ENHANCEMENT OF hFVIII ACTIVITY THROUGH LC MODIFICATIONS FOR GENE THERAPY OF HEMOPHILIA A

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ABSTRACT

Gene therapy for Hemophilia A (HA) using the recombinant Adeno-associated virus (rAAV) offers an alternative to classic treatment, which consists of FVIII protein infusions. However, due to limitations associated with rAAV and the FVIII protein itself, the end result is a transgene expression below therapeutic limits. One approach to improving the therapeutic value of rAAV gene therapy for HA is to engineer a more active FVIII protein through genetic modifications. Preliminary testing revealed that canine FVIII Light Chain (kLC) enhances coagulation activity, and that it would be possible to improve FVIII activity through modifications of the light chain. Through the process of engineering, evaluation, and negative selection of kLC, a final construct was engineered.

The hLC-K12 is a human Light Chain (hLC) construct containing 12 amino acid changes that work together to enhance coagulation activity. A comparison of the FVIII clotting activity to the amount of protein produced determined that hLC-K12 produced a 3.28 fold increase in specific activity over hLC *in vitro*. Similar *in vitro* results were observed when hLC-k12 was tested with the X5 heavy chain (X5HC), a heavy chain that has been genetically modified to enhance production. CD4KO/HA mice were injected with a rAAV vector carrying the hLC-K12 gene in conjugation with a rAAV vector carrying the X5HC gene. Replacing the hLC vector with the hLC-K12 vector produced an average 7.43 fold increase in FVIII clotting activity. An ELISA assay revealed no significant difference between productions of the heavy or light chains at any time point. By comparing the clotting activity to the amount of protein produced, it was determined that the increase in coagulation activity was due to an increase in specific activity. In fact, replacing the hLC vector with the hLC-K12 vector resulted in an average 5.8 fold increase in FVIII specific activity.

The K12 modifications were evaluated using a single chain FVIII conformation. *In vitro*, the addition of the K12 mutations to the human heavy chain, hHCK12BDD, resulted in a 4.3 fold increase in clotting activity, but no increase in protein production. There was however, a 3.3 fold increase in specific activity of the protein. Adding the K12 mutations to the X5 heavy chain, X5K12BDD, *in vitro*, resulted in a 2.7 fold increase in clotting activity and a 1.42 fold increase in specific activity of the protein.

Single chain rAAV vectors were packaged and delivered to CD4KO/HA mice. Compared to mice injected with hFVIIIBDD, the hHCK12BDD produced an average 4.6 fold increase in clotting activity. An ELISA revealed no significant difference in production between these two groups. However, mice injected with hHCK12BDD produced FVIII with an average of 4.13 fold increase in specific activity. Similarly, when compared to mice injected with X5FVIIIBDD, the X5K12BDD produced an average 2.14 fold increase in clotting activity. An ELISA assay demonstrated no significant increase in protein production between these two groups. However, when compared to X5BDD, mice injected with the X5K12BDD vector produced FVIII with an average 1.98 fold increase in specific activity.

Results demonstrate that the K12 light chain modifications are able to enhance clotting activity of hFVIII both *in vitro* and *in vivo,* using either a dual chain or single chain delivery method. In order to determine the mechanism of enhancement, hFVIIIBDD and hHCK12BDD protein was partially purified and tested for activity. Results demonstrated that the hHCK12BDD protein produced a specific activity of 39,153.69 Units/mg, which is a 6.28 fold increase over hFVIIIBDD specific activity, which was 6,237.92 Units/mg. Measurement of conversion from FX to FXa revealed that the hHCK12BDD protein generated a higher amount of FXa at a quicker rate. In conclusion, these results provide evidence that the K12 modifications enhance specific activity through an increase in FXa generation.

This thesis is dedicated to my father, John Firrman.

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CHAPTER 1

INTRODUCTION

Hemophilia A results from a defect in the coagulation FVIII protein

Hemophilia A phenotype

Hemophilia A (HA) is a recessive, X-linked, genetic, bleeding disorder that affects 1:5000 males worldwide (1-5). Female cases do occur, although they are rare(1, 6). Common symptoms include easy bruising, episodes of frequent and spontaneous hemorrhaging, and an excessive amount of bleeding during and after injury or surgical procedures (7, 8). HA persists throughout life, with both chronic and acute consequences. Over time, chronic internal bleeding can cause damage to joints, muscle, and the surrounding soft tissue. Acute and severe symptoms, such as intra-cranial bleeding, can be fatal (3, 7, 8). Intra-cerebral hemorrhaging occurs in 4-7.5% of HA positive infants, most often within the first week of life (6).

HA is classified as mild, moderate, or severe, with the degree of severity being directly proportional to the amount of residual clotting activity (2-5). With a mild case of HA, a patient retains 5-50% of normal clotting activity and the symptoms manifest as prolonged bleeding after trauma or surgery. A moderate case of HA is defined as having 1-5% of normal clotting activity and results in moderate bleeding with some spontaneous episodes. A severe case of HA is classified as having <1% of normal clotting activity and results in frequent and spontaneous internal bleeding (3-5). Of these three classifications, approximately 50% of HA cases are classified as severe, 10% are moderate and 30-40% of cases are mild (2).

Current treatment of HA relies on IV infusions of the FVIII protein. This is administered either prophylactically or as needed, in an effort to decrease the occurrence of bleeding episodes and the subsequent damage to surrounding tissues and joints (8). However, FVIII protein replacement therapy has multiple, severe limitations (3, 5). These include a relatively short half-life of the FVIII protein (~12 hours), a high cost for treatment ($100-150,000.00/ per patient/year), and the possibility of inhibitor development. Furthermore, while protein treatment is able to restore the normal bleeding phenotype, it cannot reverse joint damage (3, 8).

The clotting cascade

The disorder HA is caused by mutations in the coagulation FVIII gene that produces either ineffectual FVIII protein or no FVIII protein at all (1, 2, 9). Regardless of genetic determinant, the end result is a non-functional intrinsic clotting cascade, which is a central pathway in blood coagulation (4, 5, 7, 10-13). Under normal circumstances, the clotting cascade consists of two pathways: the extrinsic cascade, also known as the cell injury pathway, and the intrinsic cascade, also recognized as the contact pathway. These two pathways are both essential and work together to produce blood coagulation **(Ill. 1**) (4, 14).

The extrinsic cascade is first initiated after injury, when tissue factor is exposed to the blood plasma, binds coagulation protein FVII, and converts FVII to FVIIa. FVIIa then converts coagulation protein FX to FXa, which leads to the downstream conversion of prothrombin to thrombin, and fibrinogen to fibrin **(Ill. 1)** (4, 14). The intrinsic cascade is initiated by the conversion of coagulation protein FXII to FXIIa after contact with a negatively charged surface. FXIIa triggers the conversion of coagulation protein FXI to FXIa, and then FXIa initiates the conversion of coagulation protein FIX to FIXa (4, 14, 15).

ILLUSTRATION 1.

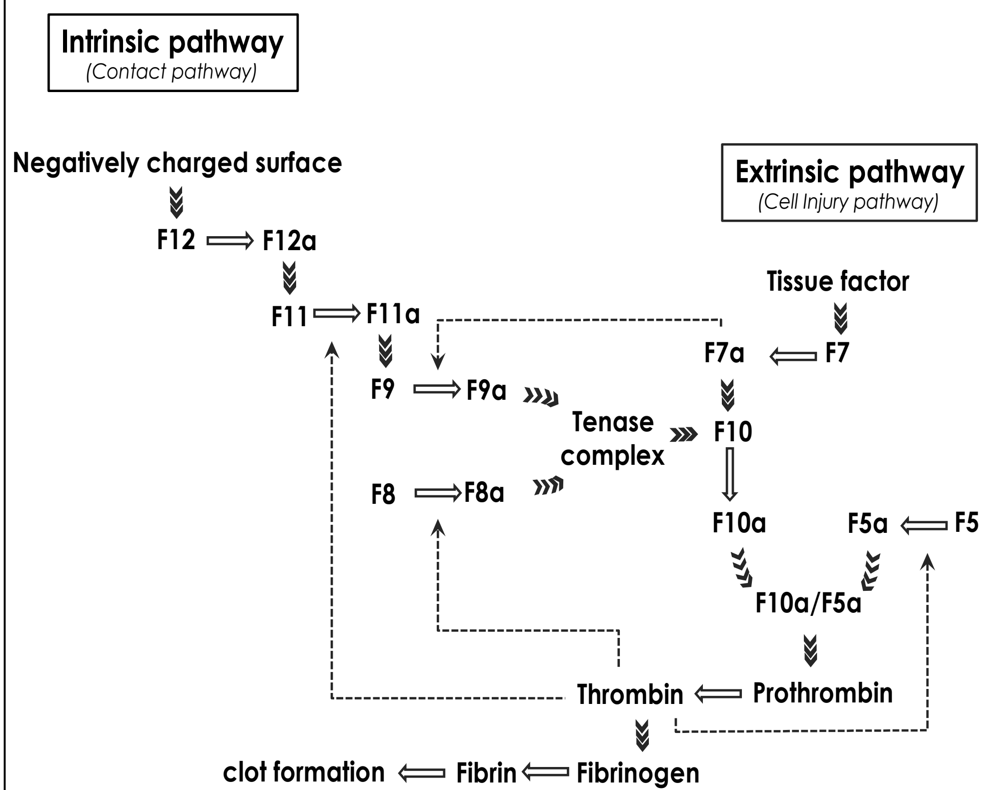


Illustration 1. The clotting cascade. The clotting cascade consists of the intrinsic and extrinsic pathways. Although both can be activated through separate means, they converge with the activation of F10 and the conversion of F10 to F10a. The end result is the conversion of fibrinogen to fibrin and the formation of a clot. The disorder Hemophilia A occurs when a defect in the coagulation F8 protein leads to an ineffectual intrinsic clotting cascade.

Additionally, FVIIa can modulate the intrinsic clotting cascade through the conversion of coagulation protein FIX to FIXa **(Ill. 1)**. The coagulation protein FVIII is converted to FVIIIa through activation by thrombin and joins FIXa as a cofactor in the Tenase complex (2). The Tenase complex is then able to trigger the conversion of coagulation protein FX to FXa **(Ill. 1)** (4, 14, 16).

The extrinsic and intrinsic clotting pathways converge through the activation of FX and its subsequent conversion to FXa. Coagulation protein FV is converted to FVa through activation by thrombin. It functions as a cofactor for FXa in the conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin (**Ill. 1**) (4, 14, 16). In the absence of functional FVIII protein, the tenase complex does not form and an insufficient amount of FXa is generated (3, 4, 5, 7, 10-13). Without a sufficient amount of FXa, enough prothrombin is not converted to thrombin, nor fibrinogen converted to fibrin, to support the formation of a fibrin clot (4, 10, 14).

The Coagulation FVIII gene and protein

The coagulation FVIII gene is located on the long arm of the X chromosome at position Xq28, from base pair (bp) 154,835,787 to 155,022,722 (**Ill. 2A**) (4, 9, 10, 17). The gene consists of approximately 186k nucleotides that encode 26 exons, varying from 69 to 3,106 bp in length, and 25 introns, varying from 200 to 32k bp in length **(Ill. 2A)** (17-20). It codes for three transcripts: FVIII, FVIIIA and FVIIIB, although the FVIII transcript is the only one comprised of all 26 exons. Both the FVIIIA and FVIIIB transcripts contain only a portion of the FVIII gene. The FVIIIA gene lies entirely within intron 22, in opposite orientation to the FVIII gene, and produces only a 1.8 kb transcript (2). The FVIIIB gene has the same orientation as FVIII and, while it begins within intron 22, its transcript also includes exons 23-26 (**Ill. 2A**) (2). The functions of the FVIIIA and FVIIIB proteins have not yet been elucidated although they can be detected in circulation (17).

ILLUSTRATION 2.

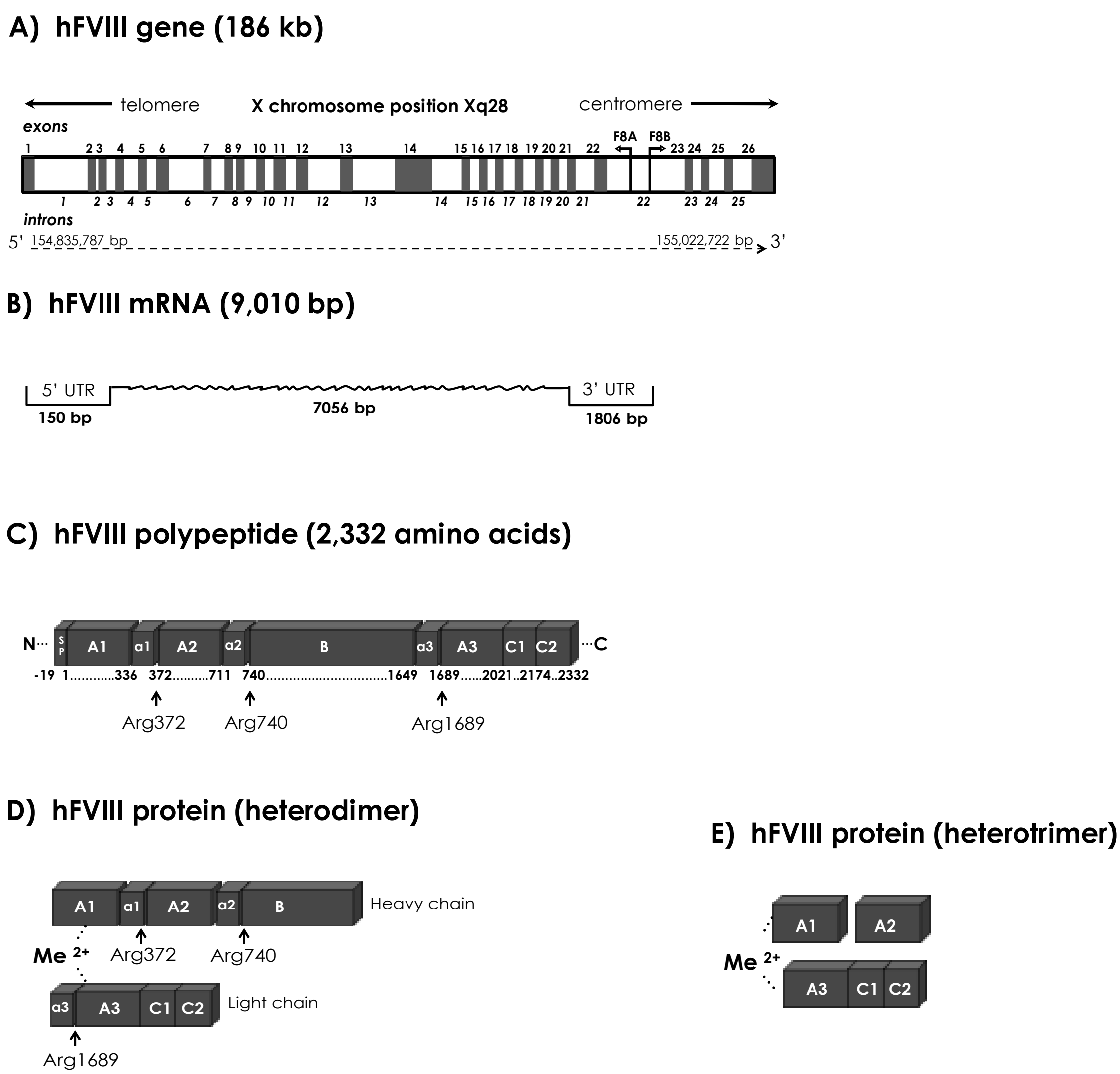


Illustration 2. The FVIII gene and protein. The FVIII gene is transcribed and translated into the FVIII protein. A) The FVIII gene is 186 kb in size, containing 26 exons. B) Post pre-mRNA processing, the FVIII mRNA is 7,056 base pairs in length. C) The FVIII polypeptide is 2,351 amino acids in length prior to cellular secretion. D) Post cellular secretion the FVIII protein circulates in the blood plasma as a heterodimer. E) After activation the FVIII protein takes on a heterotrimer conformation.

Post pre-mRNA processing, the FVIII mRNA is 9,010 bp in length, composed of a 7,056 bp coding region situated between a 150 bp 5' UTR and a 1,806 bp 3' UTR **(Ill. 2B)** (10, 17, 18). Once translated, the FVIII polypeptide is comprised of 2,351 amino acids. This includes a 19 amino acid secretory peptide sequence followed by 2,332 amino acids divided into six domains designated as A1, A2, B, A3, C1, and C2 **(Ill. 2C)(17, 18)**. Located between the A domains are short, acidic, peptide sequences containing sulfated Tyrosine residues that provide sites for thrombin cleavage, which are designated a1, a2, and a3 **(Ill. 2C)** (20).

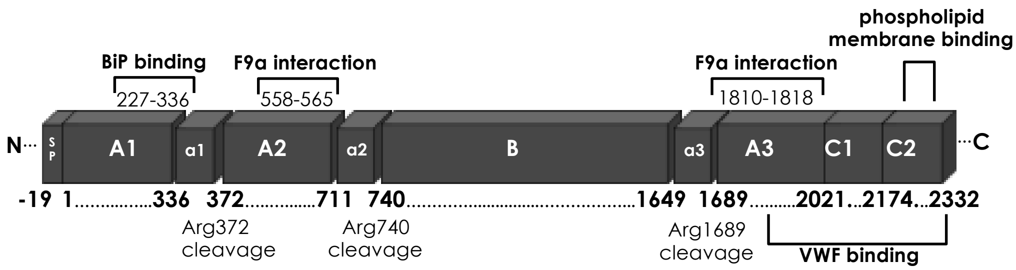
Prior to cellular production, the FVIII protein is subject to multiple, post-translational modifications. Upon entry into the ER the FVIII protein binds to the chaperone BiP and the signal peptide is removed (20). The LMAN1 receptor recruits the protein and initiates transport to the Golgi via the ER-Golgi intermediate compartment (17). Once the protein enters the Golgi body it undergoes modifications to N-linked oligosaccharides, O-linked glycosylation, sulfation of Tyrosine residues, and copper binding (17, 20). The protein is subjected to proteolytic processing through furin/PACE cleavage at positions R1313 and R1648, which divides the protein into heavy and light chains (**Ill. 2D**) (15, 17, 19). The expression of FVIII is tissue specific. It is primarily manufactured in liver sinusoidal cells, but is also manufactured to some extent in hepatocytes and Kuffer cells (2, 17, 19, 21). The importance of the liver in FVIII production is supported by the observation that a liver transplant will cure HA (17, 18).

The mature FVIII protein circulates in the bloodstream as a heterodimer of heavy chain and light chain in a metal ion complex, with a total molecular weight between 170 and 280 kDa, at a concentration of approximately 200 ng/ml (17, 21). It complexes with the protein Von Willebrand factor (VWF), which functions to stabilize the protein and prevents non-specific binding of FVIII to endothelial cell membranes (4, 21). The FVIII/ VWF complex circulates as a zymogen and becomes activated after cleavage by thrombin at positions Arg 372, Arg 740, and Arg 1689 (4, 21). Cleavage of Arg 1689 is particularly important because it removes VWF allowing FVIIIa to interact with the phospholipid surface of the platelet (17). FVIIIa binding to VWF or the phospholipid surface of a platelet is mutually exclusive (17). Post cleavage, the protein takes on a heterotrimeric conformation, with the light chain bound to A1 through metal bonding and to A2 through electrostatic interactions **(Ill. 2E)** (17, 22). Once activated, the FVIIIa cofactor loses function rapidly from dissociation of the A2 domain (19).

The FVIII protein consists of 6 domains designated as A1, A2, B, A3, C1, and C2. Both the A and C domains of FVIII are homologous to each other and to those that are found in the FV coagulation protein (2, 21). Each domain is necessary and contains at least one important site for FVIII function (**Ill. 3A**). The chaperone protein BiP binds to the A1 domain at amino acids 227-336 and Phe 309, allowing for proper movement of FVIII through the ER to the Golgi body (17, 21, 22). FIXa interacts primarily in the A2 domain at amino acid positions 558-565, but also interacts in the A3 domain at amino acids 1810-1818 (**Ill. 3A**) (17, 21, 22). The VWF protein binds to FVIII in both the A3 and C2 domains; the C1 domain enhances the VWF/C2 interaction (22). The C2 domain also provides sites for FXa binding and contains the hydrophobic hairpin loops, Met2199-Trp2200 and Leu2251-Lue2252, which interact with the phospholipid membrane of platelets (**Ill. 3A**) (22, 23).

ILLUSTRATION 3.

A) hFVIII- Full length (2,332 amino acids)



B) hFVIII- BDDSQ (1,439 amino acids)

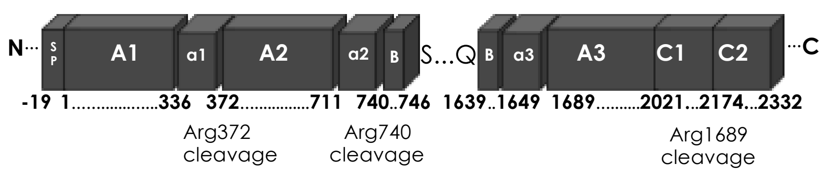


Illustration 3. The FVIII protein structure. The FVIII protein consists of six domains, A1, A2, B, A3, C1 and C2. Situated in between the A domains are acidic regions referred to as a1, a2 and a3. A) Native FVIII consists of a polypeptide 2,351 amino acids in length, which includes a 19 amino acid signal peptide and a 909 amino acid B domain. B) The hFVIII-BDDSQ construct is recombinant protein based on the original FVIII protein in which the B domain amino acids 746-1639 are removed.

The FVIII B domain is 909 amino acids long and is involved in intracellular processing, not FVIII activation(17, 22). It is completely removed upon activation of FVIII by thrombin, which indicates that some of the sequence might be dispensable. Research observed that deletions of the B domain produced protein with the same activation profile of full length FVIII, yet had increased production properties(21). Today the predominantly used versions of engineered, recombinant FVIII protein have regions of the B domain removed and are referred to as hFVIII B-Domain Deleted (hFVIIIBDD). The hFVIII-BDDSQ is a popular variant that has amino acids 746-1639 of the B domain removed, yet retains the appropriate cleavage sites for activation by thrombin (**Ill. 3B**) (15, 17). BDD variants of FVIII not only have enhanced production, but also are much smaller than full-length FVIII. For example, hFVIII-BDDSQ is 893 amino acids, or 2,679 nucleotides smaller than its full-length counterpart (**Ill. 3B**) (15).

The intron 22 inversion

Forty to forty-five percent of severe HA cases, or 20% of all HA cases combined, are directly caused by an inversion of intron 22 in the FVIII gene, which prevents contiguous transcription beyond exon 22 (2, 24, 25). This inversion occurs through homologous recombination of a 9.5 kb region within intron 22 (int22h1) with one of two regions containing 99.9% sequence similarity. These regions are found ~300 kb towards the telomere designated int22a (proximal) and int22b (distal) (**Ill. 4**) (2, 6, 24-26). Recombination of int22h1 with int22h2 or int22h3 results in relocation of exons 1- 22, moving them closer to the telomere and flipping their orientation (**Ill. 4**). The inverted portion of the gene contains the FVIII promoter, and, although it is inverted, it will actively transcribe exons 1-22 (26). Although this is not a full FVIII transcript, the mRNA produced does not undergo nonsense mediated mRNA decay (26). Instead, it is translated and a truncated FVIII protein containing the first 22 exons at normal FVIII levels is generated (26). The intron 22 inversion mutation has a deleterious effect on the FVIII gene; however, it does not affect the FVIIIB gene (**Ill. 4**). Because the FVIIIB gene is still complete it actively transcribes exons 23-26 at normal levels, resulting in a 216 amino acid protein (2, 26). Proteins containing both the FVIIIB exons 23-26 and the inverted exons 1-22 have been identified in the peripheral blood mononuclear cells and in liver sections (26).

ILLUSTRATION 4.

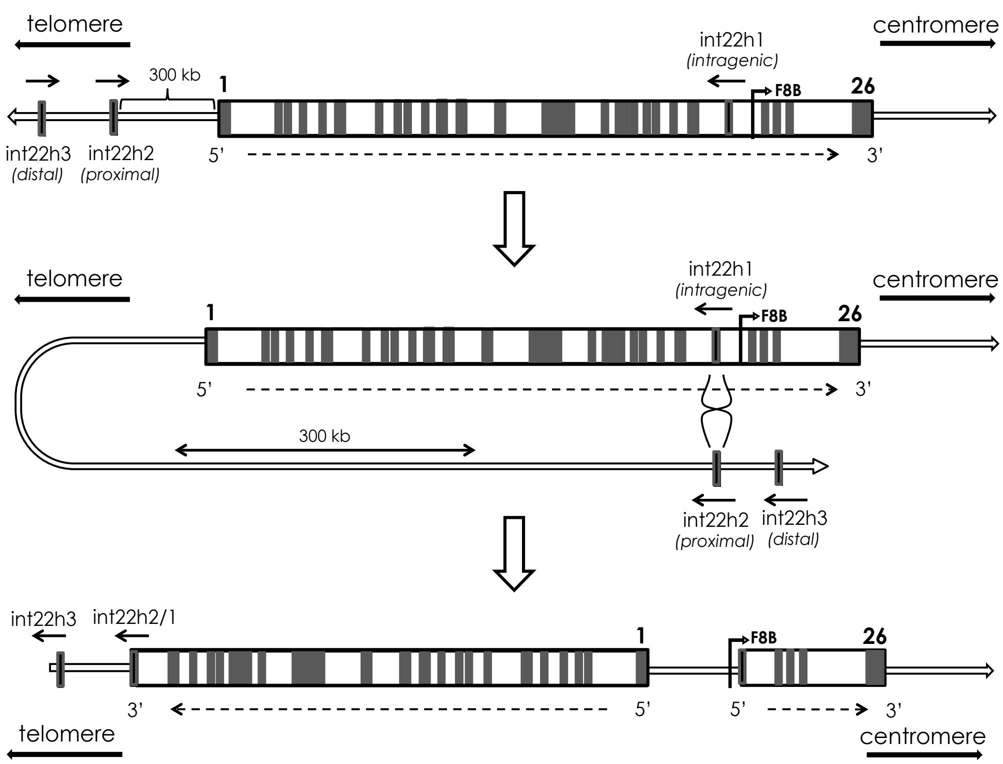


Illustration 4. The intron 22 inversion. The intron 22 inversion occurs when homologous recombination between Int22h1 and either Int22h2 or Int22h3 relocates and inverts exons 1-22. This produces a truncated F8 transcript containing exons 1-22 and a separate transcript containing exons 23-26. There are no full transcripts containing all 26 exons produced and therefore, no functional F8 is formed.

Although separated, the entire FVIII protein sequence is present (26). The two segments of FVIII cannot combine to a form functional FVIII gene and restore the clotting cascade; however, it is thought that their presence may induce tolerance (26). In fact, HA resulting from an intron 22 inversion produces no circulating FVIII; Furthermore, only 20% of these patients develop inhibitors after receiving FVIII infusions (26).

Inhibitor development

Through modern technology and medicine, FVIII protein can be produced and administered to people suffering from HA, reversing the bleeding phenotype (1). However, the introduction of FVIII to the system often leads to inhibitor formation, drastically reducing the efficacy of protein therapy (1, 27, 28). Inhibitors, which typically are neutralizing antibodies that inhibit FVIII activity, develop in ~25% of patients with severe HA after exposure to FVIII protein (1, 2, 27, 29, 30). There are some cases in which inhibitors do not neutralize FVIII function, but do enhance the removal of the FVIII protein from circulation (27). Inhibitor formation occurs because the immune system recognizes the protein as non-self and mounts a retaliatory response. This includes B-cell activation and anti FVIII IgG antibody production, usually against cofactor epitopes (6, 27). The probability of inhibitor formation relies on multiple factors, both environmental and genetic. Environmental factors include age, type of FVIII treatment regime, whether it is prophylactic or as needed, and amount administered (31). Genetic factors involve ethnicity, a family history of inhibitor development, and causative mutation (1, 31).

Research on inhibitor formation established that there is a direct correlation between the underlying genetic mutation responsible for the development of HA and the incidence of inhibitor development (1, 6, 26, 28). In general, those suffering from severe HA have the highest risk of inhibitor development and those diagnosed with mild HA have the lowest risk (2, 29). The hypothesis is that FVIII polypeptide synthesis during lymphocyte development promotes tolerance (1). In this situation the immune system of patients that have produced some FVIII, although it is mutated, may recognize infused FVIII protein as self (1, 2, 26, 29). For example, a missense mutation in the FVIII protein may result in HA, but production of the mutated FVIII can promote tolerance. This hypothesis is supported by the fact that only ~10% of patients with missense mutations develop inhibitors (26, 28). Conversely, mutations causing the absence of any protein production, such as large deletion and/or rearrangement events have the highest risk of inhibitor development (2, 6, 29). For example, a mutation that results in termination of transcription or translation will produce no FVIII protein and no ability to promote tolerance. This is supported by the fact that 68-88% of patients with large deletions develop inhibitors (26, 28). The risk of developing inhibitors remains static over time; however, it is recognized that patients with severe cases of HA that have been previously treated with infusions of FVIII, have a reduced chance of suddenly developing inhibitors (31).

Once inhibitors develop, treatment becomes more difficult, since the FVIII protein infusions become less effective. A Bethesda assay has been developed to detect and quantify inhibitor production. Therefore, inhibitors are measured in terms of Bethesda units (BU/ml) (32). One BU will result in a 50% reduction of FVIII activity (33). If less than 5 BU/ml are detected, a patient is classified as a low responder and if more than 5 BU/ml are detected a patient is classified as a high responder (34). For low responders, infusions of FVIII can still be administered, but must be done with an increased amount of FVIII proteins. The dose must be high enough to neutralize the present antibodies and still remain therapeutic (34). In the case of high responders, FVIII infusions are not adequate and bypassing agents like rFVIIa must be administered (34). Administering a bypassing agent works because FVIIa functions independently of FVIII in the clotting cascade, and is not affected by FVIII antibodies. Still, using a bypassing agent is not as practical as using recombinant FVIII protein because FVIIa has a half-life of only 2.3-2.7 hours and it is associated with side effects such as DIC, myocardial infarction, and stroke (30, 34).

