# Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Children's Hospital of Philadelphia

2. **Reporting Period (start and end date of grant award period):** 1/1/2009 – 12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Nicole M. Young,B.S, CRA

4. **Grant Contact Person's Telephone Number:** 267-426-7747

5. **Grant SAP Number:** SAP 4100047628

6. **Project Number and Title of Research Project:** 2 - Gene Transfer of Activated FVII for the Treatment of Inherited Coagulation and Platelet Disorders

7. **Start and End Date of Research Project:** 01/01/2009 – 12/31/2012

8. **Name of Principal Investigator for the Research Project:** Katherine A. High, MD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 1,590,804.00

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

| Last Name | Position Title | % of Effort on Project | Cost |
|---|---|---|---|
| Margaritis | Co-investigator | 100 (Yr 1-3)<br>70 (Yr 4) | $320,012.32 |
| Qu | Technical Director of Vector Core | 35 | $259,492.88 |
| Faella | Technician | 100 | $185,344.31 |

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

| Last Name | Position Title | % of Effort on Project |
|---|---|---|
| High | PI | 5 |

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

| Type of Scientific Equipment | Value Derived | Cost |
|---|---|---|
| None | None | None |

**10. Co-funding of Research Project during Health Research Grant Award Period.** Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No_____X_____

If yes, please indicate the source and amount of other funds:

**11. Leveraging of Additional Funds**

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes____X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If

you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

| A. Title of research project on grant application | B. Funding agency (check those that apply) | C. Month and Year Submitted | D. Amount of funds requested: | E. Amount of funds to be awarded: |
|---|---|---|---|---|
| Novel FVII-based strategies for the treatment of coagulation disorders | ☐NIH<br>☐ Other federal (specify:_____ _____)<br>☒ Nonfederal source (specify: Pfizer Inc._____) | 06/2012 | $250,000 | Not funded |
| Cell surface interactions with FVIIa during FVIIa-enhanced hemostasis | ☐NIH<br>☐ Other federal (specify:_____)<br>☒ Nonfederal source (specify: Bayer Hemophilia Awards_) | 03/2011 | $200,000 | $200,000 |
| Gene therapy for hemophilia using muscle-expressed FVIIa (Project 2) | ☒NIH<br>☐ Other federal (specify:_____ _____)<br>☐ Nonfederal source (specify: _____) | 01/2010<br>5 year grant (8/5/11-6/30/16) | $227,378/yr | $222,475/yr |

**Cell surface interactions with FVIIa during FVIIa-enhanced hemostasis**
Data from Aim 1 of this grant were used as preliminary data in the Bayer Hemophilia Awards grant application.

**Gene therapy for hemophilia using muscle-expressed FVIIa**
Data from Aim 1 of this grant were used as preliminary data in an NIH Project Program Grant application

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

The researcher is planning to apply for funding that aims to expand the data obtained in Aim 3. This will ensure that the researcher can obtain significantly longer efficacy as well as safety data in dogs with FVII deficiency, using gene transfer. This will provide further support for FVII gene transfer as a potential treatment for Factor VII deficiency.

The researcher is also planning to apply for funding using the murine FVIIa reagents that were utilized in Aim 1. The purpose of this future grant application will be the elucidation of the mechanism of action of FVIIa in hemophilia as it relates to certain structural aspect of FVIIa. This may provide additional information that can be used to design novel FVIIa-based therapeutics, either protein or gene-based.

**12. Future of Research Project.** What are the future plans for this research project?

During the grant's duration, the researcher developed a variety of FVIIa transgenes as well as vector constructs and essential data documenting the efficacy of FVIIa based gene therapy for coagulation defects. Several aspects of the generated data (such as transgene and vector constructs developed in Aim 2) are now used in preclinical studies in mice and dogs that aim to optimize the researcher's FVIIa gene transfer approach. Such approaches are either targeting the liver (as in this grant) or other tissues that can support secretion of coagulation factors (e.g., muscle). Notably, muscle is the target tissue in the reseacher's recently awarded NIH grant "*Gene therapy for hemophilia using muscle-expressed FVIIa*". Moreover, reagents that the reseacher has used in Aim 1 are also utilized in the grant "*Cell surface interactions with FVIIa during FVIIa-enhanced hemostasis*" that the researcher plans to re-submit as a new grant looking at the mechanism of action of FVIIa in hemophilia. Lastly, as a result of the encouraging data in Aim 3, the researcher plans to further extend the gene transfer approach for FVII deficiency, utilizing a bigger cohort of FVII deficient dogs that will give a larger set of pre-clinical data. Such work will also form part of a future grant application.

**13. New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X_____ No_____

If yes, how many students? Please specify in the tables below:

| | Undergraduate | Masters | Pre-doc | Post-doc |
|---|---|---|---|---|
| Male | | | | |

| | | | | |
|---|---|---|---|---|
| Female | | 1 | | |
| Unknown | | | | |
| **Total** | | **1** | | |

| | Undergraduate | Masters | Pre-doc | Post-doc |
|---|---|---|---|---|
| Hispanic | | | | |
| Non-Hispanic | | 1 | | |
| Unknown | | | | |
| **Total** | | **1** | | |

| | Undergraduate | Masters | Pre-doc | Post-doc |
|---|---|---|---|---|
| White | | 1 | | |
| Black | | | | |
| Asian | | | | |
| Other | | | | |
| Unknown | | | | |
| **Total** | | **1** | | |

**14. Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No_____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

**15. Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____ No_____X_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

**16. Collaboration, business and community involvement.**

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes____X_____ No_____

If yes, please describe the collaborations:

Dr. Timothy Nichols is the Director of the Francis Owen Blood Research Laboratory at the University of North Carolina Chapel Hill. Dr. Nichols has been a long-standing collaborator of Dr. Katherine A. High on multiple projects that involve protein infusions or gene transfer with recombinant adeno-assocoiated viral vectors in hemophilia A and B dogs located at his dog colony (Francis Owen Blood Research Laboratory). Dr. Nichols has extensive experience in performing gene transfer experiments as well as obtaining and analyzing samples from gene transfer treated dogs. In addition to the hemophilia A and B dogs that Dr. Nichols regularly maintains and were planned to be used in Aim 2, he has recently managed to obtain dogs with FVII deficiency, that were used in Aim 3. Specifically, Dr. Nichols infused a recombinant adeno-associated viral vector to a FVII deficient dog and collected blood samples (pre- and after treatment with viral vector) for analysis. This analysis consisted of determination of in vitro parameters that were performed at his laboratory. He also sent these samples to the researcher's laboratory for further analysis. Collectively, the work in here utilized Dr. Nichols's facility and experimental animals, as well as animal procedures and analyses related to vector infusion and collection of blood/tissue samples (prior/after vector infusion).

