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Infusing Factor Viii-Expressing Platelets Or Megakaryocytes As A Novel Therapeutic Strategy For Hemophilia A

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Abstract

Approximately 1:5000 males have the most common inherited form of severe bleeding, hemophilia A, a deficiency of functional coagulation factor VIII. Patients with severe hemophilia A suffer from recurrent bleeding with significant morbidity and mortality with 20-30% of these patients developing antibodies to infused Factor (F) VIII therapy. One area of on-going research for treatments for these patients is ectopically expressing FVIII in megakaryocytes and platelets. This FVIII, termed pFVIII, is stored in alpha granules of platelets and is capable of restoring hemostasis in FVIII^{null} mice, even in the presence of circulating inhibitors. pFVIII has been proposed to be used for gene therapy for patients with hemophilia A, intractable inhibitors, and life-threatening bleeds. However, prior studies by us have shown that high levels of pFVIII can injure developing megakaryocytes. Combined with the known risk of prolonged thrombocytopenia following bone marrow transplantation, this may limit its utility of this strategy. Because of these limitations, we now propose an alternative therapeutic pFVIII strategy of infusing pFVIII-expressing megakaryocytes or platelets. We envision that such a product would be generated beginning with induced-pluripotent stem cells (iPSCs). iPSC-derived megakaryocytes, termed iMks, that are modified to express pFVIII may then be used to improved hemostasis in problematic inhibitor patients with hemophilia A. As proof-of-principle, we demonstrate that improved hemostasis can be achieved in vitro and in vivo with human pFVIII-expressing murine platelet. Infusion of such platelets can provide several days of improved hemostasis in FVIII^{null} mice. They were effective in the presence of inhibitors, and the efficacy of pFVIII was enhanced by recombinant factor VIIa. Human pFVIII-expressing iMks also improved hemostasis in vitro and derived platelets from infused human pFVIII-iMks improved hemostasis in FVIII^{null} mice. These studies indicate the potential therapeutic use of recurrent pFVIII-expressing megakaryocyte or platelet infusions with prolonged hemostatic coverage that may be additive with present-day bypassing agents in hemophilia A patients with clinically relevant neutralizing inhibitors.

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**INFUSING FACTOR VIII-EXPRESSING PLATELETS OR MEGAKARYOCYTES
AS A NOVEL THERAPEUTIC STRATEGY FOR HEMOPHILIA A**

Randolph B. Lyde Jr.

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DEDICATION

I dedicate this thesis to my parents, Randolph and Cynthia Lyde, who have always supported me and have given me the opportunity to follow my dreams.

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ABSTRACT

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Randolph B. Lyde Jr.

Mortimer Poncz M.D.

Approximately 1:5000 males have the most common inherited form of severe bleeding, hemophilia A, a deficiency of functional coagulation factor VIII. Patients with severe hemophilia A suffer from recurrent bleeding with significant morbidity and mortality with 20-30% of these patients developing antibodies to infused Factor (F) VIII therapy. One area of on-going research for treatments for these patients is ectopically expressing FVIII in megakaryocytes and platelets. This FVIII, termed pFVIII, is stored in alpha granules of platelets and is capable of restoring hemostasis in FVIII^{null} mice, even in the presence of circulating inhibitors. pFVIII has been proposed to be used for gene therapy for patients with hemophilia A, intractable inhibitors, and life-threatening bleeds. However, prior studies by us have shown that high levels of pFVIII can injure developing megakaryocytes. Combined with the known risk of prolonged thrombocytopenia following bone marrow transplantation, this may limit its utility of this strategy. Because of these limitations, we now propose an alternative therapeutic pFVIII strategy of infusing pFVIII-expressing megakaryocytes or platelets. We envision that such a product would be generated beginning with induced-pluripotent stem cells (iPSCs). iPSC-derived megakaryocytes, termed iMks, that are modified to express pFVIII may then be used to improved hemostasis in problematic inhibitor patients with hemophilia A. As proof-of-principle, we demonstrate that improved hemostasis can be achieved in vitro and in vivo with human pFVIII-expressing murine platelet. Infusion of such platelets can provide several days of improved hemostasis in FVIII^{null} mice. They were effective in the presence of inhibitors, and the efficacy of pFVIII was enhanced by recombinant factor VIIa. Human pFVIII-expressing iMks also improved hemostasis in vitro and derived platelets from infused human pFVIII-iMks improved hemostasis in FVIII^{null} mice. These studies indicate the potential therapeutic use of recurrent pFVIII-expressing megakaryocyte or

platelet infusions with prolonged hemostatic coverage that may be additive with present-day bypassing agents in hemophilia A patients with clinically relevant neutralizing inhibitors.

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Chapter 1 – Introduction

Hemophilia A

Hemophilia A is a congenital X-linked recessive disease caused by a deficiency in functional Factor (F) VIII protein that affects 1 in 5000 males¹. While the role of FVIII deficiency in this disorder was discovered in the 20th century, the first accounts of hemophilia were written in the Talmud, dating back to 2nd century AD². The Talmud describes two male babies that died after circumcision and instructs that the third male baby not be circumcised. By the 20th century, doctors largely believed that the bleeding seen in hemophilic patients was due to weak vessels^{3,4}. It was not until the early 1900s that investigators like Sahli and Addis realized that the major hallmark of hemophilia was a delay in clotting of whole blood^{5,6}. In 1917, Addis showed that small amounts of “prothrombin” prepared from whole blood could markedly decrease the clotting defect. However, subsequent studies by Govaerts and Gratia showed that prothrombin could not be the ultimate culprit whose absence caused hemophilia⁷. By 1937, Patek and Taylor, re-examined the small fraction of normal plasma used by Addis and found that this fraction contained FVIII, which they then called “anti-hemophilic globulin”, that could markedly improve the clotting defect seen in hemophilic blood⁸.

Hemophilia A results from loss or reduced function and activity of the FVIII protein due to mutations in *F8* gene¹. Mutations in *F8* gene that cause hemophilia A include: point mutations, small and large deletions, splice mutations, and inversions. The level of residual FVIII activity found in patients' blood corresponds to the severity of the disease, which ranges from mild (5-50% of FVIII activity) which makes up 25% of this patient population, moderate (1-5% of FVIII activity) affecting 10% of patients, to severe (<1% of FVIII activity) affecting 65% of hemophilia A patients^{9,10}. Nearly half of all severe

hemophilia A patients have an intron 22 inversion in their *F8* gene¹¹. Severe patients experience recurrent bleeds in their bodily cavities including their joints, muscles, and internal organs. These recurrent bleeds can ultimately lead to muscular atrophy and loss of joint support known as chronic synovitis¹². In addition, one of the major complications of severe hemophilia A is that about 30% of these patients will develop neutralizing antibodies to infused FVIII therapy, the current mainstay treatment for hemophilia A patients^{1,13}.

FVIII structure and processing (from gene to plasma)

The *F8* gene is located on the distal portion of the long arm of the X chromosome, maps to position Xq28, and covers 186kb of genomic DNA¹⁴. Until recently, the cellular origin of circulating plasma FVIII within the body was unknown. Cell isolation studies indicated that liver sinusoid endothelial cells (LSECs) were the main source of circulating plasma FVIII¹⁵⁻¹⁸. While LSECs are the primary source of FVIII within the body, *F8* mRNA is found in many cell types including monocytes and hepatocytes¹⁹. Proper mRNA and protein processing is essential for excretion and function of the protein^{1,20,21}.

The *F8* gene of this large plasma glycoprotein consists of 26 exons that range from 29 to 3,106 base pairs in length^{14,21}. Fully processed *F8* mRNA contains 9 kb of nucleotides, and in the endoplasmic reticulum (ER), it is translated into a 2,351 amino acid (267kDa) protein. This single-chain peptide contains 6 domains: 3 A-domains, 2 C-domains and one large B-domain. Domains A1, A2, and A3 vary little in size with 336, 345, and 335 amino acids respectively and are homologous to ceruloplasmin and factor

V (FV) A-domains^{22,23}. C1 and C2 domains are 155 and 152 amino acids respectively and are homologous to the C domains of FV^{24,25}. The A2 and A3 domain is separated by the large B-Domain, 980 amino acids, which is the only domain within FVIII that does not share homology with FV^{23,26}. The B-Domain makes up 40% of FVIII protein. In addition, FVIII contains 3 acidic regions, a1, a2, and a3. Upon full translation within the ER, the resulting orientation of full-length FVIII is A1-a1-A2-a2-B-a3-A3-C1-C2^{24,25}.

Inside the ER, the FVIII protein is decorated by N-glycosylation at 25 N-glycosylation sites, 19 of which are found on the B-Domain^{25,27,28}. These sites have been shown to affect overall activity of the functional protein²⁸. During its formation in the ER, large mannose branched sugars are added to the developing protein. These sugars work to stabilize and prevent aggregation of newly forming protein domains. To further facilitate proper folding, the newly formed FVIII binds to chaperone proteins: calnexin (CNX), calreticulin (CRT), or immunoglobulin binding protein (BiP)²⁹⁻³¹. FVIII shuttles between ER proteins: CNX (an ER bound protein) and CRT (a soluble protein in the ER lumen) until its final glucose is trimmed from its oligosaccharide units that are attached to FVIII's asparagine residues²¹. This CNX/CRT cycle ensures proper folding of the large protein and helps retain FVIII in the lumen of the ER until this process is complete. If this process is successful, FVIII detaches from the two lectins and is permitted to leave the ER and enter the golgi^{29,31}. If FVIII is not folded correctly it goes back to the CNX/CRT cycle until it is ultimately released to the golgi or sent to be degraded.

Inside the lumen of the ER, FVIII forms a stable complex with BiP, a key participant in the unfolded protein response pathway (UPR)³²⁻³⁴. In fact, overexpression of FVIII in cultured cells results in activation of UPR and increased expression of BiP. BiP binds to the hydrophobic beta sheet within the A1 domain, which helps retain FVIII

within the ER lumen. Mutations in these BiP binding sites have been associated with increased FVIII secretion^{35,36}.

Properly-folded FVIII binds to a cargo processing complex containing ERGIC-53 (also known as LMAN1) and Multiple Coagulation Factor Deficiency 2 (MCFD2) within vesicles in order to leave the ER and enter the golgi³⁷. Mutations in ERGIC-53 or MCFD2 result in a combined FV and FVIII deficiency disorder, which result in FV and FVIII levels ranging from 5% to 30% of normal³⁸. Upon exiting the ER, FVIII is shuttled to the golgi apparatus where it is further processed and matured. Inside the golgi, FVIII undergoes proteolysis and is further matured by key enzymes (Figure 1.1). However, before this can take place, FVIII's N-linked oligosaccharides are modified into branched, complex structures. In addition, sulfation of tyrosine residues and O-linked glycosylation of serine and threonine residues within the B-Domain are added²¹. Tyrosine sulfation is important for full FVIII activity and interaction with thrombin. In fact, mutations of 6 key tyrosines to phenylalanine have been shown to decrease the efficacy at which thrombin can cleave FVIII which results in reduced FVIII activation³⁹.

Before FVIII can be released into the plasma, it undergoes proteolysis by Paired-basic Amino acid Cleaving Enzyme (PACE/Furin) within the golgi^{40,41}. This intracellular protease cleaves proteins at arginine-X-X-arginine (R-X-X-R) motifs. Inside the golgi, PACE cleaves the FVIII B-Domain arginine residues 1313 and 1648 to release the heavy 200kD (A1-a1-A2-a2-B) and light 80kD (a3-A3-C1-C2) chains of FVIII^{40,42,43}. After cleavage by PACE, FVIII is excreted from the cell into the plasma in this inactivated form. This heterodimer is held together by coordinated metal ions. Both copper and calcium or manganese ions are essential for association of the heavy and light chains as well as pro-coagulant function^{44,45}. While calcium or manganese do not contribute to the

two chains maintaining a dimeric structure, they are important for proper configuration and protein function^{44,45}.

Copper on the other hand, is essential for proper heavy and light chain dimerization of the inactive and active forms. FVIII copper binding pockets are located within the A1 and A3. Mutations in the copper-binding site C310F result in severe hemophilia^{46,47}.

Once inside the blood stream FVIII circulates in its inactivated form at a concentration of 100-200 ng/ml with ~96% of circulating FVIII bound to its carrier protein von-Willebrand factor (VWF), synthesized and secreted by endothelial cells^{48,49}. FVIII's half-life (~11 hrs) is largely associated with the half-life of VWF⁵⁰. Patients with severe VWF disease type 3 have low levels of both vWF and FVIII as the half-life of the free FVIII is ~2 hours⁵¹⁻⁵³. VWF binds to the C2 and acidic a3 FVIII domains. This VWF-FVIII interaction works to stabilize FVIII and prevent untoward activation and unintended thrombosis by preventing FVIII from binding to phospholipids on activated platelets⁵³. The VWF-FVIII complex also prevents FVIII proteolytic degradation by activated Protein C (aPC) and activated Factor X (FXa)⁵⁴⁻⁵⁶. Additionally, VWF provides FVIII to sites of vascular injury by binding activated platelets to vascular sub-endothelium^{57,58}.

The non-covalent VWF-FVIII interaction is disrupted after FVIII is activated by thrombin (FIIa). FIIa cleaves FVIII at three arginine (Arg) sites: Arg372, Arg740, and Arg1689⁵⁹. The first cleavage separates the A1 from the A2 domain. The second separates the A2 from the B domain and final cleavage cuts the acidic a3 domain releasing FVIII from VWF to form the activated heterotrimer (FVIIIa). After FVIII is activated and dissociates from vWF, FVIIIa is able to participate in the coagulation cascade^{60,61}. FVIIIa is short-lived, being unstable, its A2 dissociates rapidly⁶². In addition, FIIa, aPC and FXa tightly regulate its activity and inactivation through proteolysis (Figure

1.2). Ultimately hepatic elimination of FVIIIa is accomplished by low-density lipoprotein receptor-related protein (LRP), which binds to the dissociated A3, C2, and A2 domain to ensure efficient clearance⁶³. These interactions are mediated by heparin sulfate proteoglycans⁶⁴.

Coagulation cascade and FVIII co-factor activity

Hemostasis is a delicate and intricate balance of clot formation and lysis, involving a myriad of key blood proteins that regulate this process. In response to vascular injury, a series of serine proteases perform proteolytic cleavages to activate platelets and other blood components to promote blood coagulation and stop excessive bleeding. Activated thrombin (FIIa) sits at the center of this important physiological process⁶⁵. Multiple feed-forward loops ensure that sufficient amounts of FIIa are able to induce the activation of coagulation factors, leading to the formation of stable platelet-fibrin plug at the site of vascular injury. Two pathways (intrinsic and extrinsic) converge to ensure that sufficient amounts of FIIa are amplified⁶⁵. FVIII is an important component of the intrinsic pathway (Figure 1.3).

Upon damage to the vascular wall, tissue factor (TF), a major component of the subendothelium is exposed to blood circulation. FVIIa binds to TF to activate FX which with FVa forms the prothrombinase complex (FXa:FVa). This complex is essential for converting inactive thrombin (FII) into FIIa. FIIa is then able to influence its own activation by converting factors VIII, V, VII, and XI into their active forms. FIIa also tightly regulates the inactivation of these proteins through direct proteolysis of cofactors as well as activation of aPC. FIIa also activates platelets; it is on the surface of these activated

platelets that FVIIIa binds to FIXa to exert its full co-factor activity. The binding of FVIIIa to FIXa forms the Xase complex (FVIIIa:FIXa), which converts inactive factor X (FX) to FXa at a rate of 5 orders of magnitude higher than FIXa alone. This FXa is then able to bind to more FVa to produce more FIIa as a feed-forward cycle that eventually leads to a stable platelet-fibrin plug⁶⁵.

Role of platelets in hemostasis

Platelets have been implicated in a myriad of important bodily functions ranging from wound repair, angiogenesis, to inflammation, but their main function is in hemostasis⁶⁶. Platelets are anucleated cell fragments that are capable of forming aggregates in response to vascular injury. Platelets can range from 2-5 μm in size and circulate at a concentration of 150-450K particles per microliter of blood in healthy individuals^{67,68}.

Platelets are cytoplasmic fractions of megakaryocytes. While megakaryocytes make up less than 0.1% of nucleated cells in the marrow, they are the largest cells within the bone marrow niche, growing up to 100 μm in diameter. Some reports indicate that each megakaryocyte can make up to 10^4 platelets^{69,70}. The process by which these cells make platelets has been an area of rigorous study. After maturation, the megakaryocytes become polyploid through a process known as endomitosis. In addition, megakaryocytes develop a demarcation membrane system that functions as an intricate membrane system that allows for proplatelet formation⁶⁸. These mature megakaryocytes then extend their proplatelets, long strands of megakaryocyte cytoplasm, into the sinusoidal vessels of the bone marrow niche where they can shed mature functioning platelets. The process from megakaryocyte to proplatelet to mature

platelet is a complex one that involves many transcription factors and subcellular players⁶⁸. Recent studies have shown that megakaryocytes migrate from the marrow to the lungs intact and shed platelets in that microvascular system⁷¹.

Once platelets are released into the circulation, they travel in the blood stream as non-activated discoid particles that have a half-life of ~10 days⁷². Endothelial cells act as a physical barrier, in part, to prevent platelet attachment to the subendothelium. In addition, the endothelial layer releases factors to keep platelets quiescent. Both endothelium derived relaxing factor (Nitric Oxide, NO) and prostacyclin (PGI₂) released from endothelial cells work to prevent platelet adhesion, activation, and aggregation⁷³. Upon vascular disruption, platelets become activated to prevent excessive bleeding.

One key activator of platelets and initiator of clot formation is FIIa, which is present in high amounts upon exposure of TF and initiation of the coagulation cascade. FIIa activates platelets by cleaving the tethered ligands on protease-activated receptors 1 (PAR-1) and 4 (PAR-4) on the platelet surface. Cleavage of these G-coupled protein receptors (GPCRs) activate downstream second messengers that cause conformational and cytoskeletal changes that allow platelets to better adhere to vWF, collagen, and other platelet receptors to form a tight plug at the site of vascular injury⁷³.

These cellular signals also increase intracellular Ca²⁺ concentration within platelets which allows for the release of their content from platelet granules^{74,75}. Platelets have three distinct granules within their cytoplasm: alpha (α) granules, dense (δ) granules, and lysosomes (λ). These granules contain different proteins and small molecules either built up during the life span of the megakaryocyte or taken up from the blood stream. α-granules contain a wide array of proteins both made in the megakaryocyte or taken up in circulation including vWF, FV, and the platelet-specific C-X-C motif chemokine ligand 4 (CXCL4) also known as platelet factor 4 (PF4)^{76,77}. δ-

granules contain smaller molecules like serotonin, divalent metal cations Mg^{2+} and Ca^{2+} , and ADP and ATP. In addition, platelets release thromboxane A2 (TXA2), which binds to GPCRs on activated and surrounding quiescent to further act as a feed-forward mechanism of activation that further increases intracellular Ca^{2+} .

Key glycoprotein receptors on the platelet surface are imperative for proper activation and adhesion to other platelets and injured endothelium. Nearly 25,000 copies of the glycoprotein (GP) $Ib\alpha/\beta$ -IX (GPIb-IX-V, CD42) receptor are found on the platelet surface, making it the second most abundant receptor on platelets^{78,79}. GPIb-IX-V binds to vWF released from endothelial cells to slow platelets from circulation and attach them to damaged endothelium, and collagen in a process called adhesion⁷⁹. GPIIb-IIIa (αIIb , CD41) is essential in platelet-to-platelet binding, mediated by fibrinogen linkages in a process called aggregation. Both ADP and TXA2 induce a conformational change in αIIb - $\beta 3$ that aids in its binding to fibrinogen and thus other platelets to form compact plugs. About 80,000 copies of the αIIb - $\beta 3$ receptors decorate the platelet surface, making it the most abundant platelet receptor⁸⁰.

Platelets can even be used as a delivery system for potential therapeutics. Quebec platelet disorder (QPD), a rare autosomal dominant platelet disorder where urokinase-type plasminogen activator (uPA) is ectopically expressed in platelet α -granules, causes a mild bleeding diathesis in affected individuals⁸¹. Our group, created a QPD transgenic mouse where uPA is ectopically expressed in platelets using the *Cxcl4* promoter. Infusing these platelets into WT mice at 5% circulating platelets was able to prevent full thrombus formation in $FeCl_3$ carotid injury assays⁸². These experiments demonstrate that platelets can be used as a delivery system for therapeutic agents in order to treat disease.

FVIII variants

Ever since the successful cloning of the *F8* gene in 1984 by several groups¹⁴, researchers have tried to modify the gene and protein to ensure better intracellular processing, excretion, half-life, and activity of recombinant FVIII. In 1992, full-length recombinant FVIII was approved and came onto market for treatment of patients. The full function of the FVIII B-domain is not currently known, however it has been speculated that it could play a role in FVIII synthesis, quality control, secretion, plasma activation, platelet binding, and even clearance²⁸. In the mid 1990's, scientists discovered they could delete the B-domain in FVIII to ensure more efficient cloning of the gene while sparing functionality and half-life of the protein^{83,84}. To improve production and yield of the protein, scientists and companies have removed the B-domain. By 2000, B-domainless FVIII was available for clinical use^{85,86}.

Our studies utilize variations of the B-domain deleted FVIII mentioned above. The first is a human B-domainless FVIII (hBDFVIII) that contains a residual 14 amino acid peptide from the B-domain that links heavy and the light chain and allows for proper processing by PACE/Furin enzyme. This previously described modification has minimal effect on circulation half-life and activity of FVIII⁸⁴. The other FVIII variants utilized in our studies have a mutation in the PACE/Furin cleavage site within this residual 14-amino acid domain that prevents PACE/Furin from cleaving the single chain protein into the heavy and light chain, hBDFVIII^{R1645H (RH)}, and FVIII^Δ⁴². hBDFVIII^{RH} has a mutation in the furin cleavage site while FVIII^Δ has a 4-amino acid deletion from amino acids 1645-1648 of the entire PACE/Furin recognition sequence. These mutations cause the resulting

FVIII to be predominately expressed as a single chain which has been shown to cause a delay in A2 domain dissociation and therefore increased biological activity^{42,87}.

Previous studies by Dr. Sabatino showed that canine (c) BDFVIII was secreted primarily as a single chain when expressed in baby hamster kidney (BHK) cells. In addition, this cBDFVIII was more stable and had higher biological activity. Interrogation of its amino acid sequence revealed one key difference in its PACE/Furin cleavage site when compared to hBDFVIII^{42,88}. cBDFVIII contained a histidine at amino acid 1645 (HHQR) instead of an arginine like in humans (RHQR)⁸⁸. Further studies of cBDFVIII indicated that it was this amino acid difference that allowed for suboptimal cleavage by PACE/Furin and the excretion of this protein in its single chain form resulting in higher biological activity⁸⁸. Taking these cues from cBDFVIII Sabatino and colleges developed hBDFVIII^{RH}.