Current treatment for inhibitor development relies on inducing tolerance, known as Immune Tolerance Induction (ITI) (1, 30, 34). This method of initiating tolerance requires frequent infusions with a high dose of rFVIII over the course of weeks to years, which is expensive and time consuming (1). Typically, ITI treatment is started within a year of inhibitor detection and is successful in 60-80% of cases, depending on inhibitor titer at the start of treatment (1, 30, 34). Patients that begin ITI with less than 10 BU/ml have an 85% success rate over the course of 11 months (34). If inhibitor titer is over 10 BU/ml, the success rate drops to 33% over the course of 15 months (34). Patients with an inhibitor titer greater than 500 BU/ml are typically unable to induce tolerance (34). Alternatively, immunosuppressive drugs have also been used in an attempt to reduce inhibitor titer (1, 30). This method is often used for those who have failed to induce tolerance, or do not qualify to use ITI therapy (30). For example, rituximab is an immunosuppressive drug that depletes circulating B cells and is often administered to patients with FVIII inhibitors (30). The full benefits of rituximab administration have yet to be elucidated, although recent studies indicate it reduces the inhibitor titer in ~25% of patients (30).

Adeno-associated virus gene therapy for HA

The Adeno-associated virus (AAV)

The Adeno-associated virus (AAV) is a small, non-enveloped, single stranded DNA virus that belongs to the Dependovirus genus of the Parvoviridae family (35-38). It is ubiquitous and capable of infecting a variety of species and cell types, both dividing and non-dividing (35). AAV is considered to be non-pathogenic, because it has not been associated with any known diseases (35, 39). It is a small icosahedron that is ~260 Å in diameter and carries a 4.7 kb genome (35, 36, 40, 41). Due to size restrictions it is unable to carry the genetic sequence and elements necessary to initiate replication (38). Therefore, to introduce a productive infection requires a helper virus, such as the Adenovirus or Herpesvirus (**Ill. 5**) (37, 38, 41). Without a helper virus the AAV virus becomes latent. It

ILLUSTRATION 5

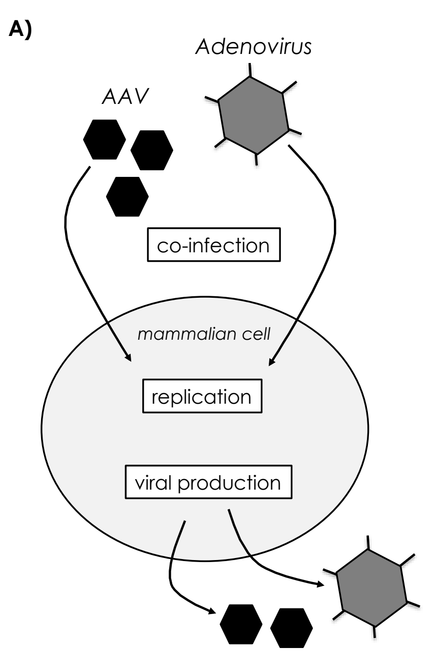


Illustration 5. AAV requires a helper virus for replication. Infection of cells with the AAV virus will only be productive in the presence of a helper virus. A) Mammalian cells infected with both AAV and the Adenovirus (a known helper virus) will lead to the replication and production of both AAV and Adenovirus. B) In the absence of a helper virus, AAV cannot replicate. The virus remains dormant and may integrate into the host's chromosome, but is not able to produce viral progeny.

can persist in either an episomal state or integrate site specifically at chromosome 19q13.4, also referred to as AAVS1 (**Ill. 5**) (36, 37, 41).

The AAV genome consists of two genes, rep and cap, flanked on either end by a 145 bp Inverted Terminal Repeat (ITR) (**Ill. 6**) (35, 36, 41). The ITR structure serves as both the origin of replication and the primer used to initiate second strand DNA synthesis (37). These structures also contain the Rep Binding Elements (RBE) and the Terminal Resolution Site (TRS) (37). Three promoters, P5, P19, and P40, work together to produce

ILLUSTRATION 6

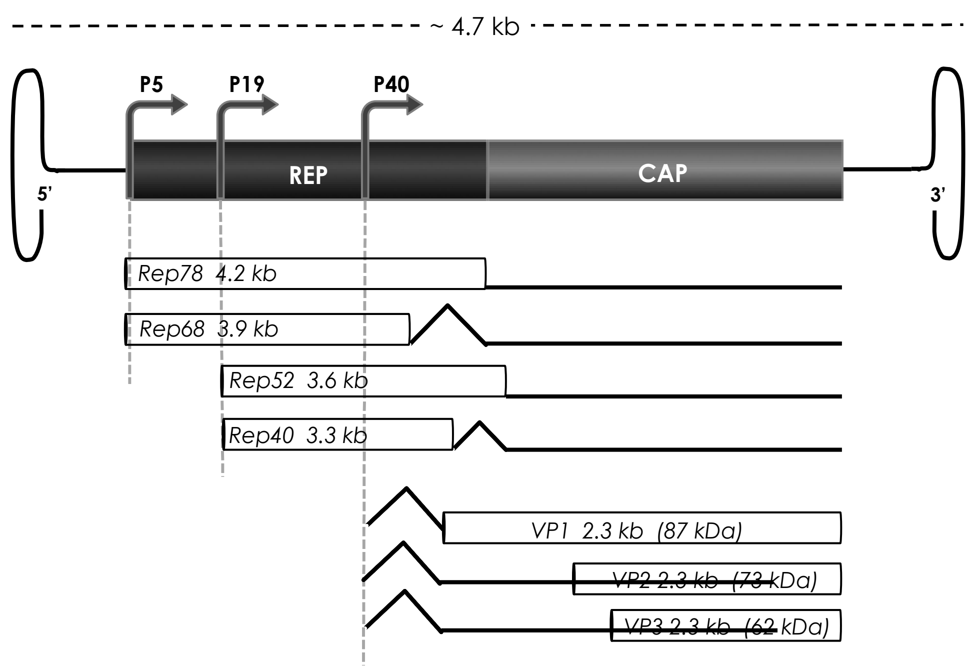


Illustration 6. AAV genes and transcripts. The 4.7 kb AAV virus carries two genes, the Rep gene and the Cap gene, flanked on either side by inverted terminal repeats (ITR). Transcription of these two genes from three separate promoters, results in the production of seven viral proteins. The Rep gene produces four proteins involved in genome replication. The Cap genes produce three proteins involved in viral capsid formation.

seven proteins, four rep proteins from the rep gene and three cap proteins from the cap gene **(Ill. 6)**. The rep gene proteins are non-structural and involved in genome replication. Rep78 and Rep68 are expressed via the P5 promoter and are involved in gene expression and DNA replication (36, 37). Rep52 and Rep40 are expressed via the P19 promoter and work to collect single stranded DNA for packaging (36, 37). Both Rep68 and Rep40 are the products of alternate splicing (36).

Post replication, both sense and anti-sense strands of DNA generated by the Rep proteins will be packaged into a viral capsid(37). The cap proteins, VP1, VP2, and VP3, are driven by the P40 promoter and are also subjected to alternate splicing (**Ill. 6**) (37). They are present at a molar ratio of 1:1:10 (36, 37). The cap proteins together form the capsid of the virus and are therefore responsible for the viral structure (36, 37, 40). The capsid proteins also determine cellular tropism and will differ between AAV serotypes (39, 40). There are multiple, well-defined, serotypes of AAV available, each having individual tropism and characteristics; however, they retain 65-99% homology to each other (36, 39, 40, 42).

AAV for gene therapy- pros and cons

AAV was first discovered in 1965 while researchers were analyzing a preparation of simian Adenovirus grown in Rhesus monkey kidney cells (43). Since then it has become popular for use in the field of gene therapy for multiple reasons (35). First, AAV is non-pathogenic and replication defective, giving it an excellent safety profile (4, 41, 44, 45). Second, there are multiple, well characterized serotypes that target a number of different cell types, both dividing and non-dividing. This makes it possible to target cells and organs precisely and limits non-specific cellular entry (4, 41). Third, it can be engineered easily through genetic cloning and requires the presence of only ~300 bp of viral DNA, limiting the amount of viral DNA that needs to be introduced to the cell (4, 41, 44). Fourth, it can establish a latent infection and produce consistent transgene expression after a single administration (41, 44, 45).

While AAV is an ideal vector choice for a number of reasons, there are two severe limitations associated with using this virus for gene therapy. First, the adaptive immune system limits the efficacy of the viral delivery and long-term gene expression (44, 46, 47). AAV is ubiquitous in nature, which means that most people have already been infected. While the virus itself is non-pathogenic, it is still a virus and recognized as foreign by the immune system. Because of this, a large number of people have pre-existing, circulating antibodies to the capsid protein, which diminishes transduction potential (46, 48). Similarly, there are pre-existing CD8+ T cells that recognize AAV capsid and become activated upon introduction of the vector, in a dose dependent manner (46, 47). Post cellular infection, the capsid can be presented via the MHC I pathway with the end result of cellular apoptosis (39, 47).

Interference of gene delivery by the immune system has been documented and is considered responsible for the disappointing outcomes from some of the recent AAV clinical trials (44, 46). Several ideas have been proposed for overcoming the immune response during and after gene delivery. One example is to decrease the amount of vector administered, since reports have indicated that the immune response is dose dependent (44, 46). A second example suggests using immunosuppressive drugs and proteasome inhibitors during vector administration (44, 46). A third example proposes using alternate AAV serotypes, selecting strains that may not have been introduced to the immune system.

This strategy has had little success due to a high degree of homology between capsid proteins of different serotypes, and subsequent T-cell cross recognition (44). It has also been suggested that candidates be screened for pre-existing immunity and treated accordingly (44).

The effect of the immune response is demonstrated in trials of AAV gene therapy for Hemophilia B (HB). HB is a monogenetic bleeding disorder caused by a defect in coagulation FIX (10). In studies using the canine animal model for HB, AAV gene therapy was able to produce and maintain 6-8% FVIII activity for over nine years (44, 47). These results were quite promising and indicated that AAV gene therapy for HB would be successful in humans. However, in clinical trials four out of five patients had <1% FIX activity, and while the fifth patient exhibited 10-12% activity, that level of activity dissipated within 10 weeks post administration (44, 47). It was determined that the poor performance of FIX-AAV gene therapy in this human clinical trial was due to an immune response to the AAV capsid and subsequent clearing of infected cells (44, 47). The end result was transgene expression too low to produce a therapeutic effect.

The second major limitation of AAV for gene therapy is vector size. It is well established that AAV has a packaging capacity of 4.7 kb (35, 36, 40, 41). Research indicates that amounts up to 5 kb can be adequately packaged but attempting to package larger constructs, and to generate oversized vectors, results in genome truncation (39). The oversized vectors are able to produce therapeutic effects; however, the vector population is heterogeneous (49). Each virus contains different and unknown amounts of viral genome. This is detrimental to gene therapy because the vector titer is typically inaccurate and production of truncated proteins may inadvertently initiate an immune response (49).

In an effort to overcome the size limitation of AAV without "overstuffing" the vector, a dual vector delivery method has been adopted (49). In this strategy, an oversized transgene is divided into multiple parts, each containing separate regulatory elements. These are then packaged into AAV separately and delivered together in one vector administration **(Ill. 7)** (42, 49-51). The therapeutic transgene is produced upon co-infection of the cell with both vectors, either through reassembly of the gene at a DNA level or association of the translated polypeptide products (42, 49-51).

ILLUSTRATION 7

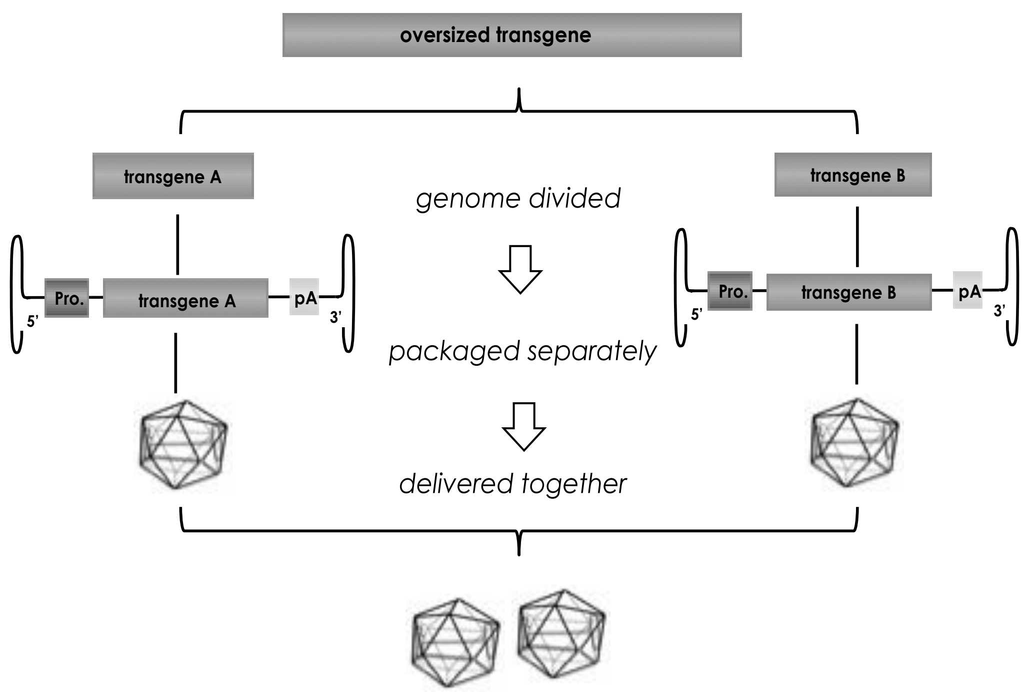


Illustration 7. The dual vector delivery strategy. The AAV vector is limited to a 4.7 – 5.0 kb piece of DNA. In cases where AAV is too small for efficient packaging, a gene can be divided into parts. For FVIII gene therapy, the FVIII gene is often divided into heavy and light chains, packaged into separate AAV vectors and then delivered together. The result is the production of fully functional FVIII protein.

AAV gene therapy for HA

HA is an excellent candidate for gene therapy because it is a monogenetic, hereditary disorder resulting from a defect of one protein that circulates in the blood at a low concentration (6, 48). The delivery of a single gene can correct the bleeding phenotype even without strict regulation of gene expression (35, 48). A therapeutic effect can be observed with as little as >1% FVIII activity. Additionally, even high levels of expression are not toxic (3, 13, 35, 48). The disease is well documented with pathways that have been fully elucidated. There are known biomarkers and endpoints that are well defined and can be measured, such as clotting activity and FVIII antigen levels (35, 48). There are also small and large animal models available for testing and screening products (3, 13, 35, 48).

To date, HA gene therapy has not been fully effective due to the size of the FVIII protein (35, 42, 51). Even the 4.3 kb B domain deleted version of FVIII becomes too large for efficient packaging when the ITR and other necessary regulatory elements are added (35, 42, 51). Attempts to package FVIIIBDD into a single vector require the use of small, minimal regulatory elements, which results in low transgene expression (45). There have been multiple studies using the dual chain delivery system by splitting the FVIII gene into heavy and light chains (35). While this method is able to produce fully functional FVIII, a chain imbalance occurs in which the light chain is produced 10-100 fold much more efficiently then the heavy chain (35, 42). This imbalance is not due to viral entry, transcription of the gene, or mRNA transport, but is due to the ineffective cellular production of the heavy chain (42, 51).

There are multiple limitations associated with the dual chain delivery of FVIII. The first disadvantage is that every cell must be transduced by two vectors, simultaneously, in order for functional FVIII to be produced (42, 51). Previous results demonstrated that simply mixing HC and LC together did not produce functional FVIII, indicating that the two chains must undergo cellular processing together (42). The second disadvantage is that cells transduced by one vector will still exhibit transgene expression, producing only half of the FVIII protein (13). This may lead to immunological consequences and inhibitor formation (13, 42).

Project objective and goals

HA is a bleeding disorder resulting from a mutation in one gene that leads to the production of one defective protein (10). Replacement of the FVIII protein, either at high or low concentrations, restores a normal phenotype (3, 8). These two basic characteristics make HA an excellent candidate for gene therapy using an AAV vector, selected based on its excellent safety profile (3). However, AAV gene therapy for HA is difficult, ultimately due to low transgene expression.

The initial objective of this project was to improve AAV gene therapy for HA, not by attempting to increase transgene expression, but instead, by directly enhancing activity of the FVIII protein produced. This is a quality over quantity approach; instead of attempting to produce a larger quantity of FVIII, the quality of FVIII is improved. In this case, even with low transgene expression, a more active protein would still deliver a therapeutic outcome. This approach makes AAV gene therapy for HA achievable without addressing the size limitation or infectivity of the AAV vector. In fact, a more active FVIII protein would be advantageous not only in context of gene therapy, but to protein therapy as well.

The idea that FVIII could be modified to have enhanced activity came from the observation that while FVIII is highly conserved between species, its activity profile is not always consistent. Canine FVIII (kFVIII) has been previously demonstrated to correct the HA phenotype in both mice and dogs, when delivered using either the dual chain or single chain strategy (45, 52). It has also been previously determined, using both a 1-stage and 2-stage APTT assay, that the kFVIIIBDD protein has a 3-fold increase in specific activity over the hFVIIIBDD protein (52). This difference in specific activity indicates that there is an element present in kFVIIIBDD, not present in hFVIIIBDD, which produces increased specific activity.

The first goal of this project was to identify the part of kFVIIIBDD responsible for the observed increase in specific activity. This was accomplished using the dual chain delivery strategy. Dividing the FVIII gene into heavy and light chains allowed for easy modifications and subsequent identification of a region in canine Light Chain (kLC) that enhanced specific activity. The second goal of this project was to successfully transfer this region to the human Light Chain (hLC), creating a human FVIII molecule with increased activity. The third goal of this project was to package this construct into AAV and test its function *in vivo* through dual chain delivery in conjugation with the X5 heavy chain (X5HC), a heavy chain modified to have enhanced production properties. The fourth goal of this project was to combine the light and heavy chain modifications into a single chain FVIII construct, package it into an AAV vector and test its function *in vivo.*

CHAPTER 2

MATERIAL AND METHODS

Genetic engineering and construct design

Human/ Canine hybrid construction

The FVIII Human Light Chain (hLC) and Canine Light Chains (kLC) share ~80% sequence homology (**Ill. 8**), yet in transfection experiments using HEK293 cells, replacing hLC with kLC results in a 3 fold increase in FVIII specific activity. In order to determine which portion of kLC was responsible for enhancing FVIII activity, human/canine hybrids were engineered to contain different portions of human and canine light chain sequences. Eight sets of primers were initially designed that annealed to regions of shared nucleotide sequence between both human and canine light chains (**Ill. 8**). Primers sequences are listed in **Table 1**.

The following steps were taken to create these hybrid constructs: PCR amplification of both human and canine light chain using Vent polymerase, purchased from New England Biolabs, run for 35 cycles in a Biometra thermocycler resulted in seven distinct fragments of light chain sequence. (Each fragment contained overlapping sequences that could be used to anneal them together). Multiple fragments were mixed together at a molar ratio of 1:1 and heated to 95 C to denature the double strands. Cooling to 55 C annealed the overlapping sequences of the fragments together to form a full light chain sequence. Vent polymerase and 10mM DNTP mix was used to fill in the single stranded regions.

The resulting sequence was then used as a template for PCR amplification with Vent polymerase for 35 cycles in a Biometra thermocycler.

ILLUSTRATION 8.

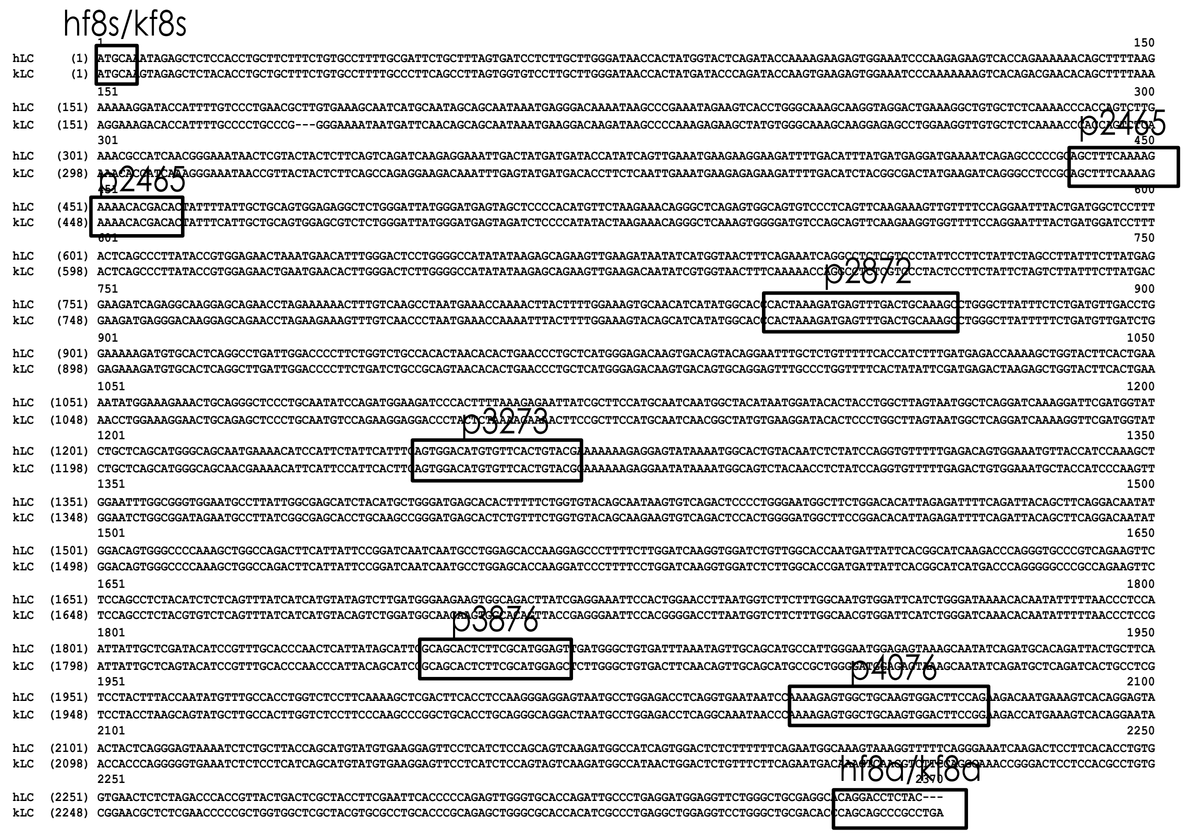


Illustration 8. Human and canine sequence alignment and primer design. The nucleotide alignment of human to canine light chain is depicted above. The location where primers anneal are enclosed with a black box. The primer sequences and information can be found in Table 1. The name of each primer is listed above its box.

TABLE 1.

set	name	orientation	sequence
set 1	2465s	forward	gcagctttcaaaagaaaacacgac
	2465a	revserse	gtcgtgttttcttttgaaagctgc
set 2	2872s	forward	cccactaaagatgagtttgactgcaaa
	2872a	revserse	tttgcagtcaaactcatctttagtggg
set 3	3273s	forward	cagtggacatgtgttcactgtac
	3273a	revserse	gtacagtgaacacatgtccactg
set 4	3512s	forward	cagcttcaggacaatatggacagtgggc
	3512a	revserse	gcccactgtccatattgtcctgaagctg
set 5	3876s	forward	cgcagcactcttcgcatggag
	3876a	revserse	ctccatgcgaagagtgctgcg
set 6	4076s	forward	caaaagagtggctgcaagtggacttc
	4076a	revserse	gaagtccacttgcagccactcttttg
set 7	hf8s	forward	tacccgcggccgcttttcaaaatgca
	hf8a	revserse	cgtcaattgtcagtagaggtcctgtg
set 8	kf8s	forward	tacccgcggcggccgcttttcaaaatgca
	kf8a	revserse	atccaattgatgtctgctcgaagcgg

Table 1. hLC and kLC hybrid construct primers. Primers were designed based on areas of shared nucleotide sequence between hLC and kLC. Each set of primers has one forward and one reverse orientation. The primer set is listed in the first column on the left, followed by the name of the primers in the second column, the orientation of the primer (forward or reverse) in the third column, and the nucleotide sequence of each primer in the fourth column.

The final product was digested with Not1 and BstB1 restriction enzymes purchased from New England Biolabs and purified on a 0.8% agarose gel using a Thermo Fisher gel extraction kit. The resulting insert was cloned into an AAV backbone containing the human β-actin promoter with a CMV enhancer, a SV40 intron, and a polyA tail using a ligation kit purchased from New England Biolabs. Each construct was tested *in vitro* through transient transfection of HEK293 cells and sent out for DNA sequencing using Genescript to ensure cloning accuracy.

hLC[1857-2147kLC] negative selection

The hLC[1857-2147kLC] construct was further analyzed for enhancing properties. Each of the 26 canine amino acids were individually tested for their role in increasing FVIII specific activity through a process of negative selection. The negative selection method uses hLC[1857-2147kLC] as a template and switches one canine amino acid with its human counterpart at a time, resulting in a total of 26 constructs. The following steps were taken to create these hybrid constructs: Primers were designed to match the sequence of the hLC[1857-2147kLC] template, however, in each construct the DNA sequence coding for the amino acid of interest was replaced with the corresponding hLC sequence. Primer sequences are listed in **Tables 2A and 2B**. These primers were used to amplify portions of the hLC[1857-2147kLC] sequence using Taq polymerase purchased from Sigma Aldrich, which has no proof reading capability. The fragments were then combined to form full light chain sequences by heating to 95 C to denature the double stranded DNA, then cooling to 55 C to anneal them together. The single stranded regions were filled in using Vent polymerase and 10 mM DNTP mix. The resulting product was utilized as a template for PCR amplification using Vent polymerase. The final product was digested with Not1 and BstB1 restriction enzymes purchased from New England Biolabs and purified on a 0.8% agarose gel using a Thermo Fisher gel extraction kit. The resulting insert was cloned into an AAV backbone containing the human β-actin promoter with a CMV enhancer, SV40 intron, and polyA tail using a ligation kit purchased from New England Biolabs. Each construct was tested for activity through transient transfection of

HEK293 cells *in vitro*, and sent out for DNA sequencing using Genescript to ensure cloning accuracy.

TABLE 2.

A)

Name	Primer sequence
JFGG1s	ccccttctggtctgccgcagtaa
JFGG2s	cttctgatctgccacagtaacacac
JFGG3s	gatctgccgcactaacacactgaa
JFGG4S	ggagtttgccctgtttttcactatattc
JFGG5S	cttcactgaaaacatggaaaggaact
JFGG6S	tccctgcaatatccagaaggagg
JFGG7S	caatgtccagatggaggaccta
JFGG8S	ggaccctacttttaaagaaaacttccg
JFGG9S	ctaaaagaaaactatcgcttccatgc
JFGG10S	tcaacggctatataaaggatacactc
JFGG11S	acggctatgtgatggatacact
JFGG12S	tcaggatcaaagggttcgatggt

Name	Primer sequence
JFGG13S	aggatcaaaagattcgatggtatctg
JFGG14S	tataaaatggcactgtacaacctctatc
JFGG15S	tgctaccatccaaagttggaatc
JFGG16S	accatcccaagctggaatctggc
JFGG17S	aatctggcgggtggaatgcctt
JFGG18S	cgagcacctgcatgccgggatg
JFGG19S	ggtgtacagcaataagtgtcagac
JFGG20S	gagcaccaaggagccctttttcc
JFGG21S	ttcacggcatcaagacccagg
JFGG22S	ccagcctctacatctctcagttta
JFGG23S	ctggatggcaagaagtggca
JFGG24S	aacaagtggcagagttaccga
JFGG25S	aagtggcacacttaccgaggg
JFGG26S	ccgattattgctcgatacatccg

B)

Name	Primer sequence
JFGG1a	TTACTGCGGCAGACCAGAAGGGG
JFGG2a	GTGTGTTACTGTGGCAGATCAGAAG
JFGG3a	TTCAGTGTGTTAGTGCGGCAGATC
JFGG4a	GAATATAGTGAAAAACAGGGCAAACTCC
JFGG5a	AGTTCCTTTCCATGTTTTCAGTGAAG
JFGG6a	CCTCCTTCTGGATATTGCAGGGA
JFGG7a	TAGGGTCCTCCATCTGGACATTG
JFGG8a	CGGAAGTTTTCTTTAAAAGTAGGGTCC
JFGG9a	GCATGGAAGCGATAGTTTTCTTTTAG
JFGG10a	GAGTGTATCCTTTATATAGCCGTTGA
JFGG11a	AGTGTATCCATCACATAGCCGT
JFGG12a	ACCATCGAACCCTTTGATCCTGA

Name	Primer sequence
JFGG13a	CAGATACCATCGAATCTTTTGATCCT
JFGG14a	GATAGAGGTTGTACAGTGCCATTTTATA
JFGG15a	GATTCCAACTTTGGATGGTAGCA
JFGG16a	GCCAGATTCCAGCTTGGGATGGT
JFGG17a	AAGGCATTCCACCCGCCAGATT
JFGG18a	CATCCCGGCATGCAGGTGCTCG
JFGG19a	GTCTGACACTTATTGCTGTACACC
JFGG20a	GGAAAAGGGCTCCTTGGTGCTC
JFGG21a	CCTGGGTCTTGATGCCGTGAA
JFGG22a	TAAACTGAGAGATGTAGAGGCTGG
JFGG23a	TGCCACTTCTTGCCATCCAG
JFGG24a	TCGGTAACTCTGCCACTTGTT
JFGG25a	CCCTCGGTAAGTGTGCCACTT
JFGG26a	CGGATGTATCGAGCAATAATCGG

Table 2. hLC[1857-2147kLC] negative selection primers. The canine amino acids in hLC[1857-2147kLC] were evaluated for their ability to enhance F8 activity. Hybrids were constructed using the primers above. A) Forward primers used to engineer the 26 constructs. B) Reverse primers used to engineer the 26 constructs.

hLC-K12 construction

Based on results from hLC[1857-2147kLC] negative selection, the hLC-K10 construct was designed. The hLC-K10 is a human light chain construct that contains 10 canine amino acids. This construct was engineered using primers that annealed to the hLC sequence but replaced the desired amino acids with canine amino acids. The fragments were joined by heating to 95 C to denature the double stranded DNA, and then cooled to 55 C to anneal them together. The resulting product was used as a temple for PCR using Vent polymerase. The final hCL-K10 PCR product was digested with Not1 and BstB1 restriction enzymes purchased from New England Biolabs and purified on a 0.8% agarose gel using a Thermo Fisher gel extraction kit. The resulting insert was cloned into an AAV backbone containing the human β-actin promoter with a CMV enhancer, SV40 intron, and polyA tail using a ligation kit purchased from New England Biolabs. Multiple clones were tested *in vitro* through transient transfection of HEK293 cells and sent out for DNA sequencing using Genescript to ensure cloning accuracy. However, sequencing revealed two extra amino acid mutations in the hLC-K10 sequence at positions 2157 and 2159. These amino acids were neither human nor canine amino acids. This construct containing 12 amino acid changes was designated hLC-K12.

