechanisms occurred through independent evolution to generate a similar phenotype. A possible explanation is the positive selection of FVIIIa stability despite differing mutations due to a conserved evolutionary pressure.

The discovery and refinement of anti-human inhibitors could be advanced as a result of these studies. We identified a single residue functional epitope for the entire inhibitory effect of the most potent monoclonal antibody. The use of orthologous FVIII antigenicity information absent that of ancestral sequences could not have identified residue 434 as the major inhibitory epitope of 4A4. Similarly, the use of ancestral FVIII sequences alone could not, at present, identify this epitope either. The combination of both ancestral and extant FVIII sequences, in this case, was required and necessary for rational engineering of FVIII. Moving forward, analysis of the gain or loss of epitopes throughout phylogenetic branches will again provide a limited number of residues to investigate. Coupled with the existing knowledge of the FVIII domain possessing the epitope through homolog scanning [106, 109, 289], as well as the shared epitopes between similar MAbs [98, 100], the identification of functional epitopes for each MAb can be resolved to the amino acid level.

The utility of ASR provides not only a platform for future bioengineering efforts, but also the discovery of potentially translational cDNA and protein products. The greatest limitation in curative efforts for hemophilia A is the low biosynthesis of FVIII at clinically tolerated doses of nucleic acid transfer. Efforts to bioengineer novel FVIII sequences have produced modest increases in FVIII production with ET3 demonstrating the highest *in vivo* production following a lentiviral gene therapy protocol [151]. We demonstrated that an ancestral species with 95% identity to human FVIII, An-53, generates significantly higher circulating FVIII levels than the state-of-the-art ET3 in a mouse hemophilia A model. Furthermore, these increased levels were unchanged at a 4-fold lower dose while ET3 demonstrated an expected dose-response (data not

shown). This data suggests that lower nucleic acid transfer levels of An-53 are sufficient to generate therapeutic levels of FVIII in vivo. As a result, the viral requirement needed to deliver a reduced number of transgenes may also be lessened, thereby improving safety, reducing production cost, and increasing availability.

These studies provide the basis for future studies to identify mechanisms of increased biosynthesis, FVIIIa stability, and immunogenicity. The results of these studies can be used to engineer novel molecules for improved protein replacement therapies as well as gene therapy. Currently, human/ancestral and ancestral/ancestral hybrid molecules are being generated and tested for predicted biochemical properties. Studies into the immunogenicity of these ancestral constructs and the refinement of anti-human inhibitors are underway. Purification of An-53 has resulted in highly concentrated fractions (5.75mg/ml) that remain soluble without the addition of surfactants. Through collaboration, we are pursuing a crystal structure for this molecule to enhance our understanding of FVIII structure/function. Finally, both AAV and lentiviral mediated gene transfer techniques are being investigated using ancestral sequences An-53-57 compared to human and ET3 FVIII. We predict that these studies will dramatically revolutionize our understanding of FVIII, and result in improved therapeutics for the treatment and potential cure for hemophilia A.

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