FVIII inhibitors

One major complication of FVIII therapy in severe hemophilia A patients is the development of alloimmune neutralizing antibodies (inhibitors) to FVIII. Inhibitors are a mixed composition of immunoglobulin G (IgG) subtypes 1-4 with IgG₄ being the most prevalent. These inhibitors are broadly classified into two types based on effects on FVIII. Type 1 completely inhibits FVIII activity through second-order kinetics. Type 2 partially inhibits FVIII activity. Type 1 inhibitors are more prevalent in patients with severe hemophilia, while type 2 is more common in mild and acquired hemophiliacs⁸⁹. Inhibitors are detected and quantified via Bethesda assay or the Nijmegen-modified Bethesda assay⁹⁰. These assays only detect inhibitors that reduce FVIII activity. Each assay uses serial dilutions of inhibitor plasma incubated with normal plasma. A Bethesda

unit (BU) is the amount of inhibitor to reduce FVIII activity level by 50%, this measurement is on log scale. Patients with a BU titer of 5 or below are considered low titer, a BU of greater than 5 is considered high-titer⁹¹.

Twenty-Thirty percent of patients with severe hemophilia A develop inhibitors. The risk of developing inhibitors is highest 10-15 days after first exposure to FVIII¹³. Specific genetic mutations are correlated to higher risk of inhibitor formation. Patients with large FVIII deletions have the highest rate of inhibitor formation. In contrast, patients who have mutations that result in loss of function, but where their FVIII retains the majority of its structure have lower risk of inhibitor formation. Race and ethnicity can also play a role in inhibitor formation. African-Americans and Hispanics have higher rates of developing an inhibitor than other ethnic groups^{92,93}.

Many patients that develop inhibitors respond to immune tolerance induction (ITI) with FVIII. ITI is accomplished through high dose (>200 IU/kg/day in some protocols) infusions of FVIII twice a day, once a day, or every other day depending on the protocol^{94,95}. Newer ITI protocols have included immunomodulating/immunosuppressive therapies like cyclophosphamide in combination with high-dose FVIII⁹⁶. However, a significant number of patients do not respond and have persistent high-titer intractable inhibitors with recurrent, sometime life-threatening bleeds⁹⁵. Bypassing agents, such as activated prothrombin complex concentrate (FIEBA), recombinant FVIIa, or potentially emicizumab (ACE910), which heterodimerizes FIX and FX are not always effective, have short half-lives (FIEBA and FVIIa)⁹⁷, or have a prothrombotic risk (all three)^{97,98}.

Induced pluripotent stem cells (iPSCs) and hematopoietic applications

We utilized iPSCs to derive megakaryocytes and platelets. iPSCs were obtained by reprogramming somatic cells either from peripheral blood of patients or healthy donors and transducing them with a set of stem cell transcription factors. In 2006, Takahashi and Yamanaka, successfully reprogrammed adult mouse fibroblasts into iPSCs through viral transduction with transcription factors Oct-4, Sox-2, Klf4 and c-Myc. The subsequent cells had similar morphology and subcellular characteristics as embryonic stem (ES) cells^{99,100}.

One important characteristic that these iPSCs share with ES cells is that they are pluripotent, being able to give rise to a myriad of cell types. When subcutaneously injected into mice, iPSCs form teratomas. These tumors developed cells from all three germ layers: ectoderm, mesoderm and endoderm⁹⁹. This quality of iPSCs meant they had the potential to be differentiated into many different cell types. In our case, we showed that these cells could be differentiated into the hematopoietic lineage. Previous studies by our group showed that iPSCs could also be gene manipulated to carry transgenes for enhanced green fluorescent protein (eGFP), Friend leukemia integration 1 transcription factor (FLI-1), and $\alpha\text{IIb-}\beta\text{3}$ under the control of the murine *Gp1ba* promoter. Upon differentiation into megakaryocytes and platelets, the transgene of interest was expressed at high levels. Differentiation into other lineages did not lead to expression of the protein^{101,102}.

In addition, these cells are able to self-renew indefinitely⁹⁹. This allows researchers to have a near-unlimited supply of starting material to study complex and rare diseases. Even more important in the case of hemophilia A therapy, one would only need to remove and reprogram cells once and have a limitless supply of transfusable material for the patient at will. Additionally, iPSCs have the therapeutic advantage of

potentially being derived from patients' own cells. This could ultimately limit the risk of graft-vs.-host disease and eliminate potential immune rejection.

Platelet gene therapy for hemophilia A

Gene therapy is the transduction of normal or modified genes into a patient's cells to treat a disease. Ideal gene therapy for patients with hemophilia A would involve long-term correction of plasma factor (F) VIII levels by establishing FVIII expression in tissues that physiologically express this protein^{103,104}. Progress has been made to this end; however, if the patient has a pre-existing inhibitor, plasma correction is not likely to be effective with the following caveat: if plasma FVIII's continued expression suppresses inhibitor formation or if the immune suppression used in some forms of gene therapy (e.g., bone marrow engraftment) is successful in eliminating the inhibitor, then such gene therapies may be useful^{105,106}.

Previous studies by our laboratory and others have demonstrated that ectopically expressing FVIII within platelets (pFVIII) could be a viable alternative to plasma correction for effective therapy in patients with significant hemophilia A and intractable inhibitors¹⁰⁷⁻¹⁰⁹. Through several transgenic mouse models of expressing pFVIII, these groups were able to demonstrate that this approach differs from plasma correction: pFVIII expressed in platelet α -granules and is released only upon platelet activation and degranulation¹¹⁰. Thus the pFVIII would be localized to sites of bleeding, potentially making it more efficacious at lower doses^{108,109}. Storing FVIII within platelets would also extend its circulatory half-life to that of the half-life of platelets, ~10 days in humans¹¹¹.

Finally, the sequestered pFVIII may be protected from inhibitors until released within the clot and be especially effective in patients with inhibitors.

Patients can receive pFVIII in two ways. The first, and most direct approach being pursued by my group and others, is long-term expression through permanent, genetic modification of hematopoietic stem cells (HSCs) so that FVIII is specifically turned on in megakaryocytes and platelets. HSCs have the capability of differentiating into all blood lineages, most importantly megakaryocytes and platelets¹¹². In addition, HSCs have the ability to self-renew and can continue to make megakaryocytes expressing pFVIII for life. This long-term approach would be accomplished through autologous bone marrow transplant (BMT). The second approach is accomplished through infusions of modified platelets derived from HSCs. These HSCs can be obtained from a myriad of sources including peripheral blood mobilized adult bone marrow¹¹³, from umbilical cord blood, from fat cells, or from iPSCs¹¹⁴. All can be genetically modified to express pFVIII upon differentiation into megakaryocytes, but only cells like cord blood expanded with valproic acid¹¹⁵, fat cells^{116,117} and iPSCs can be markedly expanded to have practical utility.

Previous studies have used tissue-specific promoters to target FVIII transgene expression to megakaryocytes and platelets. This may have the advantage of reducing potential immune response to FVIII leaking out of other cell lines at low levels, preventing the further development of inhibitors. These promoters include but are not limited to *GPIBA*¹⁰⁸, *ITGA2B*¹¹⁸, *CXCL4*¹¹⁹, *GPV*¹²⁰, and *GPVI*¹²¹. Each promoter is active during megakaryopoiesis and is fairly well expressed.

Previous studies from our laboratory have demonstrated the therapeutic efficacy of pFVIII. Using the murine platelet specific-promoter *Gp1ba*, hBDFVIII was expressed solely in platelets and megakaryocytes of transgenic mice on a FVIII^{null} background (Line

h38)¹⁰⁸. We verified that Line h38 mice expressed a FVIII equivalent of 3% normal plasma activity and 9% antigen level (as human FVIII has ~30% specific activity in mouse plasma)¹⁰⁸. This pFVIII level of expression was able to correct the hemophilia A phenotype in FVIII^{null} mice in several hemostatic challenges. In a mouse BMT study, we used the platelet-specific murine *Cxcl4* promoter to express hBDFVIII^{RH} in platelets of FVIII^{null} mice¹⁰⁹. When comparing these mice to mice that expressed hBDFVIII in their platelets, hBDFVIII^{RH} showed greater hemostatic efficacy in both the FeCl₃ carotid and cremaster laser injury models, consistent with the known greater biological activity of hBDFVIII^{RH}^{42,109}.

pFVIII also shows efficacy in the presence of FVIII inhibitors. We previously showed that pFVIII in Line h38 mice was efficacious in FeCl₃ injury after infusion of a 1:5 mixture of two FVIII inhibitors, ESH8 and GMA-8021¹⁰⁷. In another study, Dr. Shi and group demonstrated that transgenic mice expressing pFVIII was efficacious in the presence of high-titer inhibitors using a tail-clip exsanguination model¹¹⁸, but we have argued that this model may be too sensitive to low levels of released pFVIII under non-physiologic (almost static) setting.

To date, one study has demonstrated pFVIII efficacy in a canine model of hemophilia A. While this study showed that long-term canine pFVIII expression (5-9 mU/10⁸ platelets) could be achieved, it failed to test its efficacy in the setting of a hemostatic challenge model. Furthermore, this pFVIII was not tested in an inhibitor setting. In addition, only three dogs were studied, each having limited FVIII levels within their platelets¹²².

Limitations of pFVIII

While pFVIII is a potential therapy for Hemophilia A patients with inhibitors, there are several limitations of expressing pFVIII by BMT gene therapy. Currently, assessing the degree of pFVIII protection in the presence and absence of inhibitors has been difficult in animal models. No model showing bleeds comparable to those affecting patients is available. Hemostatic assays such as the murine tail-clip exsanguination assay tend to be especially sensitive to low levels of pFVIII. Released pFVIII from α -granules is not temporally or spatially available as plasma FVIII, which may explain its efficacy in the presence of inhibitors and its greater efficacy in some hemostatic models, but not in others. This temporal difference is likely the cause of potential emboli in pFVIII cremaster models of thrombosis our group has previously reported. To expose a patient to BMT gene therapy with no assurance of efficacy with and without circulating inhibitors in a hemophilia A patient with significant bleeds in targets including joint, retroperitoneal, and intracranial would be risky.

Additionally, achieving high-levels of pFVIII per platelet has been problematic with one report by Dr. Shi's group describing an ~ 0.2 U/ml antigen correction in mice¹²³. Whether the mice were accompanied with thrombocytopenia is not clear, as platelet counts in those animals and response of these mice to platelet production challenges were not reported¹²³. Ectopically expressed FVIII can cause injury to the expressing cell due to poor intracellular processing of the FVIII. We showed this to occur in developing Mks, especially expressing canine FVIII and less so for hBDFVIII¹⁰⁹. Achieving high levels of pFVIII expression is a desired goal, but may result in injured developing Mks and worsen post-BMT thrombocytopenia. A combination of thrombocytopenia and FVIII deficiency with inhibitors may be clinically challenging. Therefore, pFVIII BMT therapy has three theoretical limitations: 1) The post-BMT thrombocytopenia may be worsened by pFVIII, 2) Efficacy of pFVIII in target organs of patients with hemophilia A has not

been established, and 3) whether pFVIII may be effective in these target organs in the presence of inhibitors has yet to be tested in spite of the existence of a FVIII^{null} dog model of pFVIII.

Motivation for studies

While pFVIII has many advantages it also has its limitations, particularly in the setting of BMT. The purpose of these studies is to try to address some of the limitations of pFVIII in the setting of BMT, persistent inhibitors, and life threatening hemophilia A disease, offering an alternative therapeutic approach for pFVIII therapy in hemophilia A. To overcome the aforementioned limitations, I propose an alternative strategy for pFVIII therapy: expressing pFVIII in in vitro-grown megakaryocytes (Mks) and using the pFVIII-Mks to generate platelets for acute or for prophylactic care in severe hemophilia A patients with uncontrollable inhibitor titer and a history of life-threatening disease. Such a strategy may be able to be given through weekly prophylactic infusions and be used in conjunction with acute treatment with bypassing agents for bleeds. Efficacy in a particular patient may also be an indication that that patient may be a good candidate for undergoing pFVIII BMT, with the potential of using pFVIII platelet infusion therapeutically during the immediate post-BMT period.

Summary

pFVIII as a potential therapy for Hemophilia A patients with intractable inhibitors has been an ongoing area of study. While strides have been made to bring this potential therapy to clinic, this approach in the setting of BMT is potentially problematic to a

subset of severe hemophilia A patients with high-titer inhibitors. This thesis seeks to address these limitations with an alternative approach of pFVIII therapy. By utilizing mouse Line h38 platelets that express hBDFVIII and hBDFVIII-containing human iPSC-derived megakaryocytes (iMks), I explored the therapeutic benefit, both in vitro in a rotational thromboelastography assay (ROTEM) and in vivo in FVIII^{null} mice. I hypothesize that the Line h38 platelets infused into FVIII^{null} mice would simulate outcomes expected in patients treated in a similar fashion with modified human platelets made in vitro. Our group has shown that infused iMks release platelets in vivo with similar biology as endogenous platelets and I hypothesize that pFVIII-iMks will give rise to platelets in vivo and these functionally derived platelets will mitigate the FVIII^{null} phenotype in mice. I show that the hemostatic effects with these approaches persist in vivo for several days. This therapeutic strategy is also effective in the presence of inhibitors, and can be additive with rFVIIa. The potential uses and limitations of this infused pFVIII approach are also discussed. Chapter 2 summarizes the potential use of infusions of FVIII containing platelets from our highest expressing hBDFVIII transgenic Line h38 mice to improve hemostasis in the presence and absence of inhibitors. In addition the ability of these platelets to have a combinatorial effect with rFVIIa bypassing agent is also explored here. Chapter 3 outlines the generation and characterization of iPSCs, viral transduction, and FVIII expressing iPS megakaryocytes iMks. This chapter also details in vitro and in vivo effects of FVIII containing iMks on clot formation and thrombosis. Chapter 4 describes other studies utilizing human IgG localization in megakaryocytes to better understand how pFVIII achieves its protection from inhibitors as well as lessons learned from adenovirus-associated site 1 (AAVS1) to express pFVIII in iPSCs. Chapter 5 will conclude this thesis with clinical implications and future directions of the outlined studies.

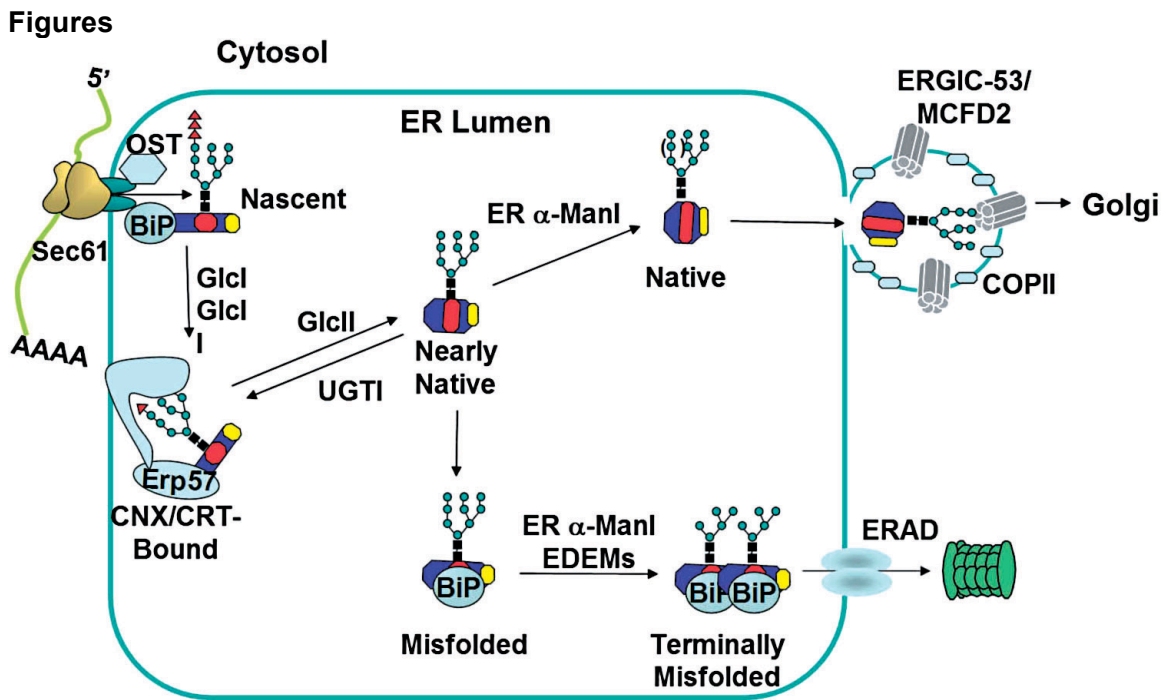


Figure 1.1: Interaction with ER proteins help to mature FVIII (adopted from Randal Kaufman²¹)
 Developing FVIII binds to critical ER chaperones: BiP, CNX, and CRT that help ensure the protein is properly folded. After being fully translated FVIII binds to CNX and CRT that help fold and prune sugars on glycoproteins. FVIII stays within this CNX/CRT complex until folded into the correct configuration. If FVIII is properly folded, it exits the ER. Upon leaving the ER, properly folded FVIII binds to ERGIC-53/MCFD2 and trafficked to the Golgi for further processing. If FVIII is not properly folded, it exits the CNX/CRT complex and binds to BiP. FVIII binding to BiP marks it for Endoplasmic-reticulum-associated protein degradation (ERAD) and FVIII is degraded.

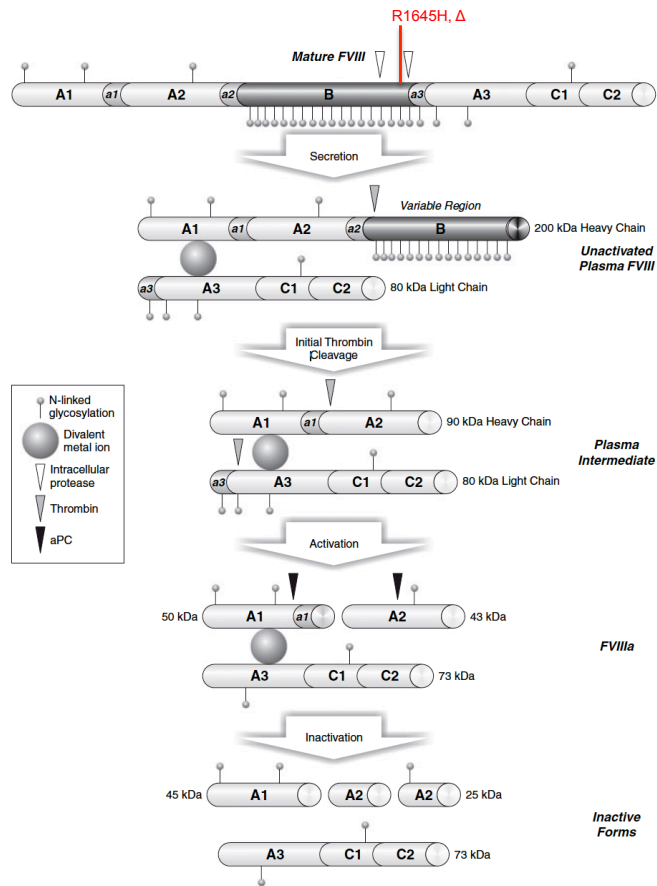


Fig. 1. Domain structure and processing of FVIII. Abbreviations: aPC, activated protein C; FVIII, factor VIII; FVIIIa, activated factor VIII.

Figure 1.2: FVIII protein structure, activation, and inactivation (adopted from Steve W. Pipe²⁸).

From top, Red line in full length FVIII indicates R1634H and Δ mutation in order to create variants. Full length FVIII is processed within LSEC cells and then secreted into the blood stream as heterodimer held together by a divalent metal ion. Thrombin then cleaves the B-domain from heavy chain of FVIII and the a3 acidic domain allowing VWF to dissociate. Upon further thrombin processing FVIII becomes a heterotrimer and the activated FVIIIa, at which point it can exhibit its co-factor activity. Inactivation occurs quickly through dissociation of A2 domain and further proteolysis by thrombin and aPC.

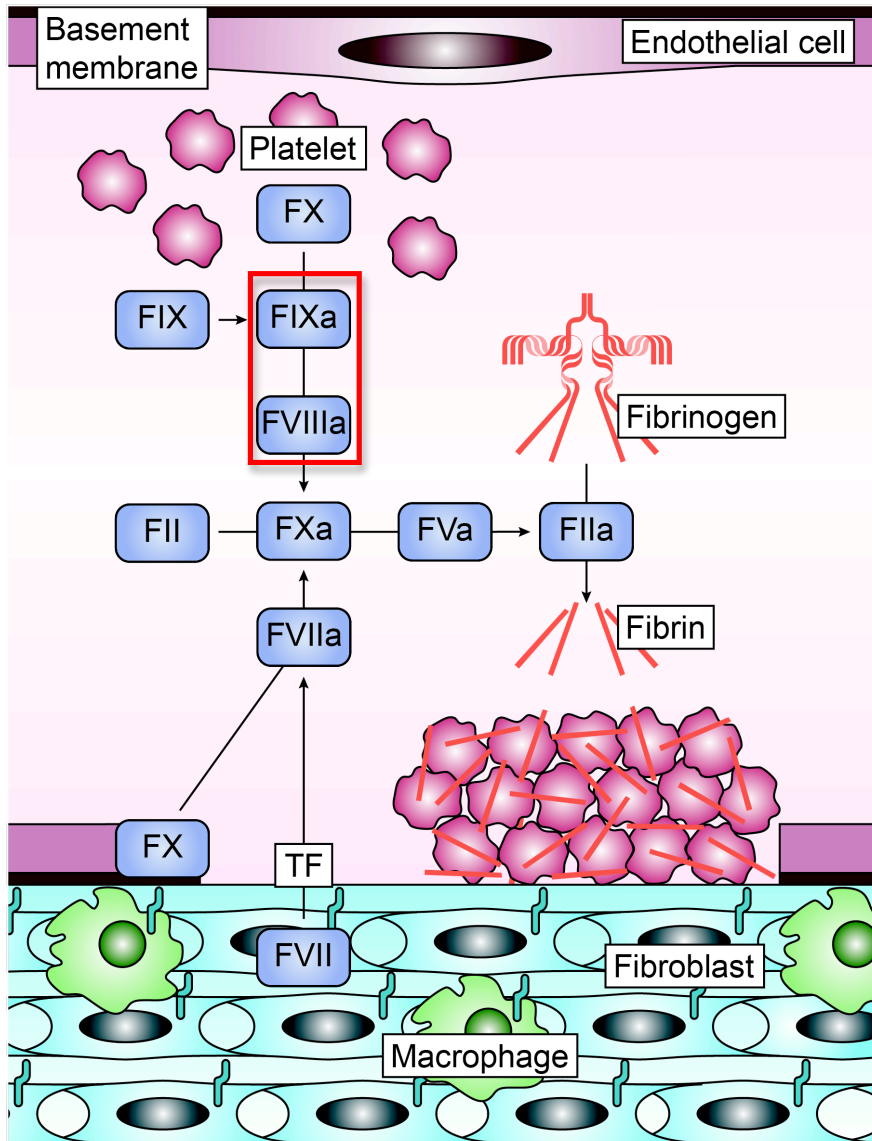


Figure 1.3: FVIII's role in hemostasis and clot formation.

FVIIIa's role in the intrinsic pathway of the coagulation cascade. Exposure of tissue factor (TF) at the site of vascular injury binds to FVIIa and activates FX. Activated FVIII works as a co-factor with FIXa to convert FX to FXa. Red box indicates FVIIIa:FIXa Xase complex. These two pathways ultimately increase the level of FIIIa, which cleaves fibrinogen to fibrin to form. This fibrin is then able to bind to platelets and form a cohesive platelet plug at the site of vascular injury.