Single chain constructs

Single chain FVIII constructs were engineered to contain the light chain mutations. This was accomplished through PCR amplification of both the light chain and heavy chain to make two fragments. These fragments were then combined to form a single chain construct by heating to 95 C to denature the double stranded DNA and cooling to 55 C to anneal them together. The single stranded regions were filled in using Vent polymerase and a 10mM DNTP mix. The resulting product was used as a template for PCR amplification using Vent polymerase for 35 cycles in a Biometra thermocycler. The final product was digested with Not1 and BstB1 restriction enzymes purchased from New England Biolabs, purified on a 0.8% agarose gel using a Thermo Fisher gel extraction kit, and cloned into an AAV backbone containing the human β-actin promoter with a CMV enhancer, SV40 intron, and polyA tail using a ligation kit purchased from New England Biolabs. Constructs were tested through transient transfection of HEK293 cells *in vitro*. Each construct was sent out for DNA sequencing using Genescript to ensure cloning accuracy.

Partial protein purification

In order to study construct mechanism and activity, protein was partially purified using the following steps: First, the protein was produced using transient transfection of 145 mm tissue culture plates of HEK293 cells with 50 ug of plasmid DNA and 150 ul of Polyjet reagent, as described in detail in the cellular transfection method section. Pure DNA was obtained through a large- scale cesium gradient purification process. After transfection, the cells were gently washed with 10ml of PBS once and 20 ml of DMEM (Invitrogen), supplemented with insulin (10mg/L), Sodium Selenite(6.7ug/L), transferrin (5.5mg/L) (Thermo Fisher Scientific Inc.), soybean and lima bean trypsin inhibitors (10mg/L) (Sigma) and 1% Tween80, was added. Media was collected and new media added at 24, 48, 72 and 96 hrs. post transfection and frozen at -80C for future use. Upon thawing, media containing FVIII was filtered using a .2 uM Whatman filter, MES was added to a final concentration of 20 mM and the pH was adjusted to 6.0. Second, the protein was partially purified using a SP Sepharose High Performance strong cation exchange column (HiTrap SP HP) (GE Healthcare) pre-equilibrated with equilibration/washing buffer (10 mM MES, 20 mM CaCl2, 200 mM NaCl, 0.01% tween 80, Ph 6.0). The column was washed with 5 column volumes of washing buffer and protein was eluted off the column with linear increase of sodium concentration to 1M. The elution was monitored using a ÄKTAFPLC system (GE Healthcare) and fractions with highest clotting activity, determined by a 1-stage APTT clotting assay, were pooled together and concentrated by Amicon concentrator (Molecular weight cut 50,000 Da) (Millipore). The purified protein was visualized using SDS PAGE electrophoresis and one or more of the following: Coomassie blue staining, western blot analysis and/or silver staining (Biorad).

AAV vector preparation

Recombinant AAV vectors used in this study were based on the liver specific AAV serotype 8. They were produced by the triple plasmid co-transfection method for recombinant AAV production. AAV vectors were purified by two rounds of cesium chloride gradient ultracentrifugation. After collection, the AAV vectors were buffer exchanged extensively against PBS with 5% D-sorbitol. The final vector was analyzed for purity and the genome titer was determined by both silver staining (Pierce silver stain kit) and real time PCR using primers targeting the FVIII heavy chain. The final vectors were stored at −80 °C before administration. Immediately prior to injection, vectors were diluted to desired concentration in 0.9% NaCl saline solution. Before use, syringes for injection were coated with a 1:10,000 pluronic F68 to inhibit binding of the AAV virus to the plastic syringe.

In vitro experiments

Cellular transfection method

All constructs were tested *in vitro* through transient transfection of HEK293 cells maintained in Dulbecco's Modified Eagles Medium, DMEM, (Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and 1% pen/strep antibiotics (Invitrogen) stored in a 37 C temperature regulated incubator supplemented with 5% CO_2 . 24 well plates were seeded 24 hrs prior to use and transfected at 80-100% confluency using Polyjet reagent purchased from SignaGen Laboratories. DNA for each construct was isolated from 4 ml of bacterial culture using a Sigma Aldrich miniprep kit, digested with BamH1 purchased from New England biolabs and run on a 0.8% agarose gel for characterization and confirmation. The concentration of each plasmid was determined via a Nanodrop instrument. Each light chain construct was transfected alone and in conjugation with either the human heavy chain (hHC) plasmid or the X5 heavy chain (X5HC) plasmid at a ratio of 1:2 (LC:HC). 16 hrs. post transfection the cells were washed with Ham's FXII media and 400 ul of Ham's FXII media (Gibco) supplemented with 2% heat inactivated fetal bovine serum was added. 24 hrs. later the media was harvested and used for subsequent testing.

mRNA isolation

HEK293 cells were seeded into 6 well plates and grown to 80-100% confluency in a 37 C incubator supplemented with 5% CO_2. Bacteria containing plasmids hFVIIIBDD, X5FVIIIBDD, X5K12BDD and hHCK12BDD was grown overnight at 37 C. The plasmid was isolated using a Sigma-Aldrich DNA plasmid miniprep kit, digested with BamHI purchased from New England biolabs for characterization and quantified using a Nanodrop instrument. Triplicate wells of cells were transfected using Polyjet reagent purchased from

Signagen Laboratories with 2ug of single chain plasmid. A media only well was used for a negative control. 16 hrs. post transfection, cells were washed with Ham's FXII media and 1ml of Ham's FXII media supplemented with 2% heat inactivated fetal bovine serum was added to each well. 24 hrs. later the media was harvested and frozen at -80C for subsequent testing. The cells were washed 2x with PBS and then removed using a cell scraper. The cells were then centrifuged for 2 minutes at 10g to remove left over PBS. Cells were frozen in liquid nitrogen until needed.

Total RNA was extracted using a Roche total RNA extraction kit, and a Nanodrop device was used to determine quantity. Reverse transcription was performed using 2 ul of RNA to make cDNA using an Invitrogen RT kit, with primers annealing to the poly A tail, selecting for mRNA only. Once total mRNA was converted to cDNA, QPCR using Fast Sybr Green mastermix was run using a Realplex PCR machine to determine CT value. QPCR primers were designed to select for FVIII heavy chain and GAPDH as a control. A ratio of the total amount of mRNA for each construct compared to the amount of GAPDH for each well was determined.

In vivo experiments

Mouse strains

Mice containing a FVIII gene exon 16 knock-out were used to test FVIII activity for all constructs. Three strains, C57BL6/129sv/HA, BalbC/HA and CD4KO/HA, are bred and maintained in a specific pathogen-free environment with a normal diet. Genotyping ensures homogeneity of strains. All surgical procedures involving animals were carried out in accordance with institutional guidelines under approved protocols at Temple

University Medical School. All anesethia was performed using isoflourane following standard protocol and procedures. Euthanasia was performed using CO_2 following standard protocols and procedures.

Hydrodynamic DNA injections

To test plasmid DNA function *in vivo*, 12 week old mice were heated for 10 minutes at 35 C and ear marked using an ear puncher. To ensure uptake of DNA by cells, the volume of injection must be high, ~10% total body weight, and the injection itself must be performed rapidly. Plasmid DNA was hydrodynamically injected into mouse tail vein using 150 ug of plasmid DNA diluted into 2 ml of 0.9% NaCl saline solution within 5-10 seconds. 48 hrs. post injection, mice were sacrificed following euthanasia procedures and blood was harvested via the hepatic artery and collected into 0.4% Sodium Citrate solution. Citrated whole blood was centrifuged at 8g for 10 minutes to separate the plasma from the red blood cells. Plasma was removed and stored at -80 until use in subsequent testing.

AAV vector injections

6-8 week old CD4KO/HA mice were heated at 35 C for 10 mins and ear marked using a ear puncher. Immediately prior to use, AAV vector was diluted in 0.9% NaCl saline solution to a total volume of 200 ul and injected into the tail vein. Total vector injection ranged from 1 -4 x 10^{11} vp/mouse. Syringes were pre-coated with 1:10,000 of pluronic F68 to prohibit AAV sticking to the plastic sides. Blood was harvested every 2-4 weeks for the course of the experiment via retro-orbital eye bleeding and collected in 0.4% Sodium Citrate. Citrated whole blood was centrifuged at 8g for 10 mins to separate the plasma from the red blood cells. Plasma was removed and stored at -80 C until use in subsequent assays.

Diagnostic assays

1-Stage activated partial thromboplastin time (APTT)

A 1-stage Activated partial thromboplastin time (APTT) was used to measure clotting activity of FVIII by measuring clot formation time in seconds. In order to determine clotting activity, 50ul of sample is mixed with 50 ul of FVIII deficient plasma (Haematologic Technologies) and 50 ul of APTT reagent (Diagnostica stago) in a standard APTT cuvette (Diagnostica stago). After the mixture is incubated for 170 seconds at 37 C, 50 ul of 25mM $CaCl_2$ is abruptly added and the time for a clot to form is measured in seconds using a Diagnostica stago Start APTT machine. The FVIII protein Kogenate (Bayer healthcare) is serially diluted 1:2 for 8-12 dilutions and clotting time for each concentration is measured. Using the Kogenate clotting time as a standard, the U/ml of FVIII in each sample can be determined based on clotting time. Samples collected from media are typically run undiluted, samples collected from mouse plasma are diluted in HEPES buffer supplemented with 1% BSA, typically at 1:10 dilution.

Enzyme linked immuno assay (ELISA)

An enzyme linked immuno assay (ELISA) is used to measure the amount of FVIII heavy and light chain antigen found in a sample. Plates are coated with human heavy chain antigen GMA-8016 (Green Mountain Antibodies) or human light chain antigen GMA-8018, (Green Mountain Antibodies) in ELISA coating buffer overnight at 4 C. The next day the coating buffer is removed and 3% BSA in PBST is added for 1 hr. After 1 hr. the wells are washed with PBST three times. Secondary antibody was diluted in PBST, for heavy chain GMA-8015 biotinylated (Green Mountain Antibodies) and for light chain GMA-8022 biotinylated (Green Mountain Antibodies), at a 1:5000 or 1:2000 dilution respectively. After 1 hr. the antibodies were removed and the wells were washed 3x with PBST.   Horseradish peroxidase was diluted in PBST at a ratio of 1:10,000 or 1:15,000 and added to wells. After 1 hr. the wells were washed 3x with PBST.   Wells were developed using a KLP brand TMB microwell peroxidase substrate system and the plate was read at 450 nm using a Molecular Devices brand Thermomax microplate reader.   Kogenate FS (Bayer healthcare) was serial diluted 1:2 and used as a standard to compare with the samples.

Western blot analysis

Partially purified FVIII protein was visualized using a western blot analysis. NuPage dye containing 50 mM DTT was added to the protein sample, loaded onto a 8% SDS PAGE gel (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) and run for 1 hr. at 180 Volts.   After running, the gel was rinsed in western blot transfer buffer for 10-20 mins and the protein was transferred to a nitrocellulose membrane. After transfer, the membrane was washed in PBST for 5 minutes 3 times. 5% non-fat milk in PBST was added as a blocking reagent. After 1hr, the membrane was washed for 5 minutes 3 times in PBST and then incubated for 1 hr. with a polyclonal sheep anti-human FVIII antibody diluted 1:2000 (Haematologic Technologies Inc.). The membrane was washed again for 5 minutes 3 times and incubated with a IRDye®800CW-conjugated donkey anti-sheep IgG (H&L) at 1:7500 dilution (Rockland Inc). An Odyssey Infrared Imaging System (LI-COR Bioscience) was used to scan the membrane and quantify the signal.

Determination of purified protein specific activity

The specific activity of partially purified protein was determined using the following steps: The hFVIIIBDD and hHCK12BDD proteins were purified in the manner described above. An ELISA was performed to determine FVIII quantity, selecting for heavy chain antigen as described above. In short, GMA-8016 was used at 1:1000 dilution as a coating antibody and GMA-8015 biotinylated was used at a 1:5000 dilution as a detecting antibody. Once the protein concentrations were determined, they were diluted to a concentration of 100 ng/ml in HEPES buffer supplemented with 1% BSA and a 1-stage APTT assay was used to determine coagulation activity. The specific activity was calculated as Units of APTT activity: mg of protein (U/mg).

FXa generation

The ability for the hFVIIIBDD and hHCK12BDD partially purified protein to form the Tenase complex with FIXa and convert FX to FXa was determined by FXa generation assay. The PC:PS (7:3, w/w) phospholipid vesicle was prepared by repeatedly extruding the lipid blends through 100nm filter membrane. Purified FX, FIXa and alpha thrombin were acquired from haematechologic Technologis inc (Essex, Vermont) and hiriduine was purchased from sigma (St Louis, MI). 1 nM hFVIIIBDD or hHCK12BDD was activated by 10nM thrombin at 37 C for 2 min in the presence of 5mM CaCl2 and reaction was stopped by adding 5 unit hirudine. 40nM FIXa were included in reaction mixture for 1 min to let formation of Tenase complex. The FXa generation reaction was started by adding different concentration of FX at 37C and reaction was terminated 2 min later by 10mM EDTA. The FXa generated were measured by its hydrolysis of chromogenic substrate S2765 (350uM). The rate of absorbance change was calculated with standard curve generated with serial diluted FXa. The rate of FXa generation was plotted against FX concentration and curve was fitted with Graphpad software using Michaelis-Menten equation to obtain Km and Vmax.

Statistical analysis

Statistical analysis was performed using a 2-tailed Student *t*-test. This was based on the comparison of the same variable between two groups in an experiment. A 2- tailed test was selected over a 1-tailed test because deviation in either direction was considered and results, either better or worse were important. The difference between groups was considered significant if the *p*-value was less .05 and not significant if the *p*-value was more than .05. The *p* value between groups is indicated in italics for each diagram under the connecting bar. Error bars for each graph were derived using standard deviation from the average of at least three points of the control group.

CHAPTER 3

RESULTS

The canine light chain enhances FVIII activity *in vitro* and *in vivo*

Canine light chain function *in vitro*

Using the dual chain delivery strategy, the coagulation activity of hFVIII was compared to that of kFVIII to determine which was more active *in vitro*. HEK293 cells were transiently co-transfected with a combination of either hLC and hHC plasmids or kLC and kHC plasmids and the amount of FVIII produced was measured using a 1-stage APTT assay. Cells transfected with hLC and hHC produced 0.16 U/ml FVIII (**Fig. 1A**). Cells transfected with kLC and kHC produced 1.13 U/ml FVIII, which is a significant 6.48-fold increase over the cells transfected with hLC and hHC (**Fig. 1A**). These results confirm the conclusion that kFVIII is more active than hFVIII *in vitro.*

In order to determine which part of kFVIII was responsible for the observed increase in clotting activity, the kFVIII gene was divided into heavy and light chains and these two chains were tested independantly. HEK293 cells were transiently co-transfected with a pair of plasmids: hLC and hHC, hLC and kHC, or kLC and hHC plasmids. Cells transfected with kHC and hLC plasmids produced no significant increase in coagulation activity compared to cells transfected with hLC and hHC plasmids (**Fig. 1B**). However, cells transfected with kLC and hHC plasmids produced 0.61 U/ml FVIII (**Fig. 1B**). This represents a 3.5-fold increase in coagulation activity over the cells transfected with hLC and hHC, which only produced 0.16 U/ml FVIII (**Fig. 1B**). These findings indicate that replacing the human light chain with the canine light chain in the dual delivery strategy enhances FVIII coagulation activity *in vitro*.

FIGURE 1

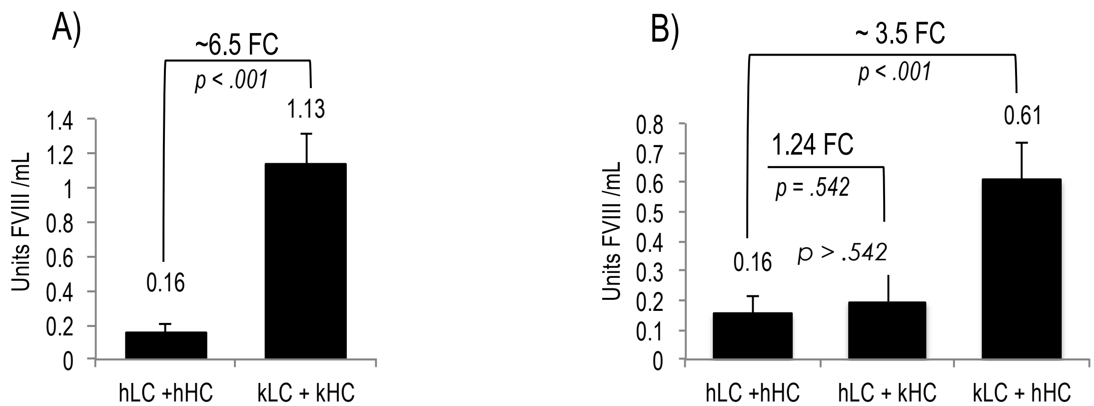


Figure 1. Canine light chain enhances FVIII coagulation activity. HEK293 cells transiently co-transfected with plasmid at a ratio of 1:2 (LC:HC). Statistical analysis was determined using a 2-tailed student *t*-test. A) Units of FVIII per mL produced determined by a 1-stage APTT assay. B) Units of FVIII per mL produced determined by an ELISA selecting for heavy chain antigen.

To further elucidate the enhancing properties of the canine light chain, HEK293 cells were transiently co-transfected with either hLC and hHC plasmids or kLC and hHC plasmids. Cells transfected with hLC and hHC plasmids produced 0.13 U/ml FVIII while cells transfected with kLC and hHC plasmids produced 0.45 U/ml FVIII (**Fig. 2A**). These results are comparable to those in Fig 1B, providing additional evidence that replacing hLC with kLC increases coagulation activity by ~3.5-fold. An ELISA measuring the amount of the heavy chain was used to analyze the amount of protein present in the media. Results found that cells transfected with hHC and hLC produced 11.4 ng of heavy chain, while cells transfected with kLC and hHC produced 25.3 ng of heavy chain (**Fig. 2B**).

FIGURE 2

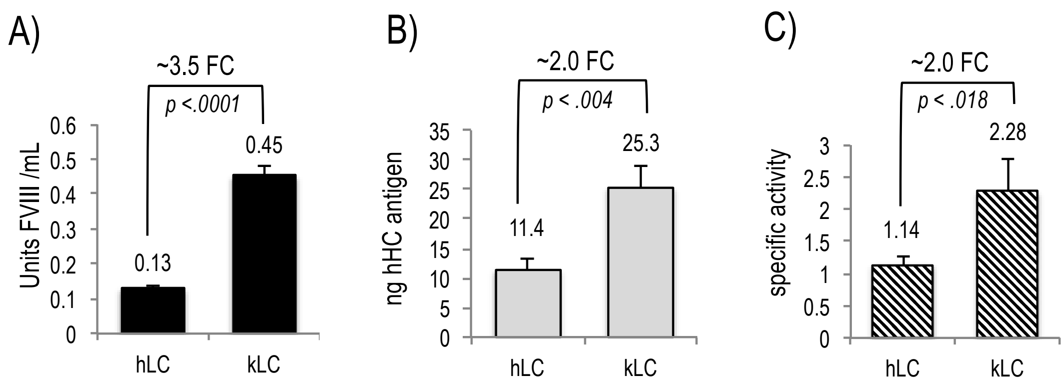


Figure 2. Canine light chain enhances FVIII specific activity *in vitro.* Activity and secretion analysis of FVIII produced from the transient transfection of HEK293 cells with the plasmids hHC and hLC or hHC and kLC. Statistical analysis was determined using a 2-tailed student *t*-test. A) Units of FVIII per mL produced from cells determined by a 1-stage APTT assay. B) Amount of heavy chain antigen detected by an ELISA. C) Specific activity of FVIII determined through the comparison of Units of FVIII per mL activity to the amount of FVIII heavy chain secreted.

This represents a 2.21-fold increase in the amount of heavy chain produced, which could explain the observed increase in coagulation activity. However, the increase in coagulation activity, determined by a 1 stage APTT assay (**Fig. 2A,**) was higher than the increase in heavy chain amount , determined by ELISA **(Fig. 2B)**, indicating that an enhancement in protein production was not the only reason for the observed increase in coagulation activity.

The specific activity of FVIII was calculated by comparing the U/ml FVIII produced, determined by a 1-stage APTT assay, to the ng of heavy chain antigen produced, determined by an ELISA. In this case, the specific activity represents a ratio of coagulation activity to the amount of protein in the media. Calculating the specific activity in this way allows for quantification of the biological activity of FVIII and provides a way to compare the activity of different FVIII constructs without having to consider a disparity in protein production. FVIII measured from cells transfected with hHC and hLC had a specific activity of 1.14, signifying that each U/ml of FVIII protein produced ~1 U/ml coagulation activity (**Fig. 2C**). FVIII from cells transfected with hHC and kLC had a specific activity of 2.28, indicating that every U/ml of FVIII protein produced ~2.28 U/ml coagulation activity (**Fig. 2C**). This represents a 2-fold increase in specific activity when the human light chain is replaced with canine light chain, indicating that the FVIII protein produced is two times more active (**Fig. 2C**). These results evidentiate that replacing the human light chain with the canine light chain significantly increases both FVIII coagulation activity and protein production, and significantly enhances specific activity of the FVIII protein itself *in vitro*.

Canine light chain function *in vivo*

Whether the canine light chain would have similar enhancing effects *in vivo* was tested through hydrodynamic injection of 12 week old, HA mice with 150 ug of plasmid DNA. The control group was injected with hHC and hLC plasmids at a 1:1 ratio (75ug hHC: 75 ug hLC) and the experimental group was injected with hHC and kLC at a 1:1 ratio (75ug hHC: 75 ug kLC). Blood was harvested from the hepatic artery via terminal bleeding 48 hours post injection; the plasma was isolated, tested for coagulation activity and the amount of FVIII protein measured. Mice in the control group produced 15.31 U/ml of FVIII, while mice in the experimental group produced 46.86 U/ml of FVIII (**Fig. 3A**).

FIGURE 3

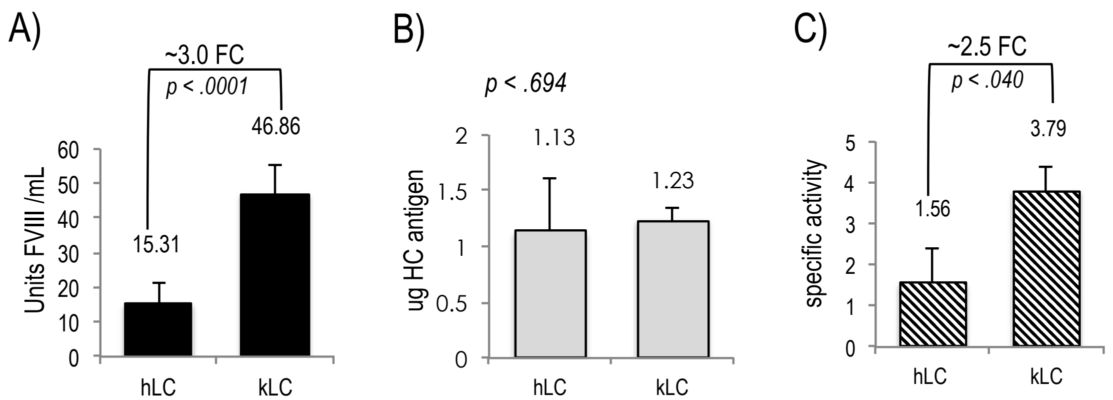


Figure 3. Canine light chain enhances FVIII specific activity *in vivo.* Activity and secretion analysis of FVIII produced from HA mice hydrodynamically injected with plasmids hHC and hLC or hHC and kLC. Statistical analysis was determined using a 2-tailed student *t*-test. A) U/ml of FVIII produced determined by a 1-stage APTT assay. B) Amount of heavy chain antigen detected by an ELISA. C) Specific activity of FVIII determined through the comparison of U/ml FVIII activity to the amount of FVIII heavy chain secreted.

This represents a 3.06-fold increase in FVIII coagulation activity, which is analogous to the *in vitro* results. An ELISA for the heavy chain found that mice in the control group produced 1.13 ug of HC antigen while those in the experimental group produced 1.23 ug of HC antigen (**Fig. 3B**).

These results indicate that there is no significant difference in the amount of heavy chain produced between mice injected with hHC and hLC or with hHC and kLC *in vivo.* This differs from the *in vitro* results reported in Fig. 2B, where replacing hLC with kLC did significantly increase protein production. However, the increase in specific activity observed *in vitro* was also apparent *in vivo*. FVIII produced from delivery of kLC and hHC had a specific activity of 3.79, which is a 2.43-fold increase over FVIII produced from the delivery of hLC and hHC, which had a specific activity of 1.56 (**Fig. 3C**). These results confirm that replacing the human light chain with canine light chain both increases coagulation activity and significantly enhances the specific activity of the protein both *in vitro* and *in vivo.*

Canine light chain amino acids 1857-2147 enhance FVIII activity both *in vitro* and *in vivo*

Human/canine hybrid construction

In order to delineate which section of canine light chain was responsible for increasing FVIII activity, the canine light chain gene was divided into regions and each region was analyzed independently. This was accomplished by designing 32 hybrid chains that contained different portions of both human and canine light chain sequences (**Table 3**). It was assumed that constructs containing canine amino acids with enhancing properties would produce FVIII activity that was similar to FVIII from cells transfected with kLC and hHC plasmids. Correspondingly, constructs without these regions would produce activity similar to FVIII from cells transfected with hLC and hHC.

TABLE 3

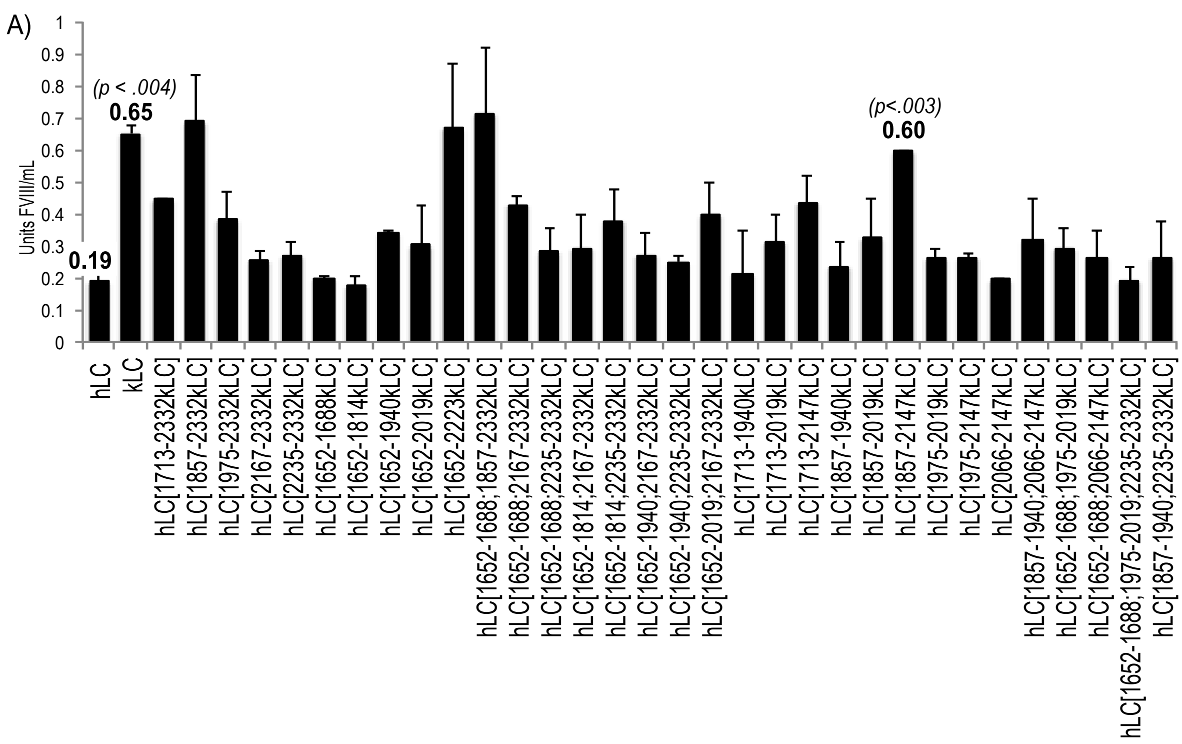
hybrid	human amino acids	canine amino acids
hLC[1713-2332kLC]	1652-1712	1713-2332
hLC[1857-2332kLC]	1652-1856	1857-2332
hLC[1975-2332kLC]	1652-1974	1975-2332
hLC[2167-2223kLC]	1652-2166	2167-2332
hLC[2235-2332kLC]	1652-2234	2235-2332
hLC[1652-1688kLC]	1689-2332	1652-1688
hLC[1652-1814kLC]	1815-2332	1652-1814
hLC[1652-1940kLC]	1941-2332	1652-1940
hLC[1652-2019kLC]	2020-2332	1652-2019
hLC[1652-2223kLC]	2224-2332	1652-2223
hLC[1652-1688;1857-2332kLC]	1689-1856	1652-1688;1857-2332
hLC[1652-1688;2167-2332kLC]	1689-2166	1652-1688;2167-2332
hLC[1652-1688;2235-2332kLC]	1689-2234	1652-1688;2235-2332
hLC[1652-1814;2167-2332kLC]	1815-2166	1652-1814;2167-2332
hLC[1652-1814;2235-2332kLC]	1815-2234	1652-1814;2235-2332
hLC[1652-1940;2167-2332kLC]	1941-2166	1652-1940;2167-2332
hLC[1652-1940;2235-2332kLC]	1941-2234	1652-1940;2235-2332
hLC[1652-2019;2167-2332kLC]	2020-2166	1652-2019;2167-2332
hLC[1713-1940kLC]	1652-1712;1941-2332	1713-1940
hLC[1713-2019kLC]	1652-1712;2020-2332	1713-2019
hLC[1713-2147kLC]	1652-1712;2148-2332	1713-2147
hLC[1857-1940kLC]	1652-1856;1941-2332	1857-1940
hLC[1857-2019kLC]	1652-1856;2020-2332	1857-2019
hLC[1857-2147kLC]	1652-1856;2148-2332	1857-2147
hLC[1975-2019kLC]	1652-1974;2020-2332	1975-2019
hLC[1975-2147kLC]	1652-1974;2148-2332	1975-2147
hLC[2066-2147kLC]	1652-2065;2148-2332	2066-2147
hLC[1857-1940;2066-2147kLC]	1652-1856;1941-2065;2148-2332	1857-1940;2066-2147
hLC[1652-1688;1975-2019kLC]	1689-1974;2020-2332	1652-1688;1975-2019
hLC[1652-1688;2066-2147kLC]	1689-2065;2148-2332	1652-1688;2066-2147
hLC[1652-1688;1975-2019;2235-2332kLC]	1689-1974;2020-2234	1652-1688;1975-2019;2235-2332
hLC[1857-1940;2235-2332kLC]	1652-1856;1941-2234	1857-1940;2235-2332

Table 3. Human/canine hybrid design. Hybrids were designed based on FVIII hLC that had different regions replaced with the corresponding kLC amino acid sequence. The regions were defined using eight sets of primers that annealed to areas of shared nucleotide sequence between hLC and kLC. The primers were used to amplify the hLC and kLC sequences, these fragments were combined to make hybrids containing different regions of hLC and kLC. In the chart above, the name of each hybrid is in the first left column. The second column labeled human amino acids indicates which amino acids are human and the third column labeled canine amino acids indicates the amino acids that were replaced with kLC. The hybrid sequence begins with the first different amino acid between hLC and kLC, which is position 1652. Amino acids 1649-1651 are the same for both hLC and kLC and therefore are the same for all constructs.

