**Furin-Enhanced In Vivo Production of Factor IX**

**BNFO 300 - Dr. Jeffrey Elhai**

**Aarthi Prakash**

**April 30, 2017**

**Introduction**

Hemophilia B (HB), also called Christmas disease, is an X-linked recessive genetic disorder that causes mutated or reduction of factor IX (FIX), which is a coagulation protein. This life-long condition affects approximately one in 30,000 males (Franchini et al., 2013). Depending on the severity, the condition can be treated transiently or could require a more aggressive prophylactic type treatment. HB can be divided into three types: Severe, less than one percent of FIX production and is about 60% of cases, moderate, in which about one to five percent FIX levels in about 15% of cases, and mild is when about six to 30 percent of FIX is produced in about 25% of cases (Hemophilia B., 2015). Often patients have bleed longer than normal for minor trauma and in severe cases can have frequent spontaneous internal bleeding. The frequency and severity are based on FIX levels and can be prevented through hematology treatments of FIX.

FIX is a clotting factor that is converted from a zymogen to a serine protease (Liu et al.). Mature FIX is known to have five distinct domains: N-terminal, Gla-domain, EGF-like domain, AP domain, and C terminal serine protease domain(citation). FIX requires various posttranslational modifications particularly the challenge is in the Gla domain to convert the zymogen to a fully functional FIX protein. One of them is gamma-carboxylation of glutamic acid residues, which is required for phospholipid and calcium bonding (Wasley et al., 1993).

Current treatments for HB have been found to be short term with recombinant FIX while long-term treatments have been found to require extensive monitoring, have adverse side effects, or have not been developed. Prophylactic treatments have to be administered every few days with a recombinant or plasma-derived FIX gene product (Franchini et al., 2013). These have been found to be transient and have reduced efficacy as well. Viral approaches have been used in gene therapy methods that avoid contamination by HIV, Hepatitis B, and even Hepatitis C. However, contamination by West Nile Virus/SARS or even prion diseases has not been investigated. In preclinical trials, using an adenoviral approach is the most promising but is limited by immune response by T-cells (CITATION).

Furin is a serine protease that processes FIX (Siner et al.). This processes converts the profactor (inactive) to mature FIX (active). Furin is a type of paired basic amino acid cleaving enzyme (PACE), a family of proteases, that is ubiquitously expressed primarily in hepatocytes (liver cells). Coexpression of FIX and furin gene has shown improved processes efficiency of profactor IX in chinese hamster ovary cells. Furin recognizes the P1 and P4 arginine residues on the peptide for efficient processing (Wasley et al., 1993).

Clinical trials of FIX expression using adeno-associated virus (AAV) vectors injected into patients has been shown to increase FIX expression with few side effects (Nathwani et al. 2011). They had infused three different doses of the AAV vectors and found FIX levels were found to be 2 to 11% of normal values in all of the six participants. Four of the six patients were able to stop their prophylactic treatments while avoiding any spontaneous hemorrhaging (Nathwani et al., 2011). Nathwani et al. (2014) continued analysis of these patient as well as added patients for assessing the safety and efficacy of that gene therapy method. The patients had dose-dependent increase of FIX of 1% to 6% of normal values over about three years. The high dosage group had an increase of about 5.1% which showed a reduction of spontaneous bleeding by 90%. A case study of a patient with an AAV gene therapy infection was found to have active FIX production after ten years (Buchlis et al., 2012). They transferred human FIX encoding AAV into skeletal muscle of a male with hemophilia B. After this patient died 10 years later, FIX production was still persistently expressed in the muscle tissue where the AAV was injected. (Buchlis et al., 2012). Wasley et al. (1993) looked at PACE processing of FIX in Chinese hamster ovary cells and found FIX was completely processed when both were co-transfected into the cells. PACE recognized both P1 and P4 arginine after they found s point mutation at P1 of arginine to threonine eliminated PACE’s ability to process FIX. There pathway analysis implicates the possibility of exploring the enzymes processing ability in vivo (Wasley et al., 1993).