Chapter 2 – In Vitro and In Vivo Hemostatic Therapeutic Efficacy of FVIII-Containing Mouse Platelets

Abstract

pFVIII is able to correct the bleeding phenotype seen in FVIII^{null} transgenic animals, however, to date this has been accomplished largely by bone marrow transplant (BMT) which can be problematic in patients with hemophilia A and intractable inhibitors. We tested an alternative pFVIII-approach using platelets containing hBDFVIII at a fairly high level to examine its potential therapeutic value. We envision that our studies will show that infused pFVIII platelets can provide hemostatic efficacy for a relatively prolonged period of time and can be used additively with a bypassing agent and can do so in spite of the presence of an inhibitor. In this chapter, I describe the in vitro and in vivo efficacy of infusing FVIII containing platelets as a therapy for Hemophilia A, their therapeutic efficacy in the presence of FVIIa, and the efficacy of pFVIII in the presence of inhibitors with and without FVIIa. First, I describe the transgenic mice used in these studies, FVIII^{null} and our murine transgenic Line h38 expressing hBDFVIII in their platelets on FVIII^{null} background. Line h38 is the highest expressing hBDFVIII transgenic line available to us. It has been bred onto the FVIII^{null} background. Efficacy of pFVIII line h38 platelets were studied in an in vitro ROTEM assay with and without a bypass agent. The ability of infused pFVIII platelet infusion to abrogate the FVIII^{null} phenotype seen in in vivo FeCl₃ carotid assay, and tail-vein exsanguination assay were done and the half-life of the observed hemostatic improvement was followed. Finally, the effects of combined efficacy of FVIIa and infused pFVIII in FeCl₃ assay in the presence and absence of FVIII inhibitors was examined. These studies showed that pFVIII infused platelets can provide a relative prolonged therapeutic benefit that was effective in the presence of inhibitors and that was enhanced by inclusion of a bypass agent.

Introduction

Genetically modified animals or transgenic organisms are a powerful research tool utilized by many laboratories. Dr. Ralph Brinster in collaboration with Dr. Richard Palmiter at the University of Pennsylvania was one of the first researchers to successfully create a transgenic mouse that was capable of passing its genetically altered genes to its offspring^{124,125}. While Brinster and Palmiter first used this technology to express oncogenes in mice, their techniques and ideas proved to be translatable to an array of genes that allow researchers to mimic clinical disease states and to study potential therapeutic interventions.

To this end, our laboratory has utilized a FVIII^{null} mouse model of hemophilia A, first created and described by Dr. Kazazian, that exhibits the prolonged bleeding time seen in hemophilia A patients^{126,127}. These mice have a disruption in exon 16 of their FVIII gene that results in a truncated dysfunctional form of the FVIII protein. This mutation ultimately causes a severe form of the disease with <1% FVIII activity¹²⁶. While these mice have no residual FVIII activity, they do not develop spontaneous bleeds into their joints as humans do. However, following a number of hemostatic challenges, these mice can bleed to death. For example, tail transection of these animals causes them to expire a few hours post clipping¹²⁸.

One major hallmark of severe hemophilia A in human is the occurrence of significant arthropathy of the synovial cavities of the large joints^{129,130}. For patients that lacked regular FVIII replacement therapy, by age 25 years over 85% of these patients have major morphology changes in key joints¹²⁹. An injury model of chronic synovitis has been developed for these mice where mechanical trauma is induced in these joints¹³¹.

These models have been used to test the effect of bypassing agents in these animals; however, there are several limitations with this model the first being these bleeds are not spontaneous as in humans¹³². The second is that these models do not mimic weight on these joints as seen in humans¹³³. Like humans, however, these mice develop antibodies to infused FVIII. Their immune response to infused FVIII is very similar to that in severe hemophilia A patients that develop inhibitors and has been extensively studied and used to develop potential therapeutic¹³⁴.

Our laboratory has utilized these mice to test the efficacy of a platelet delivery system for FVIII. In 2003, Dr. Yaravoi developed transgenic mice lines that expressed hBDFVIII solely in their megakaryocyte and platelets, driven off of the murine *Gp1ba* promoter. The highest expressing transgenic line, Line h38, had an antigenic FVIII level of 9% and an activity level of 3% determined by ELISA and Coatest assays, respectively¹⁰⁸. This level of expression is able to almost completely rescue the hemophilia A phenotype in a FeCl₃ carotid artery model. These mice achieved occlusion similar to wild-type mice and to FVIII^{null} mice infused with a 50% soluble hBDFVIII correction dose. Furthermore, these studies indicated that in all the transgenic lines, FVIII was in the platelet α -granules with none detected circulating in the plasma after they were crossed onto a FVIII^{null} background. The pFVIII was shown to colocalize with vWF, presumably in α -granules, and could be released upon platelet activation¹⁰⁸.

The efficacy of these platelets was directly compared to infused recombinant hFVIII in the presence of an inhibitor mixture consisting of ESH8 and GMA-8021. Gewirtz et al. showed that Line h38 mice were able to protect against FVIII inhibitor inactivation better than plasma FVIII. The efficacy seen in the FeCl₃ was modest, 6-7 fold improvement in BU per mL compared to hFVIII infusion¹⁰⁷. These results were

slightly different than those seen with the pFVIII mouse developed by Dr. Shi's group. Using the tail-vein exsanguination model, they showed that pFVIII was efficacious in the presence of high-titer inhibitor and protective to >20 BUs¹¹⁸.

Obtaining high levels of pFVIII in transgenic animal models as well as cell lines has been challenging. One reason is that FVIII is toxic to the expressing ectopic cell and expressing it at high levels can lead to ER stress and apoptosis¹³⁵. We showed that expressing canine FVIII injured megakaryocytes, and that hBDFVIII was also deleterious¹⁰⁹. Canine pFVIII expression in mice through lentiviral BMT showed that canine FVIII could inhibit platelet production from donor-derived megakaryocytes. To date, Dr. Shi has developed a transgenic mouse that expresses ~ 0.2 U/ml antigen correction, roughly twice that seen in Line h38¹²³. We utilized our Line h38 mice in our studies because it is the transgenic line with the highest level of pFVIII that is available to us and has been well characterized, including measurements of the platelet count and response to various hemostatic challenges^{108,110}.

Human FVIII inhibitors

About twenty-three percent of patients with severe hemophilia A that have received recombinant FVIII therapy will develop inhibitors to the infused therapy. Type 1 inhibitors inactivate FVIII through potentially binding to the A2 and A3 domains of FVIII⁸⁹. These sites are close to the binding sites for FIXa. Another main epitope of FVIII inhibitors is the C2 domain that binds to the surface of platelets. After inhibitors bind and inactivate FVIII, the FVIII is quickly cleared. Some inhibitors even bind to FVIII-associated proteins such as: FIXa, FXa, and VWF. The inhibitor studies outlined in this chapter are a mix of two inhibitors commonly used in inhibitor studies and that are well-characterized, ESH8 and GMA-8021. ESH8 is a mouse monoclonal antibody that binds

to the human FVIII C2 domain in the presence of VWF^{136,137}. GMA-8021 is a mouse monoclonal antibody also originally known as R8B12¹³⁸ binds to the C2 domain of FVIII^{139,140}. We previously showed that a 1:5 ($\mu\text{g}:\mu\text{g}$) could fully inhibit plasma FVIII when infused into a FVIII^{null} recipient mouse at 1ug/gram¹⁰⁷.

Bypassing agents

Current treatment for hemophilia A patients with inhibitors is ITI with high levels of infused FVIII with or without immunosuppression therapy⁹⁵. Where high-titer inhibitors persist, bleeds are treated with bypassing agents. Currently there are two FVIII bypassing agents that are clinically used to treat Hemophilia A patients with inhibitors, rFVIIa and activated prothrombin complex concentrates (aPCCs), notably FEIBA. A potential third therapy is emicizumab (ACE910), which heterodimerizes FIX and FX, which is currently undergoing clinical trials.

The first two bypassing agents are used for either prophylactic treatment or for acute events. aPCC is produced from pooled plasma that contains factors all of the vitamin K-dependent factors: VII, II, IX, and X. rFVIIa is a purified recombinant protein that has reported efficacy in patients with inhibitors with acute bleeds^{141,142}. rFVIIa doses for inhibitor patients ranges from 35-90 $\mu\text{g}/\text{kg}$. Its expense and its short half-life of 2-3 hours are its most challenging limitations¹⁴³⁻¹⁴⁵. aPCCs also have reported efficacy in patients with inhibitors. rFVIIa and aPCC has a reported efficacy rate of 85% and 80% in inhibitor patients respectively. While both have significantly improved quality of life for hemophilia A patients with inhibitors, these bypassing agents are not as effective as FVIII at treating acute bleeds and their short half-lives limits their prophylaxis use^{141,142}.

To note, ACE910 is currently in phase three clinical trials and has shown promise as a bypassing agent¹⁴⁶. This humanized bispecific antibody mimics activated FVIIIa

binding FIX and FX. ACE910 has good bioavailability after subcutaneous injection and has a half-life of up to 5 weeks¹⁴⁷. A 12-week study showed 72% of patients enrolled in the study with inhibitors had no bleeding events while on the therapy. Dose regimens included 0.3, 1.0, or 3.0 mg/kg once a week¹⁴⁶. Unfortunately, the success was marred due to a patient's death post conclusion of the initial study. While on ACE910, this patient being treated for a rectal hemorrhage, developed signs of thrombotic microangiopathy and died⁹⁸. Whether the occurrence of thrombotic microangiopathy on this drug will be a common complication in a significant number of patients needs further study.

Materials and Methods

Mice lines

FVIII^{null} mice with exon 16 disruption in *F8* gene^{126,127}, the murine transgenic Line h38 expressing hBDFVIII in their platelets¹⁰⁸, and the transgenic h α IIb-expressing line^{148,149} were previously described. Line h38 mice are on the FVIII^{null} background, but are designated simply as Line h38. All mice have been previously backcrossed onto a C57Bl6 background for >10 generations. In all studies, 6-10 weeks of age littermate mice of either sex were studied.

Isolation of whole blood and platelets

Whole blood of FVIII^{null}, Line h38, and h α IIb-expressing mice were collected from the inferior vena cava in 3.8% sodium citrate as described^{150,151}. Mice were anesthetized using pentobarbital (80 mg/kg, Akorn Pharmaceuticals) and vena cava exposed. Up to

1ml of whole blood was then collected into 1 ml BD syringes using a 23-gauge needle (precision glide, BD, New Jersey). 3.8% Sodium citrate was added to the whole blood to obtain 10% of total volume. Whole blood was used directly in ROTEM studies or for in vivo infusion studies after isolation of platelets using differential centrifugation as previously described. To isolate platelets, 1 μ l of PGE-1 was added to whole blood to prevent platelet activation. Whole blood was then centrifuged for 10 minutes at 860 RPM to first isolate platelet rich plasma (PRP). The PRP was then spun at 3000 RPM for 8 minutes, and the resulting platelet pellet was washed twice and then re-suspended in PBS or platelet re-suspension solution. In some studies, supernatant from PRP was tested for the presence of FVIII as previously described^{108,152} (data not shown). Total blood and platelet counts were determined using a HemaVet counter (Triad Associates) prior to study. Whole blood and platelet suspensions were used within 1 hour for both in vitro and in vivo infusion studies. In some studies, Line h38 whole blood was mixed with that of FVIII^{null} mice to obtain decreased percentages of Line h38 platelets.

ROTEM studies for pFVIII

Whole murine blood was recalcified to 10 mM with 0.2 M CaCl₂, and then 110 μ l of whole blood was transferred to 37°C ROTEM minicups (Werfern) for assessment via ROTEM thromboelastometry using an INTEM-based assay, as previously described^{153,154}. INTEM reagent (Werfern), an intrinsic pathway activator of kaolin¹⁵⁵, was used to activate mouse whole blood. In some studies, supplemental rFVIIa (1-50 nM, final concentration, NovoNordisk) was added to FVIII^{null} whole blood ROTEM cups. In other studies, iMks expressing either hBDFVIII or the hBDFVIII^{RH} variant, eGFP, or no added protein non-transduced control (NTC) were added to FVIII^{null} whole blood in ROTEM minicups. Care was taken to insure that no more than 10 μ l of cell suspension

was added to whole blood. In other studies, human full-length rFVIII (0.01-1.0 ng/ml final concentration, Advate, Shire) was added to FVIII^{null} whole blood as a positive control. The thromboelastometry assay was carried out for up to 30 minutes, and data were collected and analyzed via ROTEM software (Werfern). Typical ROTEM readout and parameter collected for these experiments are illustrated in [Figure 2.1](#).

rFVIIa

rFVIIa utilized in these studies in vitro and in vivo studies was obtained from NovoNordisk (1mg doses). 1.1 ml histidine diluent, supplied, was used to reconstitute lyophilized powder. Protein was used immediately for infusion studies or aliquoted and stored at -80°C.

Mouse platelet half-life studies

To define platelet half-life and total percentage of infused circulating platelets post infusion, 4×10^8 α IIb-expressing or calcein AM-loaded platelets were infused into FVIII^{null} mice and analyzed over 72 hours via flow cytometry. Platelets were infused via the tail-vein and blood collected from the retro-orbital sinus at various time points. Blood was collected using Microhematocrit Capillary Tubes (Fisherbrand) into minicapillary blood collection tubes prepared with EDTA DI potassium salt (RAM Scientific Inc.). 1 μ l of blood was added to 100 μ l antibody solution containing anti-CD41a-allophycocyanin (APC), and anti-CD42b-phycoerythrin (PE) (both BD Biosciences) antibodies. Antibody/blood samples were allowed to incubate for 20 minutes at room temperature. 400 μ l of phosphate buffered saline (PBS, Gibco) was added to each sample after

incubation, and the samples were analyzed via flow cytometry using a BD FACSCanto II (BD Biosciences) and further analyzed using FlowJo software.

Tail-clip exsanguination assay

The tail-clip exsanguination assay were performed in FVIII^{null} mice after they had received up to 4×10^8 Line h38 or FVIII^{null} control platelets in 100 μ l in PBS, 0.5-72 hours prior to the injury. Controls also included FVIII^{null} mice that received 100 μ l of PBS. In some studies, FVIII^{null} mice were infused with 0.25, 1 and 4 mg/kg rFVIIa retro-orbitally, up to 2 hours prior to injury. After these infusions, mice tails were resected to 2mm in diameter to allow for significant bleeding¹¹⁸. Mice were allowed to bleed overnight and then checked up to 16 hours post tail resection. The primary endpoint was the number of mice surviving over night.

FeCl₃ carotid injury assay

The FeCl₃-induced carotid artery injury assay in FVIII^{null} mice was previously described by us and shown to correlate to the amount of recombinant hFVIII infused^{108,109,152}. Mice were transfused with either $2-4 \times 10^8$ Line h38 or FVIII^{null} platelets 0.5-72 hours after being anesthetized using pentobarbital (80 mg/kg). The right carotid artery was then isolated and exposed to a 20% (W/V) FeCl₃-saturated 1 X 2 mm Whatman 1 filter paper for 3 minutes. Upon removal of the FeCl₃-saturated paper, the open surgical site was filled with PBS. Blood flow was then measured using a Doppler flow probe (Model 0.5VB, Transonic Systems). Total flow was recorded for 30 minutes, and the volume of blood flow over that time-frame was the primary endpoint.

In some FeCl₃ carotid artery injury studies, 0.1-2.0 μ g of inhibitors per gram of mice in a total of 50 μ l of water (see next paragraph) were infused via the jugular vein of

mice prior to the FeCl₃ carotid injury assay. In other injury studies, rFVIIa at a concentration of 1-4 mg/kg was infused immediately prior to the FeCl₃ carotid injury assay. In other studies, rFVIIa was infused via the jugular vein into FVIII^{null} mice at a concentration of 0.25-4.0 µg/kg concurrent with Line h38 platelet infusion and 0.1-2.0 µg inhibitor mixture.

FVIII inhibitors

For several studies, FVIII inhibitors were infused. Based on a prior study from our group showing optimal inhibition of plasma FVIII and pFVIII biology, a cocktail of two FVIII inhibitors consisting of 1 µg of ESH8 (BioMedica Diagnostics) plus 5 µg of GMA-8021 (Green Mountain Antibodies) was used¹⁰⁷. GMA-8021 is an IgG_{2a} anti-human FVIII antibody that has specificity to the A2 domain¹³⁸. ESH8 is a potent inhibitor that binds to the FVIII C2 domain in the presence of VWF and is also an IgG_{2a} isotype^{137,156}. 0.1-2.0 µg of inhibitors mixture per gram of mice in a total of 50 µl of water were infused into the mouse via the jugular vein.

Animal study approval

All studies were carried out with the approval of the Children's Hospital of Philadelphia's Institutional Animal Care and Use Committee.

Statistical analysis

Statistical differences between arms were determined using a two-sided Student's t-test or analysis of variance (ANOVA) with Bonferroni, Dunnett's or Tukey's corrections of multiple comparisons, when appropriate. PRISM 7.0 (Graphpad) was used to calculate statistical significance. P-Values ≤0.05 were considered significant.

Results

ROTEM studies with recombinant full-length hFVIII

To evaluate the hemostatic efficacy of pFVIII, we first determined the hemostatic efficacy of hFVIII added at varying concentrations of 0.01, 0.1, and 1.0 ng/ml in FVIII^{null} mouse whole blood on ROTEM analysis (Figure 2.2). ROTEM is particularly sensitive to deficiencies in whole blood and plasma. Using this method allowed us to notice small changes in FVIII that may be efficacious in FVIII^{null} whole blood. Each concentration of soluble FVIII showed some level of clot formation. Results were seen in a dose-dependent manner with 0.01 displaying the least amount of clot formation.

In vitro studies using Line h38 platelets and rFVIIa

ROTEM analysis was then used for in vitro studies of transgenic mouse lines, FVIII^{Null} and Line h38. Wild-type mice were also run as controls. FVIII^{null} mice whole blood showed no hemostatic efficacy in this system. Line h38 blood partially corrected the hemostatic defect of FVIII^{null} murine blood (Figure 2.3A). To determine the amount of Line h38 blood that was required for this effect, Line h38 whole blood was diluted with FVIII^{null} blood, and ROTEM was performed (Figure 2.3B). Clot formation was detected in as little as 1% Line h38 blood while 20% Line h38 blood was enough to bring clotting close to that seen with whole Line h38 blood.

rFVIIa was then studied as present-day alternative therapy for patients with hemophilia and inhibitors^{1,144} and the potential combination use of rFVIIa and pFVIII. rFVIIa was added to FVIII^{Null} whole blood to obtain at final concentration of 10 and 25 nM and then analyzed via ROTEM assay (Figure 2.4A). 25 nM or 90 ug/kg is a

pharmacological dose for inhibitor patients^{157,158}. While both concentrations of rFVIIa were able to induce clotting to some extent in this assay, only 25 nM was able to bring parameters back near to that of wild-type; however, delay in clot time remained suggesting that sufficient time is needed to generate sufficient FXa for hemostasis to occur. The effect of adding both rFVIIa and Line h38 platelets in combination was also investigated in this assay. 10 nM of rFVIIa in combination with Line h38 whole blood was able to have an additive effect that was greater than Line h38 whole blood alone (Figure 2.4B).

In vivo studies of infused Line h38 platelets

To better understand the hemostatic value of pFVIII in vivo in FVIII^{null} mice, the half-life of infused mouse platelets was determined via flow cytometry (Figure 2.5). Isolated mouse WT platelets were either labeled with calcein-AM prior to infusion or infused transgenic h α IIb mouse platelets were labeled with an anti-h α IIb antibody after collection from the recipient FVIII^{null} mice. Blood was collected at various time points and analyzed for percentage of remaining infused platelets. The data indicates that isolated and infused mouse platelets had a half-life of about 22 hrs consistent with other studies by our group and others^{151,159}.

The hemostatic effects of infusing pFVIII-expressing Line h38 platelets into FVIII^{null} recipient mice was tested after infusing sufficient platelets so that line h38 platelets represented ~15% of the total circulating platelets. This number of platelets would represent the number of platelets that is given in a straight-forward platelet transfusion in clinical setting¹⁶⁰, and could be tolerated on a recurring basis. Hemostatic efficacy was tested in the recipient mice in the tail-clip exsanguination assay that many

groups, including ours, have used to study corrected hemostasis in FVIII^{null} mice^{118,161} (Figure 2.6). Mice were infused with Line h38 or FVIII^{null} platelets and then their tails were resected to 2 mm in diameter. Mice that received Line h38 platelets, but not FVIII^{null} platelets survived the overnight hemostatic challenge (Figure 2.6A). This hemostatic effect lasted at least 72 hours post-platelet infusion. The hemostatic effects of rFVIIa were also tested in this model (Figure 2.6B). rFVIIa was infused in FVIII^{null} mice at concentration of 0.25, 1.0, and 4.0 mg/kg. As with other studies, high doses of rFVIIa were needed to have an effect in hemophilia A mice tail bleeding and FeCl₃ models^{153,162}. Only the highest dose was able to rescue the mice, and by 2 hours post-infusion, the response in the tail-clip exsanguination assay had lessened.

PFVIII hemostatic efficacy was also tested in the FeCl₃ carotid artery injury model in which we had previously shown a clear defect in FVIII^{null} mice and a gradient of increased hemostatic efficacy at increasing infusions of hFVIII¹⁰⁸. Infusion of Line h38 platelets – but not non-pFVIII platelets – into FVIII^{null} mice was effective in decreasing blood flow up to 48-hours post-infusion (Figure 2.7A). rFVIIa was also effective in improving hemostasis in this model, but an additive effect with Line h38 platelets was seen in recipient FVIII^{null} mice (Figure 2.7B). Line h38 mice and prophylactic pFVIII infusions in the presence of inhibitors were tested in this model (Figure 2.7C and 2.7D). Prior to platelet infusion, we injected mice with a single dose of an inhibitor mixture that we previously showed fully inhibited pFVIII function in Line h38 mice (Figure 2.7C). We confirmed these findings and defined an inhibition dose that did not inhibit hemostasis in Line h38 mice, but did completely inhibit an infusion of Line h38 platelets to 15% (Figure 2.7D); however, the addition of rFVIIa to this pFVIII therapy was able to decrease this inhibition.

Discussion

The delivery of ectopic proteins by platelets to sites of vascular injury had been proposed by our group more than 15 years ago¹⁰⁸. Our group has designed a platelet-specific thrombolytic agent delivery strategy involving the storage of urokinase-type plasminogen activator (uPA) within α -granules and their release at nascent thrombi as a thromboprophylaxis agent⁸². These studies showed the potential of using platelets as a potent delivery system without systemic sequelae. This type of delivery system was also proposed to be applicable to the delivery of FVIII in hemophilia A patients with inhibitors as well¹⁰⁷.

In these studies, we propose that infusions of pFVIII-containing platelets may be an effective alternative treatment for patients with severe hemophilia A and intractable inhibitors for acute or prophylactic care. This therapy would be useful in place of bypassing agents. These platelets would provide coverage for a longer period of time than present-day FVIII-bypassing agents and may provide additive therapeutic potential with inhibitors especially in the presence of inhibitors. Moreover, it would be therapy that would avoid the potential life-threatening complication of doing platelet-directed bone marrow gene therapy and/or can be used to help patients survive the challenges of that process.