Human/canine hybrids function *in vitro*

HEK293 cells were transiently co-transfected with hHC and each individual hybrid at a 2:1 ratio (350ng HC: 175 ng LC). 24 hours post transfection the media was harvested and used for analysis. Constructs hLC[1652-2223kLC], hLC[1652-1688;1857-2332kLC], hLC[1857-2332kLC], and hLC[1857-2147kLC] all produced FVIII activity similar to cells transfected with kLC and hHC (**Fig 4A**). All constructs produced similar heavy chain antigen levels, indicating that the difference in FVIII activity was not necessarily due to an increase in protein production (**Fig. 4B**).

FIGURE 4



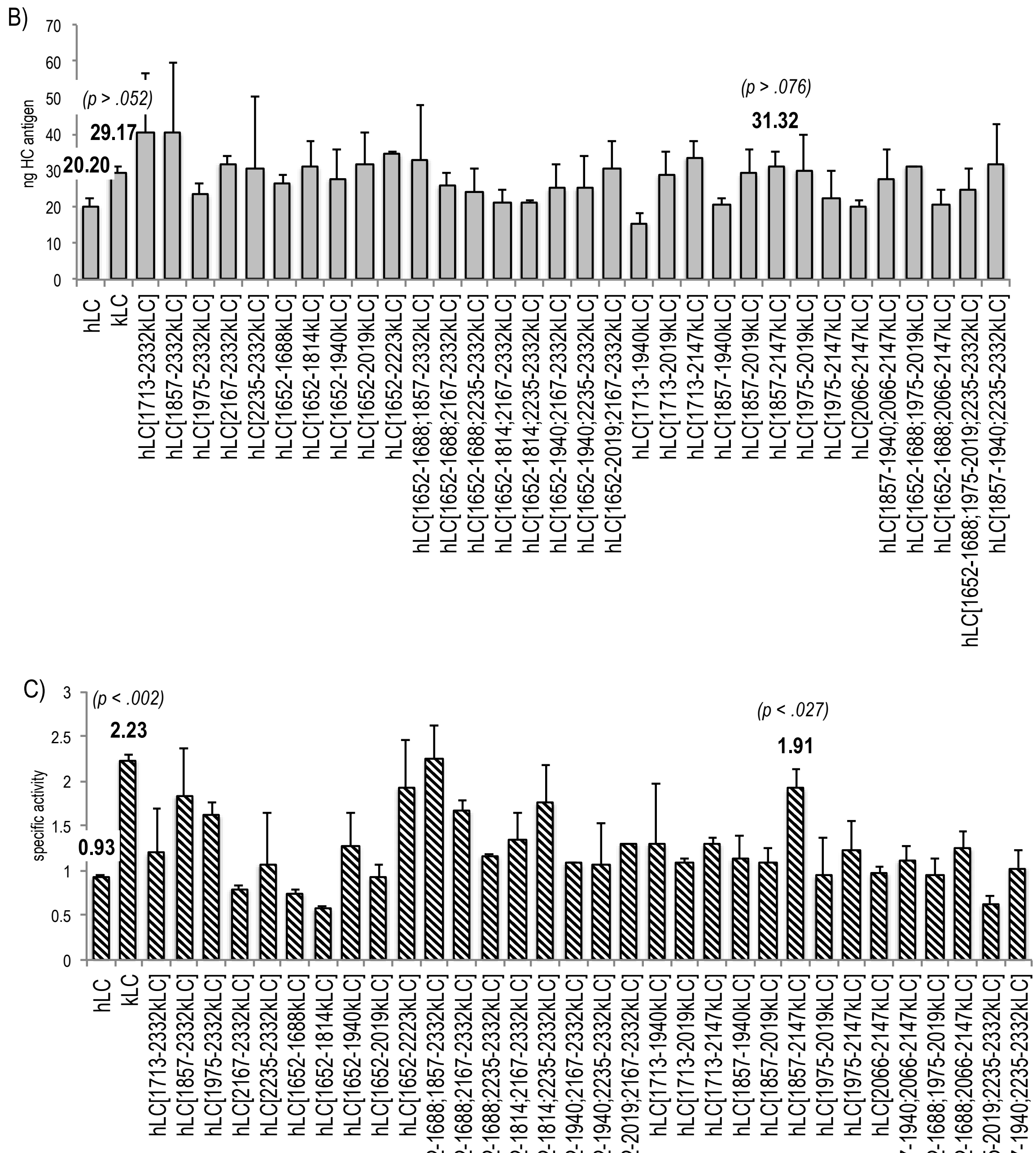


Figure 4. FVIII activity and secretion of human/canine hybrids *in vitro*. HEK293 cells were transiently transfected with hHC and hLC/kLC hybrids plasmids. Media was harvested 24 hrs post transfection and used to analyze the FVIII properties of each hybrid construct. Statistical analysis is based on a 2-tailed student *t*-test. A) U/ml FVIII produced by each hybrid, determined via a 1-stage APTT assay. B) Amount of heavy chain detected by ELISA for each hybrid construct. C) Specific activity of each hybrid.

Results indicated that the difference in FVIII activity between the hybrid constructs was based on specific activity. Constructs hLC[1652-2223kLC], hLC[1652-1688;1857-2332kLC], hLC[1857-2332kLC], and hLC[1857-2147kLC] all produced specific activity similar to FVIII from cells transfected with kLC and hHC (**Fig. 4C**). Although constructs hLC[1652-2223kLC], hLC[1652-1688;1857-2332kLC], hLC[1857-2332kLC], and hLC[1857-2147kLC] produced similar levels of FVIII activity *in vitro*, construct hLC[1857-2147kLC] was selected for further analysis because it contained the fewest number of canine amino acids.

Construct hLC[1857-2147kLC] enhances FVIII activity *in vitro*

The FVIII coagulation properties of hLC[1857-2147kLC] were further analyzed through *in vitro* testing. HEK293 cells were transiently transfected with hHC and either hLC, kLC, or hLC[1857-2147kLC]. 24 hours post transfection the media was harvested and used for analysis. Cells transfected with hHC and hLC[1857-2147kLC] produced 0.37 U/ml FVIII (**Fig. 5A**). This was analogous to cells transfected with kLC and hHC, which produced .43 U/ml FVIII. However, this was a 3-fold increase in FVIII activity over cells transfected with hLC and hHC, which only produced .12 U/ml FVIII (**Fig. 5A**). These results indicate that the hLC[1857-2147kLC] construct contains a portion of canine light chain that enhances FVIII clotting activity.

An ELISA detecting human heavy chain found that cells transfected with hLC[1857-2147kLC] and hHC produced 21.85 ng of heavy antigen (**Fig. 5B**). This was comparable to cells transfected with kLC and hHC, which produced 21.28 ng of heavy chain antigen. However, cells transfected with hHC and hLC[1857-2147kLC] or hHC and kLC both produced a significantly more heavy chain when compared to cells transected with hLC and hHC, which only produced 11.75 ng of heavy chain (**Fig. 5B**).

FIGURE 5

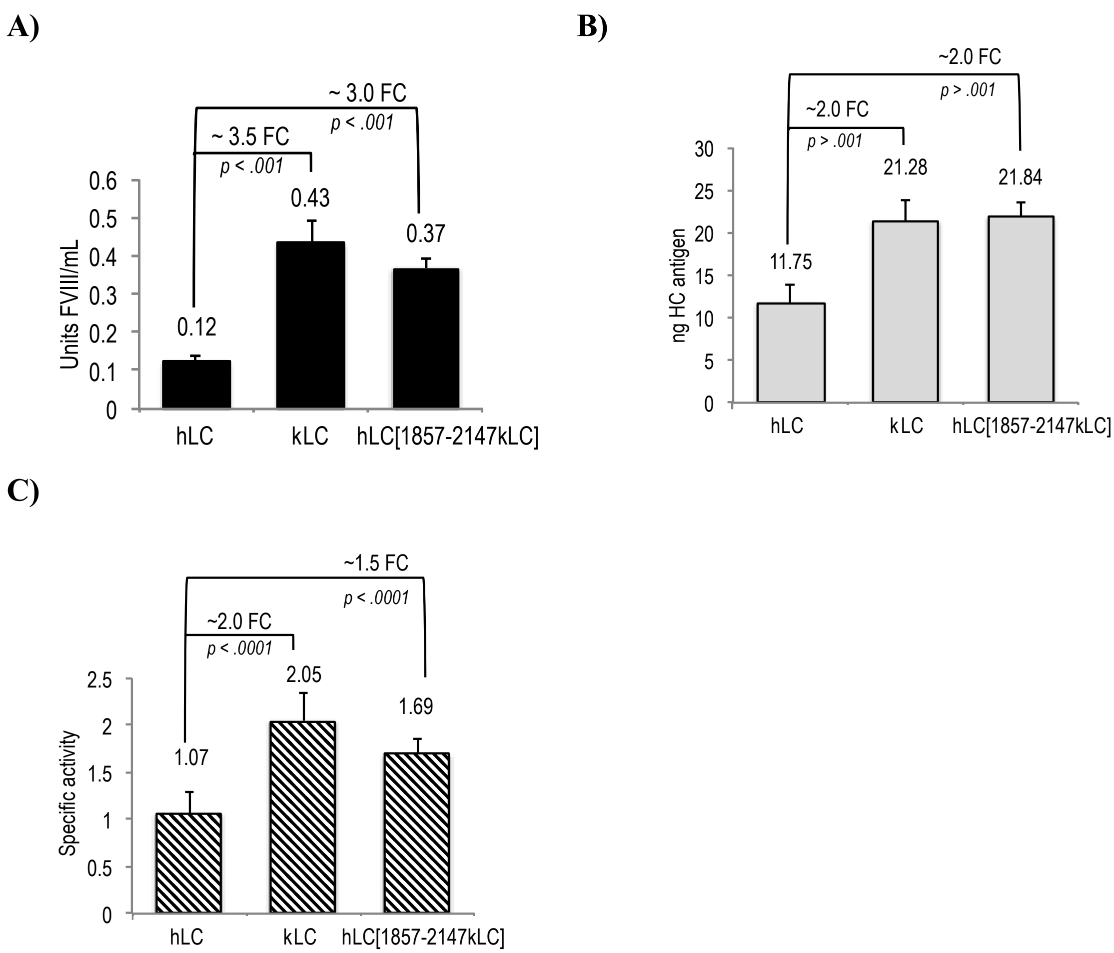


Figure 5. Construct hLC[1857-2147kLC] enhances FVIII activity *in vitro*. HEK293 cells transiently with plasmid at a ratio of 1:2 (LC:HC). Statistical analysis was run using a 2-tailed student *t*-test. A) Results from a 1-stage APTT assay used to determine FVIII clotting activity. B) Measurement of heavy chain secretion as determined by ELISA. C) Specific activity for each combination calculated as a ratio of FVIII U/ml activity to FVIII heavy chain antigen secreted.

The specific activity of FVIII from cells transfected with hHC and hLC[1857-2147kLC] was calculated at 1.69 (**Fig. 5C**). This amount of activity was similar to cells transfected with hHC and kLC, yet significantly higher than the specific activity of FVIII from cells transfected with hHC and hLC (**Fig. 5C**). Taken together, these results suggest that although the hLC[1857-2147kLC] construct does effect protein production, it primarily enhances protein specific activity *in vitro.*

Construct hLC[1857-2147kLC] enhances FVIII activity *in vivo*

The ability for construct hLC[1857-2147kLC] to enhance activity *in vivo* was tested through hydrodynamic injection of 12 week old, HA mice with 150 ug of plasmid DNA. Mice designated as the negative control group were injected with hLC and hHC at a 1:1 ratio (75ug hLC and 75 ug hHC), mice in the positive control group were injected with kLC and hHC at a 1:1 ratio (75 ug kLC and 75ug hHC), and mice in the experimental group were injected with hLC[1857-2147kLC] and hHC at a 1:1 ratio (75ug hLC[1857-2147kLC]and 75 ug hHC). Blood was harvested from the hepatic artery in a terminal bleed 48 hours post injection; plasma was isolated and subsequently tested for activity and the amount of FVIII protein was measured.

Mice in the experimental group produced 66.30 U/ml FVIII, which was a 2.69-fold increase over the negative control group, which only produced 24.86 U/ml FVIII (**Fig. 6A**). The coagulation activity of the experimental group was similar to the positive control, which produced 88.19 U/ml FVIII (**Fig. 6A**). These results are consistent with *in vitro* data, verifying that kLC amino acids 1857-2147 enhanced FVIII coagulation activity. There was no significant difference in the amount of heavy chain produced between these three groups, as determined by an ELISA (**Fig. 6B**), substantiating the conclusion that the increased coagulation activity is not due to an increase in overall FVIII protein production.

FIGURE 6

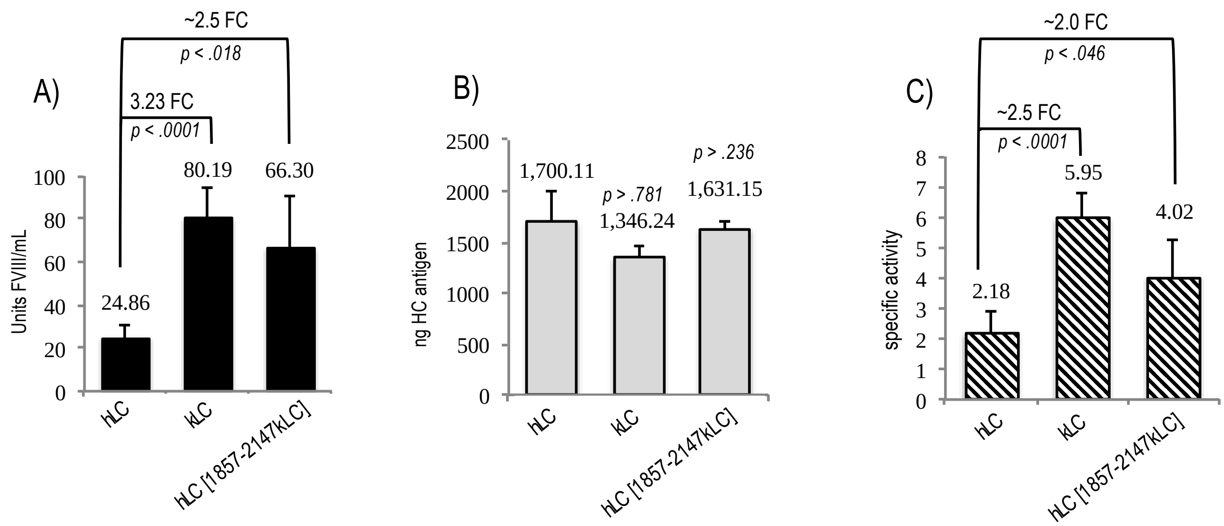


Figure 6. Construct hLC[1857-2147kLC] enhances FVIII activity *in vivo*. Three groups of HA mice were hydrodynamically injected with 150 ug of plasmid DNA. The first group was injected with plasmids hHC and hLC at a ratio of 1:1 (75ug hHC and 75 ug hLC). The second group was injected with plasmids hHC and kLC at a 1:1 ratio (75 ug hHC and 75 ug kLC). The third group was injected with hHC and hLC[1857-2147kLC] at a 1:1 ratio (75 ug hHC and 75 ug hLC[1857-2147kLC]). Blood was harvested 48 hrs. post injection from the hepatic artery. Statistical analysis is based on a 2-tailed, student *t*-test. A) U/ml FVIII produced from each group determined by a 1-stage APTT assay. B) Amount of heavy chain antigen detected by an ELISA. C) Specific activity of FVIII produced from all three groups.

The specific activity of FVIII from the experimental group was calculated to be 4.02 (**Fig. 6C**). This was significantly higher than FVIII from the negative control group, which had a specific activity of 2.18 and was similar to the positive control group, which had a specific activity of 5.95 (**Fig. 6C**). Taken together, data collected from both *in vitro* and *in vivo* experiments concludes that kLC amino acids 1857-2147 are able to enhance coagulation activity by producing a FVIII protein with increased specific activity.

Negative selection of hLC[1857-2147kLC] identified 12 amino acids that enhance FVIII activity *in vitro*

26 canine amino acids are tested for activity enhancing properties

The construct hLC[1857-2147kLC] contains 26 canine amino acids that span the A3-C1 regions of the hFVIII protein. Data collected from both *in vitro* and *in vivo* experiments have proven that together these amino acids are capable of enhancing FVIII activity. However, whether or not all 26 amino acids were required to produce this therapeutic effect was not yet clarified. It was expected that some of the 26 amino acids would not be necessary, and that only specific amino acids would be required to produce the enhancing effect. A process of negative selection was used in an effort to distinguish between the canine amino acids that were dispensable and those that were essential.

In this strategy, the contribution of each individual canine amino acid was assessed by systematically replacing each with its human counterpart in the hLC[1857-2147kLC] construct. Replacing essential canine amino acids resulted in a decrease in FVIII activity; however, replacing dispensable amino acids resulted in no change in FVIII activity.

A total of 26 constructs were designed based on the negative selection concept, each examining the effect of a single, canine to human, amino acid change. The engineered constructs were tested *in vitro* through transient transfection of HEK293 cells in conjunction with hHC at a 1:2 ratio (LC:HC). 24 hrs post transfection media was harvested and a 1-stage APTT assay was performed to determine U/ml FVIII activity for each construct (**Fig. 7A**).

FIGURE 7

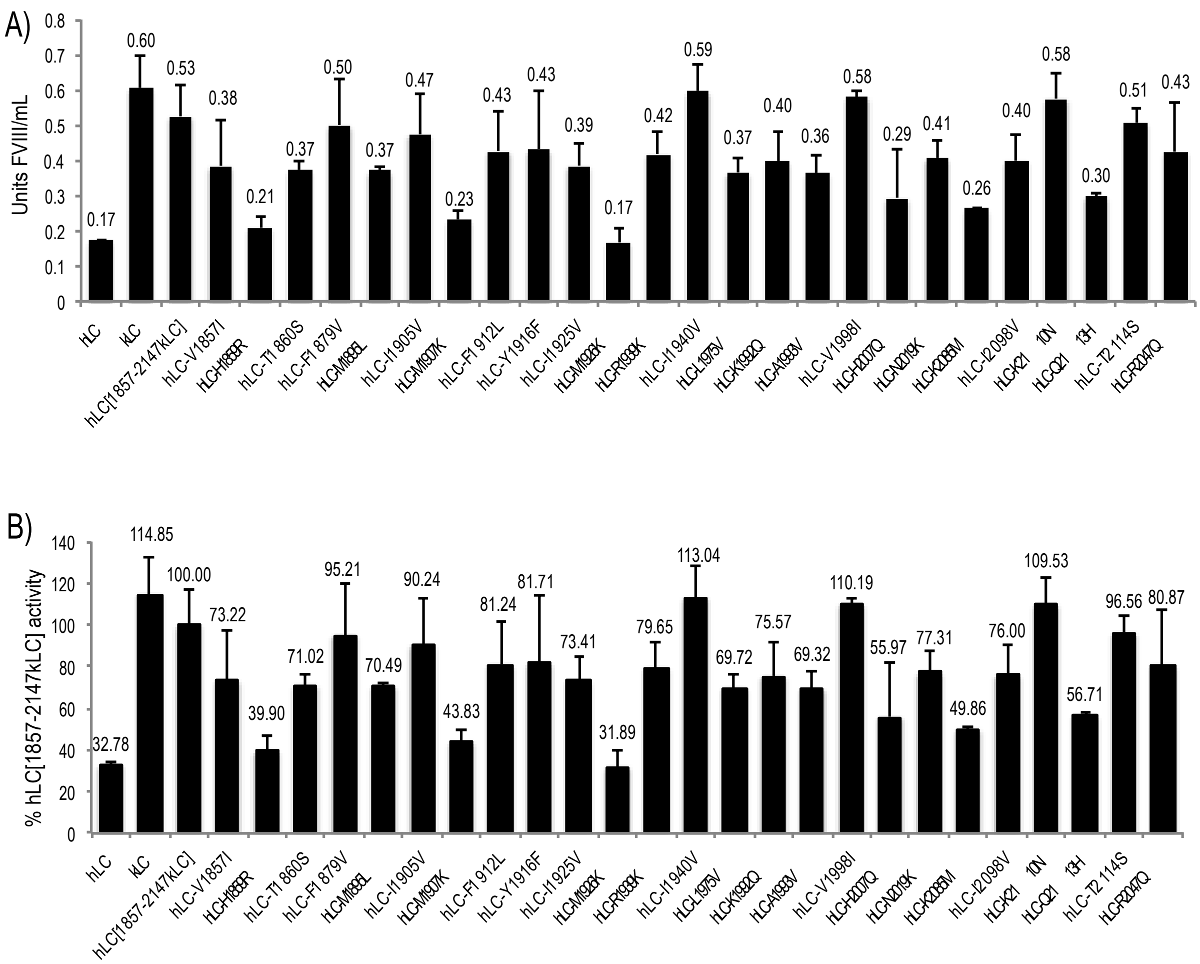


Figure 7. Negative selection of Construct hLC[1857-2147]. HEK293 cells were transiently transfected with negative selection constructs in conjunction with the hHC. A) U/ml FVIII activity produced by each construct as determined by a 1-stage APTT assay. B) Percent U/ml FVIII activity of each hybrid compared to hLC[1857-2147kLC].

As expected, cells transfected with hLC and hHC produced .17 U/ml FVIII while cells transfected with hHC and either kLC or hLC[1857-2147kLC] produced FVIII with 3.1-3.5 times more activity (**Fig. 7A**). The hybrid constructs all produced FVIII with activity that fell within the range of .17 -.59 U/ml FVIII (**Fig. 7A**).

In order to enumerate the difference in FVIII activity between the hybrid constructs, percent activity was calculated for each construct with hLC[1857-2147kLC] representing 100%. Compared to hLC[1857-2147kLC], cells transfected with hLC and hHC produced 32.78% activity and cells transfected with kLC and hHC produced 114.85 % activity (**Fig. 7B**). Most of the hybrid constructs tested produced between 70-100% activity. In fact, 17 constructs produced 70% or higher hLC[1857-2147kLC] activity, and only 4 constructs produced lower than 50% hLC[1857-2147kLC] activity **(Fig. 7B)**.

Based on the negative selection data, it was determined that constructs hLC-V1857I (73.2% activity), hLC-H1859R (39.9% activity), hLC-M1907K (43.8% activity), hLC-M1926K (31.9% activity), hLC-L1975V (69.7% activity), hLC-A1993V (69.3% activity), hLC-H2007Q (56.0% activity), hLC-E2066D, hLC-K2085M (49.9% activity) and hLC-Q2113H (56.7% activity) displayed the most decrease in FVIII activity. Out of the original 26 canine amino acids in construct hLC[1857-2147kLC], 10 were designated as essential and 16 amino acids were identified as dispensable (**Fig. 8A**).

FIGURE 8

A)

```
                      1 23                  4               5         6 7    8   9       10 11           12 13
hLC               GPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHS
hLC[1857-2147kLC] GPLLICRSNTLNPAHGRQVTVQEFALVFTIFDETKSWYFTENLERNCRAPCNVQKEDPTLKENFRFHAINGYVKDTLPGLVMAQDQKVRWYLLSMGSNENIHS
                    18571859                                            1907                 1926

                                    14                15 16   17        18           19
hLC               IHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGS
hLC[1857-2147kLC] IHFSGHVFTVRKKEEYKMAVYNLYPGVFETVEMLPSQVGIWRIECLIGEHLQAGMSTLFLVYSKKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGS
                                   1975              1993          2007

                         20                  21             22           23 2425                            26
hLC               INAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRST
hLC[1857-2147kLC] INAWSTKDPFSWIKVDLLAPMIIHGIMTQGARQKFSSLYVSQFIIMYSLDGNKWHSYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIAQYIRLHPTHYSIRST
                        2066                 2085                         2113
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B)

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              1 2                                                3                  4
hLC       GPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHS
hLC-k10   GPLLICRTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQKEDPTFKENYRFHAINGYIKDTLPGLVMAQDQRIRWYLLSMGSNENIHS
           1857 1859                                          1907               1926

                             5                6      7
hLC       IHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGS
hLC-k10   IHFSGHVFTVRKKEEYKMAVYNLYPGVFETVEMLPSKVGIWRVECLIGEHLQAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGS
                            1975             1993   2007

                 8                  9                          10
hLC       INAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRST
hLC-k10   INAWSTKDPFSWIKVDLLAPMIIHGIMTQGARQKFSSLYISQFIIMYSLDGKKWHTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRST
               2066                2085                         2113
```

Figure 8. Alignment representing positions of 10 essential canine amino acids. Amino acid sequences were aligned using the vector NTI program. Yellow represents identical amino acids and white represents different amino acids. A) Alignment of hLC and hLC[1857-2147kLC] constructs. All 26 amino acids appear white and are identified with the number at the top and the amino acid position underneath. Numbers above the 10 amino acids identified as essential are in bold and their position on the light chain is underlined. B) Alignment of hLC and hLC-K10. All 10 amino acids are listed and numbered above with their relative positions on the light chain listed below.

12 amino acids enhance FVIII activity

One final construct was designed based on the human light chain sequence that contained the 10 essential canine amino acids, designated hLC-K10. The amino acid changes in hLC-k10 are the following: V1857I, H1859R, M1907K, M1926K, L1975V, A1993V, H2007Q, E2066D, K2085M and Q2113H (**Fig. 8B**). After engineering the hLC-K10 construct, multiple clones were screened for FVIII activity through transient transfection of HEK293 cells in conjugation with hHC at a ratio of 1:2 (LC:HC). One clone, clone hLC-K10 (10), produced .96 U/ml FVIII, which represented activity superior to both kLC and hLC[2857-2147kLC] (**Fig 9**). Sequencing results of hLC-K10 (10)

FIGURE 9

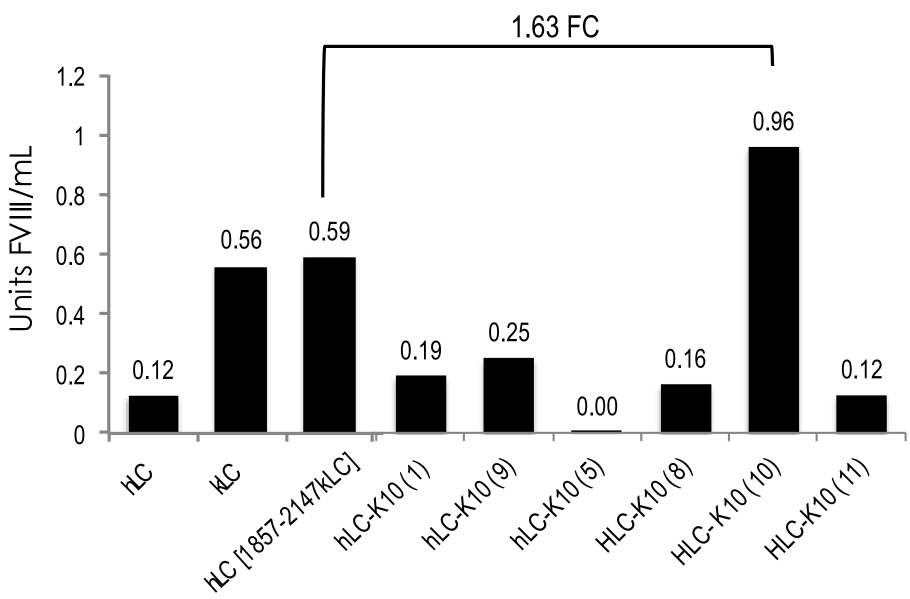


Figure 9. A single hLC-K10 clone demonstrates enhanced activity. HEK293 cells were transiently transfected with different clones of hLC-K10 and hHC. FVIII activity was determined via a 1-stage APTT assay.

identified two extra amino acid mutations that were not part of the originally identified 26 amino acids. The two novel mutations are S2157N and R2159H, and they are neither human nor canine amino acids (**Fig 10**). Since sequencing revealed this construct to actually contain 12 amino acid changes, it was designated hLC-K12.

FIGURE 10

```
            1 2                                                   3                  4
hLC       GPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHS
hLC-k12   GPLLICRTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQKEDPTFKENYRFHAINGYIKDTLPGLVMAQDQRIRWYLLSMGSNENIHS
           1857 1859                                          1907               1926

                           5                  6             7
hLC       IHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGS
hLC-k12   IHFSGHVFTVRKKEEYKMAVYNLYPGVFETVEMLPSKVGIWRVECLIGEHLQAGMSTLFLVYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGS
                           1975               1993          2007

                 8                  9                           10                                        11 12
hLC       INAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIRST
hLC-k12   INAWSTKDPFSWIKVDLLAPMIIHGIMTQGARQKFSSLYISQFIIMYSLDGKKWHTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYNIHST
                 2066               2085                        2113                                      2157 2159
```

Figure 10. Construct hLC-K12 amino acid sequence. The vector NTI program was used to align hLC and hLC-K12 amino acid sequences. The number of amino acids is labeled above and the position on the light chain is identified below. The two novel mutations, S2157N and R2159H are listed in bold.