Dr. Timothy C. Nichols (MD)
Professor of Medicine and Pathology
Director, Francis Owen Blood Research Laboratory
Francis Owen Blood Research Laboratory
Department of Pathology and Laboratory Medicine
UNC School of Medicine
125 University Lake Rd. CB# 3114
Chapel Hill, NC 27516

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

**17. Progress in Achieving Research Goals, Objectives and Aims.**

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

**There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.**

**Project Goals:**

A major complication in the treatment of hemophilia (Factor VIII [FVIII] or Factor IX [FIX] deficiency) using protein replacement is the development of neutralizing antibodies against the infused factor (FVIII or FIX) that occurs in a considerable number of severe cases of hemophilia. Such patients, as well as those with congenital FVII deficiency or inherited platelet disorders, have been treated successfully with recombinant human activated Factor VII (rFVIIa). An alternative to protein-based treatment is a gene-based approach where FVIIa is the therapeutic *transgene product*. Such an approach uses continuous expression of FVIIa and, as a result, can address several shortcomings of the existing protein-based treatment: short half-life, multiple dosing and high treatment cost. The researcher has previously demonstrated the efficacy of such a gene–based approach in small (mice) as well as large (dogs) animal models of hemophilia. A

viral vector (adeno-associated virus [AAV]) was utilized as the delivery vehicle. In such work, continuous expression of FVIIa resulted in an improvement of the hemophilic phenotype. The goal of this Project was to provide additional preclinical data on the use of activated coagulation Factor VII (FVIIa) in a gene therapy setting for the treatment of coagulation defects.

**Project Objectives:**

The researcher's previous work with FVIIa as the therapeutic transgene in a gene-based approach provided proof-of concept data. However, aspects of this work as they pertain to a potential clinical application of this gene-based approach require additional feasibility data in settings that mimic those used with recombinant FVIIa. This encompasses two such settings: (1) hemophilia patients with neutralizing antibodies to Factor VIII or Factor IX; (2) patients with rare inherited platelet disorders. The first Aim of this Project was to address the feasibility of a FVIIa gene-based approach in such settings.

The researcher's previous work expressing FVIIa in dogs with hemophilia demonstrated efficacy that was dependent on the AAV vector dose. Assuming hemophilic dogs can predict the therapeutic dose in humans, the required effective dose for a human would impose a significant complication in terms of vector manufacture. Moreover, it would also risk a vector-directed immune response from the recipient (human). Therefore, it is important to investigate methodologies to optimize FVIIa transgene expression and reduce the therapeutic AAV vector dose. The second aim of this Project focused towards this objective.

In addition to hemophilia, FVII deficiency is also treated with recombinant FVIIa, but at much lower doses. As a result, a gene-based approach with FVIIa would require much lower vector doses than those that the researcher has used in previous work with hemophilic dogs. Moreover, since FVII deficient patients lack FVII (and as a direct result FVIIa), even non-activated FVII could be used as the therapeutic transgene. Such a transgene would offer an additional advantage over FVIIa since non-activated FVII escapes functional inhibition by circulating inhibitors that can otherwise inhibit FVIIa. Therefore, using non-activated FVII (zymogen FVII) as a transgene would also afford better expression for a given AAV vector dose vs. FVIIa or, stated differently, similar expression to FVIIa but at a lower AAV vector dose. The researcher has previously described a dog model of FVII deficiency that would be the ideal recipient to demonstrate the efficacy of a FVIIa or non-activated FVII gene-based approach. The last Aim of this Project focused towards this objective.

**Project Aims:**

(1) Using AAV-injected mice, determine whether continuous expression of FVIIa can result in phenotypic improvement in mice with inhibitors to FVIII or FIX or in mice with inherited platelet disorders.
(2) Using hemophilia mouse and dog models, generate and compare optimized vectors for delivery and expression of FVIIa that will result in a lower dose required for hemostatic efficacy.
(3) Using a dog model of FVII deficiency, determine whether continuous expression of FVIIa or zymogen FVII that can result in phenotypic correction without adverse effects.

**Project Progress: AIM 1**

This Aim is divided into two sub-aims, focusing on the feasibility of FVIIa gene transfer in (1) a setting of hemophilia with inhibitors and (2) a setting of a platelet defect. The researcher will summarize the progress for each sub-aim below:

Sub-aim 1

The researcher wanted to utilize a mouse model of hemophilia that would have inhibitory antibodies to either human Factor VIII (FVIII) or Factor IX (FIX). Subsequently, the researcher would administer an AAV driving the expression mouse FVIIa (mFVIIa), termed AAV-mFVIIa (Figure 1). The choice of the species of the FVIIa transgene was such that the recipient mice would not mount an immune response to the transgene product (mice are tolerant to mouse FVIIa).

**Figure 1**. AAV vector expressing mFVIIa (AAV-mFVIIa). ITR: inverted terminal repeat; hAAT/ApoE: human alpha1-antitrypsin promoter/apolipoprotein A enhancer; intron: synthetic intron; pA: polyadenylation signal

Following AAV-mFVIIa administration, long-term evaluation of the effects of mFVIIa expression would ensue, using in vitro and in vivo assays that the researcher had previously established. As a first step, the researcher established a working protocol so that hemophilic mice can generate an inhibitory immune response to FIX. This utilized administration of human FIX in complete and incomplete Freund's adjuvant, as shown in Figure 2A and measured anti-FIX IgG levels as well as levels of FIX inhibition by this IgG (in Bethesda units). One Bethesda unit (BU) is the amount of inhibitor that inhibits 50% of human FVIII or FIX in normal plasma. The researcher established a protocol for generating long-lasting (for at least 11 weeks) inhibitory antibodies to human FIX, as shown in Figure 2B. Subsequently, the researcher administered 1.2E12 vector genomes (vg)/mouse to hemophilia B mice with inhibitors to human FIX and monitored expression of mFVIIa using a clotting-based assay (activated partial thromboplastin time [aPTT]). The researcher also monitored the titer of the inhibitory antibodies to human FIX after AAV-mFVIIa administration. The researcher found that such titer did not diminish after AAV-mFVIIa administration (last time assayed was 9 weeks post AAV-mFVIIa administration), as shown in Figure 2C. Moreover, expression of mFVIIa even in the presence of inhibitors to human FIX still resulted in a shortening of aPTT (Figure 2D). This indicates that the effect of mFVIIa expression in such mice is unaffected by the presence of anti-human FIX inhibitory antibodies.