As a proof-of-principle for this concept of infused pFVIII platelets, we utilized Line h38 platelets which have ~0.09 U (~75 ng) of hBDFVIII antigen per ml of mouse blood¹⁰⁸. Previous in vitro studies done using ROTEM technology, showed that rFVIII can correct the hemostatic deficiency of FVIII^{null} blood in this system¹²³. Whole blood from FVIII^{null} mice expressing high levels (~0.2 U/ml of murine blood) of pFVIII have also been previously reported to partially correct hemostatic function on

thromboelastography, although still showing a prolonged initiation of clot time. We confirm these results with our Line h38 whole blood including the prolonged clot time (Figure 2.3). This delay in clot time is likely due to the time it takes for platelets in the blood to become activated and release significant amounts of their granule pFVIII content. This explanation is supported by inclusions of low doses of rFVIII where the initial effects of very low dose rFVIII has a similar prolonged initiation of clot time (Figure 2.2). Interestingly, as little as 1% Line h38 blood added to FVIII^{null} whole blood was able to have some effect in this model with 20% approximating that seen with Line h38 whole blood, supporting the potential value of therapeutic pFVIII platelet transfusions (Figure 2.5B).

To examine additive effects of pFVIII platelet infusions with bypassing agents, rFVIIa was used; one of the two present-day approved bypassing agents^{141,142,157}. We show that clot formation was detectable with as low as 10 nM rFVIIa in ROTEM studies, while the addition of 25 nM FVIIa was required to improve maximum clot formation in ROTEM comparable to WT levels but still showed slight deficit in clot time (Figure 2.5A). This prolongation is consistent with rFVIIa not directly correcting the underlying hemostatic defect, but rather bypassing the defective step in the coagulation cascade. The addition of 10 nM rFVIIa to Line h38 blood showed an additive effect with a decrease in initiation of clot time, however the lag in clot time was still present (Figure 2.5B). This failure to correct the clot time may again be in part due to the need for platelet activation and pFVIII release to begin to have pFVIII contribute to overall hemostasis. In addition this delay in hemostasis is due to low levels of either supplemental rFVIII (Figure 2.2) or rFVIIa (Figure 2.4).

Prophylactic infusions of pFVIII platelets were further tested in vivo in two distinct assay systems beginning with the tail-clip exsanguination assay. The recipient infused

FVIII^{null} mice survived an overnight tail-clip exsanguination assay, which, in agreement with prior observations, was 100% lethal in non-transfused FVIII^{null} mice or mice infused with non-pFVIII platelets. The hemostatic effects of infusing pFVIII-expressing Line h38 platelets into FVIII^{null} recipient mice was sufficient to result in ~15% of the circulating platelets being from Line h38. Such a level of infused platelet should be achievable clinically and a correction of this level had shown hemostatic efficacy in vitro (Figure 2.3). Beginning at 1 and extending to 72 hours post-infused Line h38 platelets (Figure 2.6A), there was improved survival in this tail-clipping hemostatic model in FVIII^{null} mice. This effect was not due to simply having infused platelets into the FVIII^{null} mice (Figure 2.6A). We also found that the half-life of infused platelets prepared by us was ~22 hours (Figure 2.3), consistent with half-lives reported for infused murine platelets by others. By 72-hours post-infusion of 15% Line h38 platelets, we anticipate that <2% of the circulating platelets would be Line h38-derived. In contrast to this infusion of pFVIII platelets, infusion of rFVIIa was ineffective at rescuing recipient FVIII^{null} mice from tail-clip exsanguination even 0.25-hour post-infusion unless a high dose of 4 mg/kg rFVIIa was given, but even here, hemostatic efficacy was mostly gone 1.5-hours post-infusion (Figure 2.6B) consistent with the known short half-life of human rFVIIa in mice. It is worth noting that cessation of bleeding in the high dose rFVIIa arm was observed early in the assay, after the tail clip, indicating residual levels of rFVIIa might have improved their survival (data not shown).

The FeCl₃ carotid artery injury model was also used to study in vivo hemostatic challenges in FVIII^{null} mice. Previous work from our group has shown a positive correlative relationship between infused human rFVIII and decreased blood flow¹⁰⁸. This assay can be completed within 30 minutes, and thus would allow studies of the additive

effects of pFVIII with rFVIIa. Mice infused with Line h38 platelets saw therapeutic efficacy for at least 48-hours post-infusion (Figure 2.7A).

We believe that the differences between efficacy of pFVIII platelets by the two in vivo assays is the greater sensitivity of the tail-clip exsanguination assay to low levels of pFVIII as stasis likely occurs in the tails of these animals allowing greater efficacy to the available pFVIII¹⁶¹. Nevertheless, if the half-life of these platelets and their therapeutic efficacy in mice, were extrapolated to humans, one would imagine a therapeutic window of 2-3 weeks, assuming a human platelet half-life of ~7-10 days. Line h38 platelets showed a combinatorial effect with rFVIIa (Figure 2.7B), indicating that this bypassing agent could be added to patients on pFVIII infusion prophylaxis therapy in settings of acute bleeds. More importantly, prophylactic pFVIII infusion was able to be effective in the setting of inhibitor (Figure 2.7D), this effect was also enhanced with the addition of rFVIIa.

Figures

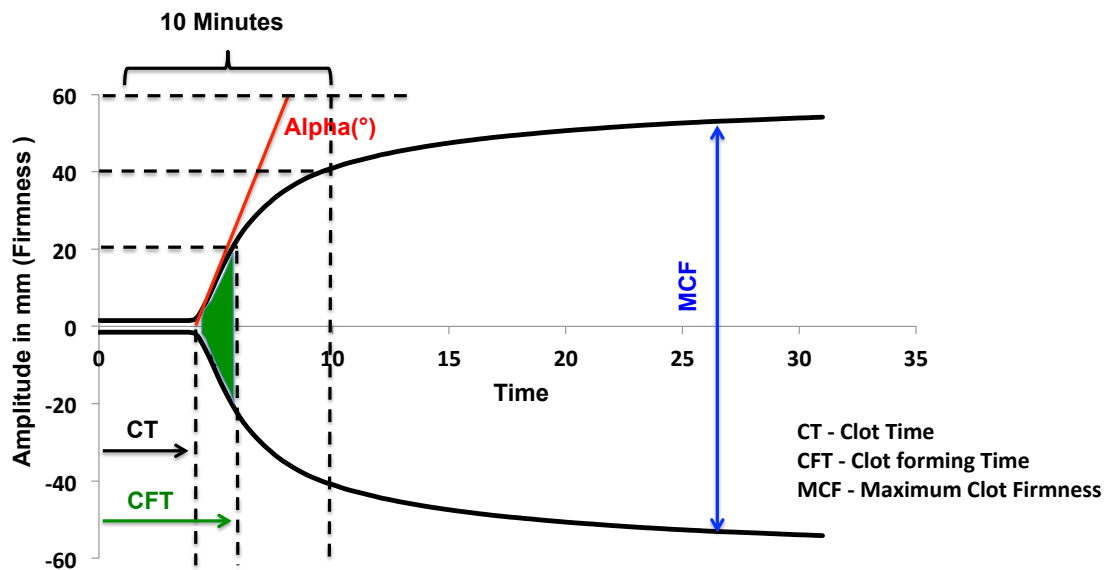


Figure 2.1 Rotational Thromboelastometry (ROTEM) parameters

Example of typical ROTEM data and parameters obtained from running in vitro experiment. Graph indicates the time it takes to initiate clotting: clot time (CT), the time it from when clotting starts to when clotting reaches 20mm of firmness (green area): clot forming time, the maximum stiffness of the clot: maximum clot firmness (MCF), and the steepness of the clotting curve: alpha angle.

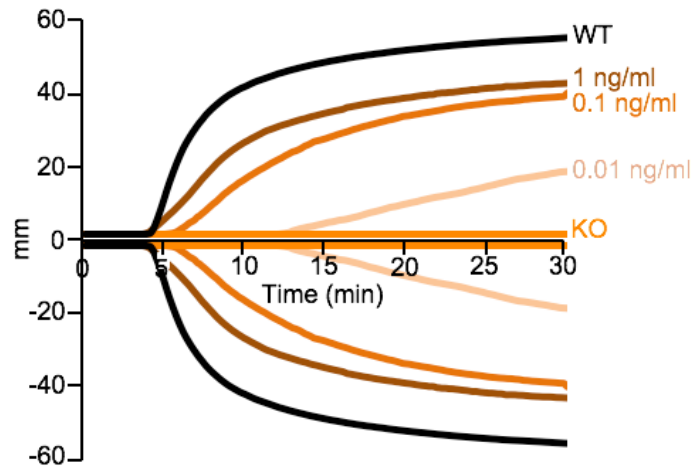


Figure 2.2 Addition of soluble rFVIII improves clotting in in vitro ROTEM assay beginning with FVIII^{null} blood.

Studies similar to Figure 2.1 except that the noted concentrations of rFVIII were added to of FVIII^{null} (KO) whole blood to achieve the concentrations noted. Each curve represents 4 independent studies.

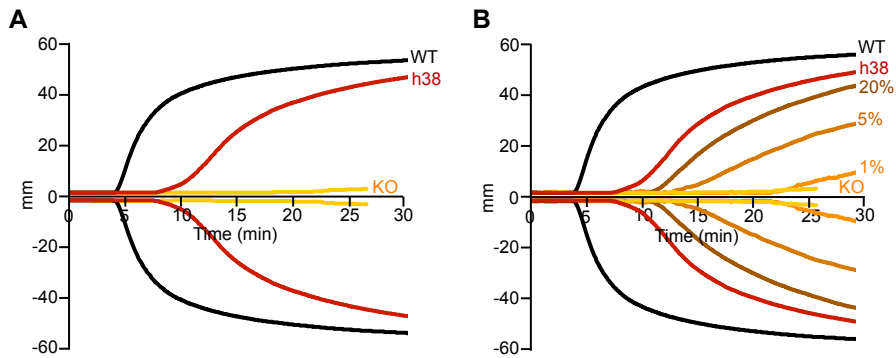


Figure 2.3 ROTEM analysis of WT, FVIII^{null} and Line h38 mice whole blood as well as studies of admixtures of Line h38 into FVIII^{null} whole blood.

Whole blood from WT (black), Line h38 (brown), and FVIII^{null} (yellow, KO) mice were studied by ROTEM in (A) and (B). (A) Each curve represents 5 independent studies. (B) Same analysis as (A) in which Line h38 whole blood was added into FVIII^{null} to result in 1, 5 or 20 percent levels of Line h38 platelets in the blood mixture. Each curve represents 4 independent studies.

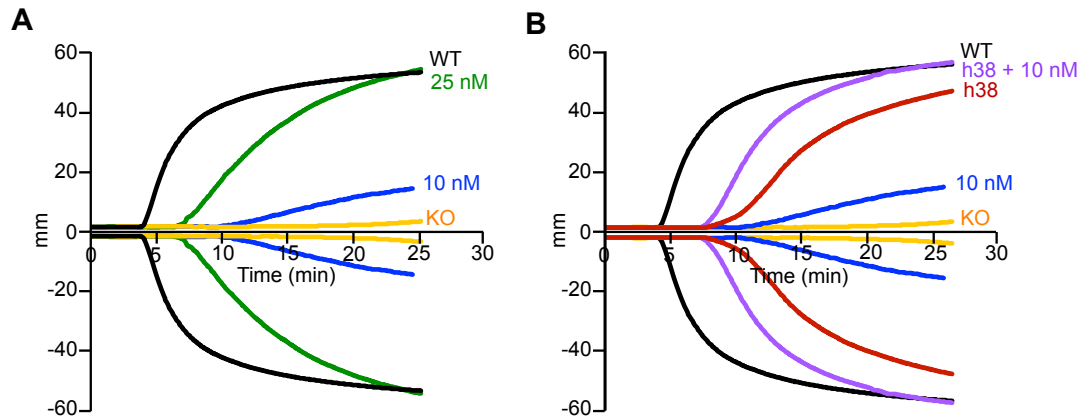


Figure 2.4 Additive effect of rFVIIa and pFVIII by ROTEM.

ROTEM analyses were performed as in Figures 2.1-2.3. (A) rFVIIa was added to FVIII^{null} mouse whole blood to bring the final concentrations to 10 and 25 nM. (B) Line h38 whole blood in the absence (red) and presence (purple) of 10 nM rFVIIa added were compared to FVIII^{null} mouse whole blood with 10 nM rFVIIa (blue). WT (black) and FVIII^{null} (yellow) mouse blood were used as positive and negative controls, respectively, in both (A) and (B). Each curve represents 4 independent studies in both (A) and (B).

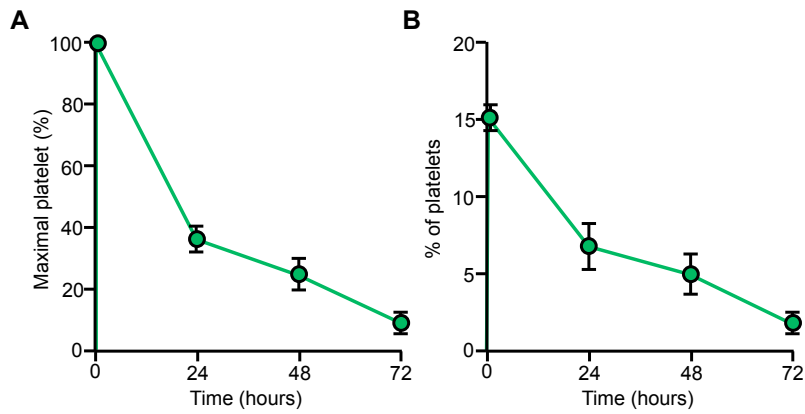


Figure 2.5 Half-life of isolated mouse platelets infused into FVIII^{null} mice.

(A) Half-life based on infusing $1-4 \times 10^8$ human α IIb-expressing mouse platelets into FVIII^{null} mice. Total α IIb-expressing platelets were tracked over 72 hours via flow cytometry. Measurement of remaining infused human platelets normalized to level at 5-minutes post-infusion. Mean \pm 1 SEM are shown. N = 4 independent studies. (B) Calcein-AM 4×10^8 loaded WT platelets infused into FVIII^{null} mice. Percent of circulating platelets that contained calcein are shown. Mean \pm 1 SEM are shown. N = 3 independent studies.

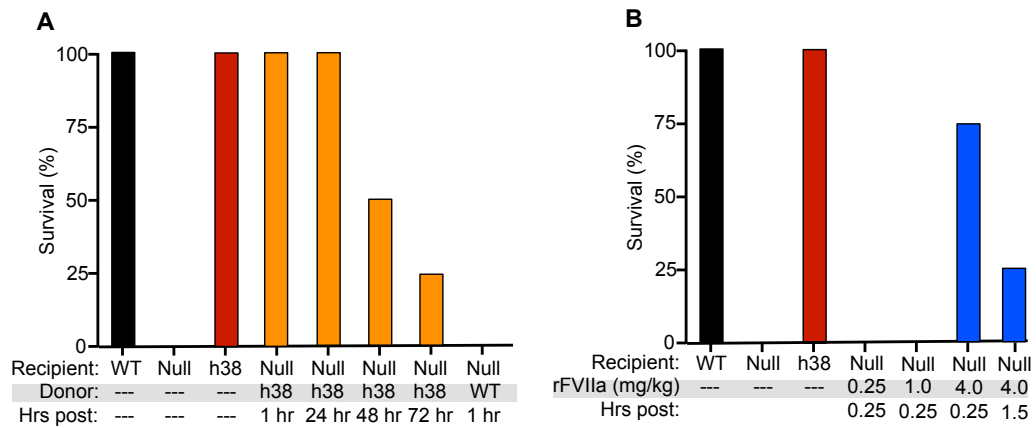


Figure 2.6 Survival post Line h38 platelet and rFVIIa infusion in the tail-clip exsanguination assay.

(A) and (B) tail-clip exsanguination assay with expiration assessed 16-hours post-tail clip. (A) On the left part of the graph, WT, FVIII^{null} and Line h38 mice underwent tail-clip exsanguination. On the right, FVIII^{null} mice were infused with 15% Line h38 platelets and then underwent tail-clip exsanguination at 1, 24, 48, and 72-hours post-infusion. Percent of animals surviving of N = 4 animals per arm is shown for each time point. (B) On the left part of the graph, WT, FVIII^{null} and Line h38 mice underwent tail-clip exsanguination. On the right, mice were infused with 0.25, 1 and 4 mg/kg rFVIIa, retro-orbitally 15 minutes prior to tail resection plus additional mice studied at 4 mg/kg rFVIIa 1.5 hours after the infusion with N = 4 animals per arm. Percent of animals surviving of N = 4 animals per arm is shown for each concentration.

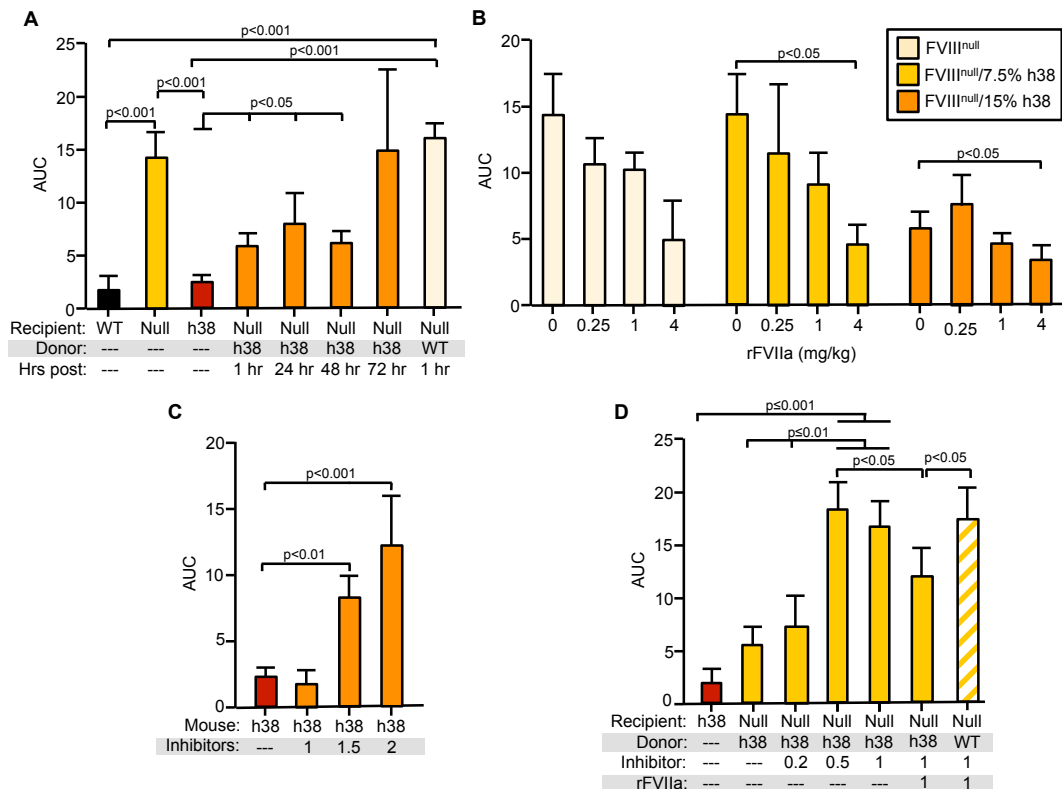


Figure 2.7 Efficacy of Line h38 platelet in the FeCl₃-carotid artery injury model.

FeCl₃-carotid artery injury studies were studied using a 20% FeCl₃ solution and a three-minute injury as described^{109,152}. Area under the curve (AUC) of subsequent blood flow was measured. In all studies, P values determined by one-way ANOVA. (A) WT, FVIII^{null}, Line h38 mice were studied as were FVIII^{null} mice infused with either WT or Line h38 platelets to achieve 20% of the circulating platelets in the recipient. Those animals infused with Line h38 platelets were studied up to 72 hours later. Mean ± 1 standard error of the mean (SEM) are shown with N = 5 animals per arm. (B) Same as in (A) with studied FVIII^{null} mice infused with the indicated amounts of Line h38 platelets and rFVIIa. Mean ± 1 SEM are shown with N = 5 animals per arm. (C) Line h38 mice were infused

with varying concentrations (1.0, 1.5, or 2.0 $\mu\text{g/g}$ of mice) of the inhibitor mixture prior to the FeCl_3 injury. Mean \pm 1 SEM are shown with N = 5 animals per arm. (D) $\text{FVIII}^{\text{null}}$ and Line h38 mice were studied in the FeCl_3 -carotid artery injury model as in (A). $\text{FVIII}^{\text{null}}$ mice were also infused with ~15% Line h38 or WT platelets and with varying concentrations of the inhibitor mixture (0.2, 0.5, or 1.0 $\mu\text{g/g}$ of mice) \pm 1 mg/kg of rFVIIa prior to the FeCl_3 injury. Mean \pm 1 SEM are shown with N = 5 animals per arm.

Chapter 3 – Generation and in vitro and in vivo characterization of iMks

Abstract

Using lentivirus to express genes in cells that normally do not express them has been a hallmark of gene therapy. To this end, I have used separate lentiviral constructs to express the three FVIII variants; hBDFVIII, hBDFVIII^{RH} and hBDFVIII^Δ and then transduce them into differentiating induced pluripotent derived megakaryocytes (iMks). hBDFVIII^{RH} and hBDFVIII^Δ have greater hemostatic efficacy and biology activity in comparison to hBDFVIII due to a mutation in a PACE/Furin cleavage site. This chapter details the generation of human iPSCs (WT control) from human bone marrow cells, their maintenance, and differentiation into iMks. In addition, this chapter will outline a protocol for making viral vectors and their use in transducing HPCs that will be differentiated into iMks. Next, I characterize these FVIII-expressing iMks (pFVIII-iMks) and investigate the effects of expressing FVIII on Mks *F8* mRNA levels, injury to the iMks and apoptosis, iMk responsiveness to agonists/activation. Lastly, I describe these FVIII-expressing iMks' efficacy in in vitro and in vivo assays of clot formation and thrombosis. Ultimately, these studies indicate the potential use of iMks in infusion therapy for severe Hemophilia A patients.

Introduction

Since the advent of iPSCs in 2006, many researchers have used this technology to model rare diseases, research areas of regenerative medicine, and develop potential therapeutics. iPSCs are made by ectopically expressing four transcription factors, Oct-4, Sox2, Klf4 and c-Myc, in somatic cells^{99,100}. Since the inception of this technology, delivery systems for the key reprogramming factors have become more efficient and safe, in part, through the development of inducible single expression cassettes.

Upon reprogramming, iPSCs share many features with ES cells and have the ability be differentiated into almost any cell type, which gives them a wide array of applications. In addition, they self-renew, allowing for a virtual unlimited supply of cells of interest after reprogramming¹⁰⁰. Because of these key features iPSCs have become attractive tools in almost all areas of research. In addition, iPSCs have the benefit of being able to be derived from somatic cells of healthy individuals as well as patients with rare diseases, allowing for their use in personalized medicine. To this end, researches have been able to further gene manipulate these cells for potential therapeutic benefit.