Results indicated that the K12 modifications to the human light chain were capable of enhancing FVIII activity **(Fig 9)**. However, whether or not the S2157N and R2159H amino acid changes were necessary for this improvement had not been determined. Since these mutations were spontaneous, they could prove to be advantageous, ineffectual or deleterious. The contribution of these mutations was evaluated by engineering a human light chain construct comprised of the human light chain sequence containing these two mutations (S2157N and R2159H), designated hLC-X2. The FVIII properties of hLC-X2 were then compared to those of construct hLC-K10, which contains the same sequence as hLC-K12 except the two extra mutations (S2157N and R2159H) have been removed.

HEK293 cells were transiently transfected with either hLC, hLC-X2, hLC-K12 or hLC-K10 in conjugation with hHC at a ratio of 2:1 (LC:HC). Cells transfected with hLC-X2 and hHC produced no significant difference in FVIII activity from cells transfected with hLC and hHC as determined by a 1-stage APTT assay (**Fig. 11A**).

FIGURE 11

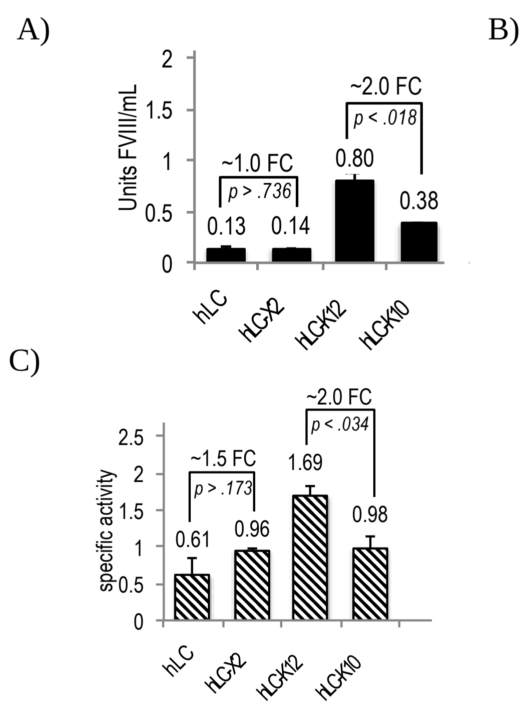


Figure 11. S2157N and R2159H mutations are required for superior FVIII activity. HEK293 cells were transiently transfected with either hLC, hLC-X2, hLC-K12 or hLC-K10 in conjugation with hLC-K10. Statistical analysis was performed using a 2-tailed student *t*-test. A) FVIII U/ml produced as determined by a 1-stage APTT assay. B) Amount of FVIII secreted as determined by an ELISA assay detecting heavy chain. C) Specific activity of FVIII produced from each construct.

These results suggest that the two extra amino acid mutations have no effect on FVIII activity. However, removing these two amino acids from hLC-K12 resulted in a 2.11-fold decrease in FVIII activity (**Fig. 11A**). Neither the addition nor removal of the two amino acids from the hLC or the hLC-K12 construct resulted in a significant change in the amount of heavy chain in the media (**Fig. 11B**). Cells transfected with hLC-X2 and hHC produced no significant difference in specific activity from cells transfected with hLC and hHC (**Fig. 11C**). However, the specific activity of cells transfected with hLC-K10 and hHC was 1.72 times lower than that of cells transfected with hLC-K12 and hHC (**Fig. 11C**). These results indicate that while mutations S2157N and R2159H do not affect activity on their own, they are able to enhance the effects of the 10 canine amino acids by ~2-fold (**Fig. 11A, C**).

The K12 light chain modifications enhance FVIII specific activity both *in vitro* and *in vivo*

The K12 modifications enhance FVIII activity *in vitro*

Negative selection of the hLC[1857-2147kLC] construct revealed 12 amino acid changes that worked together to increase FVIII coagulation activity. Using the dual delivery method, the properties of the hLC-K12 construct were further tested and analyzed *in vitro.* HEK293 cells were transiently transfected with plasmids hLC, kLC, hLC[1857-2147kLC] and hLC-K12 in conjugation with the hHC plasmids at a ratio of 1:2 (LC:HC). Media was harvested 24 hrs later and a 1-stage APTT assay was performed to determine FVIII clotting activity. Cells transfected with hHC and hLC plasmids yielded a clotting activity equal to 0.12 U/ml FVIII, kLC produced 0.43 U/ml FVIII, hLC[1857-

2147kLC]produced 0.35 U/ml FVIII and hLC-K12 produced 0.75 U/ml FVIII activity (**Fig. 12A**).

FIGURE 12

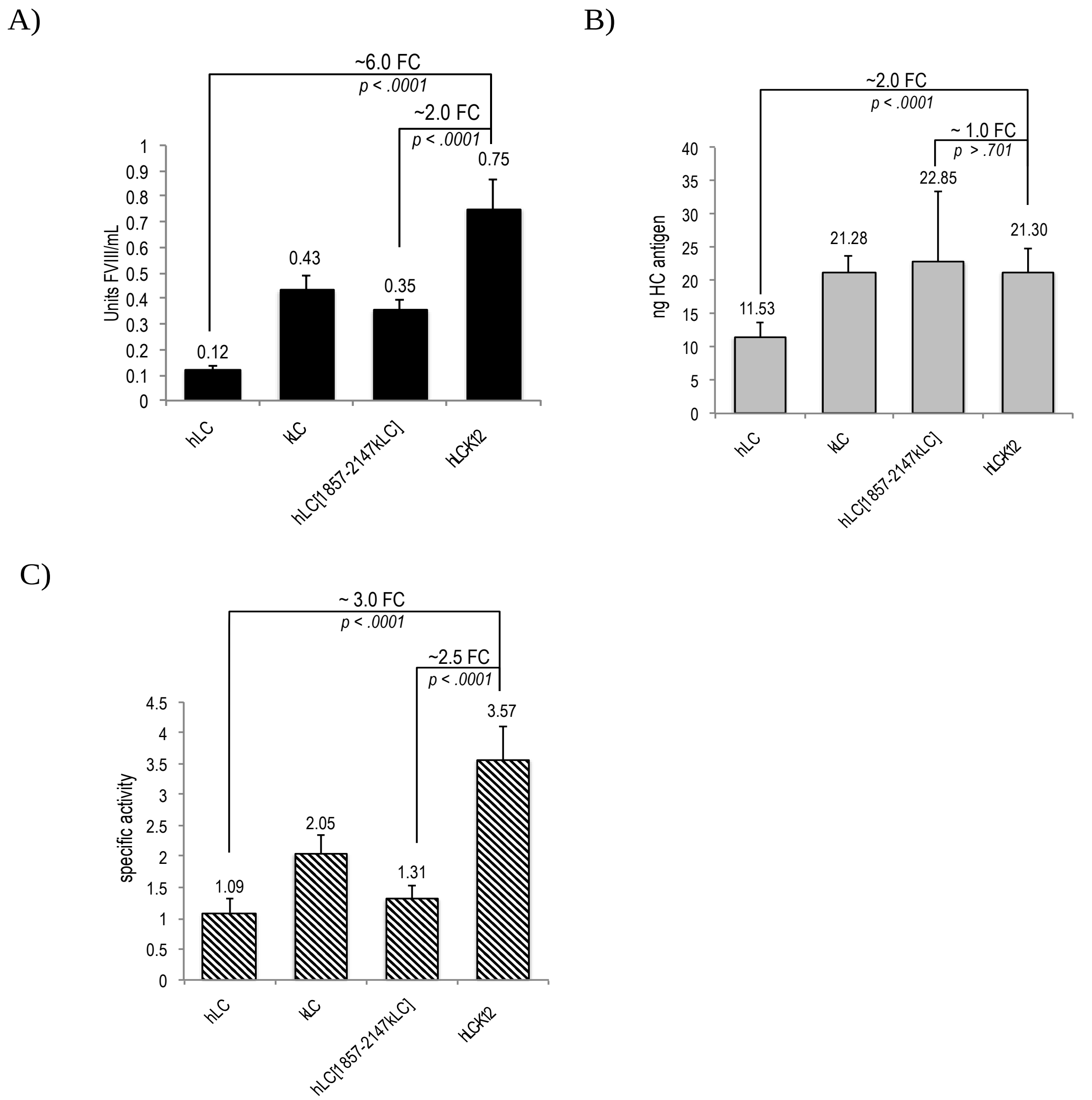


Figure 12. Construct hLC-K12 enhances FVIII activity *in vitro*. Properties of the hLC-K12 construct were tested in vitro through transient transfection of HEK293 cells. Significance was determined using a 2-tailed student *t*-test. A) U/ml FVIII activity determined via a 1-stage APTT assay. B) Amount of human heavy chain secreted determined by an ELISA detecting human heavy chain. C) Specific activity for FVIII constructs determined through comparison of U/ml FVIII clotting activity to the amount of FVIII secreted.

Cells transfected with hHC and hLC-K12 plasmids produced a 6.25-fold increase in coagulation activity over cells transfected with hLC and hHC plasmid. This also correlates to a 1.74-fold increase in FVIII activity over cells transfected with kLC and hHC plasmids, and a 2.14-fold increase in FVIII activity over cells transfected with hLC[1857-2147kLC] and hHC plasmids (**Fig. 12A**). These results demonstrate that the hLC-K12 construct has the most substantial effect on FVIII clotting activity over any of the previous light chain constructs, including canine light chain itself *in vitro*.

To detect the level of FVIII in the media, the amount of heavy chain antigen was measured using an ELISA assay selecting for FVIII heavy chain. Heavy chain from cells transfected with hHC in conjugation with hLC produced 11.53 ng, hHC with kLC produced 21.28 ng, hHC with hLC[1857-2147kLC] produced 22.85 ng and hHC with hLC-K12 produced 21.3 ng of human heavy chain (**Fig. 12B**). Replacing hLC with either kLC, hLC[1857-2147kLC] or hLC-K12 resulted in a significant increase in heavy chain production, according to a 2-tailed, student *t*-test. However, the amount of heavy chain antigen between kLC, hLC[1857-2147kLC] and hLC-K12 was of similar value (**Fig. 12B**). This indicates that substituting the hLC will results in an increase in FVIII heavy chain amount, however, this increase occurs regardless of which light chain is used. The important point is that using hLC-K12 instead of kLC or hLC[1857-2147kLC] can further enhance FVIII clotting activity, but it will not additionally effect FVIII heavy chain production *in vitro*.

The specific activity for each construct was calculated by equating the amount of FVIII produced to the amount of heavy chain produced. Cells transfected with hHC and hLC produced FVIII with a specific activity of 1.09, hHC and kLC produced FVIII with a specific activity of 2.05, hHC and hLC[1857-2147kLC] produced FVIII with a specific activity of 1.31, and hHC and hLC-K12 produced FVIII with a specific activity of 3.57 (**Fig. 12C**). This represents a 3.28-fold increase in FVIII specific activity when hLC is replaced with hLC-K12, which is also a 2.73-fold increase in FVIII specific activity over cells transfected with hLC[1857-2147kLC] (**Fig. 12C**). These results provide evidence that the hLC-K12 construct is able to enhance FVIII clotting activity by forming a more active protein, not through FVIII production *in vitro*.

The K12 and X5 modifications enhance FVIII activity *in vitro*

It is well known that the human heavy chain has a poor secretion profile. The X5 heavy chain (X5HC) is a human heavy chain construct with 5 amino acid modifications that work to enhance protein production ~5-fold. Whether or not the X5HC would work in conjugation with the hLC-K12 light chain was tested *in vitro* using the dual delivery strategy. HEK293 cells were transiently transfected with X5HC and either hLC, kLC, hLC[1857-2147kLC] or hLC-K12 plasmids. HEK293 cells were also transfected with hHC and hLC to serve as a control. A 1-stage APTT assay revealed that cells transfected with hLC and X5HC produced 1.14 U/ml FVIII clotting activity, which is a 9.5-fold increase over cells transfected with hLC and hHC, which produced only 0.12 U/ml FVIII clotting activity (**Fig. 13A**). These results demonstrate that replacing hHC with X5HC radically improves FVIII clotting activity. Cells transfected with hLC-K12 and X5HC produced 5.03 U/ml FVIII clotting activity, which is superior to cells transfected with X5HC and either the kLC or the hLC[1857-2147kLC]. This is a 4.41-fold increase in FVIII activity over cells transfected with hLC and X5HC, and a 41.92-fold increase over cells transfected with hLC and hHC (**Fig. 13A**).

FIGURE 13

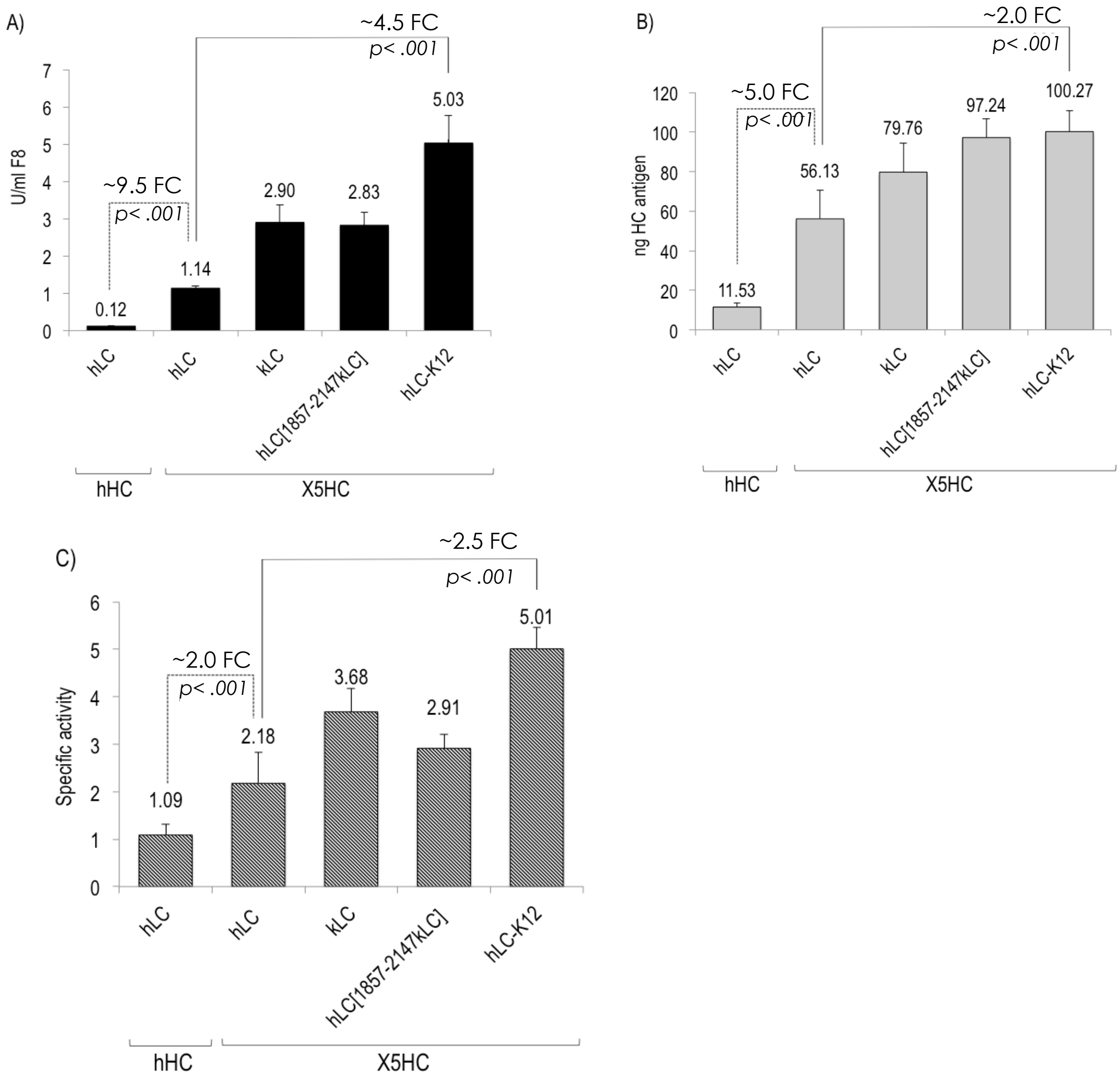


Figure 13. Construct hLC-K12 in conjugation with the modified X5HC enhances FVIII activity *in vitro*. The hLC-K12 light chain was tested in conjugation with the X5HC construct through transient transfection of HEK293 cells. Statistical significance was determined using a 2-tailed student *t*-test. A) U/ml FVIII as determined by a 1-stage APTT assay. B) Amount of FVIII antigen determined by an ELISA. C) specific activity was determined by comparing FVIII clotting activity to heavy chain production for each construct.

To measure the level of FVIII production, the amount of heavy chain was determined using an ELISA assay selecting for FVIII heavy chain (**Fig. 13B**). Cells transfected with hLC and hHC produced 11.53 ng of heavy chain while cells transfected with hLC and X5HC produced 56.13 ng of heavy chain. This is a 4.87-fold increase in heavy chain production, which signifies that the X5 modifications amplify FVIII production. When hLC-K12 is transfected with X5HC, 100.27 ng of heavy chain are detected, with is a 1.79-fold increase over the amount of heavy chain from cells transfected with the hLC and X5HC (**Fig. 13B**). This difference in production is significantly higher, however, there is no significant difference in production levels between cells transfected with X5HC and hLC-K12 and those transfected with X5HC and hLC[1857-2147kLC] (**Fig. 13B**). These results indicate that replacing the hLC will lead to an increase in heavy chain production, but that this occurs at a similar level regardless of whether the hLC-K12, kLC or the hLC[1857-2147kLC] construct is used.

Specific activity of the FVIII produced was determined by comparing FVIII U/ml clotting activity to FVIII production levels. FVIII from cells transfected with hLC and hHC has a specific activity of 1.09 while FVIII from cells transfected with hLC and X5HC had a specific activity of 2.18 (**Fig. 13C**). This suggests that although the X5 modifications mainly work to increase production levels, it is possible that these mutations also improve specific activity of the protein itself. However, when cells were transfected with hLC-K12 and X5HC plasmids, the FVIII produced had a specific activity of 5.01, which is a 2.3-fold increase over the specific activity of cells transfected with hLC and X5HC (**Fig. 13C**). This data demonstrates that the hLC-K12 modifications are able to enhance the specific activity of FVIII when transfected in conjugation with either the hHC or the X5HC plasmids *in vitro*.

The K12 modifications enhance FVIII activity *in vivo*

The hLC-K12 construct produced superior results when tested *in vitro*, however whether or not the hLC-K12 and X5HC would produce similar results *in vivo* remained untested. HA mice were hydrodynamically injected with 150 ug of DNA plasmid, 75ug of X5HC and 75 ug of light chain (**Fig. 14**). Three light chains were tested, hLC, kLC and hLC-K12. Mice were sacrificed 48 hours post injection and blood harvested through terminal bleeding from the hepatic artery. A 1-stage APTT assay was performed to determine clotting activity for each set of injections. Mice injected with X5HC and hLC produced 13.45 U/ml FVIII clotting activity, kLC produced 23.72 U/ml FVIII clotting activity and hLC-K12 produced 33.84 U/ml FVIII clotting activity (**Fig. 14A**). When hLC is replaced with hLC-K12 in hydrodynamic delivery of plasmid DNA *in vivo*, a 2.52-fold increase in FVIII clotting activity is observed. This is similar to *in vitro* findings, but with a somewhat less dramatic difference.

Whether or not this observed increase was due to an increase in FVIII protein production was determined using an ELISA assay selecting for heavy chain. Mice injected with X5HC and any of the three light chains all had similar levels of heavy chain production (**Fig. 14B**). This indicates that the increase in FVIII activity is not due to enhanced production properties. However, when the specific activity was determined by comparing FVIII clotting activity to FVIII production, it was found that mice injected with X5HC and hLC-K12 produced a specific activity of 8.03 (**Fig. 14C**). This is a 2.42-fold increase over mice injected with hLC and X5HC, which produced a specific activity of 3.32. These results are similar to those from *in vitro* testing, providing further evidence that hLC-K12 is able to enhance FVIII activity by increasing the overall specific activity of the protein itself.

Figure 14

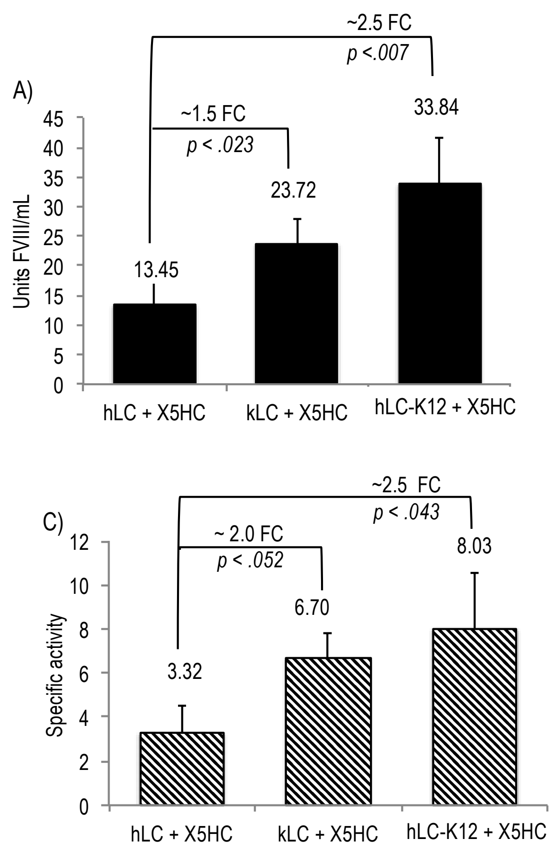


Figure 14. Construct hLC-K12 in conjugation with the modified X5HC enhances FVIII activity *in vivo*. The hLC-K12 construct was testing in conjugation with the X5HC through hydrodynamic injections of 12 week old HA mice with 150 ug of plasmid DNA. A) U/ml FVIII as determined by a 1-stage APTT assay. B) Amount of FVIII secreted as determined by an ELISA for human heavy chain antigen. C) Specific activity was determined by comparing the FVIII clotting activity to the amount of heavy chain secreted.

The rAAV-hLC-K12 vector enhances FVIII activity *in vivo*

The K12 modifications to the human light chain are able to increase FVIII clotting activity by enhancing protein specific activity. This has been demonstrated through the transient transfection of HEK293 cells *in vitro* and hydrodynamic injection of HA mice with plasmid *in vivo*. However, these results all relied on DNA delivery of the gene. In order to determine if similar results would be possible using AAV gene therapy, the hLC-K12 construct was packaged into an AAV vector and tested using the dual delivery method. The liver was targeted through the use of AAV serotype 8, a serotype known to infect the liver, and through the addition of the liver specific enhancer Apolipoprotein E (ApoE) and human α1-anti-trypsin (hAAT) promoter. This light chain vector, hLC-K12, was injected into 6-8 week old CD4KO/HA male mice in conjugation with an AAV8 vector containing the X5 heavy chain under direction of the ApoE-hAAT enhancer/promoter combination, X5HC. (CD4KO/HA mice are a double knock out strain unable to produce CD4+ T cells or express the FVIII protein. These mice were selected for their inability to mount an immune response to FVIII, which would interfere with FVIII analysis). For comparison, mice were also injected with an AAV serotype 8 vector containing the human light chain gene, hLC, and an AAV serotype 8 vector containing the canine light chain gene, kLC. These light chain vectors were delivered with the X5HC vector at a ratio of 1:4 (LC:HC) in order to achieve ultimate expression. Blood was harvested from mice every 2-4 weeks through retro-orbital eye bleeding and used for subsequent testing.

A 1-stage APTT assay was performed to determine the FVIII U/ml clotting activity for each set of mice. Delivery of the X5HC with the hLC vector produced a peak of .949 U/ml FVIII at week 6, and by week 24 FVIII expression had decreased to .296 U/ml FVIII

(**Fig. 15A**). Delivery of the X5HC with the kLC vector produced a peak of 6.00 U/ml FVIII at week 6, which was down to 1.01 U/ml FVIII by week 24.

FIGURE 15

A)

B)

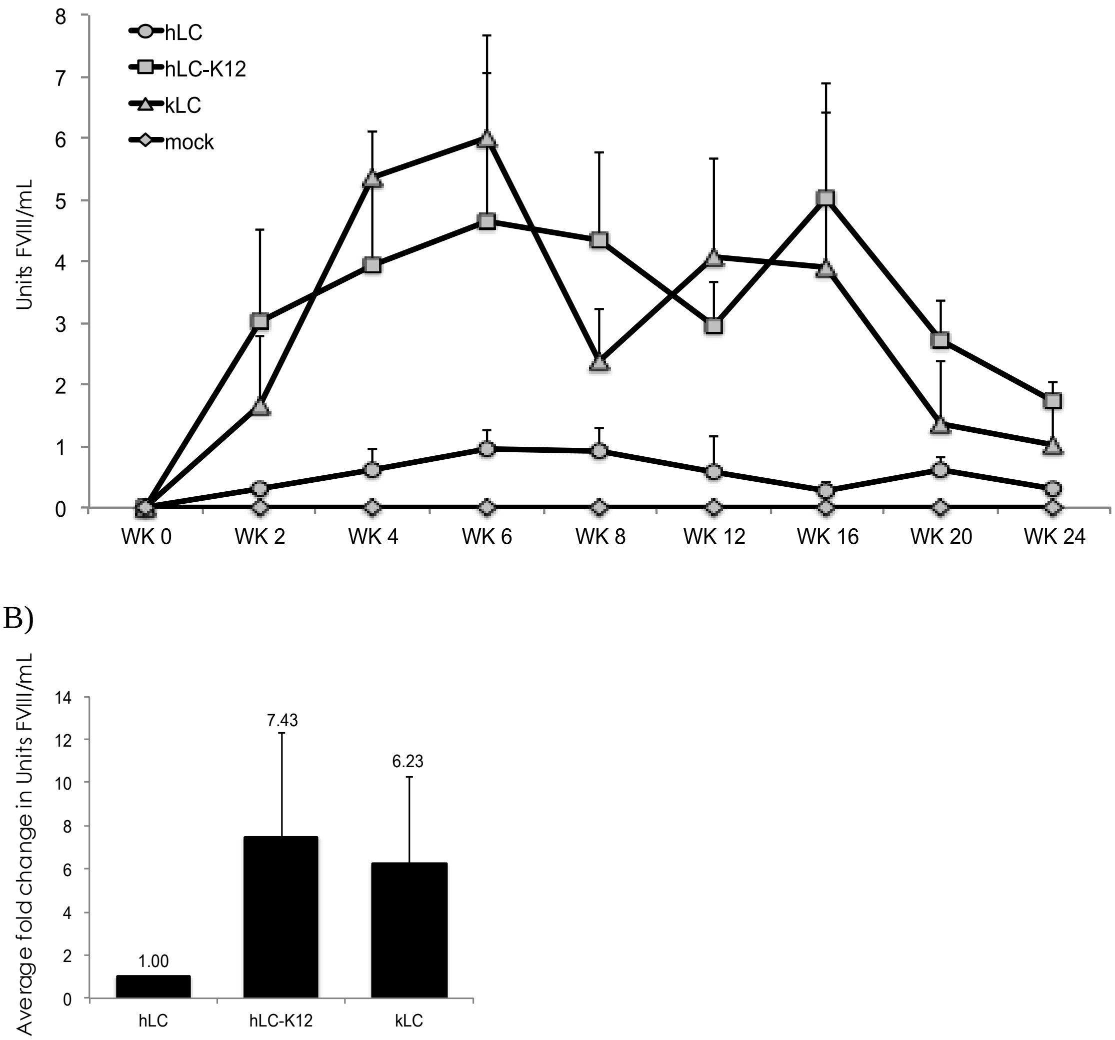


Figure 15. The hLC-K12 AAV vector enhances FVIII clotting activity *in vivo*. CD4KO/HA mice were injected with X5HC vector in conjugation with either the hLC, kLC or hLC-K12 vector. The mock group was un-injected. Blood was harvested through retro-orbital eye bleeding every 2-4 weeks. A) U/ml of FVIII as determined by a 1-stage APTT assay for each group of mice over the course of 24 weeks. B) The average fold change in FVIII U/ml over X5HC and hLC vector injected mice.

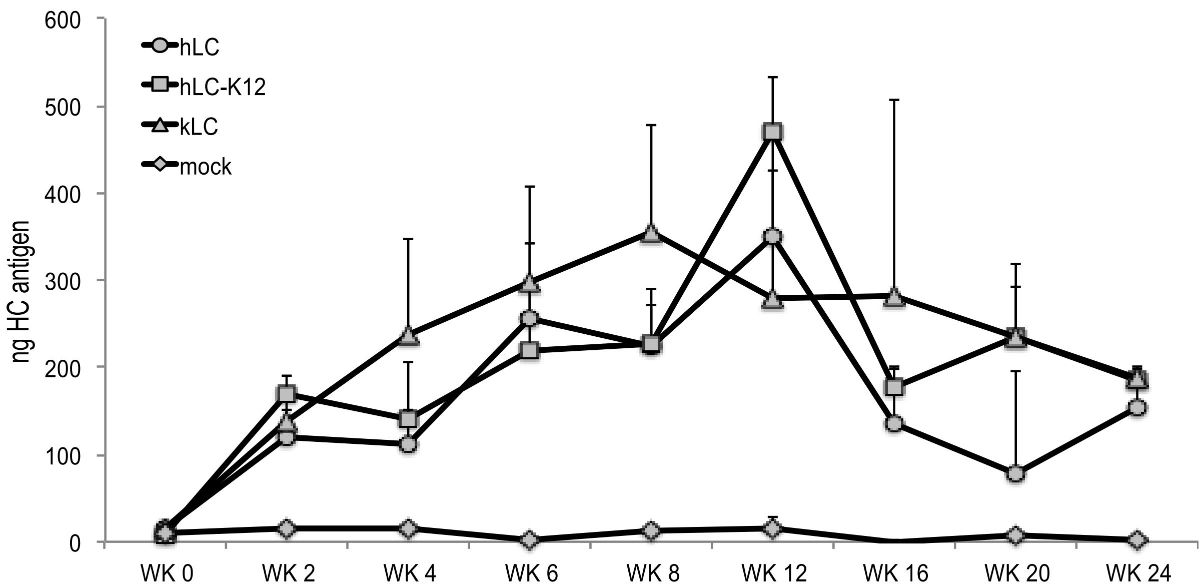
When the X5HC and hLC-K12 vectors were delivered together they produced a peak of 5.04 U/ml at week 16, which was down to 1.73 U/ml by week 24 (**Fig. 15A**). At week 6 the X5HC and hLC-K12 vectors produced 4.65 U/ml, which is similar to the 5.04 U/ml at week 16. These results signify that the all three light chains produced similar trends in FVIII clotting activity, peaking at around 6 weeks post vector delivery.

