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Philip Zakas\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ \_Jan 30, 2016\_\_\_\_\_\_\_\_

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Evolutionary approaches to coagulation factor VIII biopharmaceutical engineering

By

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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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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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**Table of Contents**

* Introduction ……………………………………………………………………………… 1
* Chapter I Engineered Hematopoietic Stem Cells as Therapeutics for

Hemophilia A (Published)……………………………………………………………… 29

* Chapter II: Development and Characterization of Recombinant Ovine

Coagulation Factor VIII (Published)

* + Abstract…………………………………………………………………………. 53
	+ Introduction ……………………………………………………………….……. 55
	+ Materials and Methods………………………………………………………...... 57
	+ Results …………………………………………………………….……………. 65
	+ Discussion ……………………………………………………………………… 78
* Chapter III: Expanding the Ortholog Approach for Hemophilia

Treatment Complicated by Factor VIII Inhibitors (Published)

* + Summary ……………………………………………………………………..… 84
	+ Introduction ……………………………………………………...…………...… 86
	+ Materials and Methods …………………………………………………………. 89
	+ Results ………………………………………………..………………...………. 93
	+ Discussion …………………………………………………..………………… 110
* Chapter IV: Bioengineering Coagulation Factor VIII through Ancestral

Sequence Reconstruction

* + Materials and Methods ………………………………………………...……… 114
	+ Abstract/ Introduction/ Results ………………………………………….……. 120
* Discussion ………………………………………………….…………………………. 145
* References ……………………………………………………………………......…… 155

**Figures and Tables**

Figure i-1: The coagulation cascade ……………………………………………….……………..4

Table I-1: Technologies for HSCT gene therapy …………….….….…….………………….… 34

Figure II-1. Expression of recombinant ovine and human FVIII …………………….….….…. 66

Table II-1. Purification of BDD oFVIII …...…………………………………………………… 68

Figure II-2. Biochemical analysis of BDD oFVIII .………………………………………….… 69

Figure II-3. Discrimination of the BDD oFVIII heavy and light chains ………………………. 71

Figure II-4. Thrombin-activated decay rate of oFVIIIa .…………….…………….…………… 73

Figure II-5. BDD oFVIII binding to VWF …………………………….………………………. 75

Figure II-6. In vivo efficacy of oFVIII in hemophilia A mice .….….…………………………. 77

Figure III-1A-C. Antigenicity and inhibitor titers for inhibitor patient plasmas ……….……… 94

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

patient plasmas ………………………………………………………………..... 99

Figure III-2. Identification of A2 and C2 domain epitopes targeted by patient plasmas .….…. 100

Figure III-3. Reactivity of anti-hFVIII A2 and C2 domain MAbs with FVIII orthologs …..… 102

Table III-2. Inhibitor titers of anti-human FVIII MAbs against ovine and porcine ……….…. 103

Figure III-4. Reactivity of anti-rpFVIII MAbs with roFVIII …………………………………. 105

Figure III-5. Inhibitor titers in pre-immunized murine hemophilia A plasmas ………………. 106

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

acquired hemophilia A ...……………………………………………...………. 109

Figure IV-1: FVIII phylogenetic tree and expression analysis ……………...…….……..…… 124

Supplemental Figure IV-S1: SDS-PAGE analysis of An-53 and An-68 following

two-step ion-exchange…………………………………………………..…….. 128

Supplemental Table IV-1. ED50 determination of An-53 and An-68 by Dixon

up-and-down method ………..………………………………………….…….. 130

Supplemental Figure IV- S2: Schematic of linear AAV expression cassette

for hydrodynamic injections ………………………………………………….. 131

Supplemental Figure IV-S3: DNA quality determination of ancestral FVIII cDNA ………… 132

Supplemental Figure IV-S4: DNA quality determination of human and ET3 FVIII cDNA .… 133

Supplemental Figure IV-S5: FVIII plasma levels following hydrodynamic injection ……….. 134

Figure IV-2: An-FVIII activation and stability …………………...……………………………136

Supplemental Table IV-2 ………………………………………………………………………138

Figure IV-3: An-FVIII immune recognition and bioengineering …………………...…………140

Supplementary Table IV-3: qRT-PCR primers for steady-state transcript determination …….143

Supplemental Figure IV-S6: Phylogram of FVIII mammalian phylogeny ………………..…..144

**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