The ability of these cells to differentiate into a myriad of functional cell types has been exploited extensively, including benign hematology^{163,164}. Protocols have been developed to differentiate iPSCs into mesoderm layer cells and HPCs. These HPCS can then be further differentiated to any subsequent lineage cell, including terminal hematopoietic cells like Mks^{165,166}. Some of the earlier hematological applications of iPSCs included the study of sickle cell anemia¹⁶⁷ and Fanconi anemia¹⁶⁸.

Our laboratory has utilized iPSCs, not only to differentiate them into Mks to study rare diseases like Glanzmann's thrombasthenia, but also to correct the underlying

defect. For Glanzmann thrombasthenic iPSCs, we showed that expression of the human *ITGA2B* cDNA under the human *Gp1ba* promoter can drive sufficient α IIb expression to correct the thrombasthenic disorder in two different patient derived lines¹⁰². The expression construct was inserted using zinc finger nuclease (ZFN) technology into the adenovirus associated vector site (AAVS) 1 “safe harbor” locus and shown to correct α IIb expression in an Mk-specific fashion. More recently, our laboratory was able to develop iPSC lines derived from a patient with Paris Trousseau syndrome (PTSx), which underexpressed FLI1. In parallel lines, using TALEN technology, FLI1 was heterozygously knocked out in a WT line and another line was established where FLI1 overexpression was driven in a Mk-specific fashion. These studies targeted FLI1 using ZFN technology to the AAVS1 locus and provided new insights into FLI1 biology, its role in PTSx and FLI1’s potential use in optimizing platelet release from iMks after infusion into recipient mice¹⁰¹.

While both these studies from my group utilized an AAVS-1 targeting method, lentiviral targeting methods have also been used to express genes into iPSCs. Gene therapy through lentiviral expression has a number of advantages for transgene expression¹⁶⁹. Lentiviral vectors are able to easily integrate into a large variety of cell types, allowing for efficient transfection of the desired genes¹⁷⁰. Once integrated, these vectors are able to express high levels of the transgene with limited silencing¹⁶⁹. These vectors can also express genes in a tissue-specific manner with the appropriate inclusion of a tissue-specific promoter¹⁰⁹. In recent years, their use has become more efficient and safe through the use of 3 and 4 vector systems for producing virus as well as the development of self-inactivating vectors¹⁷¹⁻¹⁷³. Lentiviruses, however, can cause toxicity to targeting cells when used in large quantities¹⁷⁴. Furthermore, off target integration of the vectors is also an area of concern^{175,176}.

Because of their benefits and ease of use, our group has also utilized lentiviral vectors in experiments to express FVIII variants in Mks of FVIII^{null} mice and in human and canine Mks as well¹⁰⁹. We found that significant levels of hBDFVIII could be produced within Mks and subsequently platelets of these animals and that this expression could aid in hemostasis of FVIII^{null} mice after injury. Previously, we developed 3 vector plasmids that contained hBDFVIII, inactivation resistant FVIII (IRFVIII), or canine (c) cBDFVIII under the control of the *GPIBA* promoter. Although the level of IRFVIII and cBDFVIII antigen was lower than hBDFVIII and Line h38 mice, IRFVIII and cBDFVIII showed higher hemostatic efficacy¹⁵². Noting the success of cBDFVIII in these studies, our group utilized a human FVIII variant that had an analogous sequence in a PACE/Furin cleavage site, hBDFVIII^{RH}, developed by Drs. Valder Arruda and Denis Sabatino^{42,88}. Comparing cBDFVIII, hBDFVIII, and hBDFVIII^{RH}, we found hBDFVIII^{RH} had better hemostatic efficacy than hBF8. Additionally, we showed that FVIII expressed in Mks and platelets could have adverse effects on the developing cells, especially when using cBDFVIII. Previously, cBDFVIII had shown to be expressed in BHK cells at three times the level of hBDFVIII, but we found the opposite using Mks from mice, humans or dogs¹⁰⁹. These cFVIII-Mks were more apoptotic as noted via TUNEL assay, in comparison to FVIII^{null} Mks. It was also found that thrombopoietin (TPO) levels in transgenic cBDFVIII animals were elevated, suggesting that these post-BMT mice were compensating for a detrimental effect of cBDFVIII on megakaryopoiesis¹⁰⁹. Taken together, we hypothesized that the level of cBDFVIII expressed in Mks could cause them to become apoptotic, and that these deleterious effects may need to be considered when developing pFVIII-based therapy.

To date only one report has tried to express FVIII in iMks. Using human artificial chromosome (HAC) vector, Yakura, et al., used an integration-free method to express

FVIII driven by the *CXCL4* promoter¹⁷⁷. iPSCs were made from hemophilia A mouse cells and differentiated into iMks on OP9 stromal cell cultures after HAC targeting with Sendai virus (SeV). This group showed they could get Mks-specific expression of FVIII after the addition of TPO during iMk differentiation. While they showed expression of FVIII in Mk, this detection was limited to mRNA. This group was unable to show any expression of functional FVIII protein. In addition, this study did not show the functionality and efficacy of pFVIII-iMks in any FVIII^{null} models of thrombosis or hemostasis¹⁷⁷. Because of these limitations, one is left to wonder whether or not these Mks or the FVIII they contained could produce platelets that would be able to correct the FVIII^{null} phenotype.

In an attempt to express high levels of FVIII in my iMks, I attempted to use the *Gp1ba* promoter-driven FVIII cDNA inserted into the AAVS1 in iPSCs, but this strategy did not lead to sufficient FVIII expression (but see Chapter 4). My subsequent studies in this chapter used lentiviral vector studies to drive expression in iMks of hBDFVIII or hBDFVIII^{RH} or hBDFVIII^Δ after viral transduction into HPCs from differentiated WT iPSCs. These transduced HPCs were differentiated into FVIII expressing iMks (pFVIII-iMks). pFVIII-iMks were then characterized in vitro and then tested for hemostatic efficacy both in in vitro ROTEM and in vivo FeCl₃ assays in the setting of hemophilia A.

Materials and Methods

Generation, confirmation, and maintenance of WT iPSC line

WT (WTBM1-8) iPSC used in subsequent experiments was generated as previously described^{102,178}. Bone marrow cells were purchased from the stem cell core at the University of Pennsylvania (Philadelphia, PA). CD34⁺ cells were selected and

seeded onto a 35-mm plate, pre-coated with 5 µg/ml polybrene. pHAGE-hSTEMCCA (Figure 3.1), which contains the four transcription factors, OCT4, SOX2, KLF4, and c-MYC essential for reprogramming⁹⁹. This vector was co-transfected with a vector containing reverse tetracycline transactivator protein (rTTA), pHAGE2-CMV-rTTA that allowed for cell reprogramming after the addition of doxycycline¹⁷⁸. Post-incubation, viral media was replaced with media suitable for iPSC grow, consisting DMEM/F12 (Gibco), 20% KnockOut Serum (Invitrogen), 1 mM L-glutamine (Sigma-Aldrich), 1% nonessential amino-acid solution (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), and 10 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems). To select for properly transfected cells, 1 µg/ml doxycycline was added to the iPSC media for 3 weeks. Media was removed and replaced daily until individual drug resistant clones were present. Clones were picked and allowed to culture further¹⁷⁹.

Proper reprogramming and analysis of pluripotency was confirmed through karyotyping of genomic DNA, analysis of ES cell surface markers, and teratoma formation of iPSCs. Karyotyping was performed by Cell Line Genetics (Madison, WI) (Figure 3.2A). 6×10^6 iPSCs were injected subdermally into NOD-SCID mice (strain 250, Charles River) to assess teratoma formation. Tumors were collected, fixed and stained using hematoxylin and eosin, and assessed for germ layers as previously described^{102,165,178} (Figure 3.2B). To study pluripotent markers, cell expression of stage-specific embryonic antigen (SSEA) 3, SSEA4, Tumor-related Antigen (TRA)-1-60 and TRA-1-81 antigens were investigated via flow cytometry using a BD FACSCanto II (BD Biosciences). SSEA3 Alexa Fluor (AF) 488, SSEA4 AF 647, TRA-1-60 AF 488, and TRA-1-81 AF 647 (Biolegend) antibodies were used to see relative amounts of antigens (Figure 3.2C). Proper cell morphology of iPSC colonies and cultures is indicated in Figure 3.2

Successfully reprogrammed cells were cultured and maintained as previously described¹⁶⁶. Six-well culture dishes were coated with 0.01% gelatin at 37°C for 1 hour prior to addition of irradiated mouse embryonic fibroblasts (MEF) feeder-layer. This feeder-layer created a support, secreting factors needed for iPSC survival. After addition, MEFs were allowed to adhere to the plate for a minimum of 4 hours at 37°C in 5% CO₂ prior to use of the plate. iPSC colonies were maintained on MEF plates (Figure 3.3A) and passaged every 3-4 days. To passage feeder cultures, cells were incubated with Accutase up to 5 minutes at 37°C to detach cells from the plate. Detached cells were then centrifuged at 1200 rpm for 3 minutes before passaging at the appropriate split ratios, ranging from 1:8 to 1:24, in iPSC medium. 10 µM Y-27632 dihydrochloride Rho Kinase (ROCK) inhibitor (Tocris) was added to the media overnight. The following day, iPS media lacking ROCK inhibitor was substituted and replenished daily.

After several successful passages on feeder-layer MEFs were transitioned to a feeder-free culture (Figure 3.3B). Splitting ratios on feeder free cultures ranged from 1:2 to 1:12. Prior to passaging, six-well plates were treated with diluted Matrigel hESC-qualified matrix (Corning) for 1 hour. To passage feeder-free cultures, cells were washed with DMEM/F12 media and were allowed to incubate with Dispase (STEMCELL Technologies) at 37°C for up to 6 minutes or until the edges of the iPSC colonies began to detach. Cells were then washed twice with DMEM/F12 media and then fresh mTRS-1 media was added (STEMCELL Technologies). Colonies were then removed by scarping the well with cell scraper (SARSTEDT) and then split to the appropriate ratio and transferred to matrigel plates. mTSR-1 cell media was removed and replenished daily. All iPSCs were maintained at 37°C at 5% O₂ and 5% CO₂.

Vectors used for reprogramming and lentiviral production

Previously described self-inactivating (SIN) lentiviral constructs¹⁸⁰ used in these studies included the hBDFVIII (BD), the hBDFVIII^{R1645H} (RH), the hBFVIII^Δ (Δ), and eukaryotic green fluorescent protein (eGFP), each driven by the murine *Ccx14* proximal promoter¹⁰⁹. Infectious viral stocks were produced through transient infection of human embryonic kidney 293T cells with the psPAX2 packaging vector, the pMD2G vesicular stomatitis virus glycoprotein envelope vector, and the appropriate lentiviral FVIII plasmid (Figure 3.4) at a DNA ratio of 2:1:4 respectively. Plasmids were co-transfected into 15cm dishes of 9×10^6 293T cells using polyethylenimine (PEI) (Polysciences). PEI to DNA volume ratio was 3:1. Viral particles were collected from the supernatant 48-hours post-transfection, and concentrated by ultracentrifugation at 30,000g for 2 hours (Figure 3.5). Titering of final lentiviral infectious stocks was performed at the Fred Hutchinson Cancer Research Center as follows: HT1080 cells were plated in DMEM growth medium in 12-well plates. The following day, cells were transduced with dilutions of the viral vector. The cells were subsequently kept in culture for 10 days in which they were passaged at a 1:5 split twice. DNA was extracted from cells and the percentage of viral genome copies were analyzed by qPCR as previously described¹⁰⁹.

iPSC differentiation into iMks

IPSCs cultured on feeder-free cultures were utilized to differentiate into Mks. Differentiation of WT IPS line was performed as previously described^{102,165,166}. Differentiation was an 8 day process that began with iPSCs and ended up HPCs. Prior to the start of differentiation, six-well plates were coated with 1:3 Matrigel (Corning):DMEM/F12 media and allowed to incubate at 37°C for 30 minutes. Feed-free cultures were then split onto these plates at a ratio of 1:3 to 1:12. Cells were then allowed to re-attach overnight prior to differentiation.

Differentiation began on day 0 (D0). Prior to D0 media being added to the differentiation plates, the cells were re-fed with mTSR-1 media for 2-4 hours to remove any debris. See [Table 3.1](#) for full details of the various differentiation media. Cells were washed with Roswell Park Memorial Institute media (RPMI, Gibco) before feeding with 4 ml per 35-mm dish of D0 media. At D2, cells were again washed with RPMI prior to 4 mls per 35-mm dish of D2 media being added. On D4, cells were fed 4 ml per 35-mm dish of D4 media. Two days later, D4 media was removed and D6 media added. The day after D6 media is added, the unattached, suspended HPCs were collected from the supernatant. D7 media was then added, and HPCs were collected on both D7 and D8. After D8, plates were discarded and HPCs can be either be further differentiated and matured into iMks as previously described or infected with lentivirus and then differentiated. HPCs are added into iMk differentiation media for up to 7 days¹⁶⁶.

Establishing pFVIII-iMks

HPCs ($5-10 \times 10^5$ per well) were transfected with the FVIII-expressing or control lentiviral vectors at a multiplicity of infection of 1-10 via spin infection at 2000g for 2 hours at 37°C in a 12-well non-tissue culture plate (BD Pharmingen) coated with 10-20 $\mu\text{g cm}^{-2}$ RetroNectin (Takara Bio) in iMk differentiation media as previously described^{109,152}. Viral particles were washed off the cells 24 hours post-infection. Cells were placed in fresh iMk differentiation media for 5 days, and the resulting iMks were characterized in vitro or used to study pFVIII biology in vitro and in vivo.

Viral integration number in pFVIII-iMks

DNA was extracted from pFVIII-iMks using DNeasy[®] Blood and Tissue Kit (Qiagen). Viral integration was determined via Lenti-X[™] Provirus Quantitation Kit (Clontech). Total RNA was isolated from 2×10^6 iMks using RNeasy Micro Kit (Qiagen). Total RNA (500 ng) from each sample was used to generate cDNA using High Capacity Reverse Transcription Kit (ABI) used according to manufacturer's instructions.

mRNA analysis

Message RNA level of target genes in final iMks was determined by quantitative RT-PCR using Taqman Probes with TaqMan[®] Fast Advance Master Mix (ABI) via 7900HT Real-Time Cycler. Taqman Probes used were: FVIII (*F8*) Hs00252034_m1, integrin alpha-IIb (*ITGA2B*) Hs01116228_m1, Tata Binding Protein (*TBP*) Hs00427620_m1, and platelet factor 4 (*PF4*) Hs00427220_g1. Relative transcript levels were quantified using the $2^{-\Delta\Delta CT}$ method¹⁸¹ and calculated against TBP as the standard.

Flow cytometry analysis of iMks

Day 6 pFVIII-iMks, NTC and eGFP controls were stained and analyzed surface markers and degree of apoptosis on FACS Canto (BD Biosciences) and further analyzed using FlowJo software as previously described. Antibodies used in these studies are listed in [Table 3.2](#).

pFVIII antigen level in pFVIII-iMks

FVIII antigen levels in iMks were determined using an ELISA for hFVIII as previously described. Levels were compared to recombinant full-length hFVIII (Advate, Shire) and pFVIII levels in Line h38 Mks.

Apoptotic studies of pFVIII-iMks

Apoptotic changes in pFVIII-iMks was done in part by TUNEL-labeling using the APO-bromodeoxyuridine (BrdU) TUNEL Assay Kit (Invitrogen) as per manufacturer's directions. Samples were stained and analyzed via flow cytometry using APC-labeled anti-CD42b (BD Biosciences) and AlexaFluor-488 labeled anti-BrdU for degree of apoptosis. All samples were compared to non-transduced controls. To further investigate apoptosis, 1×10^6 iMks samples were incubated with 5 μ l annexin V-FITC (BD Biosciences), 10 μ l APC-labeled anti-CD42b in 100 μ l Tyrode's buffer (Sigma) with 0.1% bovine serum albumin (BSA, Thermo) at room temperature (RT) for 30 minutes. Post incubation, cells were washed twice and then resuspended in Tyrode's buffer that contained 0.1% BSA. Annexin V binding and CD42b levels were analyzed via flow cytometry. iMks were also stained concurrently with anti-CD41 and anti-CD42b (both BD Biosciences) antibodies to measure the loss of glycolocalicin within CD42, an indicator of Mk injury¹⁸².

iMk responsiveness to thrombin

To examine in vitro-responsiveness of iMks to an agonist, 1×10^6 iMks were incubated with 1 unit of thrombin/ml (T8885-1VL, Invitrogen) in Tyrode's buffer with 0.1% BSA in a final volume of 100 μ l containing 1:2000 APC-labeled anti-CD42b antibody and FITC-labeled PAC-1 (BD Biosciences) as previously described¹⁰². The samples were incubated at 37°C for 30 minutes. Post incubation, 500 μ l of ice-cold 0.1% Tyrode's buffer was added. PAC-1 and CD42b binding were then analyzed via flow cytometry.

In vitro hemostatic studies with pFVIII-iMks

ROTEM studies of pFVIII-Mks were performed by adding 10 μ l of medium containing $5 \times 10^{3-5}$ pFVIII-iMks expressing hBDFVIII or hBDFVIII^{RH}, which has been shown by our group and others to have increased specific activity and hemostatic efficacy, to 110 μ l of whole FVIII^{null} mouse blood. Negative controls were iMks transfected with no lentivirus, the eGFP-expressing lentivirus or phosphate-buffered saline (PBS, Gibco) added to the same volume of blood. ROTEM studies were performed as described in Chapter 2.

In vivo hemostatic studies with pFVIII-iMks

In vivo FeCl₃-carotid artery injury studies using FVIII^{null} mice after xenotransfusion of human iMks were similar to the studies with Line h38 platelets, except that the recipient FVIII^{null} mice were pre-treated with clodronate liposomes (Encapsula NanoSciences) 24 hours prior to iMk infusion, to deplete macrophages and extend the half-life of the infused iMks and their released platelets. The percent human platelets circulating post-iMk infusion were determined following retro-orbital blood draws by flow cytometry using species-specific anti- α IIb antibodies, as we previously described^{151,183}. Recipient FVIII^{null} mice received $5-10 \times 10^6$ iMks expressing hBDFVIII, hBDFVIII^{RH}, or eGFP infused via the jugular vein in 200 μ l total volume of PBS.

Statistical analysis

Statistical differences between arms were determined using a two-sided Student's t-test or analysis of variance (ANOVA) with Bonferroni, Dunnett's or Tukey's corrections of multiple comparisons, when appropriate. PRISM 7.0 (Graphpa) was used to calculate statistical significance. P-Values ≤ 0.05 were considered significant.

Results

FVIII expression in pFVIII-iMks

The iPSC line that we utilized had been previously used by our group and shown to be capable of differentiating into an evaluable number of iMks^{101,102}. HPCs were transduced with the previously described *Cxcl4*-proximal promoter driven lentiviral vectors that express hBDFVIII (termed below BD), hBDFVIII^{RH} (termed below RH), hBDFVIII^Δ (termed Δ), or eGFP. A schematic showing iPSC hematopoietic differentiation, FVIII lentiviral transduction and downstream applications is shown in [Figure 3.6](#). On Day 6 of iMk differentiation, lentiviral copy numbers of transduced cells were determined and shown not to be significantly different between the four vectors, although the highest level was seen for the RH-iMks ([Figure 3.7A](#)). *F8* mRNA was highest for BD ($p < 0.001$, [Figure 3.7B](#)), while RH and Δ showed similar levels. Although BD had the highest message level, FVIII antigen per cell by ELISA was ~50% higher for RH ($p < 0.01$, [Figure 3.7C](#)). In addition, Line h38 Mks expressed at 2 orders of magnitude higher than pFVIII-iMks.

In vitro characterization of lentiviral pFVIII-iMks

Our previous studies suggested that ectopically expressed FVIII in Mks was harmful to the developing cells. To determine if damage and level of apoptosis to iMks occurred in this system, examination of cells for bromo-deoxyuridine staining via TUNEL assay ([Figure 3.8A](#)) and annexin V binding ([Figure 3.8B](#)) was completed. No difference was observed in these studies between pFVIII-iMks and controls. In addition, the health

of the pFVIII-iMks was analyzed by responsiveness to agonist stimulation and no difference was observed (Figure 3.9A). iMks were further analyzed for percent of the CD41⁺ pFVIII-iMks that were also CD42b⁺ – an indicator of undamaged Mks (Figure 3.9B) – was done, comparing pFVIII-iMks to non-transfected iMks controls. mRNA levels of endogenous genes *CXCL4* and *ITGA2B* genes were also investigated and showed no difference between FVIII-containing iMks, and NTC and eGFP controls (Figure 3.10A and 3.10B, respectively).

In vitro studies with human pFVIII-iMks

ROTEM studies were done with pFVIII-iMks added to FVIII^{null} murine whole blood and demonstrated that as few as 5x10³ pFVIII-iMks added to 110 µl of whole blood can improve hemostasis compared to the addition of iMks not expressing FVIII (Figure 3.11A and 11B). The RH-iMks showed greater efficacy than BD -iMks as indicated by a shorter clotting time and increased α -Angle (Figure 3.11A and 11B). The addition of 10 nM rFVIIa showed a combinatorial effect with the pFVIII-iMks, further improving hemostasis, but not fully correcting it as the clotting time notably remained prolonged (Figure 3.11C), supporting the Line h38 platelet data shown in Figure 2.4.

In vivo studies with human pFVIII-iMks

Infused iMks released detectable human platelets with a half-life of ~4 hours (Figure 3.12A). This short timeframe precluded tail-clip exsanguination studies, but allowed us to perform FeCl₃ carotid artery studies initiated within 30 minutes after iMk infusion. Both WT and RH iMks improved hemostasis significantly compared to non-

pFVIII-iMks, although none of these mice developed occlusive thrombi (Figure 3.12B). As in the ROTEM studies, RH iMks improved hemostasis better than BD iMks.

Discussion

Our laboratory has shown that infused Mks into mice release functional platelets after being entrapped in the lungs⁷¹. These platelets respond to platelet agonists and are able to take part in hemostasis during hemostatic challenge. These iMk-released platelets are also functional. Recently using in situ microscopy, it has shown that endogenous Mks in mice also travel to the lungs after being released from the marrow and release platelets that account for half of the daily production of platelets⁷¹. Thus infused iMks-released platelets in the lungs are likely to represent as good as platelets as one can obtain from in vitro-grown megakaryocytes and provide insights into the applicability of pFVIII-iPlatlets (iPlts) in the care of patients with hemophilia A.

In vitro characterization of pFVIII-iMk

Previous studies from our group showed that FVIII expressed at high levels within Mks, especially cFVIII, can be deleterious to the developing cell. We therefore examined using various markers of apoptosis and iMk health to see whether the pFVIII-Mks were injured by the expressed FVIII. These studies found no indication of apoptosis or other injuries (Figure 3.8-3.10). The health of these iMks is likely in part due to human pFVIII not being as injurious to Mks as cFVIII and in part to the fact that the level of human pFVIII expressed in these iMks was low perhaps 1% of that achieved in Line h38 transgenic mice Mks.