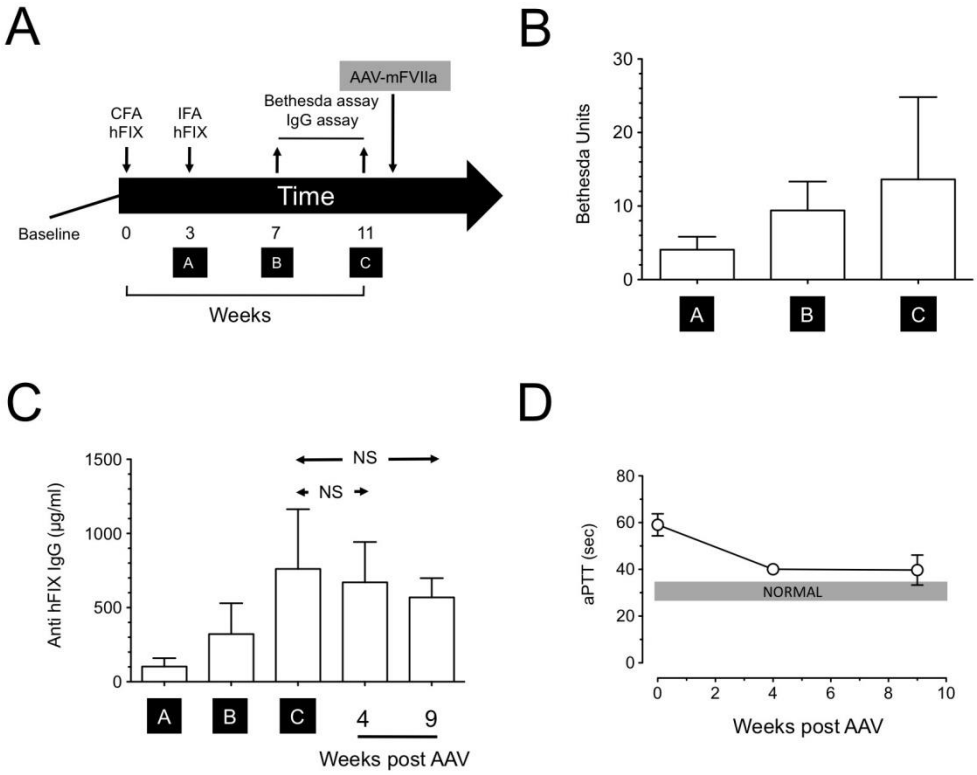



**Figure 2**. Development of inhibitory antibodies to human Factor IX in hemophilia B mice and administration of AAV-mFVIIa. **(A)** Timeline of experimental procedure. Complete (CFA) or incomplete (IFA) Freund's adjuvant is co-administered with human FIX (hFIX) at the specified timepoints (downwards arrows). Sampling (upwards arrows) is used to determine IgG levels and BU titers. AAV-mFVIIa administration follows last sampling timepoint. **(B)** Bethesda units of inhibitory antibodies to hFIX in HB mice at the specified timepoints shown in (A). **(C)** Total anti-hFIX IgG in HB mice with inhibitory antibodies to hFIX. NS: non-significant difference. **(D)** aPTT in mice with inhibitory antibodies to hFIX following AAV-mFVIIa administration. Hemostatically normal mice have an aPTT of ~ 30sec, as shown by the grey box.

Since severe hemophilia A patients have a larger chance of inhibitory antibody formation than hemophilia B (~30% vs. ~5%, respectively), the researcher wanted to demonstrate the efficacy of mFVIIa administration in hemophilia A mice with inhibitors to human FVIII. Initial experiments with weekly administration of human FVIII showed that 54% of hemophilia A mice mounted an inhibitory response to human FVIII with a titer of > than 5 BU. However, this response was not sustained. Further attempts to improve this inhibitory response to FVIII using a similar approach shown for FIX in Figure 2A was unsuccessful with mice showing a very low response to the antigen, for reasons unknown (Figure 3). Despite this setback, the obtained data from hemophilia B mice with inhibitors to human FIX represent an important finding that fulfills the goal of this sub-aim, suggesting that a gene-based FVIIa approach in the setting of hemophilia inhibitors remains efficacious.

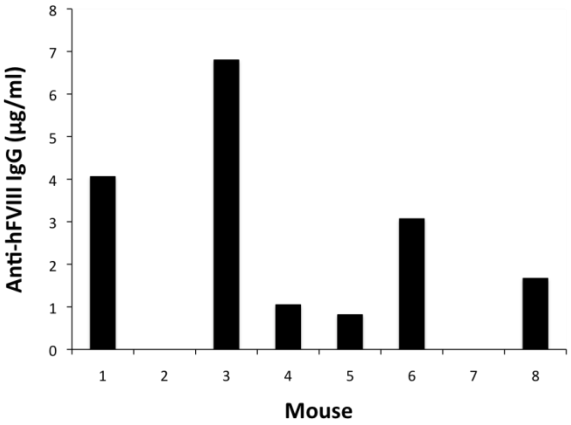



**Figure 3**. Total IgG against hFVIII assayed 2 weeks post IFA-human FVIII administration in hemophilia A mice. The researcher attempted to generate inhibitors in mice with hemophilia A, using infusion of recombinant human coagulation Factor VIII (ReFacto®, Wyeth Pharmaceuticals) complexed with Freund's complete and, subsequently, incomplete adjuvant (CFA and IFA, respectively), as described in Figure 2A. Two weeks post IFA administration, the researcher collected blood from the mice for determination of total anti-FVIII IgG, using an enzyme-linked immunosorbent assay. The results shown in here demonstrate a substantially low response to the antigen in the range of ~0-4 µg/ml, approx. This is ~100 fold lower than the researcher observed in similar experiments using human Factor IX with hemophilia B mice (compare to Figure 2C).

Sub-aim 2

Inherited platelet disorders can be as debilitating as hemophilia and they result from mutations in a variety of genes that form essential receptor complexes on the surface of platelets. As a result, there is no single protein replacement therapy. Rather, they rely on management of bleeds that is most effective by platelet transfusions. Patients with Bernard-Soulier syndrome (BSS) have a defective platelet adhesion to the vessel wall following injury as a result of a dysfunctional glycoprotein Ib-IX-V complex (present on the platelet surface). On the other hand, patients with Glanzmann's thrombasthenia (GT) have defective platelet aggregation due to a dysfunctional complex of glycoprotein IIb/IIIa (also expressed on the platelet surface). Despite the lack of protein replacement therapies, case reports of successful treatment of bleeds in such patients with bolus infusion of recombinant FVIIa suggests the possibility that a gene-based FVIIa approach may also be suitably effective. To demonstrate this, the researcher utilized two mouse models of platelet defects with the intention of administering an AAV vector expressing mFVIIa and then monitoring the effects of continuous mFVIIa expression using established in vitro and in vivo assays.

Mice that have a deletion in the glycoprotein Ib alpha (GPIbα) or GPIb beta (GPIBβ) subunit of the glycoprotein Ib/X/V complex represent a mouse model BSS. The researcher used an existing mouse colony of BSS mice in the researcher's facility to establish baseline hemostatic parameters prior to the administration of AAV expressing mFVIIa, shown in Table 1 below:

| Mouse (# of mice sampled) | Prothrombin time (sec) [average ± 1 standard deviation (SD)] [1:160 dilution] | Prothrombin time (sec) [average ± 1 standard deviation (SD)] [1:320 dilution] | Platelet count in K/µl (average ± 1 SD) |
|---|---|---|---|
| BSS (14) | 26.5 ± 2.2 | 30.1 ± 3.1 | 325.5 ± 94.1 |

**Table 1.** Baseline parameters of BSS mice. Plasma dilutions were used for prothrombin time measurements, as indicated

Subsequently, BSS mice were either administered an AAV (serotype 8) expressing mFVIIa from a liver-specific promoter/enhancer (entire construct is termed AAV-mFVIIa, Figure 1) or were untreated. A vector dose of 1.2E12 vg/mouse was used in the treated BSS cohort. Mice were monitored for mFVIIa expression through the use of a prothrombin time (PT) assay that is sensitive to levels of FVIIa in the plasma. The researcher had previously shown that overexpression of mFVIIa in hemophilic mice results in increased mortality due to thrombosis (Aljamali MN et al., J Clin Invest 2008 May;118[5]:1825-34). Therefore, the safety of the AAV-mFVIIa treatment was monitored by platelet counts as well as levels of thrombin-antithrombin (TAT) complexes. Excessive coagulation in the recipient mice from the AAV-delivered mFVIIa would result in a drop in platelet counts and an increase in TAT levels. Lastly, functional correction of the BSS phenotype was assessed by a tail clip assay; BSS mice show an excess of blood loss in that assay. The results of the AAV-mFVIIa experiment are shown in Figure 4. They demonstrate 3 important findings: (1) expression of mFVIIa was long-term and maintained at relatively stable levels, as shown by sustained reduction of the PT. Of note, the reduction of the PT is due to the presence of mFVIIa in mouse plasma that result in a faster clot time. (2) Despite its procoagulant nature, mFVIIa expression did not result in an increase in TAT levels (determined at 9 weeks post AAV administration) or further change of platelet counts. (3) Expression of mFVIIa resulted in statistically significant reduction in blood loss following tail clip, performed at 11 weeks post AAV administration.