Replacing the hLC vector with either the kLC or the hLC-K12 vectors significantly increased FVIII clotting activity. Replacement of hLC with the kLC vector lost significance at week 16, however, replacement with the hLC-K12 vector was significant throughout the course of 24 weeks. There was no significant difference in clotting activity between mice injected with the X5HC and either the kLC or the hLC-K12 vector at any time point (**Fig. 15A**). The fold change in FVIII clotting activity over mice injected with X5HC and hLC vectors was determined for both kLC and hLC-K12 at each time point (**Fig. 15B**). Replacing the hLC vector with the hLC-K12 vector produced an average of 7.43 times more FVIII clotting activity, ranging from a 4.44-fold increase at week 20 to a 18.81-fold increase at week 16 (**Fig. 15B**). Replacement with the kLC vector produced an average of 6.23 times more FVIII clotting activity, ranging from a 2.23-fold increase at week 20 to a 14.54-fold increase at week 16 (**Fig. 15B**). These results reveal that replacing the hLC vector with either the kLC or the hLC-K12 vectors enhanced FVIII clotting activity, which is similar to previously established hydrodynamic, *in vivo,* and cellular, *in vitro,* results.

Production of the FVIII protein was measured using an ELISA selecting for the FVIII heavy chain. Delivery of the X5HC in conjugation with any of the three light chains produced no significant difference in heavy chain amount (**Fig. 16A**).

FIGURE 16

A)



B)

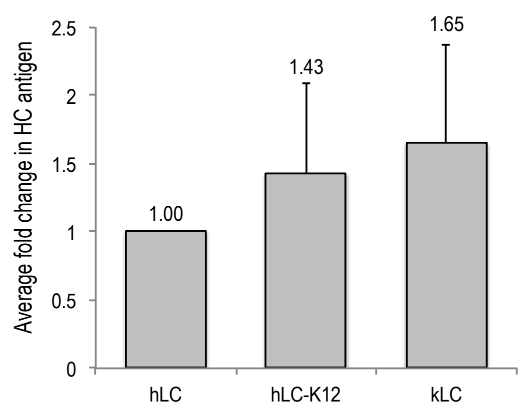


Figure 16. The hLC-K12 AAV vector does not effect heavy chain secretion *in vivo*. CD4KO/HA mice were injected with X5HC vector in conjugation with either the hLC, kLC or hLC-K12 vector. The mock group was un-injected. Blood was harvested through retro-orbital eye bleeding every 2-4 weeks. A) Amount of heavy chain antigen measured for each group, determined by an ELISA selecting for heavy chain. B) The average fold change in heavy chain antigen for mice compared to X5HC and hLC vector injected mice.

The fold change in heavy chain production of mice injected with the X5HC and either hLC-K12 or kLC over those injected with the X5HC and hLC vectors was calculated for each time point (**Fig. 16B**). Mice injected with X5HC and hLC-K12 vectors produced a range of 0.86- 2.99 times more heavy chain, with an average of a 1.42-fold increase. Mice injected with X5HC and kLC vectors produced a range of 0.80- 3.0 times more heavy chain, with an average of a 1.65-fold increase (**Fig. 16B**). This demonstrates that replacing hLC with either the hLC-K12 or kLC vectors does not affect production of the FVIII heavy chain. These results are important because they provide evidence that the observed increase in FVIII activity associated with the hLC-K12 construct is not due to a change in heavy chain production. Therefore, the K12 modifications function independently of the X5 modifications *in vivo* when using AAV vector delivery.

Whether or not there was a difference in light chain production was also determined through an ELISA selecting for the FVIII light chain. (Mice injected with the X5HC and kLC vectors were excluded from this assay because the light chain antibodies do not bind to the kLC with specific affinity). Mice injected with X5HC and hLC vectors produced a range of 434.6 -1252.9 ng LC and those injected with the X5HC and hLC-K12 produced a range of 432.4-1470.8 ng LC (**Fig. 17A**). There was no significant difference in light chain production for these groups at any time point. The fold change in light production of the group injected with hLC-K12 over those injected with hLC ranged from .995-1.26 over 24 weeks, with an average of a 1.12-fold difference (**Fig. 17B**). These results indicate that the K12 modifications do not effect FVIII light chain production.

FIGURE 17

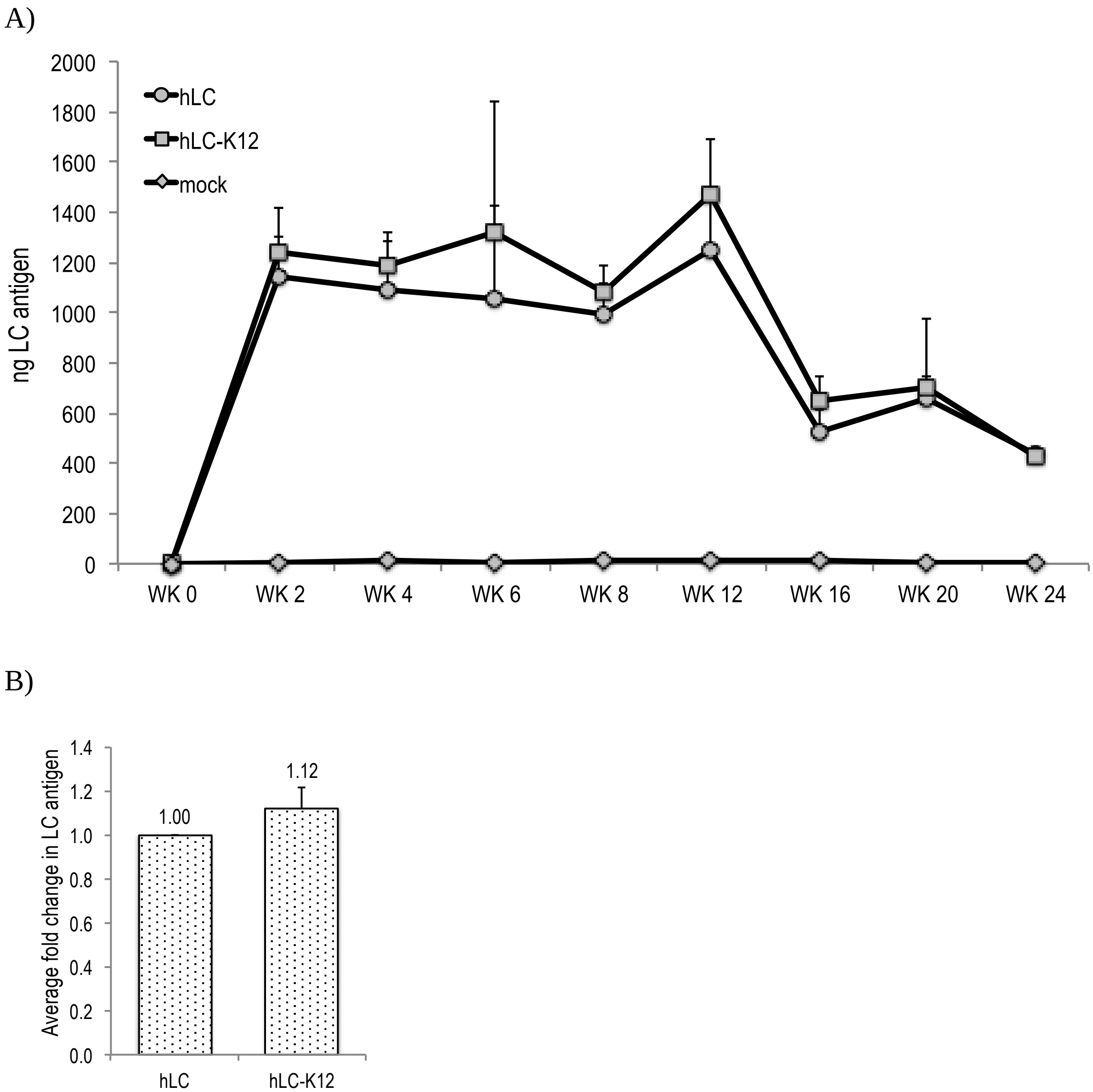


Figure 17. The hLC-K12 AAV vector does not effect light chain secretion *in vivo*. CD4KO/HA mice were injected with X5HC vector in conjugation with either the hLC or hLC-K12 vector. The mock group was un-injected. Blood was harvested through retro-orbital eye bleeding every 2-4 weeks. A) Amount of light chain antigen secreted for each group, determined by an ELISA selecting for light chain. B) The average fold change in ng of light chain antigen over X5HC and hLC vector injected mice.

The specific activity was determined by comparing the U/ml FVIII activity, to the amount of heavy chain produced (**Fig. 18A**). Mice injected with X5HC and hLC produced FVIII with a specific activity that ranged from .150 at week 12 to .540 at week 4. Mice injected with the hLC-K12 vector produced FVIII that had a specific activity that ranged from .637 at week 12 to 2.77 at week 4. The group of mice injected with the kLC vector produced FVIII with a specific activity that ranged from .526 at week 24 to 2.39 at week 4 (**Fig. 18A**). According to a 2-tailed student *t*-test, the increase in specific activity observed with both the hLC-K12 and kLC vectors compared to the hLC vector was significant at every time point, with the exception of kLC at week 24. There was no significant difference in FVIII specific activity between mice injected with hLC-K12 or kLC at any time point.

The fold change in specific activity between mice injected with the X5HC and hLC vectors and those injected with the hLC-K12 or kLC vectors was calculated for each week (**Fig. 18B**). Fold change in specific activity when hLC was replaced with the hLC-K12 vector ranged from 3.28 -14.27; with an average 5.78-fold increase. The fold change in specific activity when hLC was replaced with kLC vector ranged from 2.05-9.73; with an average 4.53-fold increase (**Fig. 18B**). Taken together, these results provide conclusive evidence that the K12 modifications are able to yield a higher amount of FVIII clotting activity through the formation of a FVIII protein that is more specifically active. These modifications have no effect on heavy chain or light chain production *in vivo*.

FIGURE 18

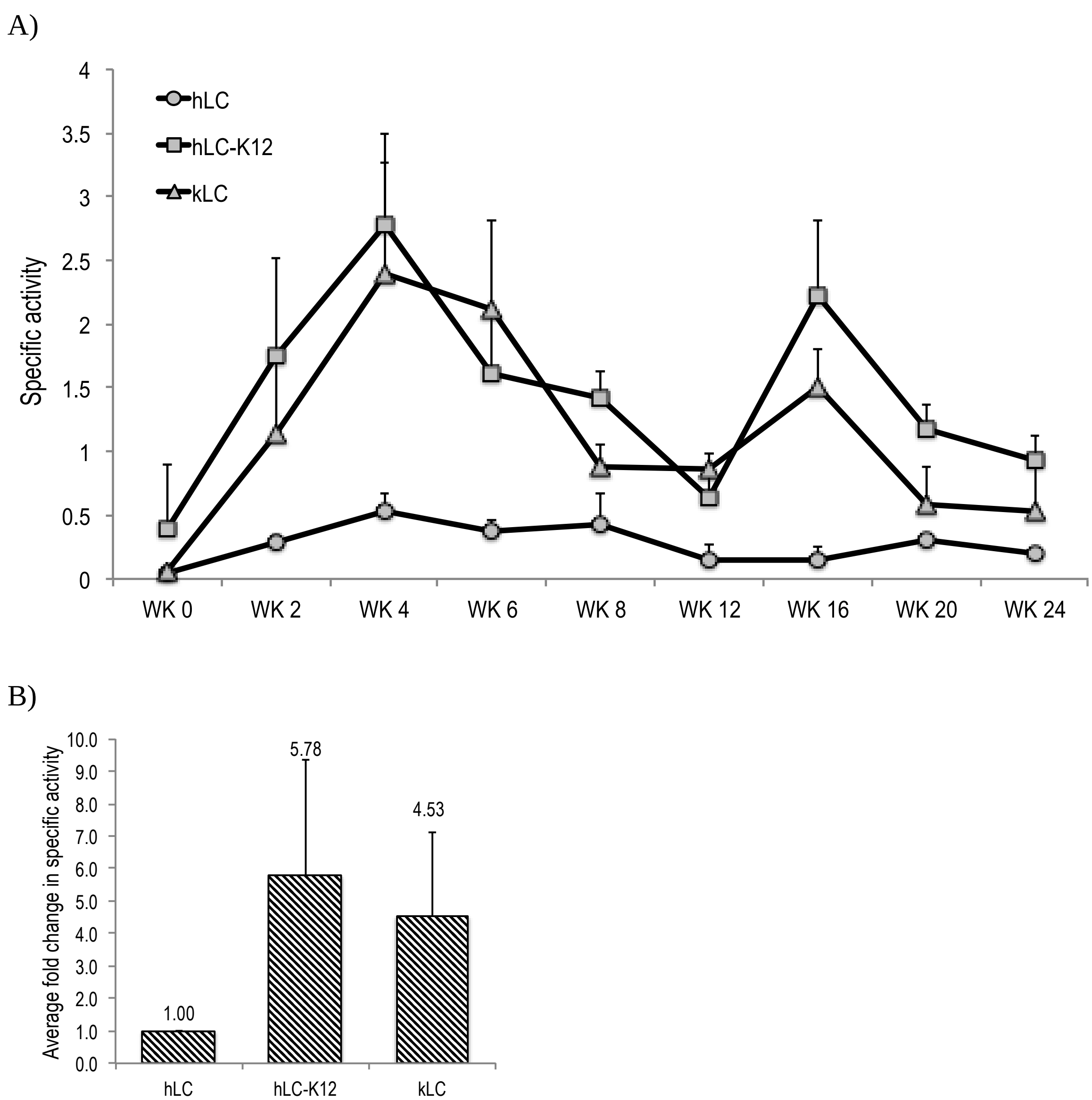


Figure 18. The hLC-K12 AAV vector enhances FVIII specific activity *in vivo*. CD4KO/HA mice were injected with X5HC vector in conjugation with either a hLC, kLC or hLC-K12 vector. The mock group was un-injected. Blood was harvested through retro-orbital eye bleeding every 2-4 weeks. A) FVIII specific activity was calculated for each time point. B) The average fold change in specific activity over X5HC and hLC vector injected mice.

The K12 modifications enhance FVIII activity in a single chain form

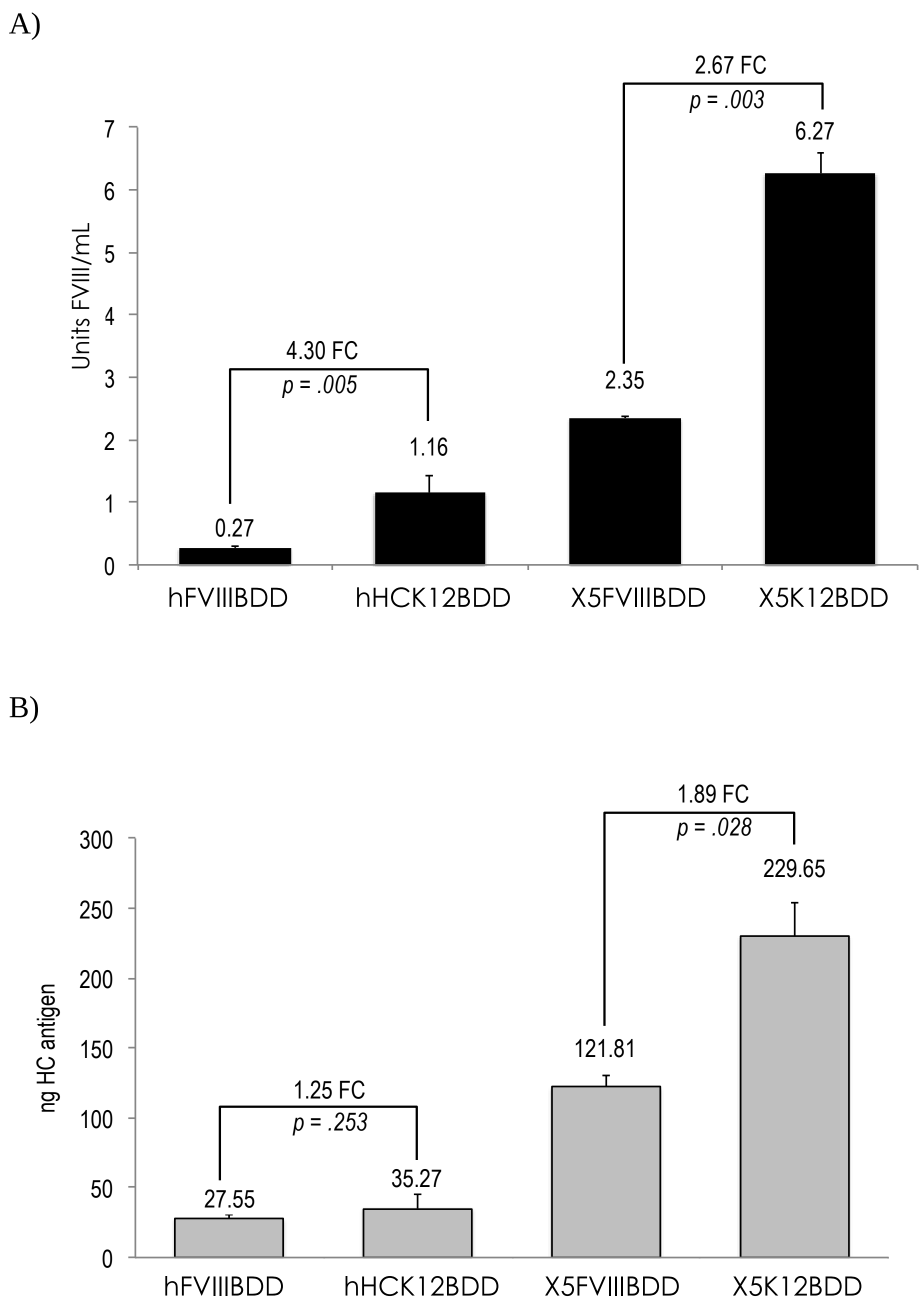
Light chain modifications enhance single chain FVIII activity *in vitro*

Results of *in vitro* and *in vivo* testing have provided conclusive evidence that the K12 modifications enhance FVIII clotting activity. However, this is limited to use with the dual chain delivery strategy. Whether or not these mutations would have similar effect when used as a single chain FVIII molecule was not yet established. Single chain FVIII constructs were engineered to contain either hLC or the hLC-K12 light chain. Two sets of single chain constructs were designed, one set combined these light chains with the human heavy chain, and the other set combined these light chains with the X5 modified heavy chain.

Single chain constructs were first tested *in vitro* through transient transfection of HEK293 cells, 24 hrs. later media was harvested and used for subsequent testing. According to the results of a 1-stage APTT assay, cells transfected with hFVIIIBDD yielded 0.27 U/ml FVIII and hHCK12 yielded 1.16 U/ml FVIII (**Fig. 19A**). Compared to hFVIIIBDD, the K12 mutations produced a 4.3-fold increase in FVIII U/ml. These findings are similar to *in vitro* and *in vivo* results using the dual chain delivery strategy, where the addition of the light chain modifications increases FVIII clotting activity.

Cells transfected with X5FVIIIBDD produced 2.35 U/ml FVIII, which represents a 8.7-fold increase over hFVIIIBDD. This demonstrates that the X5 heavy chain mutations are able to enhance FVIII activity independantly (**Fig. 19A**). When cells were transfected with X5K12BDD they produced 6.27 U/ml FVIII, which is a significant 2.67-fold increase in U/ml FVIII compared to X5FVIIIBDD (**Fig. 19A**). Combining the X5 and K12 mutations into construct X5K12BDD resulted in a 23.2-fold increase in U/ml FVIII over the wild type hFVIIIBDD construct (**Fig. 19A**). These results imply that the X5 and K12 modifications work together to enhance FVIII clotting activity *in vitro*.

FIGURE 19



C)

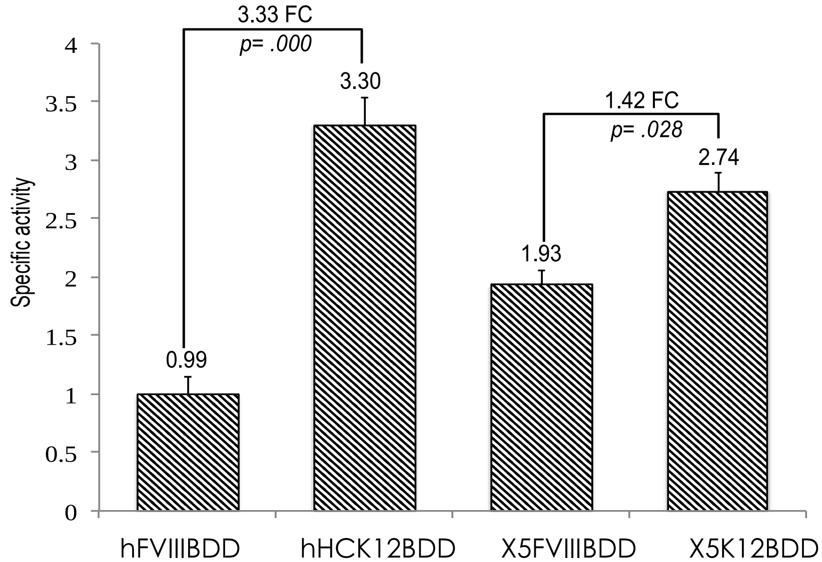


Figure 19. The K12 light chain modifications enhance hFVIIIBDD single chain activity *in vitro*. HEK293 cells were transiently transfected with single chain constructs, 24 hrs later media was harvested for testing. Statistical analysis is based on a 2-tailed student *t*-test. A) U/ml FVIII clotting activity as determined by a 1-stage APTT assay. B) Measurement of heavy chain antigen detected using an ELISA. C) Specific activity of the FVIII protein produced from each single chain construct.

The amount of FVIII protein produced for each construct was determined using an ELISA selecting for heavy chain antigen (**Fig. 19B**). hFVIIIBDD yielded 27.6 ng heavy chain and hHCK12BDD yielded 35.3 ng heavy chain. The total amount of heavy chain produced for \these constructs were similar, with no statistical difference according to a 2-tailed, student *t*-test. Cells transfected with the X5FVIIIBDD construct produced 121.8 ng of heavy chain antigen, which is 4.26 times more than hFVIIIBDD (**Fig. 19B**). This demonstrates that the X5 heavy chain mutations are able to enhance overall FVIII protein production. X5K12BDD yielded 229.7 ng of heavy chain antigen, a significant 1.89-fold increase over X5FVIIIBDD (**Fig. 19B**). This implies that the K12 light chain modifications may further modulate the FVIII production properties when used in combination with the X5 mutations *in vitro*.

The specific activity for each construct was calculated by comparing the FVIII clotting activity to FVIII protein production. Cells transfected with hFVIIIBDD produced FVIII with a specific activity of 0.99, and hHCK12BDD produced a specific activity of 3.30 (**Fig. 19C**). Addition of the K12 light chain modifications produced FVIII with a specific activity 3.33 times higher than hFVIIIBDD (**Fig. 19C**). This indicates that the K12 modifications are able to produce FVIII protein that has an increased specific activity. Cells transfected with the X5FVIIIBDD yielded FVIII with a specific activity of 1.93, which is a 1.95-fold increase over FVIII from cells transfected with hFVIIIBDD (**Fig. 19C**). This implies that the X5BDD construct is able to not only enhance FVIII production, but may also play a role in specific activity. This is similar to previous results using the X5HC in the dual delivery strategy. Addition of the K12 mutations, X5K12BDD, resulted in FVIII with a specific activity of 2.74 (**Fig. 19C**). This is a 1.42-fold increase over X5FVIIIBDD, revealing that combining the K12 and X5 modifications together resulted in a 2.77-fold increase in specific activity over wild type hFVIIIBDD *in vitro* (**Fig. 19C**). Taken together, these results suggest that the K12 modifications are able to enhance FVIII specific activity in a single chain form when combined with either the human heavy chain or the modified X5 heavy chain.

mRNA expression levels are similar for single chain constructs *in vitro*

Assessments of the FVIII modifications to this point have involved testing the FVIII protein after subjection to cellular processing and production. It is possible, however, that these mutations effect FVIII mRNA status. An increase in mRNA levels of FVIII would lead to an increase in cellular production of the protein. Therefore, it seemed important to determine whether or not these mutations had an effect on mRNA (**Fig. 20**). HEK293 cells were transiently transfected with plasmids hFVIIIBDD, X5FVIIIBDD, hHCK12BDD or X5K12BDD. 24 hrs later the cells were washed with PBS, harvested and stored in liquid nitrogen until needed. Total RNA was extracted from these cells and reverse transcription to make cDNA was completed using primers that selected for mRNA only. QPCR was performed using the cDNA as a template and one set of primers that targeted the human heavy chain and a separate set of primers that targeted GAPDH. The CT values for the heavy chain were divided by the CT values for GAPDH to calculate a ratio of heavy chain: GAPDH (**Fig. 20**).

FIGURE 20

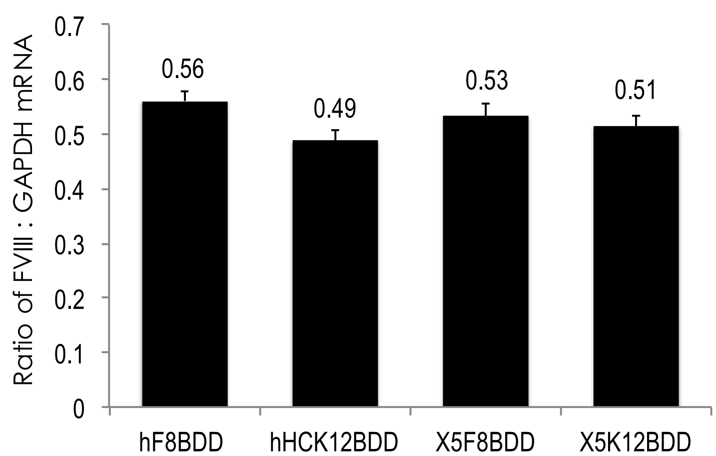


Figure 20. mRNA levels for all single chain constructs are similar *in vitro*. mRNA levels for cells transfected with each single chain construct were analyzed. HEK293 cells were transiently transfected with single constructs, RNA was extracted and cDNA was synthesized selecting for mRNA only. QPCR determined the CT value for the human heavy chain and for GAPDH. A ratio of heavy chain to GAPDH was calculated for each construct.

If mRNA levels for FVIII were the same as GAPDH the ratio would equal 1, indicating that there are minimal amounts of FVIII mRNA present in the cells. Conversely, as mRNA levels for FVIII increase, the ratio decreases. A ratio closer to 0 means that the cells are producing more FVIII mRNA in relation to GAPDH.

Cell transfected with hFVIIIBDD produced a ratio of 0.56, X5BDD ratio was 0.53, X5K12BDD ratio was 0.51 and the hHCK12BDD ratio was 0.49 (**Fig. 20**). hFVIIIBDD presented with the highest ratio of all four groups, which means that hFVIIIBDD had the lowest amount of FVIII mRNA detected. Conversely, hHCK12BDD presented with the lowest ratio of all 4 groups, which means that hHCK12BD had the highest amount of FVIII mRNA detected. The ratios of heavy chain to GAPDH for all four groups were similar, all within 15% of each other (**Fig. 20**). These results demonstrate that the difference in FVIII protein production and activity is not due to a change in mRNA levels, and that the X5 and K12 modifications have no effect on FVIII mRNA status.

The K12 light chain modifications enhance single chain FVIII activity *in vivo*

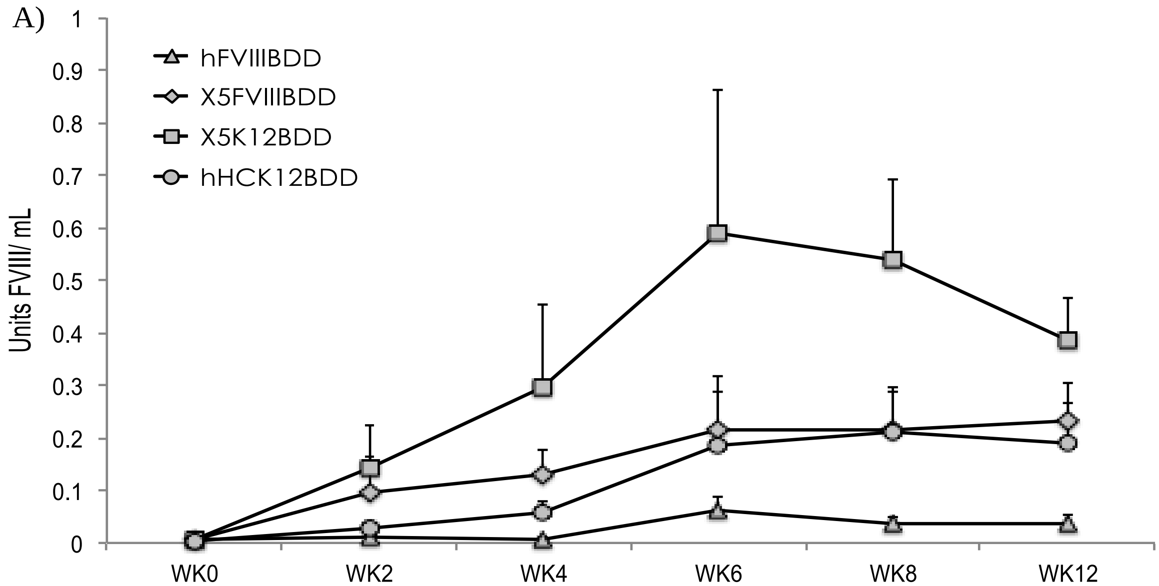
The K12 light chain modifications have been determined to enhance specific activity of the FVIII protein when delivered using the dual chain method *in vitro* and *in vivo*, and as a single chain construct *in vitro.* Whether or not these modifications would enhance single chain FVIII *in vivo* had not yet been determined. In order to test these single chain constructs *in vivo*, they were packaged into AAV serotype 8 vectors and delivered to 6-8 week old CD4KO/HA at a dose of $1x10^{11}$ vp/mouse. Because size is limited for these constructs, the liver specific Transthyretin (TTR) promoter was used instead of the ApoE/hAAT enhancer/promoter combination. Blood was harvested through retro-orbital eye bleeding every 2-4 weeks post vector injection for testing.