In vitro hemostatic studies with human pFVIII-iMks

ROTEM studies were completed with pFVIII-iMks added to FVIII^{null} murine whole blood and demonstrated that as few as 5×10^3 pFVIII-iMks added to 110 μ l of whole blood can improve hemostasis compared to the addition of iMks not expressing FVIII. This would be consistent with as few as 10^5 iMks being sufficient to achieve hemostatic effect in a 30 gm mouse. If each iMk released ~ 100 iPlts that would mean that $\sim 10^7$ human iPlts or $\sim 1\%$ of the concurrent $\sim 2 \times 10^9$ murine platelets would be sufficient to demonstrate hemostatic efficacy in the intact mouse. Importantly, these in vitro hemostatic studies also supported that RH pFVIII were more effective than WT pFVIII released from iMks. The released pFVIII from iMks showed combinatorial hemostatic improvement with rFVIIa, further supporting the Line h38 platelet data shown in [Figure 2.4](#) and the utility of pFVIII infusion in the care of patients with hemophilia A.

In vivo hemostatic studies with human pFVIII-iMks

During these experiments, there was no available FVIII^{null} murine model on an immune compromised background that was available for infusion studies, so we infused iMks into clodronate liposome-treated FVIII^{null} mice to improve human platelet half-life by reducing phagocytic removal of the human cells and their released platelets. This was a successful model system although it is likely that circulating pre-activated platelet-like particles released in culture as we previously described are also present, though our prior studies suggest that they poorly contribute to hemostasis¹⁸³. Clearly, these studies should be repeated in FVIII^{null} mice that are on an immunocompromised background and such studies are underway having obtained such mice from Dr. Shu-Wha Lin at Taiwan University.

The relatively short half-life of the iPIts in clodronate liposome-treated FVIII^{null} mice precluded tail-clip exsanguination studies, but allowed us to perform FeCl₃ carotid artery studies initiated within 30 minutes after iMk infusion. Both hBDFVIII and hBDFVIII^{RH} iMks improved hemostasis significantly compared to non-pFVIII-iMks, although none of these mice developed occlusive thrombi (Figure 3.12B). As in the ROTEM studies, hBDFVIII^{RH} iMks improved hemostasis better than hBDFVIII iMks, further supporting the application of this variant or others showing greater specific activity in any therapy involving pFVIII treatment in hemophilia A.

Tables

Table 3.1 Composition of iPSC and megakaryocyte differentiation medias

Component	Working Concentration	Final Concentration	Volume (1 plate)
Day 0 (D0)			
RPMI (P/S+Glut)			24 mL
Ascorbic Acid (fresh)	5 mg/mL	10 µL /mL	240 µL
MTG (fresh)	26 µL/2 mL IMDM	3 µL /mL	72 µL
BMP4	10 µg/mL	5 ng/mL	12 µL
VEGF	50 µg/mL	50 ng/mL	24 µL
CHIR (GSK b3 inhibitor)	10mM	1 µM	2.4 µL
Day 2 (D2)			
RPMI (P/S + Glut)			12 mL
SP34 (P/S + Glut)			12 mL
Ascorbic Acid (fresh)	5 mg/mL	10 µL/mL	240 µL
MTG (fresh)	26 µL/2 mL IMDM	3 µL/mL	72 µL
VEGF	50 µg/mL	50 ng/mL	48 µL
BMP4	10 µg/mL	5 ng/mL	12 µL
bFGF-100	100 µg/mL	20 ng/mL	4.8 µL
Day 4 (D4)			
StemPro 34 (P/S+Glut)			24 mL
Ascorbic Acid (fresh)	5 mg/mL	10 µL/mL	240 µL
MTG (fresh)	26 µL/2 mL IMDM	3 µL/mL	72 µL
VEGF	50 µg/mL	15 ng/mL	7.2 µL

Day 6,7,8 (D6,7,8)

Component	Working Concentration	Final Concentration	Day 6 (1 plate)	Day 7 (1 plate)	Day 8 (1 plate)
SFD			18 mL	24 mL	30 mL
Ascorbic Acid (fresh)	5 mg/mL	10 µL/mL	180 µL	240 µL	300 µL
MTG (fresh)	26 µL/2 mL IMDM	3 µL /mL	54 µL	72 µL	90 µL
VEGF	50 µg/mL	50ng/mL	18 µL	24 µL	30 µL
bFGF-100	100 µg/mL	100ng/mL	18 µL	24 µL	30 µL
TPO	50 µg/mL	50ng/mL	18 µL	24 µL	30 µL
IL-6	10 µg/mL	10ng/mL	18 µL	24 µL	30 µL
Flt3L	50 µg/mL	25ng/mL	9 µL	12 µL	15 µL
SCF	100 µg/mL	25ng/mL	4.5 µL	6 µL	7.5 µL

Table 3.1 Cont.

iMk differentiation media

Component	Working Concentration	Final Concentration	Volume (1 plate)
SFD			12 mL
Ascorbic Acid (fresh)	5 mg/mL	10 µL/mL	120 µL
MTG (fresh)	26 µL/2 mL IMDM	3 µL/mL	36 µL
SCF	100 µg/mL	25 ng/mL	3 µL
TPO	50 µg/mL	100 ng/mL	24 µL

Ascorbic acid (Wako Chemicals)

BFGF: Basic fibroblast growth factor (R&D Systems)

BMP4: Bone Morphogenic Protein 4 (Stemgent)

CHIR: Synthase Kinase 3 inhibitor CHIR (Tocris)

FLT-3L: Tyrosine Kinase 3 Ligand (Gibco)

Glut: L-glutamine (Sigma-Aldrich)

MTG: monothioglycerol (Sigma-Aldrich)

RPMI: Roswell Park Memorial Institute media (Gibco)

SCF: Stem Cell Factor (Gibco)

SP34: StemPro-34 serum-free mediums (R&D Systems)

TPO: Thrombopoietin (R&D Systems)

VEGF: Vascular Endothelial Growth Factor (R&D Systems)

SFD: Serum-free differentiation medium (5% IMDM, 1% B-27, 0.5% N-2 supplements (Invitrogen), 1 mM (1%) L-glutamine, 0.5% BSA, and 22% Hams F12 medium (Corning).)

Table 3.2. Summary of commercial antibodies used in this paper including their indicated usage.

Target	Source	Reactivity	Label	Company	Catalog Number	Usage
CD42b	Monoclonal Mouse	Human	APC	BD Biosciences	551061	Flow Cytometry
CD42b	Monoclonal Mouse	Human	PE	BD Biosciences	555473	Flow Cytometry
CD41a	Monoclonal Mouse	Human	APC	BD Biosciences	559777	Flow Cytometry
CD41a	Monoclonal Mouse	Human	PE	BD Biosciences	555467	Flow Cytometry
CD42a	Monoclonal Mouse	Human	PerCP	BD Biosciences	340537	Flow Cytometry
CD42a	Monoclonal Mouse	Human	PE	BD Biosciences	558819	Flow Cytometry
Annexin V	Bacteria	Human	FITC	BD Biosciences	556420	Flow Cytometry
PAC-1	Monoclonal Mouse	Human	FITC	BD Biosciences	340507	Flow Cytometry
BrdU [PBR-1]	Monoclonal Mouse	Human	Alexa Fluor 488	Invitrogen	A23210	Flow Cytometry
IgG	Polyclonal Goat	Human	Alexa Fluor 488	Thermo	A-11013	IFM
vWF	Monoclonal Mouse	Human	N/A	Thermo	MA5-14029	IFM
Mouse IgG	Donkey	Mouse	Alexa Fluor 594	Thermo	A-21203	IFM

PE: Phycoerythrin
 APC: Allophycocyanin
 FITC: Fluorescein isothiocyanate
 IFM: Immunofluorescence microscopy

Figures

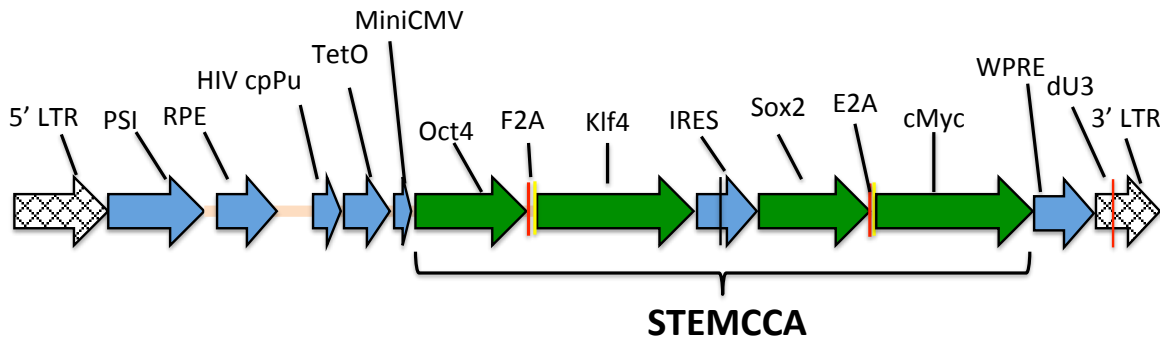


Figure 3.1 Lentiviral vector schematic of inducible pHAGE-STEMCCA (adapted from Sommer et al., *Stem Cells*. 2009¹⁷⁸).

iPSCs were generated using a lentiviral vector that contained the 4 reprogramming transcription factors: Oct-4, Klf4, Sox2, and cMyc. Illustration indicates the single multicistronic mRNA cassette. Expression of the 4 genes is under the control doxycycline inducible promoter, TetO-miniCMV. cpPu: central polypurine tract. dU3: deleted U3. HIV: human immunodeficiency virus. IRES: internal ribosome entry site. LTR: long terminal repeat. miniCMV: mini-cytomegalovirus. PSI: packaging signal. RRE: rev responsive element. STEMCCA: stem cell cassette. WPRE: woodchuck hepatitis virus post-transcriptional regulatory element.

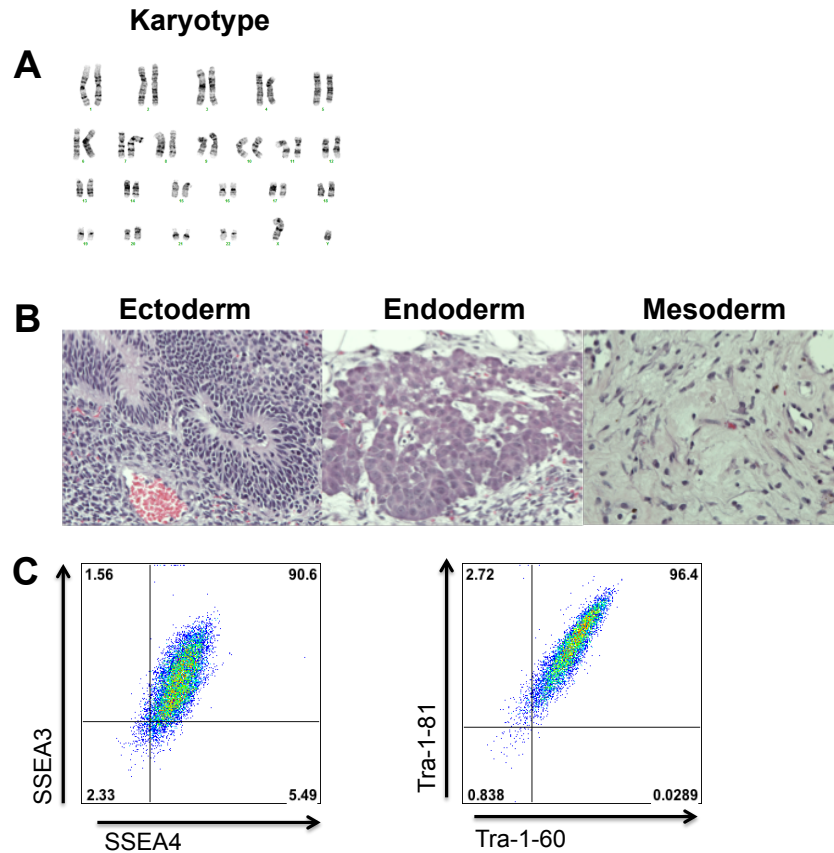


Figure 3.2 Confirmation of WT iPSC (WTBM1-8) pluripotent state.

(A) Karyotype of the WT iPSC line showing a normal human chromosomal pattern 23 chromosomal pairs and no abnormalities. (B) Teratoma formation and germ layer analysis indicate the presence all three germ layers. (C) Flow cytometry of early ES cell proteins and pluripotency markers in WT iPSCs using SSEA3, SSEA4, Tra-1-60, and Tra-1-81¹⁷⁹. WT iPSC line exhibited high levels of each marker after reprogramming as desired. SSEA: Stage-specific embryonic antigen. TRA: Tumor-related antigen.

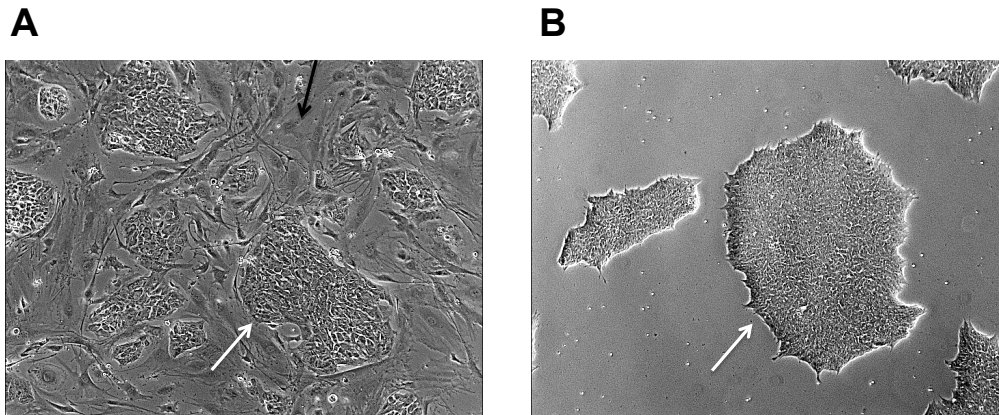


Figure 3.3 iPSC morphology.

(A) iPSCs on feeder layer on brightfield imaging. (B) Same as in (A), but iPSCs in feeder-free culture. Images show representative iPSC colonies that were used for culture and differentiation. White arrows indicate iPSC colonies and black arrows indicate irradiated mouse embryonic fibroblast feeder layer. Brightfield images taken at 4X using Zeiss, Axio, Observer z.1 microscope.

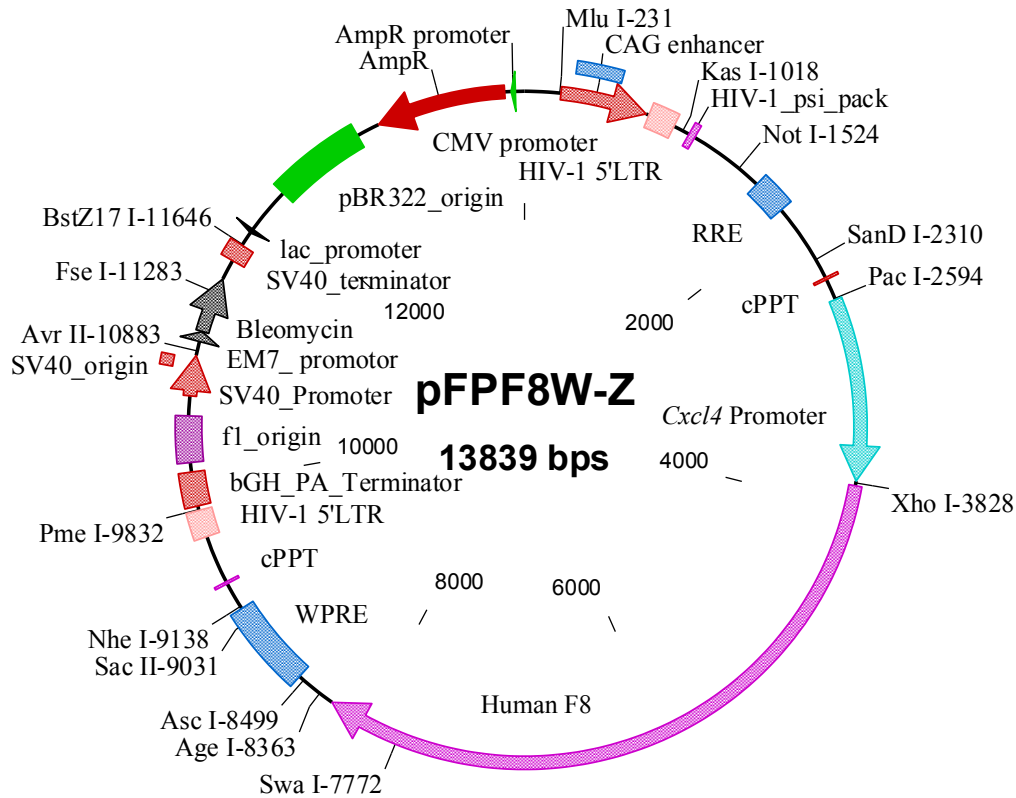


Figure 3.4 Lentiviral schematic used for pFVIII expression

FVIII-containing viruses were made using the above lentiviral plasmid that contained *F8* cDNA and mouse *Cxcl4* (PF4) promoter. HPCs were transduced with lentivirus that carried a transgene for one of three F8 cDNA variants: hBDFVIII, hBDFVIII^{RH} and hBDFVIII^A. Expression of FVIII was controlled and specific to Mks and platelets through the use of the *Cxcl4* proximal promoter. Illustration shows the components of the viral vector. The hBF8 constructs were made by Dr. Teeshell Greene based on the plasmid pFUGW¹⁵², inserting the murine *Cxcl4* promoter and human WT B-domainless F8 cDNA replacing the eGFP cDNA in pFUGW. pFPF8W was then modified by Dr. Guohua Zhao in our group to remove second Xho I site and insert an Nhe I site in same position and an Age I site at position 8363 after the F8 cDNA to facilitate insertion of hBDFVIII^{RH} and hBDFVIII^A.

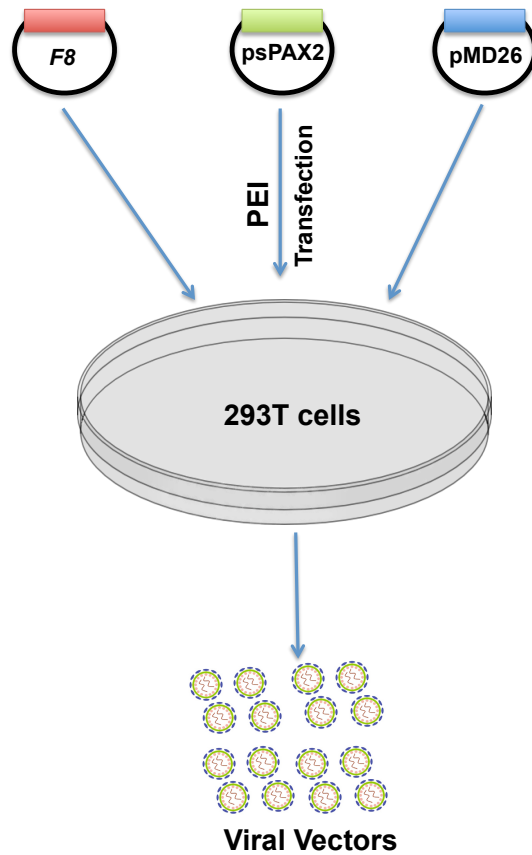


Figure 3.5 FVIII lentivirus production.

Lentiviral vectors were made using a three-vector system¹⁸⁴. 293T cells were co-transfected using PEI with vectors: *F8* cDNA vector (Figure 3.5), psPAX2 viral packing vector, and pMD26 viral envelop vector. 48-hours post infection, viral vectors were collected from the supernatant and concentrated through ultracentrifugation as described^{109,152}.

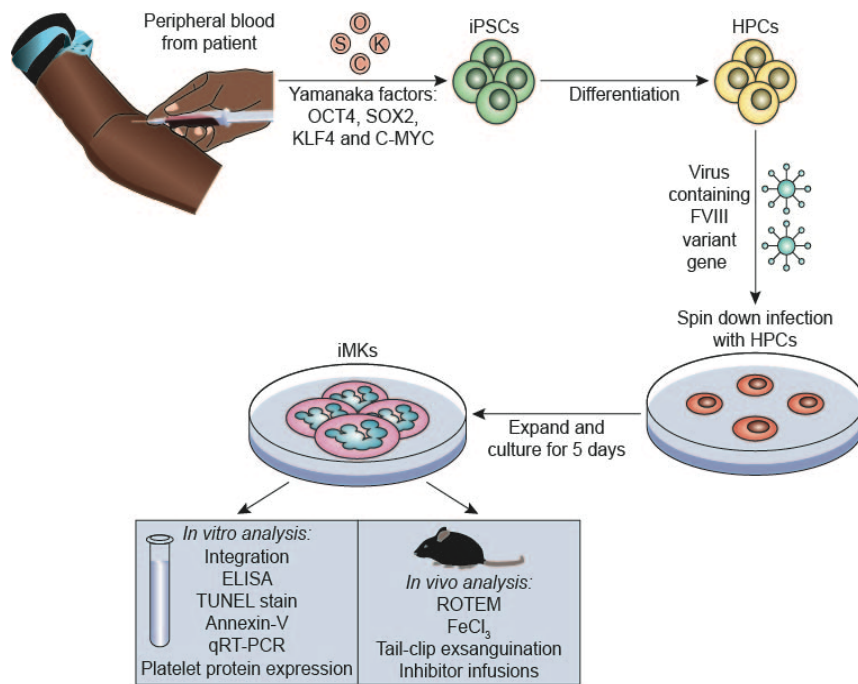


Figure 3.6 Schematic of experimental design using iMks to study pFVIII biology.

Peripheral blood mononuclear cells from patients were reprogrammed into iPSCs as previously described^{178,179}. iPSCs were differentiated into HPCs. HPCs were transfected with a SIN lentiviruses expressing pFVIII or controls, and the HPCs were then differentiated into iMks. After differentiation and maturation of pFVIII-iMks, these cells were used for studies of pFVIII biology in vitro and in vivo.

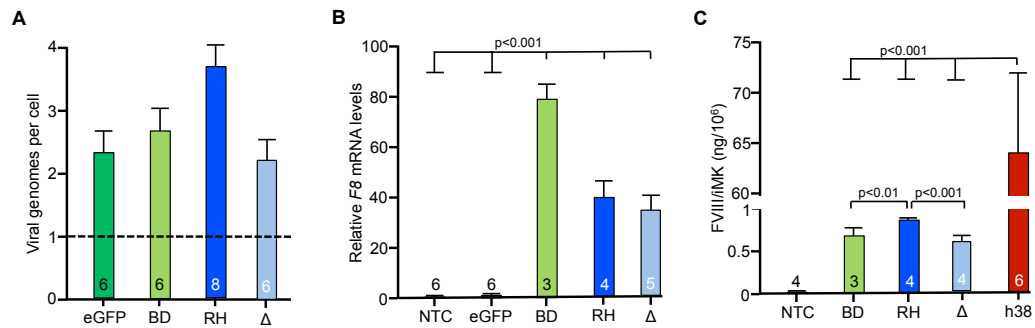


Figure 3.7 FVIII expression in iMks.