Given the encouraging results from BSS mice, the researcher investigated the effects of mFVIIa expression in GT mice. The researcher obtained GT as well as hemostatically normal mice from heterozygous GT mouse breedings. Such mice were utilized in experiments where an AAV serotype 8 vector expressing mFVIIa (AAV-mFVIIa, Figure 1) was administered intravenously via the tail vein (1.2E12 vg/mouse). Three cohorts of mice were used: GT mice treated with AAV-mFVIIa (GT-AAV); GT untreated; wildtype littermates untreated. As a measure of mFVIIa expression, the researcher used a PT assay and hemostatic effects of continuous expression of mFVIIa were monitored by a tail clip assay. These results are shown in Figure 5 and point to 3 findings: (1) AAV delivery of mFVIIa resulted in sustained expression of mFVIIa in GT mice (based on the reduction on the PT assay); (2) there was no statistical difference in the amount of blood loss between AAV-mFVIIa treated and untreated GT mice (tail clip assay); (3) expression of mFVIIa did not result in changes in survival of the AAV-treated mice.

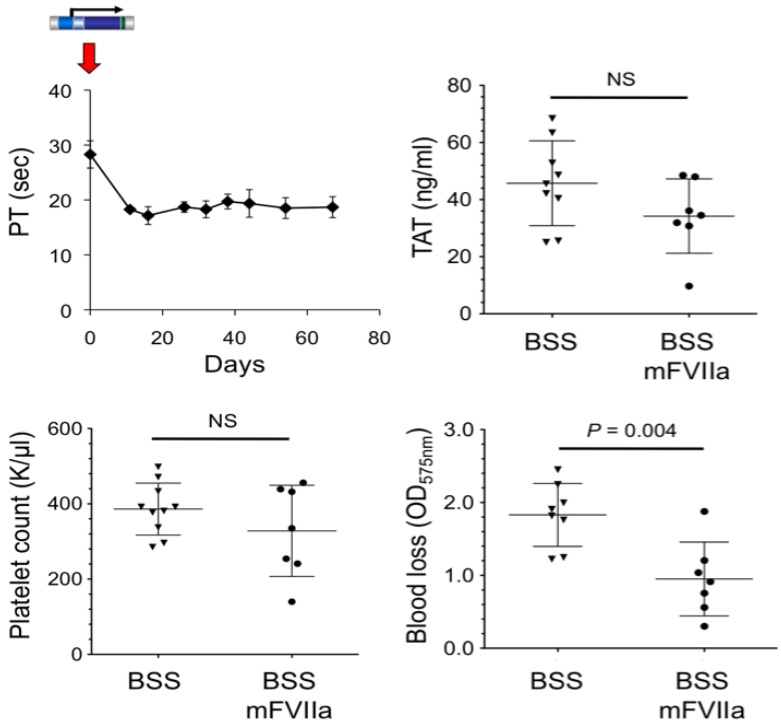



**Figure 4**. BSS male mice were injected with 1.2E12 vector genomes/mouse of AAV-mFVIIa (serotype 8) via the tail vein (red arrow). Untreated mice were used as controls (BSS). Expression of mFVIIa in AAV-mFVIIa injected mice (BSS-mFVIIa) was assessed by a PT assay. Measuring TAT levels and platelet counts monitored the safety of this approach (both assayed at 9 weeks post-AAV administration). The phenotypic effects of mFVIIa expression were assessed by a tail clip assay that measures blood loss over a 10-minute period following tail transection (3mm diameter).

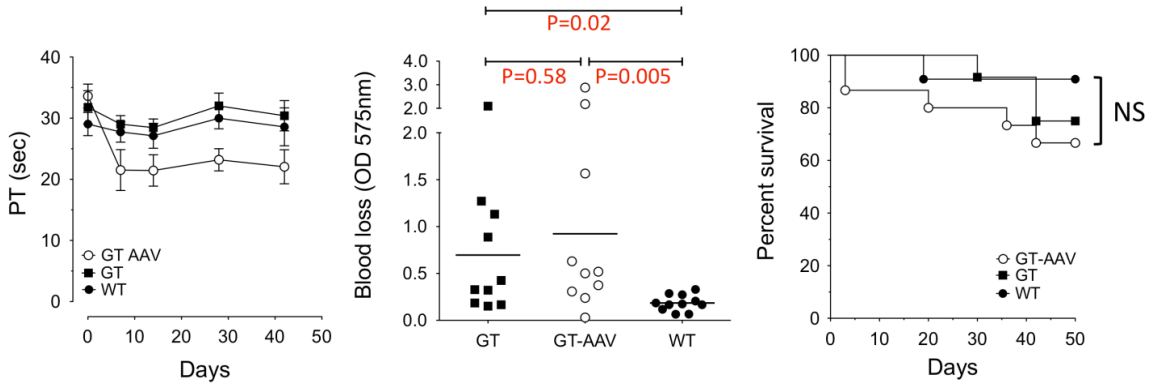



**Figure 5**. AAV-mFVIIa was administered IV in mice with GT (1.2E12 vector genomes/mouse; GT-AAV). Wild type littermates or untreated GT mice were used as controls. Murine FVIIa expression following AAV administration resulted in supraphysiological reduction in the PT. Despite this, GT-AAV mice did not show an improvement in the tail clip assay (blood loss) compared to untreated GT controls. Continuous expression of mFVIIa did not result in changes in survival among the cohorts for the duration of observation.

Overall, these data clearly suggest that sustained expression of mFVIIa in a setting of GPIb deficiency via AAV-delivery results in a phenotypic improvement without resulting in excessively procoagulant state (at least as monitored by TAT/platelet counts). This supports the therapeutic potential of FVIIa gene delivery in patients with GPIb deficiencies. However, no phenotypic beneficial effects of continuous expression of mFVIIa were observed in mice with Glanzmann's thrombasthenia, suggesting that the effects of mFVIIa were insufficient to enable hemostasis (no change in blood loss). As a result, this mode of therapy may not be optimal for GT. Since the overall aim of this work was to evaluate the potential of FVIIa delivery by AAV for the treatment of platelet defects, the researcher has reached these goals.