A 1-stage APTT assay was performed at each time point to determine U/ml FVIII clotting activity. Mice injected with the hFVIIIBDD construct produced a range of .012-.061 U/ml FVIII, with peak activity at week 6. Mice injected with the hHCK12BDD construct yielded a range from .029 - .213 U/ml FVIII, with peak activity at week 8 (**Fig 21A**). Mice injected with the X5FVIIIBDD construct produced a range of .097- .231 U/ml FVIII, with peak activity at week 12 (**Fig 21A**). Those injected with X5K12BDD produced a range of .144-.590 U/ml FVIII with peak expression at week 6 (**Fig 21A**). Compared to hFVIIIBDD, mice injected with either hHCK12BDD, X5FVIIIBDD or X5K12BDD produced a significant increase in FVIII clotting activity at each time point.

The fold change in U/ml FVIII activity over hFVIIIBDD was calculated for mice injected with hHCK12BDD, X5FVIIIBDD or X5K12FBDD (**Fig 21B**). Addition of the K12 modifications to hFVIIIBDD resulted in 2.51-6.85 times more U/ml FVIII, with an average of a 4.6-fold increase. The X5FVIIIBDD construct resulted in 3.53-15.61 times more U/ml FVIII, with an average of a 7.85-fold increase (**Fig 21B**). These results demonstrate that when the hFVIIIBDD is modified with either the K12 mutations or the X5 mutations alone, there is an increase in FVIII clotting activity. When these two sets of modifications are added together, X5K12BDD construct, there is a 9.59-35.85-fold increase in FVIII clotting activity, which is an average of 16.46 times more FVIII U/ml over hFVIIIBDD (**Fig 21B**). Compared to mice injected with the X5FVIIIBDD construct, the X5K12BDD groups produced a range of 1.49-2.72 times more U/ml FVIII, which is an average 2.14-fold increase in clotting activity (**Fig.21B**). This was a significant increase in FVIII clotting activity, after week 2.

FIGURE 21

A)



B)

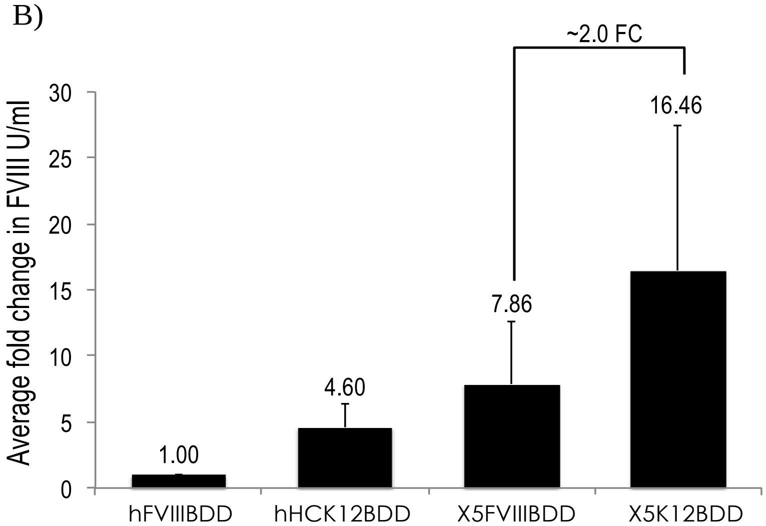


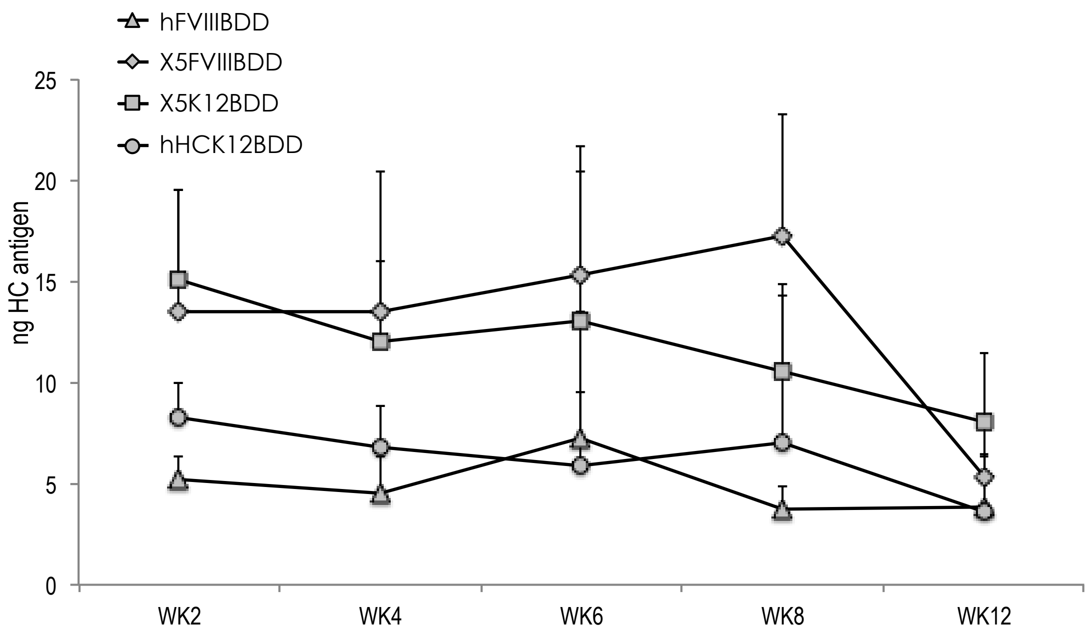
Figure 21. The K12 modifications enhance FVIII clotting activity in CD4KO/HA mice. 8 week old CD4KO/HA mice were injected with single chain vectors. Blood was harvested every 2-4 weeks via retro-orbital eye bleeding. A) U/ml of FVIII produced over 12 weeks determined by a 1 stage APTT assay. B) The average fold change in U/ml FVIII clotting activity produced by mice injected with the single chain mutants compared to hFVIIIBDD.

In order to determine if production of the FVIII protein contributed to the increase in FVIII clotting activity, an ELISA selecting for heavy chain was performed. Mice injected with hFVIIIBDD produced a range of 3.73 – 7.30 ng of heavy chain and mice injected with hHCK12BDD yielded a range of 3.56-8.31 ng of heavy chain (**Fig. 22A**). The difference in heavy chain production between these two constructs was not significant, except for at week 8, according to a 2-tailed student *t*-test. Mice injected with X5FVIIIBDD produced a range of 5.37-17.27 ng of heavy chain and X5K12BDD yielded a range of 8.06- 15.04 ng of heavy chain (**Fig. 22A**). Compared to hFVIIIBDD, mice injected with these constructs produced a significant increase in heavy chain production up until week 12, according to a 2-tailed student *t*-test. However, there was no significant increase in heavy chain production between mice injected with X5FVIIIBDD and X5K12BDD, except for week 8 (**Fig. 22A**).

The average fold change in heavy chain production compared to hFVIIIBDD was calculated for mice injected with hHCk12BDD, X5FVIIIBDD and X5K12BDD. Mice injected with hHCK12BDD yielded 0.80 - 1.89 times more FVIII protein, with an average of a 1.34-fold increase in FVIII production (**Fig. 22B**). Mice injected with X5FVIIIBDD produced 1.41-4.62 times more FVIII heavy chain, with an average of a 2.74-fold increase over hFVIIIBDD. Likewise, mice injected with the X5K12BDD vector produced 1.78-2.89 times more heavy chain, with an average 2.45-fold increase in FVIII production (**Fig. 22B**). These results indicate that the X5 modifications enhance FVIII production, however, the K12 modifications do not. This is demonstrated by the increase in heavy chain production when the X5 modifications are added (X5FVIIIBDD). However no significant increase in production is observed by the addition of the K12 modifications to the human

FIGURE 22

A)



B)

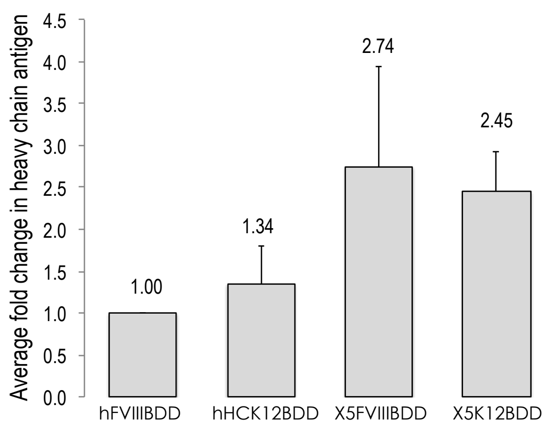


Figure 22. The K12 modifications do not effect FVIII secretion in CD4KO/HA mice.

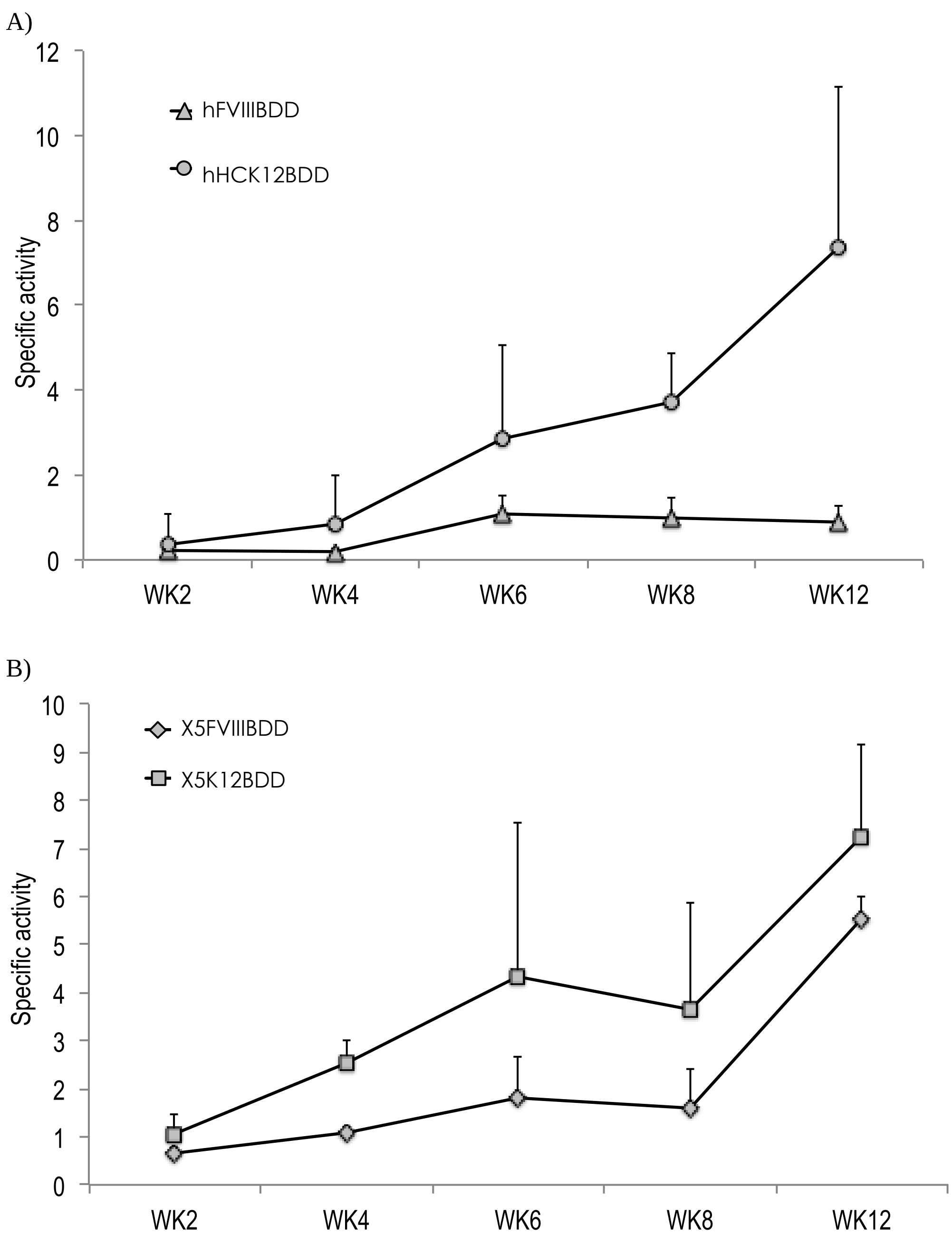
8 week old CD4KO/HA mice were injected with single chain vectors. Blood was harvested every 2-4 weeks via retro-orbital eye bleeding. A) Amount of FVIII produced over 12 weeks determined by an ELISA selecting for heavy chain antigen. B) The average fold change in FVIII heavy chain antigen produced by mice injected with the single chain mutants compared to hFVIIIBDD.

heavy chain or to the X5 heavy chain (X5K12BDD).

The specific activity for mice injected with each vector was calculated by comparing the U/ml FVIII activity to the amount of FVIII protein produced. Mice injected with hFVIIIBDD produced FVIII with a specific activity that ranged from .197-.987, which peaked at week 8 **(Fig. 23A**). Mice injected with hHCK12BDD produced FVIII with a specific activity that ranged from .358- 7.36, which peaked at week 12 **(Fig. 23A**). FVIII specific activity from hHCK12BDD injected mice was significantly higher compared to FVIII from hFVIIIBDD injected mice at every, time point except for week 6, according to a 2-tailed student *t*-test (**Fig. 23A**). The specific activity of FVIII from mice injected with X5FVIIIBDD ranged from .652-5.52, which peaked at week 12 **(Fig. 23B**). X5K12BDD injected mice produced FVIII with a specific activity that ranged from 1.06-7.25, which peaked at week 12 **(Fig. 23B**). Specific activity of FVIII from mice injected with X5K12BDD was significantly higher than those injected with X5BDD, except weeks 2 and 12, according to a 2-tailed student *t*-test **(Fig. 23B**).

The average fold increase in specific activity of mice injected with hHCK12BDD compared to hFVIIIBDD was calculated for each time point. FVIII from mice injected with hHCK12BDD produced a range of 1.55-8.36 times higher specific activity compared to hFVIIIBDD, with an average of a 4.13-fold increase (**Fig. 23 C**). These results provide further evidence that the K12 modifications alone enhance FVIII clotting activity by producing a FVIII protein that has a more specific activity. The fold change in specific activity for mice injected with the X5K12BDD vector over those injected with the X5BDD vector was calculated for each time point (**Fig. 23D**). Mice injected with X5K12BDD yielded FVIII with a specific activity that ranged from 1.32-2.37 times higher than FVIII from mice injected with X5FVIIIBDD (**Fig. 23D**). This represents an average of a 1.98-fold increase in specific activity when the K12 light chain modifications are added to the X5BDD construct (**Fig. 23D**). These results substantiate the hypothesis that the K12 modifications enhance FVIII protein specific activity *in vivo.*

FIGURE 23



C) D)

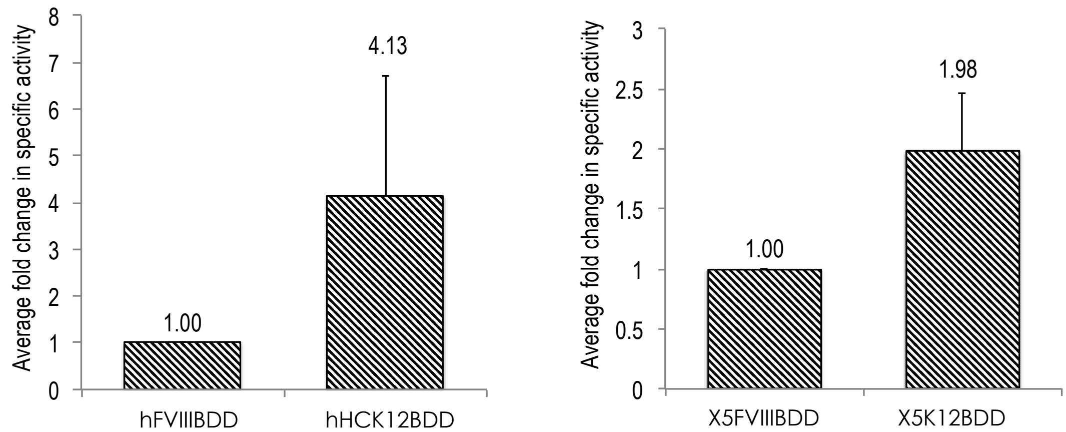


Figure 23. The K12 modifications enhance FVIII specific activity in CD4KO/HA mice. 8 week old CD4KO/HA mice were injected with single chain vectors. Blood was harvested every 2-4 weeks via retro-orbital eye bleeding. A) Specific activity of protein produced from mice injected with hFVIIIBDD compared to mice injected with hHCK12BDD over 12 weeks. B) Specific activity of protein produced from mice injected with X5FVIIIBDD compared to mice injected with X5K12BDD over 12 weeks. C) The average fold change in specific activity produced by mice injected with the hHCK12BDD compared to hFVIIIBDD. D) The average fold change in specific activity produced by mice injected with the X5K12BDD compared to X5FVIIIBDD.

The hHCK12BDD purified protein exhibits enhanced specific activity

Results so far have established that the K12 modifications enhance FVIII coagulation activity in both dual and single chain delivery, *in vitro* and *in vivo.* It has been determined from data analysis that these modifications do so by increasing the specific activity of the protein itself. However, this was determined rudimentarily through direct comparison of clotting activity to the amount of protein produced. The exact mechanism affected by the K12 modifications was not yet identified. In order to understand how the K12 modifications are able to increase coagulation activity, hFVIIIBDD and hHCK12 BDD proteins was partially purified through a SP Sepharose High Performance strong cation exchange column and used in subsequent testing.

After purification, the protein was visualized using western blot analysis. The sample was first incubated with sheep polyclonal antibodies to FVIII, and then incubated with donkey anti-sheep antibodies. The resulting western blot revealed that both the hFVIIIBDD and hHCK12BDD proteins produced the same banding pattern (**Fig. 24**).

Figure 24

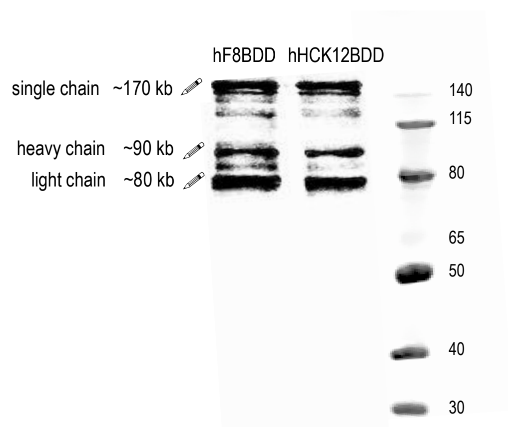


Figure 24. Western blot analysis of partially purified protein. hFVIIIBDD and hHCK12BDD proteins were partially purified using an SP Sepharose High Performance strong cation exchange column. A western blot analysis was performed using sheep polyclonal antibodies to hFVIII as a primary antibody, and donkey anti-sheep as a secondary antibody. The membrane was scanned using an Odyssey Infrared Imaging System.

The single chain form of both the hFVIIIBDD and hHCK12 BDD proteins are the same size, approximately170 kDa (**Fig. 24**). They both have a heavy chain band approximately 90-95 kb and a light chain band approximately 75-80 kb in size (**Fig. 24**). This pattern is similar to previously published results, and demonstrates that the K12 modifications do not effect FVIII protein light and heavy chain heterodimer formation.

Once the proteins were purified and confirmed via western blot analysis, the specific activity was determined by comparing the amount of protein quantified by ELISA to U/ml FVIII produced by a 1-stage APTT assay. The hFVIIIBDD protein produced a specific activity of 6,237.92 Units/mg and the hHCK12 protein produced a specific activity of 39,153.69 Units/mg (**Fig. 25**).

Figure 25.

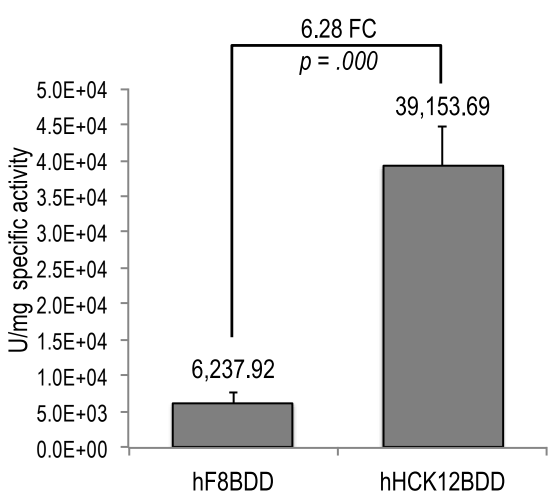


Figure 25. hHCK12BDD has a higher specific activity compared to hFVIIIBDD. The specific activity for hFVIIIBDD and hHCK12BDD was determined through comparison of protein concentration to coagulation activity. Statistical analysis is based on a 2-tailed student *t*-test.

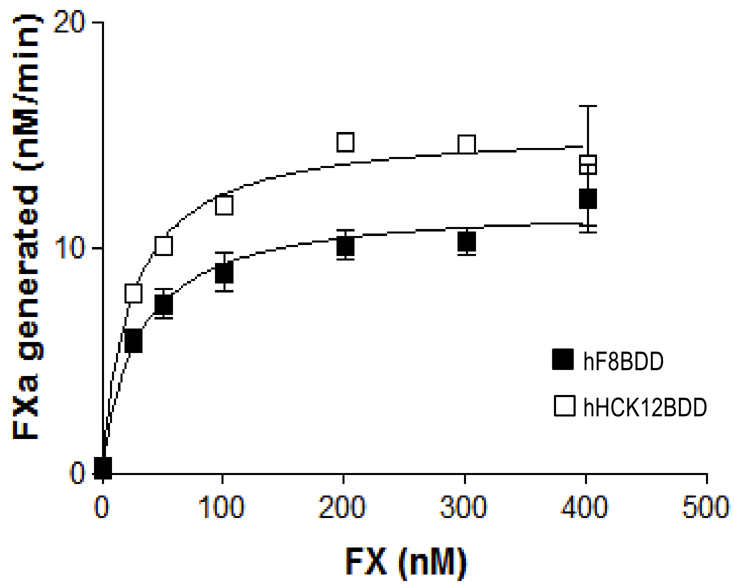
This represents a 6.28 fold increase in specific activity compared to hFVIIIBDD. These results substantiate previous results, providing evidence that the K12 modifications enhance coagulation activity by producing a more specifically active protein.

Testing of the purified hHCK12BDD confirmed that the K12 modifications were able to enhance specific activity of the protein in a purified form. The next logical step was to determine how these modifications were able to do this. It was hypothesized that the K12 modifications increased specific activity either because it has higher affinity to FIX, or it has higher cofactor activity in FIXa/FVIIIa complex, which leads to conversion of FX to FXa at an increased rate. The effects of the K12 modifications on the tenase complex were evaluated by performing a FXa generation test, in which the rate of catalysis of FX to FXa is measured. Results of this experiment show that the Tenase with hHCK12BDD as cofactor is more efficient in converting FX to FXa than its counterpart with hFVIIIBDD (**Fig. 26A**). A Michaelis-Menten kinetics analysis revealed that for the protein hFVIIIBDD V_{max} was 11.93, however, for the protein hHCK12BDD the V_{max} was 15.33. This proves that compared to hFVIIIBDD, the hHCK12BDD protein is able to convert FX to FXa at a quicker rate (**Fig. 26B**). For the protein hFVIIIBDD, the K_m was 29.14. However, for the protein hHCK12BDD the K_m was 24.29 (**Fig. 26B**). This means that compared to hFVIIIBDD, Tenase complex with hHCK12BDD binds to its substrate, i.e., FX with a higher affinity.

Results from this assay substantiate the hypothesis that the K12 modifications enhance FVIII activity through an increase in protein specific activity. They also provide evidence that this occurs when the Tenase complex interacts with FX. These modifications produce a FVIII protein that has better coagulation activity which helps the Tenase complex not only bind to FX with a higher affinity but convert FX to FXa at a quicker rate.

Figure 26

A)



B)

	K_m	V_{max}
hF8BDD	29.14	11.93
hHCK12BDD	24.29	15.33

Figure 26. The K12 modifications increase FXa generation. The conversion of FX to FXa was measured and analyzed using partially purified protein. A) Generation of FXa using different starting concentrations of F10. hHCK12BDD converts more FX to FXa at a quicker rate compared to hFVIIIBDD. B) Michaelis-Menten kinetics analysis of FXa generation provided the K_m and V_{max} for the hFVIIIBDD and hHCK12BDD proteins.

CHAPTER 4

DISCUSSION

Review of results

We first hypothesized that the human FVIII protein could be engineered to have enhanced coagulation properties based on the observation that orthologs of FVIII have varying coagulation profiles. Most orthologs share 80% or more sequence similarity to hFVIIIBDD, this estimate excludes the B domain sequences, which vary drastically between species (53, 54). However, regardless of sequence similarity, analysis of several FVIII orthologs revealed that they manifested different coagulation properties (55). For example, it has been previously demonstrated that Ovine FVIIIBDD has a 2-fold increase in specific activity when compared to hFVIIIBDD (55). Porcine FVIIIBDD has enhanced protein production; with up to a 14 fold increase compared to hFVIIIBDDD (53). Previously published work using purified protein had already established that kFVIIIBDD produced a 3-fold increase in specific activity over hFVIIIBDD (56).

It was logical to conclude that the disparities between these orthologs were the result of differences in their amino acid composition. Our hypothesis was that the amino acids responsible for augmenting the FVIII coagulation properties could be identified, and once identified, incorporated into hFVIIIBDD. Our objective was to develop a FVIII protein with enhanced specific activity for use in AAV gene therapy. For this purpose, we selected kFVIIIBDD to use as our template, since it was previously determined to have the highest specific activity of the known orthologs (53, 56). We began by testing the properties of kFVIIIBDD using the dual chain delivery method. Preliminary testing of kLC delivered to cells in conjugation with KHC revealed a 6.48 fold increase in FVIII clotting activity over hLC and hHC (*Fig. 1A*). This supported data from previous published work, in that kFVIIIBDD has enhanced FVIII activity and that the kFVIIIBDD could be delivered using a dual chain delivery strategy (52, 56). Whether or not the observed increase in clotting activity derived from properties of the heavy or light chain was still unknown. We found that *in vitro* delivery of kHC and hLC produced only a 1.24 fold increase in clotting activity, however, delivery of the kLC and hHC produced a 3.5 fold increase in clotting activity (*Fig 1B*). This preliminary data suggested that the kLC enhanced FVIII clotting activity, and that the kLC could be used in conjugation with the hHC. This initial assessment led to further evaluation of the combined delivery of the hHC and kLC, both *in vitro* and *in vivo*.

Additional testing of kLC and hHC yielded a 3.45 fold increase in clotting activity, a repeatable and consistent result, *in vitro* (*Fig. 2A*). While an increase in coagulation activity was desirable, the underlying reason for this increase was not yet known. For an increase in coagulation activity to occur there must be either an increase in protein amount or an increase in specific activity of the protein itself. It was possible that the increase in coagulation activity was the direct result of an increase in FVIII protein production. It was also possible that the protein produced was more active. An ELISA measuring heavy chain antigen levels was used to compare FVIII production levels between cells transfected with hLC and hHC and cells transfected with kLC and hHC. Results found that cells transfected with kLC and hHC produced a 2.21 fold increase in protein production over hLC and hHC (*Fig. 2B*). This indicated that kLC was able to enhance FVIII production to some extent, although, this increase wasn't enough to fully explain the activity increase. The specific activity of the protein was determined by comparing the amount of clotting activity to the amount of protein produced. This allowed for the direct comparison of clotting activity by removing production variability. The specific activity of the protein produced by the combination of kLC and hHC was 2 fold higher than hLC and hHC (*Fig. 2C*).

These *in vitro* results were somewhat ambiguous, indicating that the kLC is able to effect both production and specific activity. In an attempt to clarify this matter, the kLC and hHC plasmids were tested *in vivo.* The hydrodynamic delivery of kLC and hHC plasmids to HA mice resulted in a 3.06 fold increase in clotting activity with no significant increase in protein production *(Fig. 3A and B).* Calculation of the specific activity determined that protein from mice injected with kLC and hHC produced a 2.43 fold increase in specific activity (*Fig. 3C). In vivo* results supported the *in vitro* conclusion that the kLC enhances specific activity of the FVIII protein. However, in the case of FVIII protein production, *in vitro* and *in vivo* results differed (*Fig. 2B and 3B*). This implied that *in vitro* results might not always accurately mirror *in vivo* outcomes. Since our ultimate goal was to develop FVIII for use in gene therapy, our primary concern is with what occurs *in vivo*. *In vivo,* there is no difference in production between the use of kLC or hLC, and the increase in coagulation activity seems to be the direct result of a more active FVIII protein. From this initial set of testing, we concluded that the kLC is capable of increasing FVIII clotting activity, not by enhancing FVIII production, but my producing a FVIII protein that has more activity (*Fig. 2C and Fig. 3C*).

An alignment of the kLC and hLC amino acid sequences revealed that they are ~80% homologous to each other, and in fact, they only differ by ~118 amino acids (*Ill. 8*).

Because they have such a high degree of similarity, it was postulated that functional hLC/kLC hybrids could be engineered to contain varying portions of kLC and hLC. Thirty-two constructs were designed and engineered, each containing both hLC and kLC sequences. *In vitro* testing of these constructs in conjugation with the hHC revealed a pattern, in which adding increasing amounts of kLC amino acids to hLC increased activity, but adding increasing amounts of hLC amino acids to kLC decreased FVIII activity (*Fig. 4A*). The amount of FVIII protein produced remained fairly consistent between all the hybrid constructs, indicating that any increase in FVIII clotting activity was not the result protein production (*Fig. 4B*). Four constructs, hLC[1652-2223kLC], hLC[1652-1688;1857-2332kLC], hLC[1857-2332kLC] and hLC[1857-2147kLC], were determined to have a significant increase in specific activity compared to hLC and hHC (*Fig. 4C*). All four of these constructs contained the canine amino acids 1857-2147. Therefore, the hLC[1857-2147kLC] construct was selected for further analysis.

hLC[1857-2147kLC] contains 26 canine amino acids that span the A3-C1 domain of the FVIII protein. *In vitro* and *in vivo* testing of this construct revealed coagulation properties similar to kLC (*Fig. 5A-C, Fig. 6A-C)*. Whether or not all 26 amino acids were necessary was evaluated next through a method of negative selection. In this process each amino acid position is analyzed independantly by switching from the canine amino acid back to the human amino acid. If an amino acid has an enhancing effect, than removing it will result in a decrease in FVIII activity (*Fig. 7)*. Using this approach, 10 amino acids were selected for their potential contribution to enhancing FVIII activity (*Fig. 8 A and B*), and a final construct, hLC-K10, was designed and tested.