(A) DNA from 10^6 iMks was collected and analyzed for lentiviral integration. Viral genomes per cell were expressed relative to non-transduced control (NTC, dashed line). Mean \pm 1 SEM are shown with the number of independent experiments indicated in each bar. No difference was noted by ANOVA analysis between the lines. (B) 500 ng of total RNA was analyzed for *F8* message. NTC and eGFP-expressing iMks were used as negative controls. Mean \pm 1 SEM are shown with number of independent experiments indicated in each bar. P values determined by one-way ANOVA. (C) 2×10^6 iMks were lysed to extract cellular content. Whole cell lysates were analyzed using human FVIII ELISA. Mean \pm 1 SEM are shown with number of independent experiments indicated in each bar. P values determined by one-way ANOVA. BD = hBDFVIII-iMks, RH = hBDFVIII^{RH}-Mks, Δ = hBDFVIII ^{Δ} -iMks, and h38 = Line h38 mouse platelets.

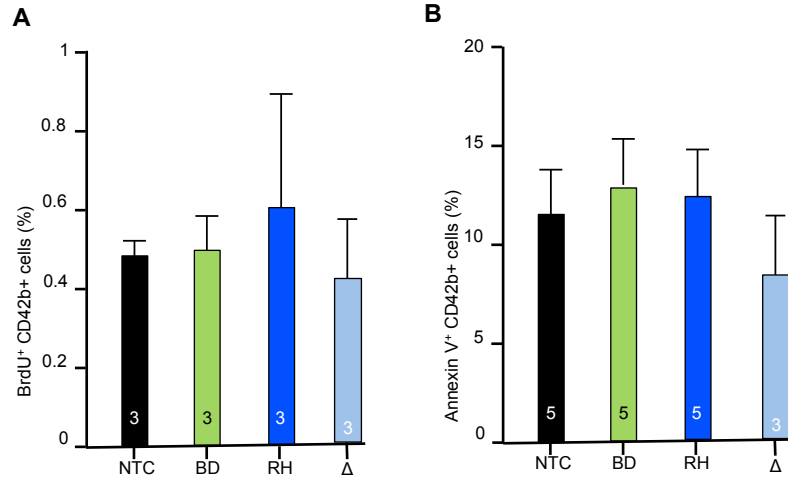


Figure 3.8 Apoptotic effects of FVIII on iMks.

(A) TUNEL-stained for BrdU and co-stained for CD42b to examine for pFVIII-iMks apoptosis. Mean \pm 1 SEM are shown with number of independent experiments indicated in each bar. No difference was noted by ANOVA analysis between the examined iMk lines. **(B)** Annexin V binding as an indicator of apoptosis of iMks. Mean \pm 1 SEM are shown with number of independent experiments indicated in each bar. No difference was noted by ANOVA analysis between the examined iMk lines.

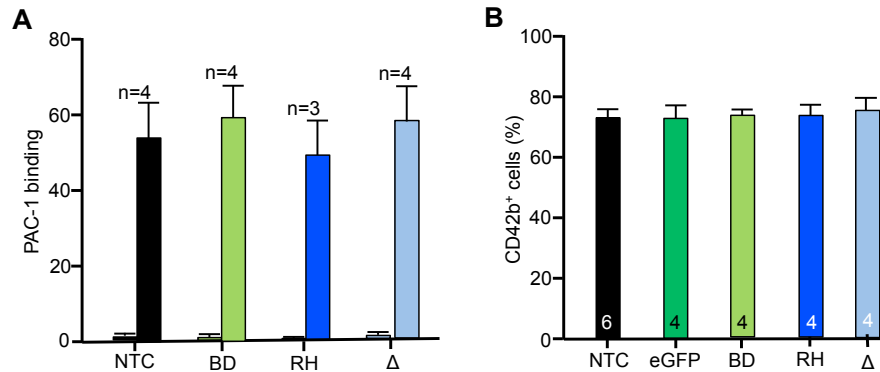


Figure 3.9 pFVIII effects on iMks.

(A) Agonist responsiveness of iMks to 1 unit of thrombin/ml and measuring FITC-labeled PAC-1 antibody binding. For each iMk, the left bar is pre-thrombin and the right is post-thrombin activation. Mean \pm 1 SEM are shown with number of independent experiments for each iMk condition is shown. $P < 0.001$ for pre- versus post-thrombin activation for each iMk condition by one-way ANOVA, but no difference was noted by ANOVA analysis between the examined iMk lines. (B) Studies of iMk injury with loss of CD42b⁺ signal within the CD41⁺ as an indication of metalloproteinase cleavage of the extracellular glyocalicin domain of the GPIIb α chain¹⁸² are shown. Mean \pm 1 SEM are shown with number of independent experiments indicated in each bar. No difference was noted by ANOVA analysis between the examined iMk lines.

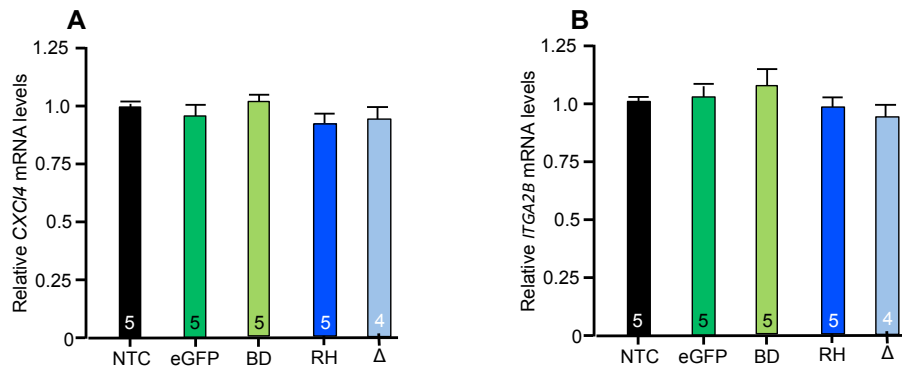


Figure 3.10 mRNA expression of Mk and platelet genes in the studied iMk lines.

Examination of relative mRNA levels compared to NTC by quantitative RT-PCR¹⁸⁵ for *CXCL4* (A) and *ITGA2B* (B). In both (A) and (B), 500 ng of total RNA was analyzed for message as described¹⁸⁶. NTC and eGFP-expressing iMks were used as negative controls. Mean \pm 1 SEM are shown with number of independent experiments indicated in each bar. Values calculated against TBP standard. (A) *CXCL4* and (B) *ITGA2B* genes showed no difference in comparison to controls.

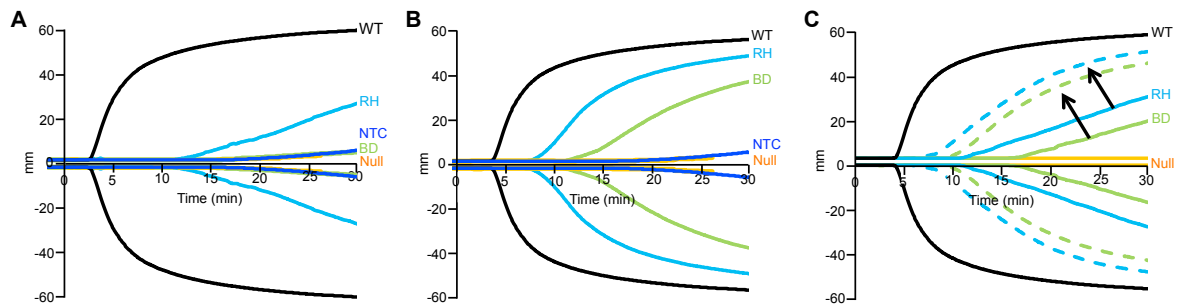


Figure 3.11 In vitro efficacy of pFVIII-iMks in ROTEM studies.

ROTEM studies as in [Figure 2.3](#), but with added iMks instead of Line h38 platelets. BD = BDFVIII-iMks added; RH = BDFVIII^{RH}-iMks added; and NTC = non-transfected control iMks added. Each curve represents 5 independent studies. **(A)** Addition of 5×10^3 iMks to 110 μ l of whole blood from FVIII^{null} mice. WT mice blood and FVIII^{null} (KO) mice blood were studied as positive and negative controls, respectively. **(B)** Same as in (A) with 5×10^4 BD or RH iMks. **(C)** Same as in (A), but with (dotted lines) or without (solid lines) 10 nM rFVIIa also added to the samples. Arrows indicate the paired studies without and with rFVIIa.

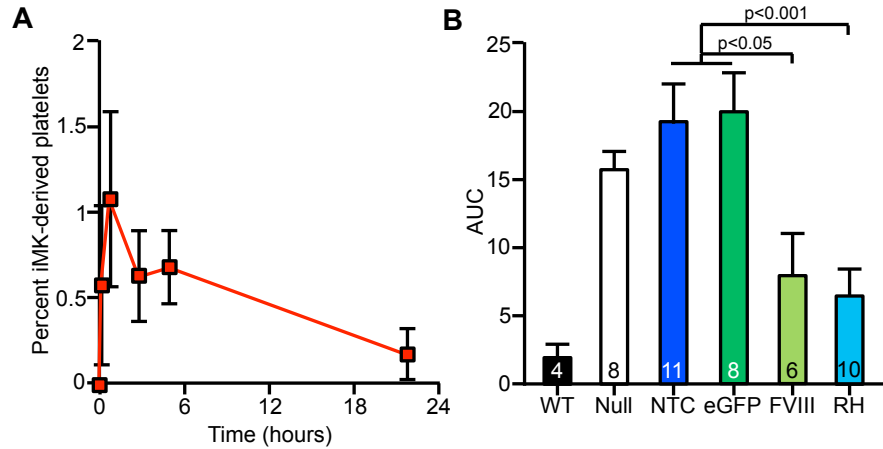


Figure 3.12 pFVIII-iMk infusion efficacy in FVIII^{null} mice using the FeCl₃-carotid artery injury model.

5-10X10⁶ iMks were infused into clodronate liposome-treated FVIII^{null} mice. **(A)** Human platelets released from infused iMks were analyzed up to 24-hours post iMks infusion via flow cytometry. Mean ± 1 SEM are shown with 5 independent studies. **(B)** Similar FeCl₃-carotid artery injury studies as in [Figure 2.7](#) with the primary endpoint being remaining blood flow as measured by AUC. Mean ± 1 SEM are shown with the number of independent studies noted in the bar graphs. P values were done by one-way ANOVA.

Chapter 4 – Megakaryocyte IgG uptake and AAVS1 FVIII targeting of iMks

Abstract

Megakaryocytes and platelets take up specific proteins from blood circulation and store them in their α -granules. This uptake includes a general uptake and storage of IgG. This uptake should include neutralizing inhibitors of FVIII, which are virtually all IgGs. This would imply that inhibitors are localized into the same granules as FVIII and should either neutralize the pFVIII within the granules or neutralize FVIII after release if the IgG and FVIII are stored in distinct α -granules. However, pFVIII remains effective upon release at sites of vascular injury. Whether IgG taken up by Mks is stored in different granules than FVIII has yet to be explored. This chapter outlines preliminary studies to localize human IgG taken up by Mks using fluorescence microscopy, and indicates that IgG does not localize with endogenous Mk vWF. Additionally, in this chapter, an alternative approach to express pFVIII in iMks is explored through FVIII AAVS-1 correction of hemophilia A iPSCs. These studies seek to enhance pFVIII expression in iMks as well as note some of limitations of using iMks.

Introduction

Human FVIII inhibitors storage in α -granules

Mks and platelets store endogenously made proteins and small molecules within three distinct granules: α , δ , and λ . α -granules contain proteins both made in the Mk or taken up in circulation. α -granules contain proteins such as vWF, PF4, other chemokines, and many more¹⁸⁷. They also take up FV (in humans), albumin, fibrinogen, and IgGs from their surroundings^{188,189}. The exact method of uptake is an area of ongoing study, but it is thought to be receptor-mediated and clathrin-dependent^{189,190}. To date, the exact receptor that is responsible for uptake of plasma proteins has yet to be identified.

It is hypothesized that once these proteins are taken up from circulation and then are stored within the α -granules. After the development of our Line h38 transgenic mice in 2003, our group showed that pFVIII was present in granules that overlapped with granules that contained vWF, suggesting that pFVIII is largely stored in α -granules and released upon platelet activation^{108,152}. In the absence of vWF, our group has shown that pFVIII is still found at ~70% of the level in vWF-sufficient Mks, is still stored in α -granules and is functional when released¹⁹¹.

For pFVIII to be effective in the presence of high-titer inhibitors, it must be shielded from circulating inhibitors. Given this information and the fact that IgG is endocytosed and stored in α -granules, one would assume that inhibitors taken up by Mks and platelets would be stored with pFVIII. If this is true, why isn't the FVIII contained within granules with inhibitors inhibited and rendered ineffective? And if IgG is stored at high quantities and released upon platelet activation, why does the locally released IgG

not inactivate released pFVIII? We hypothesize that IgG taken up by platelets is stored in “uptake” α -granules that do not contain FVIII and that vWF and pFVIII are stored in “endogenous” α -granules. Others have suggested the stored vWF protects the pFVIII from inhibitors both in the granules and after subsequent release of both pFVIII/vWF. They confirmed our findings that pFVIII is still stored at ~70% levels in type 3 vWD mice and is functional in a FVIII^{null} setting. In addition, they also present tail-clip exsanguination data that in Type 3 vWD/FVIII^{null} mice where pFVIII loses efficacy in the presence of inhibitors. However, these results are limited to just one hemostatic model¹⁹².

AAVS-1 correction

Previous studies by our group had utilized AAVS-1-targeting to express, under a *Gplba* promoter, genes within differentiating iMks¹⁰². Our first attempt at AAVS-1 correction was by Spencer Sullivan. His report, in 2014, showed that he could correct the defect in Glanzmann thrombasthenia due to defective α IIb in iMks and iPlts by targeting *ITGA2B*-expression vector to the AAVS-1 locus. These studies demonstrated that he could express α IIb at a high level via this insertion technology and the construct utilized. Subsequently, Karen Vo using the same vector backbone and strategy, showed she could overexpress FLI1 by ~5-fold in iMks, clearly at a lower level than that achieved for α IIb, but still significantly higher than endogenous FLI1¹⁰¹.

The first part of this chapter outlines preliminary studies on the localization of human IgG in Mks after endocytosis in vitro. Purified human IgG was added to Mk media to allow for uptake into Mks. Localization was compared to vWF an endogenous α -granular protein and one of importance to pFVIII biology. This chapter also details

AAVS-1 targeting of FVIII into WT iPSCs, results and implications to using this system to express pFVIII.

Materials and Methods

Mk differentiation

iMks were differentiated as previously described in Chapter 3. Mouse Mks were cultured and differentiated from bone marrow as previously described^{82,151}.

Isolation of human IgG

Human IgG was isolated from healthy donors according to the Thermo Scientific protocol (20399). All buffers and resins were equilibrated at room temperature prior to the start of purification. 2 ml of Protein G agarose was packed into a support column (Thermo). Then 5 ml of Binding buffer (PBS) was added to resin and column and allowed to drain. Human plasma was isolated by spinning down whole blood for 10 minutes at 3000 RPM. Binding buffer and human plasma was mixed at 1:1 (V:V) and then added to the column. After the column drained, 15 mls of Binding buffer was added to wash the resin followed by 5 ml of Elution buffer (0.1M Glycine, pH 2.5). The elute was collected in 0.5 ml fractions. The pH of each fraction was adjusted using 100 μ l Neutralization buffer (1M Tris-HCl pH 8.0). The protein concentration of each fraction was determined using nano-drop, ND-1000 (NanoDrop) and bicinchoninic acid (BCA) protein assay (Thermo Scientific).

Incubation of human IgG with Mks

Human IgG was added to Mk culture and incubated for 16 hours at various concentrations (0.001 - 1 mg/ml) at 37°C with D10 mouse Mks or D5 iMks. Mks were washed the next day for intracellular IgG staining.

Intracellular staining and immunofluorescence microscopy

Washed Mks were fixed in 2% paraformaldehyde (PFA) solution for 2 minutes. Fixed cells were then seeded onto slides using cytospin protocol. 100 µl of cell mixture was loaded into specimen chamber (Cyto-Tek) with filter paper (Cyto-Tek). Cells were spun for 1 minute to allow adherence. A circle was drawn around adherent cells using a liquid blocker PAP pen (Sigma). vWF antibody (Table 3.1) was added into an Antibody solution (PBS, 0.1% (W:V) BSA, 0.2% saponin, 0.02% (W:V) NaN₃) at a 1:100 dilution (V:V) and allowed to incubate with the seeded cells for 1 hour at room temperature. Slides were washed 3 times with Antibody solution and then 1:300 anti-mouse IgG and 1:400 anti-human IgG (see Table 3.1) were added to the Antibody solution and allowed to incubate for 1 hour at room temperature. 4',6-diamidino-2-phenylindole (DAPI) solution was then added at a concentration of 1:1000 (V:V) in antibody solution and then incubate with seeded cells for 10 minutes. Cells were washed 3 times. A drop of Mounting media (Thermo) was added to cells and then a coverslip placed and sealed using clear nail polish on top of the slide. Images of Mks were taken using a DMI8 Leica Microscope. Images were taken at 40X objective and captured via Hamamatsu Photonics ORCA-Flash 4.0 sCMOS digital camera. Final images were processed using imageJ64.

AAVS1 targeting of FVIII

WT iPSCs were targeted through co-transfection of AAVS-1 targeting plasmid and the right and left zinc finger nuclease vectors (Figure 4.3)^{102,193}. Verification of correct full plasmid sequences were confirmed by sequencing. Transfected cells were grown on drug-resistant MEFs as previously described¹⁰². Cells containing the AAVS1 *F8*-expression construct were selected using 1.2µm/ml puromycin added to iPSC media (Figure 4.3)¹⁰². Subsequent colonies were picked and analyzed for gene insertion. Hemizygous or homozygous insertion of *F8* cDNA was confirmed via southern blot as previously described^{102,178,179}.

F8 mRNA and FVIII antigen

F8 mRNA and FVIII antigen levels in Mks were investigated as previously described in Chapter 3.

Results

Localization of human IgG

After incubation with IgG, Mks were visualized via confocal laser scanning immunofluorescence microscopy¹⁹⁴. iMks showed that ~ 65% of IgG-positive granules were not positive for vWF (Figure 4.1D), Mks not incubated with IgG show no background staining of IgG (Figure 4.1F). Incubation of human IgG with mouse Mks indicate similar results (Figure 4.2).

AAVS-1 correction of iPSCs

iPSCs that had the *Gplba*-promoted driven *F8* cDNA targeted to AAVS-1 locus and then differentiated into iMks show FVIII mRNA above NTC (Figure 4.4A).

hBDFVIII^{RH} showed higher mRNA levels than hBDFVIII. CXCL4 mRNA showed no difference among the groups (Figure 4.4B). Despite detectable F8 mRNA, both hBDFVIII and hBDFVIII^{RH} antigen were not detectable by FVIII ELISA (Figure 4.4C).

Discussion

FVIII inhibitors

Isolated human IgG showed uptake into human and mouse Mks after 16-hour incubation. These studies showed that this IgG was largely located in granules that were not associated with vWF (Figure 4.1C and 4.1G). While very preliminary, these data suggest that the majority of IgG was located in granules that were not associated with vWF, however a third of IgG was located in α -granules that did contain vWF, suggesting that the “uptake” and endogenous α -granules may ultimately overlap. These studies do not yet show that the uptake IgG overlaps with pFVIII containing granules, which we previously shown only partially overlaps with vWF-containing granules¹⁰⁸. Our attempts to co-stain Mks for pFVIII were stymied due to low level of pFVIII expression in AAVS-1 targeted iMks. In addition efforts have been made to try and tag endogenous FVIII either with Human influenza hemagglutinin (HA) or eGFP.

In addition to being stored in separate granules to avoid intra-granular neutralization of the pFVIII, we had postulated that the “uptake” and “endogenous” α - granules might be differentially released. This differential release would prevent the presence of neutralizing FVIII antibodies at the site of pFVIII release, perhaps initially. Previous studies have shown that ~90% of all stored IgG in platelets is released upon platelet activation¹⁹⁵. How then is pFVIII still effective in these settings? The difference in

distribution of released IgG from platelets and pFVIII may not occur on a global scale, but on a micro-scale within a growing thrombus. Perhaps, microenvironments within a growing thrombus release pFVIII from distinct granules in sufficient amounts relative to the inhibitors to feed-forward the clotting cascade before being neutralized by inhibitors. Detailed thrombus studies, perhaps with the cremaster laser injury model, may support this hypothesis. To define the role of platelet vWF in the protective process, we can repeat the in vitro and in vivo iMk studies in Chapter 3 using an iPSC line in which the VWF gene is disrupted using CRISPR/Cas9 technology.

AAVS-1 FVIII expression

The lentiviral approach used in Chapter 3 requires re-infection of cells using the appropriate expression vector with each differentiation and does not optimally take advantage of iPSC technology. Establishing a specific sub-line from the parental iPSC line WTBM1-8 that consistently expresses high levels of pFVIII without iMk injury would be ideal. We therefore tried this approach using an AAVS1 strategy with pFVIII expression driven with a strong Mk-specific promoter (*Gp1ba*), which allowed the level of *ITGA2B* mRNA expression to reach >50% of the level seen for the native *ITGA2B* gene¹⁰², which is one of the highest expressed gene in developing megakaryocytes⁸⁰. We did achieve D6 iMks that expressed *F8* mRNA at up to 4 times the amount as their non-transfected control (Figure 4.4A) however; this was not sufficient to produce detectable levels of FVIII antigen within iMks above WT background (Figure 4.4C). Our data with the lentiviral approach in Chapter 3 suggest that the FVIII mRNA levels obtained by AAVS-1 targeting is 10-fold too low an expression level to see comparable FVIII levels described there and even that may still be an order of magnitude less than desired as seen in Line h38.

Sequence analysis of the resulting AAVS1 vector did not provide an explanation of why this particular construct as opposed to the *ITGA2B* cDNA construct and the *FLI1* cDNA construct was unsuccessful. Additional targeted clones were chosen, cultured, and analyzed for FVIII expression to rule out potential sub-clone bias. Furthermore, this particular *GpIba* -promoter driven construct may not be successful and if we shuttled the *Cxcl4*-promoter driven *F8* cDNA from the lentiviral constructs, perhaps we would have achieved greater levels and success.

Figures

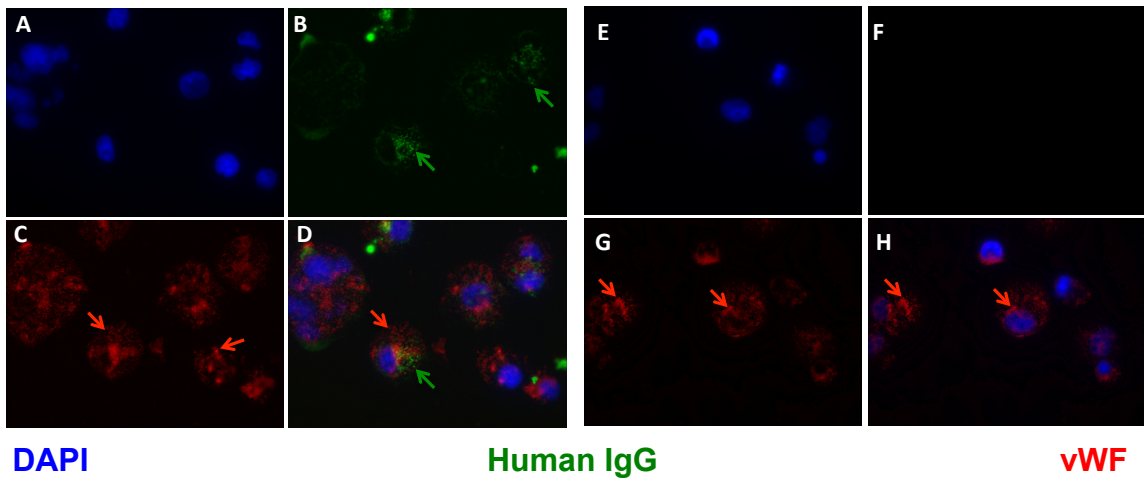


Figure 4.1 Localization of human IgG in iMks post incubation.