Project Progress: AIM 2

Optimized vectors for FVIIa gene transfer

The researcher constructed an AAV vector backbone that, upon AAV packaging, will result in a AAV vector with a double-stranded genome, as shown in Figure 6A. Given that the vector backbone did not have an intron (to enhance exression of the cFVIIa transgene), the researcher included the native intron 3 from the canine *F7* gene. In order to test the expression cassette in scAAV-cFVIIa-CO, the researcher injected the plasmid scAAV-cFVIIa-CO in C57BL/6 mice using hydrodynamic tail vein injection. The AAV-cFVIIa plasmid was used as control and expression of cFVIIa was monitored by PT. As shown in Figure 6B, the scAAV-cFVIIa-CO plasmid expresses a functional cFVIIa, confirming the functionality of the expression cassette in that construct. However, there was still a possibility that the processing of the included intron was sub-optimal, something that would result in reduced cFVIIa expression. To test this, the researcher administered 3.52E11 vector genomes/mouse of the scAAV8-cFVIIa-CO in hemostatically normal mice, harvested their livers at 8 weeks post AAV administration and extracted total RNA. Reverse transcriptase PCR from that RNA template using specific primers showed that the cFVIIa intron was properly and nearly completely spliced, as indicated by the lack of or very low signal of the unspliced mRNA (Figure 7).

In order to assess performance of the scAAV-cFVIIa-CO, the researcher used hemostatically normal mice administered with scAAV-cFVIIa-CO at a variety of doses (3.52E9 - 3.52E11 vg/mouse), compared to a single dose of single stranded AAV-cFVIIa (3.52E11 vg/mouse). The researcher used a prothrombin time clotting based assay as a measure of transgene expression and found that the scAAV-cFVIIa-CO administered at a dose of 3.52E10 vg/mouse (green arrow, Figure 8A) gave similar reduction in the prothrombin time vs. AAV-cFVIIa administered at a 3.52E11 vg/mouse dose (red arrow, $P>0.05$, Figure 8A), thereby offering a 10 fold vector dose advantage. Since the researcher used hemostatically normal mice, the prothrombotic potential of cFVIIa or cFVIIa-CO expression was monitored by changes in the platelet counts. A drop in platelet counts in the vector injected mice would indicate a prothrombotic state. However, the researcher did not observe any changes among the mouse cohorts ($P>0.5$, Figure 8B).

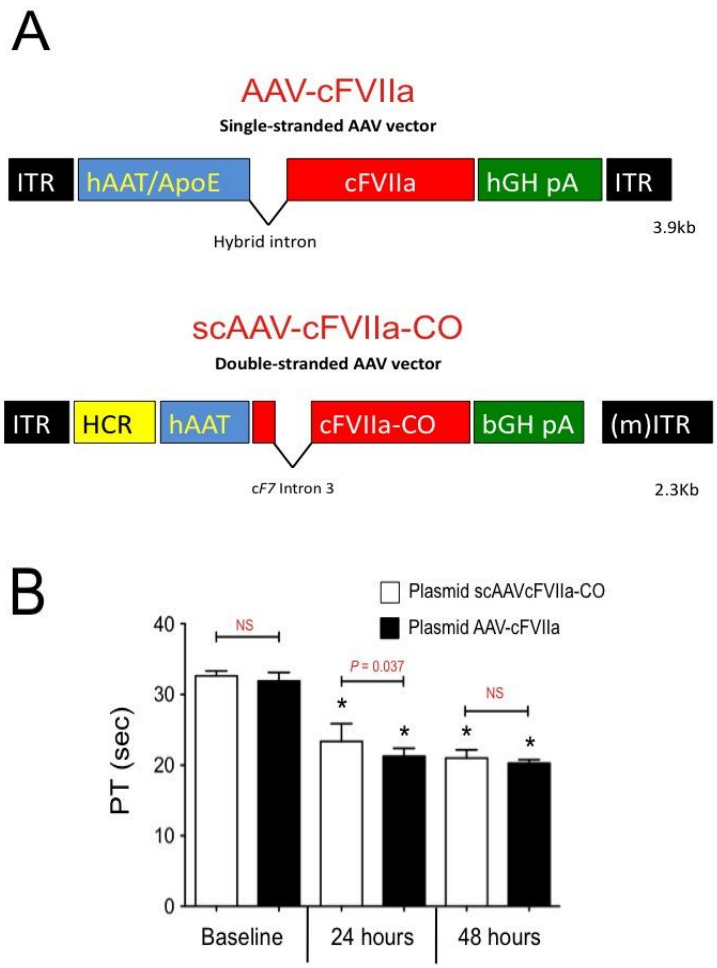



**Figure 6**. **(A)** Schematic representation of the single stranded (AAV-cFVIIa) and double stranded AAV vector (scAAV-cFVIIa-CO) expressing canine FVIIa (cFVIIa). This vector contains the human α1 antitrypsin promoter/apolipoprotein E enhancer (hAAT/ApoE), a hybrid intron, a polyadenylation signal (pA) from human growth hormone (hGH) and the whole expression cassette is flanked by inverted terminal repeats (ITRs). The double stranded AAV vector designed by the researcher, contains a hepatic control region (HCR) from ApoE (acting as an enhancer), a hAAT promoter, a codon optimized cFVIIa transgene that incorporates the wildtype intron 3 from the canine F7 gene (cFVIIa-CO) and a pA signal from bovine growth hormone (bGH). The 5' ITR is wildtype (from AAV serotype 2) whereas the 3' ITR is from AAV serotype 4 and is mutated so that during packaging the resulting AAV genome is in a double stranded form. **(B)** C57BL/6 mice were administered a hydrodynamic injection of the scAAV-cFVIIa-CO and AAC-cFVIIa (control plasmid). Plasma after 24h and 48h was collected and expression was measured by a PT clotting assay. P values from a Student's t-test between the two cohorts of mice are shown. Asterisk denotes $P<0.05$ vs baseline for each plasmid tested. NS denotes non-significant differences.

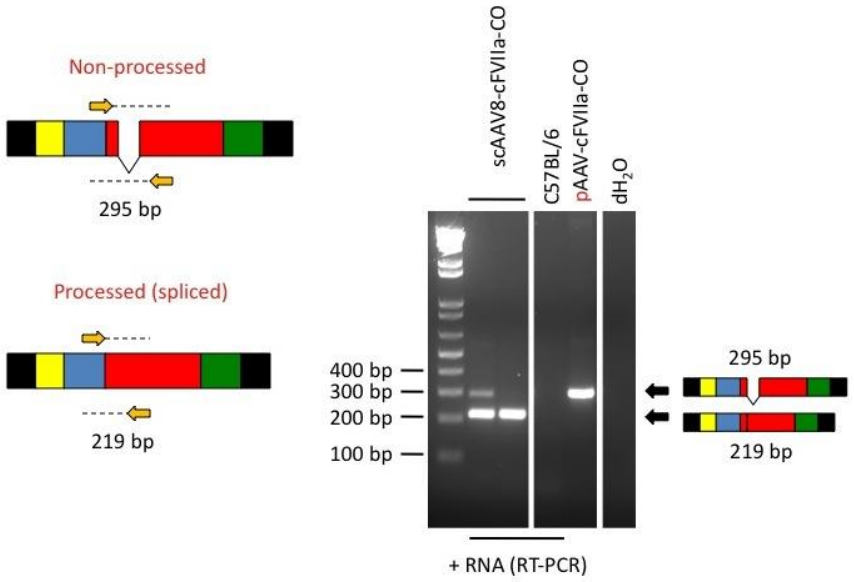



**Figure 7**. Thirty ng of total liver RNA from hemostatically normal mice injected with scAAV-cFVIIa-CO (serotype 8) to perform an RT-PCR using appropriate primers (orange) spanning the c*F7* intron. In this reaction, the size of the non-processed (unpliced) and processed mRNA (splicing of c*F7* intron) are indicated. The researcher shows 2 representative AAV-injected mice in addition to negative control mice (uninjected C57BL/6 mice) and water ($dH_2O$). As a positive control for the size of the unprocessed mRNA, the *plasmid* of scAAV-cFVIIa-CO (pAAV-cFVIIa-CO) was used.