While screening clones for function, one clone produced a drastic increase in coagulation activity, with an increase higher than kLC or the hLC[1857-2147kLC] construct (*Fig. 9*). Sequencing analysis revealed that this construct contained two extra amino acid changes, making a total of 12 mutations. These extra amino acid mutations, S2157N and R2159H, were outside the original region of interest, which were canine amino acids 1857-2147. They were also neither human nor canine substitutions (*Fig. 10*). However, the superior activity of this construct, aptly named hLC-K12, could not be ignored, and whether or not these two extra mutations truly effected coagulation activity was not yet determined. As random mutations it was possible that they could be enhancing, deleterious or non effectual.

The contribution of the mutations S2157N and R2159H to FVIII activity were assessed through *in vitro* testing. Results demonstrated that adding the two mutations to hLC did not significantly enhance coagulation activity, however, removing them from hLC-K12 resulted in a significant 2.11 fold decrease in clotting activity (*Fig. 11A*). The addition or removal of these two mutations had no effect on FVIII protein production (*Fig. 11B*). There was no change in specific activity of FVIII when the two mutations were added to hLC, however, there was a significant, 1.72 fold decrease in specific activity when they were removed from hLC-K12 (*Fig. 11C*).

The results from this set of *in vitro* testing were conclusive in that the S2157N and R2159H mutations did not significantly enhanced activity on their own. There was no evidence that these two mutations should have any enhancing properties. However, when they were added to the hLC-K10 they did significantly enhance FVIII activity. It is hypothesized that there is an amino acid - amino acid interaction or possibly a conformational change occurring that requires all 12 of these amino acids, but the reason why these two random mutations enhance FVIII activity is not currently understood. However, because these mutations had such a significant effect, the construct hLC-K12 was selected for further analysis.

In vitro testing of hLC-K12 revealed a 6.25 fold increase in coagulation activity and a significant increase in protein production (*Fig. 12A and B).* This did indicate that the K12 modifications possibly modulate protein production *in vitro*. This is similar to results with the kLC and hHC (*Fig 2B*). However, the hLC-K12 produced a 3.28 fold increase in FVIII protein specific activity, indicating that these modifications primarily enhanced coagulation activity by forming a more active protein (*Fig. 12C*). The hLC-K12 construct produced the best results for all the light chains tested, with activity superior to even kLC itself, *in vitro*.

The hLC-K12 was tested in conjugation with the X5HC *in vitro*. The X5HC is a heavy chain construct that has been modified to contain 5 amino acid mutations that enhance FVIII protein production. The X5HC is by far the best heavy chain construct, able to yield superior FVIII protein production. *In vitro*, the X5HC on its own will produce a 9.5 fold increase in coagulation activity and a 4.87 fold increase in protein production (*Fig. 13 A and B*). Results also indicate that the X5HC is also able to enhance specific activity of the FVIII protein 2 fold (*Fig 13C*). However, its primary mechanism of FVIII coagulation modulation is through enhancement of protein production. Research on the X5 modifications has already been submitted for peer-reviewed publication.

When the hLC-K12 was tested with the X5HC it enhanced coagulation activity 4.41 fold and specific activity 2.3 fold over X5HC and hLC (*Fig. 13A and C*). There was also a significant 1.79 fold increase in protein production, which parallels *in vitro* results using kLC and hLC-K12 in conjugation with the hHC (*Fig. 2B and 12B*). It seems that testing of the light chain constructs *in vitro* results in an increase in FVIII protein production. *In vivo,* the X5HC and hLC-K12 were tested together through hydrodynamic injection of HA mice. Results revealed that the hLC-K12 construct enhances clotting activity 2.52 fold and specific activity 2.42 fold over X5HC and hLC (*Fig. 14A and C*). However, here was no significant increase in protein production (*Fig. 14B*). These results conclude that the hLC-K12 is able enhance specific activity of FVIII when used with either the hHC or the X5HC.

Comparison of results from testing the hLC-K12 in conjugation with the hHC or X5HC reveals that when the hLC-K12 and the X5HC constructs are used together there is a less prominent increase in protein specific activity compared to hLC-K12 and hHC (*Fig. 12C and 13C*). This implies that there is an overlap in enhancing properties between the X5HC and hLC-K12. *In vitro,* the X5HC enhances specific activity ~ 2 fold, and the hLC-K12 is able to enhance FVIII production ~2 fold. *In vivo,* however, the hLC-K12 no longer effects protein production, but the X5HC still has an effect on protein specific activity (*Fig. 14C).* The full effect of the X5 modifications on protein specific activity has not been completely elucidated, however, current research is looking into this matter. The focus of this project are the K12 light chain modifications, and we have so far demonstrated that these modifications enhance FVIII specific activity, both *in vitro* and *in vivo,* with either the hHC or the X5HC.

The hLC-K12 and X5HC were packaged into AAV serotype 8 vectors and delivered to CD4KO/HA mice. These mice are a double knock, not only unable to produce FVIII, but also unable to generate CD4+ T cells. This is important in FVIII gene therapy, because there is always a chance for inhibitor formation against the FVIII protein. Inhibitors are typically IgG that result from a CD4+ T cell response to FVIII, and will negatively affect coagulation activity. Because the main objective of this project is to engineer a FVIII construct with enhanced activity, it would be counter intuitive to use a strain of mice that may produce inhibitors. The CD4KO/HA mouse was generated by our lab as a way of avoiding immune interference with FVIII therapy. Blood was harvested from these mice via retro orbital eye bleeding every 2-4 weeks post vector injection.

AAV injection of hLC-K12 and X5HC using the dual chain vector method produced a significant increase in coagulation activity over hLC and X5HC at every time point, with an average of 7.43 times more activity (*Fig. 15A and B*). These results were similar to AAV injections of kLC and X5HC, which was somewhat unexpected. When *in vitro* and *in vivo* results of kLC and hLC-K12 are compared, the hLC-K12 consistently out-performed kLC. This was demonstrated with either the hHC or X5HC (*Fig. 12-14*). It was expected that similar to previous results, the hLC-K12 would produce activity superior to kLC. This small discrepancy demonstrates that packaging and testing these constructs through vector injections are necessary to fully evaluate their effects in the gene therapy context.

Similar to results from hydrodynamic testing, none of the light chain constructs had any significant effect on FVIII protein production at any time point (*Fig. 16A and B, Fig. 17 A and B*). Compared to hLC, both kLC and hLC-K12 vectors produced a significant increase if FVIII protein specific activity (*Fig. 18A and B*). kLC produced an average 4.53 fold increase and hLC-K12 produced an average 5.78 fold increase. Taken together, these results provide evidence that the K12 modifications are able to enhance FVIII coagulation activity, not through production, but by producing a more active protein. These results verify that the K12 modifications will be valuable in AAV gene therapy for HA using the dual vector delivery strategy.

Up until this point, all testing of the light chain constructs were done using the dual chain delivery strategy. However, whether or not the K12 modifications would work the same in a single chain form was undetermined. In the dual chain delivery method, both chains are delivered and transcribed separately, once in the ER they come together to form a functional FVIII protein (42, 51, 52). However, the exact mechanism of how this occurs is not known (42, 51). It has been previously established by our lab that the LC effects the production of the HC in the dual delivery strategy, and that modifications to the amino acid sequence can disrupt or effect their association in the ER (42, 51). It is possible that the X5 and/or K12 modifications introduced effect the light and heavy chain association in the ER. It was possible that a single transcript would produce different results. In order to test these modifications, single chain constructs were engineered to contain the human heavy chain and K12 mutations or the X5 heavy chain and the K12 mutations. These constructs were then tested for activity *in vitro* and *in vivo.*

In vitro, when compared to hFVIIIBDD, the hHCK12BDD produced a 4.3 fold increase in coagulation activity (*Fig. 19A*). Compared to X5FVIIIBDD, the X5K12BDD produced a 2.67 fold increase in coagulation activity (*Fig. 19A*). Put together, the X5K12BDD produced a total of a 23.2 fold increase in coagulation activity over hFVIIIBDD. These results were similar to those using the dual chain delivery method, however increase for the single chain constructs were not as drastic (*Fig.12A and 13A*). *In vitro*, adding the K12 mutations to hFVIIIBDD produced no significant increase in production, however, there was a significant 1.89 fold increase in production when the K12 mutations were added to the X5FVIIIBDD (*Fig. 19B*). This indicated that the X5 and the K12 modifications potentially work together to further enhance production of the protein, *in vitro.*

Compared to hFVIIIBDD, hHCK12BDD produced FVIII with a specific activity 3.33 times higher. X5K12BDD produced FVIII with a 1.42 fold increase in specific activity over X5FVIIIBDD (*Fig. 19C*). This represents a 2.75 increase in specific activity for FVIII from X5K12BDD compared to hFVIIIBDD. These results conclude that the addition of the K12 modifications enhances specific activity of the FVIII protein, regardless of the heavy chain. However, when the K12 and X5 modifications are put together, there is a less drastic increase compared to the K12 modifications only. This provides further evidence that the X5 modifications are able to marginally enhance specific activity of the FVIII protein on its own, and that there may be an overlap in mechanism between the X5 and K12 modifications, *in vitro*.

Results of *in vitro* testing indicate that the X5 and K12 modifications enhance FVIII production. It is possible that this was the result of an increase in mRNA transcription. An increase in mRNA levels of FVIII could lead to an increase in cellular production of the protein. Therefore, it was logical to test whether or not these mutations had an effect on mRNA status. QPCR was used to determine the CT values for both GAPDH and hFVIII in mRNA samples from cells transfected with the single chain constructs. The CT values for the heavy chain were divided by the CT values for GAPDH to calculate a ratio of heavy chain: GAPDH. If mRNA levels for FVIII were the same as GAPDH the ratio would equal 1, indicating that there are minimal amounts of FVIII mRNA present in the cells.

Conversely, as mRNA levels for FVIII increase, the ratio decreases. A ratio closer to 0 means that the cells are producing more FVIII mRNA in relation to GAPDH.

Comparison of FVIII to GAPDH revealed that the levels of mRNA for all single chain constructs were similar to each other. Ratios only ranged from 0.56 for hFVIIIBDD to 0.49 for hHCK12BDD (*Fig. 20*). In fact, all single chain constructs produced mRNA levels within 15% of each other (*Fig. 20*). Due to this high degree of similarity, it was concluded that any difference in FVIII protein production and activity is not due to a change in mRNA levels. This also demonstrates that the X5 and K12 modifications have no effect on FVIII mRNA status *in vitro*.

Analysis of the single chain constructs *in vitro* indicated that the K12 modifications enhanced FVIII specific activity, however, this was yet to be established *in vivo* through AAV delivery. It was possible that even with the modifications, the FVIII concentrations would not reach therapeutic levels due to packaging inefficiency of the vector itself. The single chain constructs were reduced to ~5.1 kb in size by replacing the ApoE/hAAT enhancer promoter combination with the small, liver specific, Transthyretin (TTR) promoter, and packaged into AAV serotype 8. Eight-week old CD4KO/HA mice were injected with $1x10^{11}$ vp/mouse and blood was harvested via retro orbital eye bleeding every 2- 4 weeks.

Mice injected with hFVIIIBDD produced at best, .061 U/ml FVIII. Addition of the K12 mutations, construct hHCK12BDD, resulted in an average 4.6 fold increase in activity, with peak expression of .213 U/ml FVIII (*Fig. 21A and B*). Addition of the X5 mutations, construct X5BDD, produced .231 U/ml FVIII with an average of 7.85 fold increase in FVIII activity (*Fig. 21A and B*). When the K12 and X5 modifications are added together, construct X5K12BDD, there is an average 16.46 fold increase in FVIII activity over hFVIIIBDD, with a peak of .59 U/ml FVIII. Compared to X5BDD, X5K12BDD produced an average 2.14 fold increase in FVIII activity (*Fig. 21A and B*). These results provide evidence that while the X5 and K12 mutations are able to enhance activity on their own, they are more effective together. This also implies that they are working through separate mechanisms, although the addition of the K12 mutations to the X5 construct did not enhance activity to the same extent as when these mutations are added to hFVIIIBDD. There was no difference in FVIII production between hFVIIIBDD and hHCK12BDD or X5BDD and X5K12BDD (*Fig. 22A and B*). However, there was a significant increase in production with the addition of the X5 mutations (*Fig. 22A and B).* These results provide further evidence supporting the postulation that the X5 mutations enhance production and the K12 mutations do not.

Addition of the K12 mutations resulted in a significant increase in FVIII specific activity (*Fig. 23*). Mice injected with hHCK12BDD produced an average 4.13 fold increase in specific activity compared to hFVIIIBDD (*Fig 23 A and B*) and mice injected with X5K12BDD produced a 1.98 fold increase in specific activity compared to X5FVIIIBDD (*Fig. 23 C and D*). This increase in specific activity is independent of the heavy chain modifications, and further substantiates our original hypothesis that the K12 modifications result in a more active FVIII protein, *in vitro*.

We have so far provided strong *in vitro* and *in vivo* evidence supporting our claim that the light chain modifications can produce a FVIII protein with increased specific activity. However, we have no direct evidence demonstrating in what way this is occurring. In order to determine how the K12 modifications are able to increase specific activity, protein was partially purified and tested. Since we are primarily concerned with the K12 light chain modifications, hFVIIIBDD and hHCK12BDD proteins were selected for purification and analysis. This allowed for the pure comparison and evaluation of the K12 modifications alone, without interference of the X5 modifications.

A western blot analysis confirmed that the protein isolated was indeed FVIII (*Fig. 24*). The banding pattern for hFVIIIBDD and hHCK12BDD are similar to each other, indicating the K12 modifications have no effect on the FVIII processing and cleavage into heavy and light chains (*Fig. 24*). An ELISA was performed to quantify the protein and the specific activity was calculated. The hFVIIIBDD protein had a specific activity of 6,237.92 Units/mg. However, hHCK12BDD had a specific activity of 39,153.69 Units/mg, which is a 6.28 fold increase over hFVIIIBDD (*Fig. 25*). This is similar to previous *in vitro* and *in vivo* results, providing direct evidence supporting the hypothesis that the K12 modifications increase FVIII protein specific activity.

FVIII is an essential part of the intrinsic clotting cascade, it binds to FIXa to form the tenase complex, which then converts FX to FXa. Since this is the main function of FVIII, it was logical to hypothesize that the K12 modifications affected either binding to FX when in the tenase complex or the conversion of FX to FXa. The ability for the hFVIIIBDD and hHCK12BDD proteins to convert FX to FXa was measured using a FXa generation assay. The results of this experiment demonstrated that the hHCK12BDD protein is able to convert more FX to FXa at a quicker rate (*Fig. 26A*). Using the results of this test, a Michaelis-Menten kinetics analysis determined both the K_m and V_{max} for these proteins (*Fig. 26B*). Compared to hFVIIIBDD, hHCK12BDD has a higher V_{max} (15.33) and a lower K_m (24.29) (*Fig. 26B*). This provides evidence that the K12 modifications are able to increase specific activity by binding to FX with a higher affinity and producing more FXa compared to hFVIIIBDD.

Implications for HA protein therapy

K12 light chain modifications for use in FVIII protein therapy

The first recombinant FVIII protein concentrate was approved for use in the USA in 1992 (57, 58). Introduction of manufactured FVIII removed the threat of viral infection associated with human derived plasma FVIII, in particular, the threat of hepatitis in the 1970s and HIV in the 1980s (57, 58). Since the implementation of FVIII protein replacement therapy and the development of infusion regimens, the predicted lifespan of a person with severe HA has gone from 20 years in 1970 to the same as those who are unaffected today (57, 58). While the use of recombinant FVIII has dramatically improved the quality of life for those living with HA, low patient compliance with treatment remains a key issue due to the high frequency of required infusions (57, 59).

Current prophylactic treatment consists of infusions three to four times a week, which is necessary to maintain therapeutic value because the FVIII protein half- life is only 8-12 hrs in length (59). There have been multiple attempts to increase half-life of the FVIII protein with the working hypothesis that a longer half-life would require less frequent IV infusions (57-59). One example is ELOCTATE, which is a recombinant FVIII protein fused to the IgG constant region Fc (60). Another example is BAY 94-9027, an rFVIII BDD protein that is conjugated to a 60 kDa PEG molecule(59). Research found that both of these constructs retain normal FVIII activity and are not associated with an increase in inhibitor formation (59, 61). However, even with these modifications the FVIII protein half-life was found to be at best 18-19 hrs (61). It has since been established that this is due to the protein's association with VWF, which itself has a half-life of 16-17 hrs (59). Because FVIII circulates in conjugation with VWF, liver clearance of VWF will also clear any associated FVIII. These findings imply that unlike FIX, which can be modified to have a half-life of almost 90 hrs, the ability to increase FVIII half-life is limited (61). Therefore, in order to increase effectiveness of protein therapy, additional strategies will be necessary. As an alternative approach to exploring a longer protein half-life, we suggest that the use of a more active protein may decrease the number of required infusions, which will encourage patient compliance.

Our current research has established that a 12 amino acid modification to the human FVIII light chain results in a FVIII protein with enhanced specific activity. Results indicated that this increase was due to a more robust generation of FXa, and not due to an increase in protein half-life. While the exact half-life has not yet been determined, there have been no indications that the K12 light chain modifications affect FVIII protein half-life. In fact, if the K12 modifications substantially increased protein half-life there would have been an increase in protein amount over time that was proportional to the increase in activity, but this was not observed. However, results provided evidence both *in vitro* and *in vivo* that FVIII proteins containing the K12 modifications retained 3-5 times more specific activity.

The K12 modifications have the potential for use in protein therapy because even with only low levels of protein there remains the possibility of a therapeutic outcome. For example, a goal of 10% FVIII activity requires approximately 20 ng of human rFVIIIBDD, or approximately 4-6.6 ng of K12 modified rFVIIIBDD. The K12 modified rFVIIIBDD requires 3-5 times less protein to produce the same outcome. A 20 ng amount of K12 modified rFVIIIBDD would produce 30-50% FVIII activity. After one half-life, 20 ng of human rFVIIIBDD would successively become 10 ng and produce 5% FVIII activity. Similarly, K12 modified rFVIIIBDD would decay over one half-life from 20 ng to10 ng of protein, however, it would still produce 15-25 % activity. Over time, the levels of infused FVIII protein will decrease, yet with the K12 modifications a higher level of activity will remain. We suggest that the amount of FVIII protein in circulation is inconsequential, as long as protein activity is high enough to produce a therapeutic outcome and restore a normal bleeding phenotype. If the K12 modified rFVIIIBDD protein functions in humans as it does *in vitro,* it would theoretically require less frequent infusions. At the very least it would potentiate the effects of regular infusions.

Whether or not the K12 modifications could be used in combination with the FC fusion or PEGylation strategy has not been examined. It is possible that adding the K12 modifications to the ELOCTATE or BAY 94-9027 construct will result in a protein with a longer half-life and more specific activity. However, it is also possible that the two sets of modifications will not work well together. Our results, both *in vitro* and *in vivo*, demonstrated that adding the K12 light chain modifications to the X5 heavy chain modifications produced somewhat disappointing results. Although adding both sets of modifications together did further improve the FVIII protein activity profile, it was not synergistic. This indicates that adding two sets of modifications together may not always be beneficial. However, since our original objective was to design a FVIII construct for use in the gene therapy setting, FC fusion and PEGylation was not considered. Nevertheless, if the K12 modifications are seriously analyzed for use in protein replacement therapy this may be something worth examining.

The K12 protein modifications- implications for genetic engineering

There is a remarkable amount of information available on the genetic causes of HA. In fact, the online FVIII database contains 1,500 documented variants of the FVIII gene that have resulted in 5,472 individual HA cases (62). Over 1000 missense mutations have been identified that will cause some form of HA, although, the relationship between type and location of the missense mutation with severity of disease is not yet completely defined (63). When the available information is analyzed comprehensively, the overall conclusion is that mutations to the FVIII amino acid sequence are detrimental. Nevertheless, our original goal was to develop a FVIII protein that had enhanced activity. This strategy relied on the use of genetic engineering resulting in desirable mutations that had a positive effect. Because most FVIII mutations result in HA, it seemed unreasonable to screen random mutations looking for a positive outcome (63). Attempting to use computer modeling to identify amino acid changes that would enhance activity seemed unreliable because it cannot predict whether or not there will be a global conformational change in protein structure (63). Therefore, we designed and implemented a unique strategy to engineer FVIII based on the observation that canine FVIII has enhanced specific activity compared to human FVIII.

The coagulation properties of canine FVIII have been previously documented and are well defined. Preliminary experimentation determined that the increase in protein specific activity resulted from an unknown region of the canine light chain sequence. Using this data, we analyzed the entire canine light chain by engineering 32 human light chain constructs to contain varying canine regions. These mutants were then screened for increased coagulation activity. Once the regions of interest were selected, each canine amino acid located in that region was analyzed for potential enhancing effects.

This strategy of genetic engineering is distinctive because it does not consider protein structure or domain organization. We did not specifically target the A3, C1 or C2 domains found in the light chain, nor did we eliminate any section from consideration. We also tested regions of canine amino acids that spanned multiple domains, or regions that were found within individual domains. Position of the amino acid on the 3-D protein structure was not considered, for example, whether or not the amino acid was located on the interior or exterior surface. In the end, our construct contained 12 amino acid mutations that spanned the A3- C1 domain of the FVIII light chain. We would have been unsuccessful if we had targeted and analyzed each domain individually. Additionally, this method assigned equal weight to each amino acid, regardless of location or composition. This is innovative because not all amino acid changes are considered to be significant, this is typically seen when amino acids sharing similar properties are exchanged. For example, an Isoleucine to Valine substitution is considered to be less consequential then a change from Methionine to Asparagine.

In this study we determined, and provided evidence, that ours is the best approach to engineering FVIII. The K12 modifications can be used as an example of how to genetically engineer a protein based simply on ortholog analysis. This is significant to the field of genetic engineering because, even though we have all the techniques available to manipulate and test new sequences, it is often challenging to know where to start. This is largely due to the difficulty and ambiguity of determining the effect a single amino acid change will have on overall protein function. This becomes even more arduous when considering multiple amino acid positions at once. We suggest that this strategy can be used for other proteins in which orthologs maintain activity in the human system.

In the case of FVIII, our approach provided a unique way to engineer a better protein, specifically a more active protein. However, this strategy could be used to further expand the FVIII coagulation profile. Previous work by our lab has identified five porcine amino acids that are capable of enhancing FVIII secretion. By screening a larger number of FVIII orthologs for enhanced coagulation properties, it is possible a more superior FVIII protein could be designed. Whether or not combining different sets of mutations will have a synergistic, additive or no effect must also be considered. This would rely on studying mechanism of action using partially purified protein, as performed in this study. However, it is possible that using our method of genetic engineering, a FVIII protein with even superior function may be identified.

The K12 protein modifications- inhibitor development

Approximately 25% of patients with severe HA will develop inhibitors after exposure to FVIII protein, radically diminishing the effectiveness of both gene and protein therapy (27) (28-30, 64). As discussed in the introduction section, once inhibitors develop the only successful options are either ITI treatment or the use of bypassing agents (34, 65). Therefore, it is acknowledged that inhibitor development is the most detrimental side effect of FVIII therapy. The risk of inhibitor development is a serious consideration when deciding if an engineered FVIII protein will be approved for use as treatment. Even FVIII orthologs that have superior coagulation activity are not used in human treatment because they are considered to increase the risk of inhibitor development. This is why we do not suggest to simply deliver Canine FVIII for HA treatment and instead, developed a process to select for only a limited number of amino acid changes. Current opinion on the development and use of rFVIII variants is to use the minimal number of amino acid changes necessary to provide a beneficial outcome. In this way, the protein is as similar to the wild type FVIII protein as possible.

Whether or not the K12 modifications will effect inhibitor development is unknown. In fact, when tested *in vivo* we used CD4 knock out mice to avoid potential inhibitor development, in order to focus on protein function and activity. Our goal was to develop a more active FVIII protein and to assess its use in a gene therapy context. We have also established a novel system of engineering and selecting for protein variants. For these purposes, the immunological response was not considered. However, whether or not the K12 modifications promote inhibitor development will have to be analyzed before serious consideration for use in clinical trials. It will be important to ensure that these modifications do not introduce a new antigenic site, or 'hotspot', for inhibitor formation. Whether or not these adaptions increase the rate of inhibitor formation will also need to be measured.

An assessment on the risk of inhibitor development due to the K12 modifications to the FVIII protein will be important for both protein and gene therapy. However, there will be a considerable difference in the possibility of antibody formation when using gene therapy compared to traditional, protein therapy. Research has found that delivery of a transgene to liver hepatocytes by AAV induces immune tolerance, preventing inhibitor formation (64-66). It was hypothesized that this was due, in part, to hepatic secretion of the protein into circulation, and subsequent immune presentation at multiple, extra hepatic sites. It has since been established that transgene expression from the liver hepatocytes induces CD4+CD25+ regulatory T-cell (T-regs) formation, which actively suppresses antibody formation (64-66).

Recent *in vivo* studies on the delivery of a FIX transgene to the liver using an AAV vector resulted in immune tolerance to FIX (64-66). However, results indicated that this required a minimal level of expression, and when only low levels of FIX were expressed, inhibitors did form (66). Similarly, delivery of a codon optimized hFVIII transgene to the liver by AAV, not only produced long-term transgene expression, but also promoted tolerance (64-66). The use of a codon optimized hFVIII transgene is at least partially responsible for the observed tolerance induction, because this type of modification results in enhanced in protein secretion.

Whether or not K12 modified rFVIII will promote immune tolerance *in vivo* has not yet been determined. It is logical to conclude that delivering this transgene to the hepatocytes using an AAV vector will promote tolerance, as long as expression remains above the minimal level for T-reg induction. This may present a problem, since the K12 modifications result in a more specifically active protein and do not effect secretion or transgene expression. Whether or not the protein is secreted at a high enough level will have to be determined. It is possible that the K12 modifications can be combined with the X5 modifications in order for the level of protein to be high enough to maintain tolerance.

Due to immune system variability, it is impossible to say in absolute terms whether or not specific protein modifications will or won’t result in inhibitor formation. Even if the K12 modifications result in some inhibitor formation, this may not necessarily be problematic for all HA patients. It is well established that there is a direct correlation between the underlying genetic mutation responsible for the development of HA and the incidence of inhibitor development (6, 26, 28). HA patients that have no circulating FVIII have immune systems that should, theoretically, recognize any version of FVIII as foreign, even wild type FVIII. In this case, it will not matter if the K12 modifications change protein structure. We assert that HA patients with no circulating FVIII will have similar chances of developing inhibitors regardless of the type of FVIII protein administered. If this is the case, then the best protein should be selected for use, even if the modifications result in structure change that can be recognized by the immune system. Especially in the context of gene therapy, where there is the potential for tolerance induction.

Implications for AAV gene therapy

Transgene modifications enhance therapeutic outcome of gene therapy

HA gene therapy using an AAV vector faces multiple challenges with the end result of low transgene expression. The size limitation of the AAV vector is the primary reason that AAV-HA gene therapy has been, so far, unsuccessful. We hypothesized, and provided evidence, that AAV-HA gene therapy can produce a therapeutic outcome simply by modifying the transgene to produce a more active protein. This solution is novel because it does not actually address the size constraints associated with FVIII and AAV packaging. There have been multiple efforts to reduce the transgene size by either removing portions of FVIII or introducing the use of smaller regulatory elements. However, our data provides evidence that modifications to the transgene itself can significantly increase gene therapy efficiency. This has implications for use in AAV gene therapy, because it provides a unique answer to the size limitation of the AAV vector. It is possible that this strategy can be used for other protein as well.

FIX clinical trials

Hemophilia B (HB) is an X-linked, genetic, bleeding disorder resulting from defects in the coagulation FIX protein (10). In the absence of FIX, the Tenase complex cannot form and the intrinsic clotting cascade becomes ineffectual. HB is similar to HA in that the end result is an insufficient amount of FX converted to FXa to support the formation of a fibrin clot (67, 68). Current treatment of HB relies on the IV infusion of FIX protein, however, like FVIII protein therapy, this treatment is costly and time consuming. For these reasons, AAV gene therapy has been examined as an alternate approach to FIX protein infusion (67, 68). Research on AAV-FIX gene transfer has progressed much further compared to FVIII, mainly because FIX is small enough for efficient AAV packaging. The FIX gene itself is only 1382 bp in length, well within the 4.7 kb AAV packaging limit (10).

To date, there have been multiple, human clinical trials testing the use of AAV for FIX gene therapy. An initial clinical trial using AAV2 serotype to deliver FIX resulted in no detectable levels of FIX protein (67, 68). These results were disappointing, and it was suggested this poor performance was due to an immune response to the AAV2 serotype capsid protein. However, another clinical trial using a self-complimentary, AAV8 serotype vector to deliver a codon optimized FIX gene produced promising results. All ten patients injected produced a dose dependent increase in FIX protein levels, averaging 1-6%. Six patients injected with a high dose of vector produced an average of 5.1 +/_ 1.7% FIX activity over the course of 3.2 years (69).

The recognized success of AAV-FIX clinical trials has implications for HA gene therapy. First, these results prove that a single injection of AAV vector can establish transgene expression that lasts for years. This is important because multiple AAV injections are not feasible at this point in time due to immunological complications. Second, this indicates that once the size limitation associated with FVIII is resolved, the clinical trials should be successful. It is logical to conclude that addition of the K12 modifications to the FVIII gene will further enhance success of clinical trials, because the protein produced will have more activity.

Conclusions

In conclusion, we have developed a novel FVIII construct containing 12 amino acid changes to the light chain that are able to enhance protein specific activity. This was demonstrated both *in vitro* and *in vivo*, whether delivered using the dual chain method or as a single chain version of FVIII. These modifications work in a similar manner when used in conjugation with either the hHC or the X5HC, *in vitro* and *in vivo*. Purified protein was used to demonstrate that these modifications are able to enhance Tenase complex binding to FX and FXa generation. Packaging and delivery of FVIII carrying these modifications resulted in the production of protein with an increased specific activity, demonstrating that even low levels of protein production can produce a therapeutic effect. This construct will be beneficial to both gene therapy and protein therapy. Furthermore, it demonstrates that gene therapy can be improved through genetic manipulation of the transgene instead of attempting to modulate vector size or the immune system reaction.

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