This research proposal aims to explore the possibility of furin co-expresion with FIX through AAV recombinant vector infusion in Hemophilia B mice to improve the processing efficiency of FIX. This method could improve the hemophilic phenotype more than previously and increase the conversion of profactor FIX to mature FIX.

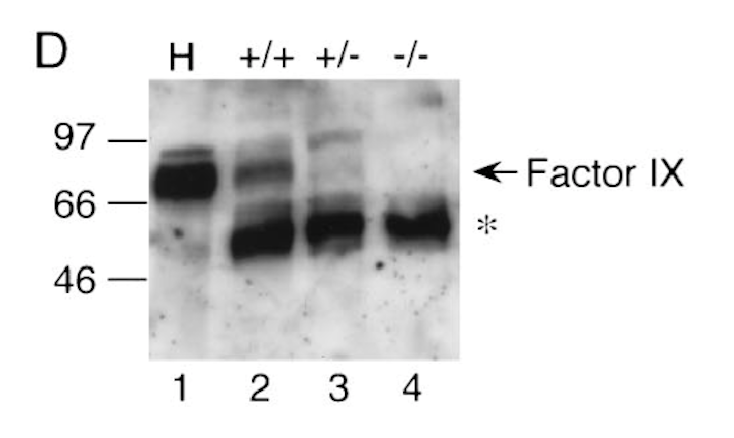
**Experiment**

To explore the activity of furin hemophilia B deficient mice will be transfected with AAV vector that is designed to coexpress both FIX and the furin genes. RT-PCR analysis will be used to observe the proper function of the vector incorporated into the liver cells. FIX will be separated as propeptide and mature FIX then analyzed through western blot gel. To quantify the amount produced, staining density will be quantified from the gel.

Mice will be developed as FIX deficient to mimic Hemophilia B conditions as previously described by Wang et al. (1997). They will be made by using a FIX targeting vector that will generate FIX-deficient mice through disruption of the gene. The AAV vector will be developed as previously described by Wang et al. (1999) with human FIX (hFIX), furin, and a strong liver-specific enhancer/promoter (LSP). This will be derived from the vector plasmid pAAV-LSP-hFIX that will be constructed in the lab. This developed vector will be injected into hepatocytes of the HB mice wThis will be injected in hepatocytes in the HB mice and analyzed through PCR initially. Reverse transcriptase Polymerase Chain Reaction (RT-PCR) and Sanger sequencing will be used to determine if the vector was transcribed properly. RT-PCR will give information about proper transfection of the vector and will be done as described by Liu et al (2014). Human FIX antigen will be detected through ELISA analysis as previously described (Wang et al., 1999) to determine presence of FIX and in what quantity.

Once it has been shown to be present in those cells a western blot analysis will be done using mouse or human plasmas as the sample. The primary antibody will be determined as an anti-hFIX antibody. Through the western blot antibodies there will be two of them, one that targets mature FIX and another that targets propeptide FIX. The western blot will react with the specified antibodies and show whether the FIX protein is there (Wang et al. 1999). The blot gel could be stained and analyzed using a densitometer to approximately quantify the amount of FIX produced. The mice liver tissue will be analyzed as well through immunofluorescence stainig to detect hFIX as well and will be done as described by Wang et al (2014). The hFIX could also be isolated and purified and then subjected to affinity chromatography to separate the propeptide from the mature as described by Liu et al. (2014). The mice will also be subject to a tail clip test similar to Wang et al. (1997) to analyze the phenotypic effect of the FIX and furin co-expression. These methods will allow hFIX and furin to be analyzed for efficacy as an in vivo approach.

**Discussion**

****

**Figure 1.** The western blot from Wang et al. (1999).

We expect the RT-PCR experiment to product results that the transcribed mRNA is the hFIX DNA. The ELISA should show that there is sufficient hFIX being transcribed and produced compared to previously described amounts. After performing all of the methods, the western blot analysis should show similar values as Figure 1. This shows the presence of FIX in the tissue analysis and would show the overall FIX production similar to the results above. We would like to find the gel to be stained and have similar amounts quantified as the previous ELISA done to see similar quantities being synthesized. Once the hFIX is determined and quantified then the immunofluorescence staining should show staining in the linings and veins rather than the actual cells as previously described by Wang et al. (1999).