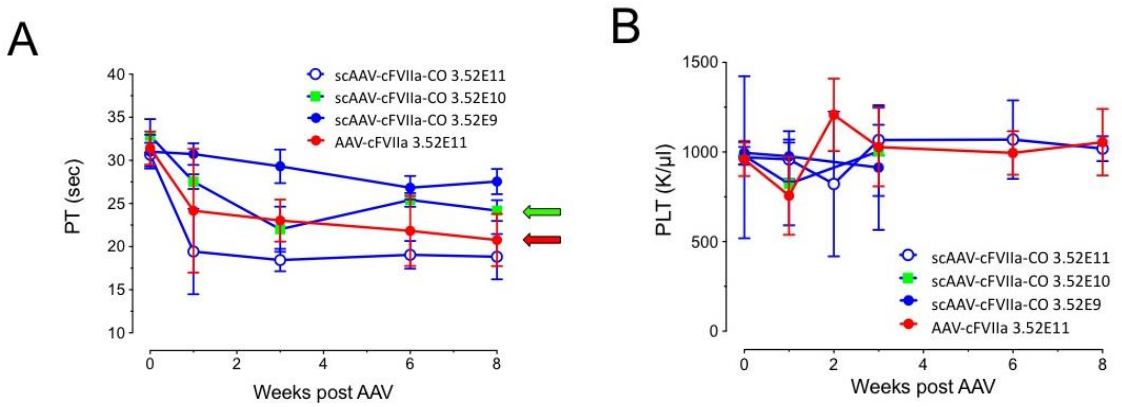



**Figure 8.** **(A)** Hemostatically normal mice were injected with scAAV-cFVIIa-CO or single stranded AAV-cFVIIa. Vector doses are shown in vg/mouse. Prothrombin time (PT) was used as a measure of transgene expression. **(B)** Platelet count (PLT) measurements in hemostatically normal mice were injected with scAAV-cFVIIa-CO or single stranded AAV-cFVIIa. Vector doses are shown in vg/mouse.

Collectively, the data obtained suggest that the combination of AAV conformation (self-complementary), promoter/enhancer and transgene modification resulted in an improved performance, as assessed by cFVIIa expression. Therefore part of the goals set forth in this Aim was reached. The researcher did not assess how the optimized scAAV-cFVIIa-CO performs in

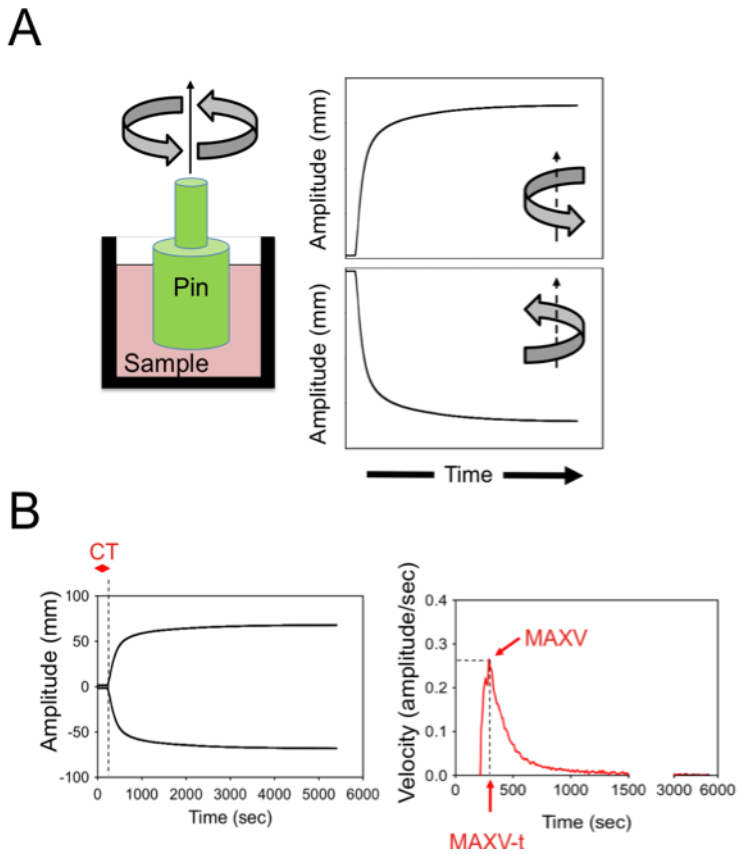
hemophilia mice or in hemophilic dogs. One reason for this was the fact that the yield of the scAAV-cFVIIa-CO preparation was very low, compared to yields of the single-stranded AAV-cFVIIa vector. This is a common attribute of self-complementary vector preparations. A new batch of the scAAV-cFVIIa-CO was initiated but was not evaluated in hemophilic mice or dogs within the period of this grant. However, given the researcher's past experience with the AAV-cFVIIa expression in hemophilic mice and dogs (Margaritis P et al., Blood 2009 Apr 16;113[16]:3682-9), the researcher would expect the beneficial effects of scAAV-cFVIIa-CO (in terms of vector dose administered) to hold true for hemophilic dogs and mice administered with that vector.

Project Progress: AIM 3

Congenital deficiency in FVII is a life-threatening bleeding disorder. The mode of treatment of FVII deficiency is by infusion of fresh-frozen plasma, plasma-derived FVII (pdFVII) concentrates (prothrombin complex concentrates [PCC] with higher FVII content) and recombinant FVIIa. In general, hemostasis can be achieved with only a mild elevation of plasmatic FVII levels to reach above 10-15% normal. In contrast to hemophilia, much lower-doses of recombinant FVIIa are necessary to achieve therapeutic efficacy (15-30µg/kg vs. 90-270µg/kg in hemophilic patients with inhibitors). From a FVIIa gene transfer standpoint, FVII deficiency has two advantages over hemophilia: (1) the lower dose of recombinant FVIIa for efficient treatment would translate to a lower FVIIa expression hence a lower dose of AAV in a gene therapy setting; (2) the therapeutic transgene can be unactivated (*zymogen*) FVII (vs. activated FVII), thus avoiding any adverse events related to the procoagulant properties of activated FVII (FVIIa). The researcher previously identified beagles with congenital FVII deficiency (FVII activity <2%) due to a G96E mutation that affected FVII secretion (Callan MB et al., J Thromb Haemost 2006 Dec;4[12]:2616-22). A colony was established at Dr. Timothy C. Nichols facility at the University of North Carolina at Chapel Hill. The goal of this aim was to demonstrate the efficacy of continuous expression of cFVIIa or cFVII zymogen via AAV delivery in dogs with congenital FVII deficiency.