(A-D) Immunofluorescence microscopy of iMks incubated with isolated human IgG for 16 hrs. Magnification at 40X. (E-F) Immunofluorescence microscopy of control iMks not incubated with human IgG. (A) DAPI (B) human IgG (C) vWF (D) Merged image. IgG. (E) DAPI (F) human IgG (G) vWF (H) Merged image. Red arrows indicate vWF, green arrows indicate human IgG.

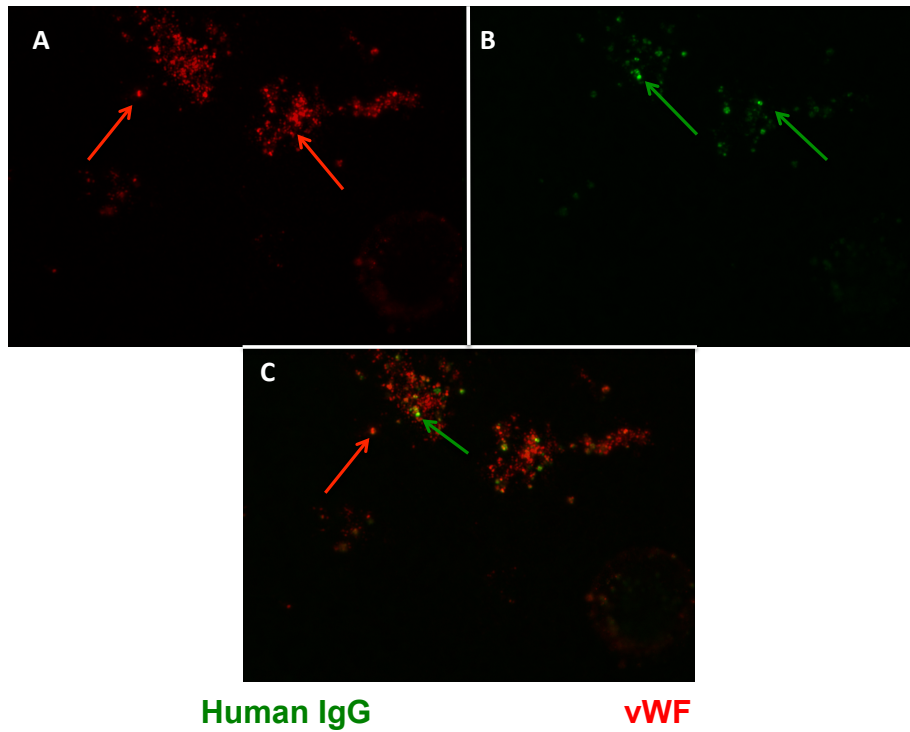


Figure 4.2 Mouse Mks incubated with human IgG.

(A-C) Immunofluorescence microscopy of mouse Mks incubated with isolated human IgG for 16 hours. Magnification at 40X. (A) vWF (B) human IgG (C) Merged image. All images show representative of experiments. Red arrows indicate vWF and green arrows indicate human IgG.

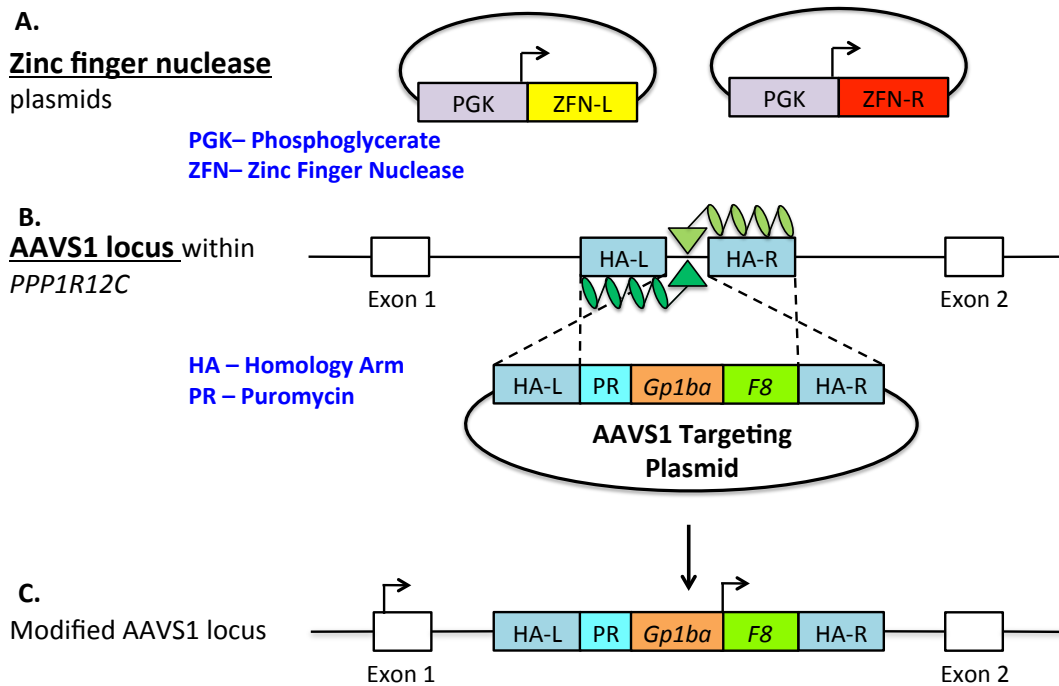


Figure 4.3 Vector Schematic of the AAVS1 targeting ZFN, targeting plasmid, AAVS-1 locus for FVIII expression (Adopted from Hockemeyer et al.^{102,193})

Components for AAVS1 targeting of F8 to iPSCs. **(A)** Vector schematic of PGK-ZFN-L and PGK-ZFN-R plasmids that contain components to target ZFNs to the HA of the AAVS1 locus. Expression of ZFNs are controlled by the PGK promoter**(B)**. AAS1 locus and AAVS1 targeting plasmid containing *F8* gene. Plasmid indicates HAs that over lab with HAs of ZFNs to apply for insertion of F8 into the AAVS1 locus. Plasmid also contains puromycin resistance and *Gp1ba* promoter to express F8 in Mks and platelets. **(C)** Schematic shows proper insertion of F8 into AAVS1 locus.

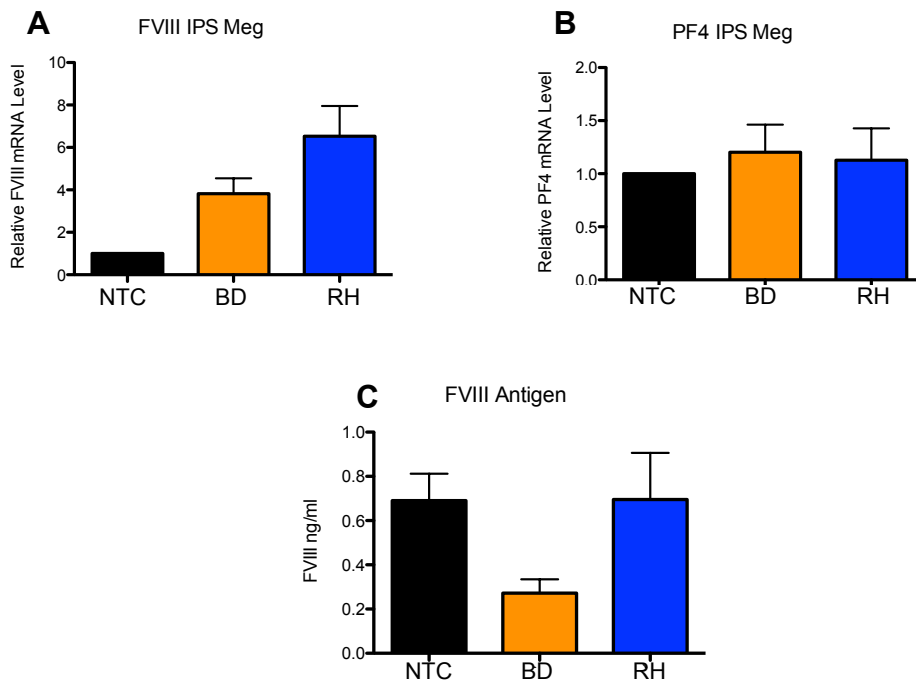


Figure 4.4 FVIII expression in WT iMks after AAVS-1 targeting.

(A) and (B) 500 ng of total RNA isolated from D6 iMks was analyzed for *F8* and *CXCL4* (PF4) message using qRT-PCR. NTC was used as a negative control. Mean \pm 1 SEM are shown. N=6 for all studies. (C) 2×10^6 iMks were lysed to extract cellular content. Whole cell lysates were analyzed using human FVIII ELISA. Mean \pm 1 SEM are shown. N=6 studies per arm.

Chapter 5 – Discussion and Future Directions

Discussion

These studies sought to address the limitations of pFVIII gene therapy by BMT and offer an alternative route of pFVIII delivery that is not permanent. In these studies, we have tested the concept of whether infusions of platelets that can deliver pFVIII could be a potential strategy for treating patients with severe hemophilia A and intractable inhibitors using Line h38 platelet infusions. We provide proof-of-principle both in in vitro and in vivo hemostatic models that human pFVIII-expressing murine platelets can improve hemostasis. The pFVIII hemostatic effect lasted several days even in mice where platelet half-life is approximately half of that in humans. This hemostatic effect was additive in the presence of rFVIIa and effective in the presence of inhibitors. To further these studies and show that infused Mks can also deliver this same therapy and that it is applicable to human Mks, we utilized iPSC-derived Mks that expressed FVIII. This approach allowed us to have a renewable resource of cells that we could gene manipulate in vitro and expanded to limitless amounts. Studies with pFVIII-iMks support Line h38 studies and suggest that once robust technologies are developed to generate clinically relevant platelets – be it from iPSCs or from another cell line such as adipose cells^{116,117} – such technology could have application beyond just providing an alternative source of platelet transfusions, but serve as a delivery system for various therapeutic agents, perhaps beginning with pFVIII in the described clinical setting.

Implications for future studies

Infusion of Line h38 platelets

Our studies seek to extend previous research done in the field of pFVIII, which was always envisioned to lead to BMT-based therapies. Thus while others had shown that pFVIII-containing platelets can improve hemostasis in a ROTEM model, FeCl₃ carotid artery injury, and tail-clip exsanguination assays, none of those studies were focused on using these for proof-of-principle of using infused pFVIII platelets as a therapy. Thus no study defined how long the hemostatic effects last, the additive effects with rFVIIa and its application in the presence of inhibitors.

On the other hand, our studies with Line h38 are also limited. While we show that infusions of 15% Line h38 platelets is sufficient to correct the bleeding phenotype seen FVIII^{null} mice in the presence and absence of inhibitor, our studies were limited by the relatively low levels of pFVIII in Line h38 platelets which have the whole blood equivalency of 3% FVIII plasma activity (as human FVIII has ~30% activity within murine plasma^{107,108}). Thus in our studies with ≤15% Line h38 platelets, the blood would have the equivalency of ≤0.6%FVIII activity. This low level of FVIII may contribute to the sub-optimal contraction seen on ROTEM studies (Figure 2.4). In the tail-clip exsanguination assay, the efficacy of pFVIII at such low levels is consistent with our prior proposal that the tail-clip exsanguination assay is especially sensitive to pFVIII. Calculated levels of plasma equivalent FVIII activity 72 hours after Line h38 platelet infusion would have levels as low as 0.02% and this was still effective in this hemostatic challenge (Figure 2.6). Finally, the low pFVIII levels may explain why a high concentration of sclerosing FeCl₃ was needed in the carotid artery studies with the endpoint of decreased arterial blood flow rather than full occlusion (Figure 2.7). Thus, if only low levels of pFVIII can be achieved in patients, its clinical value may be too limited especially in the presence of

high-titer inhibitors. Whether that low level would still be beneficial and useful in conjunction with rFVIIa would then need to be addressed. Thus clearly achieving at least the higher level seen in a recent transgenic mouse report may be of value¹²³.

pFVIII-iMks

In vitro-grown human Mks from various sources have been used to generate platelets both by direct harvesting of so-called platelet-like particles (PLPs) in culture or by using various bioreactors¹⁹⁶⁻¹⁹⁸. Many of these PLPs lack surface CD41 and are not even derived from Mks^{199,200}. Moreover of the CD41⁺-PLPs, only a small percent are non-injured annexin V negative/CD42b⁺ platelets. Not surprisingly, these PLPs have limited half-life in circulation post-infusion, are pre-activated, and have poor agonist responsiveness^{199,201}. On the other hand, PLPs released from intravenous infusion of human Mks shed within the lungs over the subsequent few hours may be functional platelets as it has been recently shown that endogenous marrow Mks travel to the lungs where they account for ~50% of normal platelet release of platelets in mice⁷¹. We previously shown that such released platelets from infused human Mks have a Gaussian-size distribution, circulating half-life and agonist responsiveness similar to well-prepared, fresh, donor-derived platelets¹⁵¹.

Remarkably, even with the low level of pFVIII in the studied iMks, the low number of platelets released per iMk and shortened released human platelet half-life, efficacy in the FeCl₃-carotid artery injury model was seen, especially with the RH variant (Figure 3.12). This better improvement with RH compared to BD pFVIII was also seen in the ROTEM studies (Figure 3.11). While RH antigen levels were higher than the BD pFVIII in iMks, this difference was small (Figure 3.7), and not likely solely responsible for the greater efficacy of RH. Moreover, this finding is also consistent with our previous studies

showing that FVIII^{null} mice expressing RH after lentiviral gene therapy had greater efficacy in improving hemostasis in the cremaster arteriole laser injury model due to increased RH stability within the core of the growing thrombus¹⁰⁹.

Our pFVIII-iMks studies also have several important limitations. iMks are known to be small with low ploidy and release ~10 platelets/iMk even upon infusion into recipient mice. At present, there was no available FVIII^{null} murine model on an immune compromised background that was available for infusion studies, so we infused iMks into clodronate liposome-treated FVIII^{null} mice to improve human platelet half-life by reducing phagocytic removal of the human platelets. To test the full potential of this model, a mouse would need to be developed to allow for long-term circulation of iMks and their released platelets. The survival of the released human platelets in clodronate liposome-pretreated FVIII^{null} mice (Figure 3.12A) was shorter than the ~12 hours seen when human iMks were infused into immunodeficient mice. Currently we are breeding Non-obese (NOD)/SCID/interferon receptor 2γ-deficient (NSG)-FVIII^{null} mice obtained from Dr. Shu-Wha Lin at Taiwan University. We plan to cross these mice with a mouse that have a mutation in their *Vwf* gene (vWFR1326H) that allows for better incorporation of human platelets into mouse thrombi as described by Tom Diacovo²⁰². This will allow us to fully test the therapeutic benefit of pFVIII-iMks over longer periods of time and in the presence of inhibitors. These mice will also allow us to test other models of thrombosis. To date, because of the low integration of human platelets into mouse thrombi, we were precluded from using cremaster laser injury models for these studies. These new mice will allow us to perform these studies and further test the details of the hemostatic efficacy of our pFVIII-iMks

Additionally, in these studies we failed to show iMks' effectiveness in the setting of infused inhibitors. Whether pFVIII-iMks will be beneficial in the FeCl₃ or tail-clip

exsanguination model has yet to be fully elucidated; however, current studies to this end are underway. More importantly, the level of pFVIII in these iMks was even lower than that of the Line h38 platelets, and clearly a strategy to achieve higher levels of pFVIII per iMk needs to be established as well as to establish a self-replicating lineage. Chapter 4 outlines other strategies utilized to express FVIII into iMks. So far our lentiviral approach gave the highest amounts of antigen. In subsequent studies, we also hope to utilize FVIII variants that have increased expression and/or biological activity in addition to the ones outlined in this thesis.

IgG localization

While our main goal was for the use of infused pFVIII-iMks as an effective hemostatic strategy in patients with high titer inhibitors, we also wanted to better understand why pFVIII was more effective than plasma FVIII in the care of patients with hemophilia A and inhibitors. In both plasma and pFVIII, FVIII is bound to vWF, so we felt that there should be an alternative explanation other than vWF binding to explain the difference efficacy in the setting of inhibitors. We hypothesized that the pFVIII was either localized in different granules than endocytosed IgG in absolute terms or in sufficient amounts to allow efficacy or that there were macro- or micro-domains within the interstices of a thrombus that contained free pFVIII able to activate the clotting cascade sufficiently before being neutralized. We began with confocal microscopy to test this hypothesis. Human IgG granular distribution only partially overlapped with that of vWF. Further studies would need to be done to test this hypothesis by looking at co-localization of pFVIII in iMks, where pFVIII expression level is increased, and co-localization with endogenously expressed vWF and endocytosed human IgG. Also iMks that have targeted disruption of vWF will need to be studied and perhaps the ability of

these two iMk populations to release platelets in FVIII^{null} mice will need to be looked on in situ microscopy. The former hypothesis is supported in a 2011 study by Kamykowski et al. This group showed that proteins with pro and anti-agonist functions stored within platelets showed very little co-localization within the same α -granules when visualized via confocal microscopy²⁰³. Understanding the basis for enhanced pFVIII resistance to inhibitors may provide insights that can lead to new strategies to improve this biology for example by enhancing vWF levels in pFVIII-iMks.

Strategies to increase pFVIII expression within iMks

In our studies, we utilized HPCs transduced with the appropriate lentiviruses to obtain pFVIII-iMks. These experiments were limited by the amount of virus we could add to the cells without having adverse effects due to viral toxicity. One approach that can be taken to not need to constantly transfect cells with lentiviruses is to accomplish the genetic modification at an earlier self-replicative stage. We described such a strategy using AAVS-1 targeting of vector constructs in iPSCs to drive megakaryocyte-specific expression using a *Gp1ba* promoter, but while this strategy had achieved high levels of eGFP expression and correction of α IIb expression in iMks, it did not drive significant expression of pFVIII (Figure 4.4). While increased *F8* mRNA was obtained due to AAVS-1 targeting, this level of mRNA was at least 40-fold too low for achieving a useful pFVIII-iMk when compared to the pFVIII-iMks that were transduced with lentivirus.

Through lenti-viral targeting of FVIII in iMks we were able to achieve modest levels of FVIII when compared to our Line h38 transgenic Mks (Figure 3.7). Interestingly, to achieve this relatively low level of pFVIII, HPCs needed to transduced with 10 viral

particles per cell that resulted in a FVIII mRNA level of at least 40 times greater than controls

We also explored other strategies to express FVIII within iMks, not outlined in this thesis. Of note, we tried to introduce hBFVIII plasmids through electroporation into HPCs to increase *F8* cDNA, mRNA, and antigen. We gave a dose of DNA that was well tolerated by Meg-01 line cells however, this dose caused cell arrest in HPCs and no production of FVIII. We also tried a 100-fold increase in lentiviral transduction which only yielded a modest increase in iMk FVIII (data not shown). Clearly, we may have challenges achieving this goal on two levels: 1. tolerance of the induction system and the amount of DNA given that allows for the survival of the iPSCs or HPCs and 2. expression levels of ectopic pFVIII and their ability to cause apoptosis of differentiating Mks.

Other limitations of the pFVIII-iMk infusion approach

One area of potential concern with pFVIII-iMks approach (beyond those that may be associated with infusion of iMks in any clinical setting) is the need to infuse large numbers of platelets into mice and potentially humans that are not thrombocytopenic. Infusing high levels of platelets may induce untoward thrombosis causing symptoms seen in thrombocytosis²⁰⁴. We tested whether a modest 15% increase in pFVIII could have a beneficial effect on thrombosis and hemostasis. These studies did not address the upper limits of infusing large amounts of platelets. While we did not indicate any adverse effect in mice receiving 15% platelet infusions, further studies would need to be completed to ensure that maximum benefit of pFVIII could be achieved without thrombotic risks. We propose that a once weekly to every other week infusion of pFVIII-iMks would provide prophylactic therapy with no notable increase in platelet counts. This may need to be tested in mice models after we establish the NSG-FVIII^{null} mice.

Clinical application

For pFVIII infusion therapy to reach clinical applications, the aforementioned limitations would have to be addressed. Once these limitations are addressed, we envision pFVIII therapy being beneficial in several areas of hemophilia A with severe inhibitors. One scenario for pFVIII infusion therapy could be that a patient with intractable inhibitors would receive products derived from “universal” pFVIII-iMks, which would release platelets missing particular human leukocyte-related antigens (HLA) on their surface²⁰⁵. This could be accomplished through reprogramming cells from healthy individuals and gene manipulating iPSCs to lack HLA antigens. Our group is beginning to work with Dr. David Russell at the University of Washington on this project²⁰⁶. This would prevent the need to reprogram each patient’s cells and allow for better selection of iPSC lines that not only give rise to the highest level of iMks, but lines that also express FVIII the best.

Alternatively, patients may have their own iPSCs established and utilized to generate pFVIII-iMks. Both strategies may avoid the development of antibodies to the infused platelets and allow long-term treatment requiring transfusion every 1-2 weeks or longer with limited increases in their total platelet counts. Applying this strategy to pFVIII delivery would provide insights into what type of clinically relevant bleeds pFVIII therapy would be useful for and whether, in a particular patient with specific inhibitors, there would be benefit from a more permanent pFVIII therapy using marrow-directed gene therapy. Furthermore, the pFVIII-iMks may then be useful to support such challenging patients during their post-BMT thrombocytopenia with its anticipated increased bleeding risks.

Conclusion

In summary, we studied the potential of an alternative approach for using pFVIII strategy to treat patients with severe hemophilia A and intractable inhibitors. Using transgenic mouse Line h38 platelets and WT iPSCs modified to express FVIII when differentiated into iMks, we show the utility of prophylactic infusions of pFVIII on achieving several days of hemostasis in FVIII^{null} mice. This pFVIII strategy was also effective in the presence and absence of inhibitors and was additive with a bypassing agent, rFVIIa. iMks expressing both hBDFVIII, hBDFVIII^{RH} were able to effect clot formation and thrombosis both in vivo and in vitro. hBDFVIII^{RH} had more of an effect in these settings than hBDFVIII. I also looked at IgG uptake into Mks and found that it largely localizes to granules that are not associated with vWF, which may further explain the efficacy of pFVIII in the setting of inhibitors. Prophylactic infusions of pFVIII has the advantage of not permanently altering the patients, can provide longer lasting prophylaxis than present-day bypassing agents, and be used in conjunction with these bypassing agents. This approach may also be a useful adjuvant for bleeding challenges post-marrow transplantation for gene therapy. Finally, it may be a first-to-clinic application of in vitro-generated megakaryocytes and their derived platelets that would justify the costs of preparing such a platelet product relative to donor-derived platelets.

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