There is a possibility to have incorrect or outlier values during the quantification of hFIX or during RT-PCR when converting into cDNA. If there is irregular amounts of FIX in the controls could indicate inconsistent or incomplete vector transfer into the mouse liver. With regards to the vector, it could have incomplete genes and or incorrect combination of the genes in the derived vector. The tail clipping method could be too broad of a measurement as well and not give bleeding times that are consistent with hemophilia B.

During experimentation there could be an immune response to the antibodies that are developed causing interference. There is possible interference by other processing events from outside or earlier in the secretory pathway. The biggest pitfall with gene therapy experiments is the possibility of uncontrolled mutations during the gene transfers and transcriptions since mRNA does not have a proofreading ability. Gene therapy transfection of the vectors are not always incorporated into the genome and therefore requires isolation of the transfected cells to determine incorporation.

Furthermore this experiment poses some limitations as it only represents a minor amount of how much can be implicated with hFIX processing. There are possible complications in health for the animals or in humans when put in clinical trials because they are voluntary. This could be ameliorated by following certain guidelines of animal experiments. The antibodies developed are uncertain and the extent of their effect is hard to determine. With regards to the mice, each mouse could react differently depending on their breeding and epigenetic relationships or interactions with inherent mouse proteins such as during the knockdown of furin as well as factor IX.

The multiple complications could possibly be controlled however some of them are unpredictable. Through this experiment we hope to see implications for improving current gene therapy methods for better processed hFIX and for better development of recombinant hFIX production for long term treatment options.

**References**

Buchlis, G., Podsakoff, G.M., Radu, A., Hawk, S.M., Flake, A.W., Mingozzi, F., & High, K.M. Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. *Blood. 119(13).* Doi: 10.1182/blood2011-09-382317.

Cardo, L.J. (1991). Human Factor IX for the Treatment of Hemophilia. *JAMA, 266(6). Pp. 794*

Children’s Hospital of Philadelphia. (2016, October 17). In crafting new treatments for hemophilia, a ‘less is more’ approach. *ScienceDaily.* Retrieved April 6, 2017 from [www.sciencedaily.com/releases/2016/10/161017140552.htm](http://www.sciencedaily.com/releases/2016/10/161017140552.htm).

Dietrich, B., Schiviz, A., Hoellriegl, W., Horling, F., Benamara, K., Rottensteiner, H., Turecek, P.L., Schwarz, H.P., Scheiflinger, F., & Muchitsch, E. (2013). Preclinical safety and efficacy of a new recombinant FIX drug product for treatment of hemophilia B. *The Japanered Society of Hematology, 98, pp 525- 532.* Doi: 10.1007/s12185-013-1448-z.

Franchini, M., Frattini, F., Crestani, S., Sissa, C., & Bonfanti, C. (2013) Treatment of hemophilia B: focus on recombinant factor IX. *Biologics: Targets and Therapy,(7), pp. 33-38.* Doi: 10.2147/BTT.S31582

Hemophilia B. (2015, July 15). Retrieved February 22, 2017, from [https://www.hemophilia.org/Bleeding-Disorders/Types-of-Bleeding- Disorders/Hemophilia-B](https://www.hemophilia.org/Bleeding-Disorders/Types-of-Bleeding-Disorders/Hemophilia-B)

Leblond, J., Gaudreau, S., Grondin, F., Laprise, M., Dubois, C.M. (2004). Release of an Endogenous Inhibitor of the Proprotein Convertase Furin in Activated Human Platelets. *Cardiovascular Pathology, pp s153.*

Liu, J., Jonebring, A., Hagstrom, J., Nystron, A., & Lovgren, A. (2014) Improved Expression of Recombinant Human Factor IX by Co-expression of GGCX, VKOR, and Furin. *The Protein Journal. 33.* Doi: 10.1007/s10930-014-9550-5.