The researcher decided to utilize an AAV serotype 8 vector expressing the zymogen canine FVII (cFVII) as the therapeutic transgene. This would avoid any adverse procoagulant events of using a cFVIIa transgene. In terms of measuring transgene expression in the AAV-treated FVII deficient dogs, the researcher would use two types of assays: prothrombin time assay and rotational thromboelastometry (ROTEM). The prothrombin time assay has been previously used by the researcher in Aims 1 and 2. ROTEM analysis is usually done on whole blood and measures several parameters of clot formation (such as elasticity) that cannot be obtained in traditional clotting based assays (such as PT). The assay is shown diagrammatically in Figure 9A. Due to the inability to have fresh blood samples from the AAV-treated dogs (they are located in the facility of Dr. Nichols in North Carolina), the researcher decided to setup a ROTEM assay using plasma samples. Preliminary ROTEM analysis using normal or cFVII deficient plasma in ROTEM assays with tissue factor as the activator of coagulation, allowed the researcher to establish two clot parameters that were significantly different between such plasma samples: clot time (CT) and time to maximum clot velocity (MAXV-t). Those two parameters are described in Figure 9B. The researcher subsequently established a relationship between cFVII activity and CT or MAXV-t using cFVII deficient plasma samples spiked with variable amounts of normal canine plasma to give defined % of cFVII activity. These results are shown in Figure 10A-C and demonstrate <u>an inverse relationship between cFVII activity and CT/MAXV-t parameters, a natural consequence of accelerated clot formation</u>.

Having established these assays, the researcher manufactured an initial AAV serotype 8 preparation expressing cFVII zymogen, shown in Figure 11A. The researcher intended to utilize hemophilia A or B mice to test the AAV vector preparation. However, due to time constrains in this research proposal and the availability of FVII deficient dogs, the researcher decided to test the AAV vector preparation directly in FVII deficient dogs.




**<u>Figure 9</u>. (A)** In a rotational thromboelastrometry instrument the sample is placed in a cup and following activation (with tissue factor for example), a pin oscillates clockwise/anticlockwise. As the clot develops, its elasticity changes and is translated as clot amplitude, for each direction of movement (positive/negative). **(B)** In the resulting thromboelastogram (combined clot amplitudes), the clot time (CT) is the time to initial fibrin formation (Left). The first derivative of the clot amplitude (velocity) can be obtained (right) and the peak is called maximum velocity (MAXV). The time to reach MAXV is called MAXV-t.

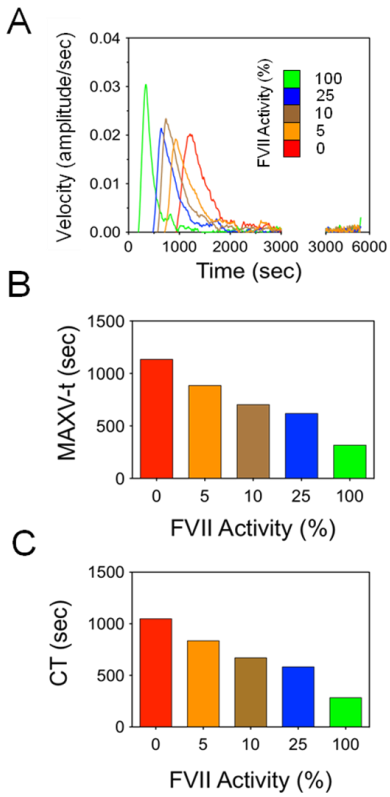



**Figure 10.** Using plasma mixtures of defined cFVII activity, rotational thromboelastometry was performed with human TF as the initiator. A graph showing velocity vs. time **(A)** and the bar chart of derived MAXV-t values **(B)** shows that MAXV-t descreases as cFVII activity increases. In a graph of CT vs. FVII activity **(C)**, CT decreases as cFVII activity increases.

The researcher administered the AAV-cFVII vector to a male FVII deficient dog at a dose of 4.95E13 vg/kg. This vector dose is within the vector dose of AAV-cFVIIa given to hemophilia dogs in the researcher's previous study (Margaritis P et al., Blood 2009 Apr 16;113[16]:3682-9), that resulted in robust expression of cFVIIa. The FVII deficient recipient dog was covered with normal canine plasma immediately prior to and after vector administration (100cc each time) as well as daily (50cc) for three days. Samples taken following AAV delivery were used to determine cFVII expression, using PT and ROTEM assays. As it can be seen in Figure 11B-D, AAV delivery of cFVII zymogen resulted in robust expression that caused a drop in the abnormal PT, CT and MAXV-t parameters *below* normal values (wildtype). This observation was sustained at relatively stable levels for at least 70 days (the observation is ongoing). These data point to two major findings: (1) AAV-mediated delivery can result in robust cFVII expression; (2) the AAV vector dose can likely be reduced further to result in normal clot parameters or even at levels of cFVII expression of 15-30% that have been show to be sufficient for treatment of FVII deficient patients.

The overall result of the experiments in Aim 3 clearly demonstrate the therapeutic potential of AAV-mediated gene transfer of zymogen FVII in a setting of FVII deficiency. Testing of the AAV-cFVII vector was not performed in hemophilia mice therefore that milestone was not

accomplished. Also, due to time constraints, the researcher did not perform additional dog experiments with lower AAV-cFVII vector doses. These are deficiencies in the goals set for Aim 3. However, the researcher acquired pre-clinical efficacy data in a large and relevant model of FVII deficiency. Thereby, the researcher believes that the impact of the large animal data obtained overrides at least the lack of hemophilic mouse data. Overall, the goals in Aim 3 were partially reached but the critical experimental validation of using gene transfer of FVII for treatment of FVII deficiency was acquired.