Nathwani, A.C., Tuddenham, E.G.D., Rangarajan, S., Rosales, C., McIntosh, J., Linch, D.C., Chowdary, P., Riddel, A., Pie, A.J., Harrington, C., O’Beirne, J., Smith, K., Pasi, J., Glader, B., Rustagi, P., Ng, C.Y.C., Kay, M.A., Zhou, J., Spence, Y., Morton, C.L., Allay, J., Coleman, J., Sleep, S., Cunningham, J.M., Srivastava, D., Basner- Tschakarjan, E., Mingozzi, F., High, K.A., Gray, J.T., Reiss, U.M., Nienhuis, A.W., & Davidoff, A.M. (2011). Adenovirus-Associated Virus Vector-Mediated Gene Transfer in Hemophilia B. *The New England Journal of Medicine. 365(20).* Doi: 10.1056/NEJMoa1108046.

Nathwani, A.C., Reiss, U.M., Tuddenham, E.G.D., Rosales, C., Chowdary, P., McIntosh, J., Della Peruta, M., Lheriteau, E., Patel, N., Raj, D., Riddell, A., Pie, J., Rangarajan, S., Bevan, D., Recht, M., Shen, Y.M., Halka, K.G., Basner- Tschakarjan, E., Mingozzi, F., High, K.A., Allay, J., Kay, M.A., Ng, C.Y.C., Zhou, J., Cancio, M., Morton, C.L., Gray, J.T., Srivastava, D., Neinhuis, A.W., & Davidoff, A.M. (2014). Long-Term Safety and Efficacy of Factor IX Gene Therapy in Hemophilia B. *The New England Journal of Medicine, 371(21)*. Doi: 10.1056/NEJMoa1407309.

Rehemtulla, A., & Kaufman, R.J. (1992). Protein processing within the secretory pathway. *Current Opinion in Biotechnology, (3), pp 560-565.*

Siner, J.O., Samelson-Jones, B.J., Crudele, J.M., French, R.A., Lee, B.J., Zhou, S., & Arruda,V.R. (2016). Circumventing furin enhances factor VIII biological activity and ameliorated bleeding phenotypes in hemophilia models. *JCI Insight, 1(16).* doi: 10.1172/jci.insight.89371.

Wang, L., Takabe, K., Bidlingmaier, S.M., Ill, C.R., & Verma, I.M. (1999). Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. *National Academy of Science, 96, pp.*3906-3910.

Wang, L., Zoppe, M., Hackeng, T.M., Griffin, J.H., Lee, K., & Verma, I.M. (1997). A factor IX-deficient mouse model for hemophilia B gene therapy. *The National Academy of Science, 94,* pp. 11563-11566.

Wasley, L.C., Rehemtulla, A., Bristol, J.A., & Kaufman, R.J. (1993). PACE/Furin Can Process the Vitamin K-dependent Pro-factor IX Precursor within the Secretory Pathway. *The Journal of Biological Chemistry, 268(12),* pp. 8458- 8465.

Wootla, B., Christophe, O.D., Mahendra, A., Dimitrov, J.D., Repesse, Y., Ollivier, V., Friboulet, A., Borel-Derlon, A., Levesque, H., Borg, J., Andre, S., Bayry, J., Calvez, T., Kaveri, S.V., & Lacroix-Desmazes, S. (2010). Proteolytic antibodies activate factor IX in patients with acquired hemophilia. *Blood, 117 (7).* Pp. 2257-2264 Doi: 10.1128/blood-2010-07-296103.

Zhang, G., Shi, Q., Fahs, S.A., Kuether, E.L., Walsh, C.E., & Montgomery, R. R. (2010). Factor IX ectopically expressed in platelets can be stored in -granules and corrects the phenotype of hemophilia B mice. *Blood, 116(8),* pp. 1235-1243. Doi: 10.1182/blood-2009-11-255162.