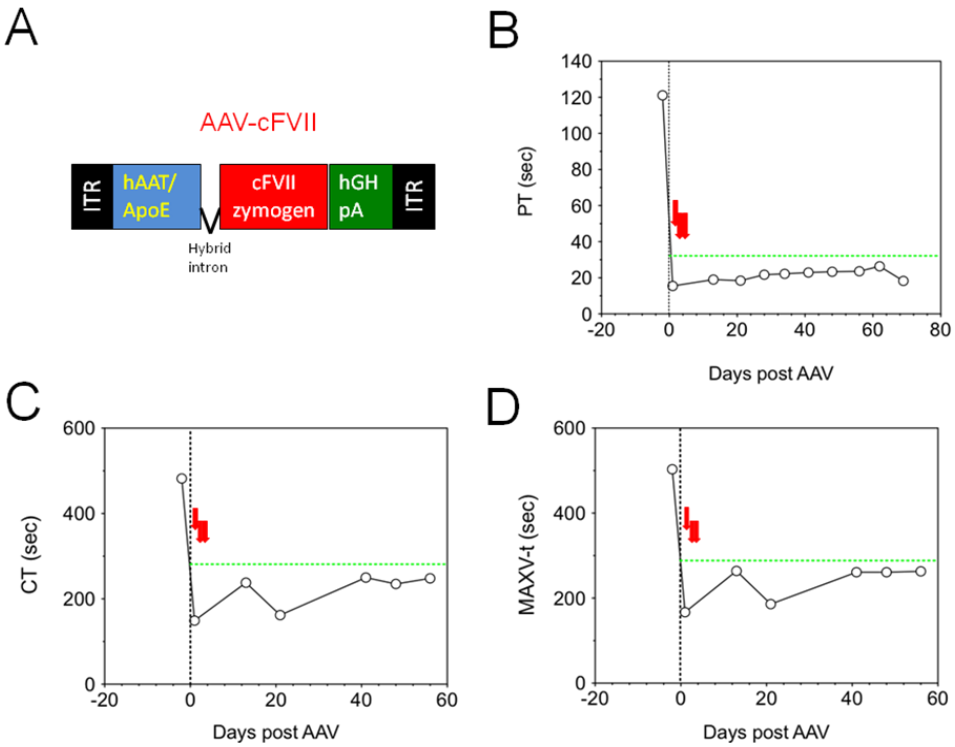



**Figure 11**. Determination of efficacy in the FVII deficient dog treated with AAV serotype 8 expressing cFVII (shown diagrammatically in **[A]**). the AAV elements are the same as shown for AAV-cFVIIa in Figure 5A. AAV-cFVII was administered at day 0 (vertical dotted line) and samples were collected at various timepoints. Normal canine plasma was given prophylactically in the first few days after AAV administration (red arrows). A prothrombin time assay **(B)** and a ROTEM assay **(C-D)** were used to monitor cFVII expression. Two ROTEM parameters are shown in, clot time (CT) and MAXV-t. A green line signifies the value of a hemostatically normal dog for each assay.

SUMMARY OF OBTAINED RESULTS

The goal of this Project was to provide additional preclinical data on the use of activated coagulation Factor VII (FVIIa) or zymogen FVII in a gene therapy setting for the treatment of coagulation defects. Through Aim 1, the researcher demonstrated the efficacy of using continuous expression of FVIIa via gene transfer in the setting of hemophilia complicated with inhibitors. This is the clinical setting where recombinant FVIIa is currently used. Moreover, the researcher demonstrated that a FVIIa gene-based approach can also be used in a setting of a

platelet defect, although not in *all* platelet defect animal models tested. In itself, this is an important finding since inherited platelet disorders can result from mutations in multiple genes, hence protein replacement therapy is not available. In the second Aim, using cFVIIa as the therapeutic transgene, the researcher demonstrated that modification of the AAV expression cassette and DNA structure can result in a tenfold advantage over the existing AAV vector expressing cFVIIa. Although the researcher did not perform studies in hemophilic dogs utilizing this optimized AAV due to manufacturing issues (low yields), the researcher believes that the overall conclusions obtained still stand. This is an important result that will form the basis of future experiments in hemophilic dogs. Lastly, in Aim 3, the researcher used gene transfer of canine zymogen FVII in a dog with FVII deficiency. The particular transgene used addressed issues of thrombogenicity that may arise from using an *activated* FVII as a transgene. The researcher demonstrated long-term expression of canine FVII using a variety of in vitro assays. All such assays showed that a vector dose of 4.95E13 vg/kg resulted in supraphysiological expression/activity. Due to time limitations, the researcher did not perform an AAV administration in a FVII deficient dog at a lower vector dose. However, the obtained data are novel and clearly support the use of gene transfer of FVII zymogen for the long-term treatment of FVII deficiency.

Scientific abstracts containing work described in this project are shown below. Funding from the Pennsylvania Department of Health was properly acknowledged in all cases. The researcher would like to note that abstracts 1 and 2 were not previously included in the annual reports.

1. E. Roy, A. Faella, H.D. Downey, L. Ivanciu, S. Zhou, K. A. High and P. Margaritis. Efficacy and safety of continuous expression of an improved variant of FVIIa on murine hemostasis with or without inhibitors. *American Society of Hematology Annual Meeting*, December 5-8, 2009, New Orleans (LA), USA.
2. E. Roy, A. Faella, H.D. Downey, L. Ivanciu, S. Zhou, K. A. High and P. Margaritis. Catalytic domain variants for murine FVIIa: effects in hemostasis under conditions of continuous expression in vivo. *Gordon Research Conference in Hemostasis*, July 25 – July 30, 2010, Waterville Valley (NH), USA.
3. P. Margaritis, A. Faella, G. Pavani, L. Couto, S. Zhou, K. A. High. AAV Vector and Transgene Modifications for Improved Efficacy in Hemophilia Gene Therapy with Continuous Expression of FVIIa. *American Society of Gene and Cell Therapy* Annual Meeting, May 16-19, 2012, Philadelphia (PA), USA.

**18. Extent of Clinical Activities Initiated and Completed**. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

**If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed.** (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

**Note**: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

**19. Human Embryonic Stem Cell Research.** Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

**20. Articles Submitted to Peer-Reviewed Publications**.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

**Note:** The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

| Title of Journal Article: | Authors: | Name of Peer-reviewed Publication: | Month and Year Submitted: | Publication Status (check appropriate box below): |
|---|---|---|---|---|
| 1. Catalytic domain modification and viral gene delivery of activated factor VII confers hemostasis at reduced expression levels and vector doses in vivo | Margaritis P, Roy E., Faella A., Downey H.D., Ivanciu L., Pavani G., Zhou S., Bunte R.M., High K. A. | Blood | 9/2010 | ☐Submitted<br>☐Accepted<br>☒Published |

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

The researcher is planning to submit the data generated using the mice with platelet defects (Aim 1) as well as the data in Aim 3 when more timepoints and dog(s) have received AAV-expressing cFVII zymogen.

**21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project.** Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

**22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment**. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

This research project has led to several major discoveries: (1) demonstrated the efficacy of a gene transfer approach using activated FVII as the therapeutic transgene for hemophilia complicated with inhibitors (a common complication in hemophilia treatment); (2) demonstrated the efficacy of a gene transfer approach using activated FVII as the therapeutic transgene for GPIb deficiency (an inherited platelet defect); (3) the project has demonstrated efficacy of using continuous expression of zymogen FVII for the treatment of FVII deficiency, a rare bleeding disorder. These pre-clinical data may form the basis for larger studies that may ultimately lead to novel gene based treatments.

**23. Inventions, Patents and Commercial Development Opportunities.**

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

**24. Key Investigator Qualifications.** Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

## BIOGRAPHICAL SKETCH

| NAME | POSITION TITLE |
|---|---|
| **HIGH, Katherine H.** | Professor of Pediatrics |
| eRA COMMONS USER NAME<br>**KHIGHCHOP** | |

EDUCATION/TRAINING

| INSTITUTION AND LOCATION | DEGREE | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| Harvard College, Cambridge, MA | AB | 1972 | Chemistry |
| UNC School of Medicine, Chapel Hill, NC | MD | 1978 | Medicine |
| Yale Univ. School of Med., New Haven, CT | Fellow | 1990 | Hematology |

**A. Personal Statement**

My research career, continuously funded by the NIH since the early 1980's, has focused on the molecular basis of hemophilia and the development of novel therapeutics for this and other genetic diseases. Our laboratory defined the mutations responsible for hemophilia B in a number of patients, and in the canine hemophilia B model maintained at UNC-Chapel Hill. We have subsequently explored the use of recombinant adeno-associated viral (AAV) vectors to effect transfer of the *F9* gene into mice, hemophilic dogs, and humans with hemophilia B. We have used both skeletal muscle and liver as targets for gene transfer, and have shown long-term expression at therapeutic levels in both mouse and dog models of hemophilia B for both target tissues. We conducted the first clinical trial of AAV-mediated gene transfer to skeletal muscle, and showed that while we could demonstrate gene transfer and expression of Factor IX, the circulating F.IX levels were too low to ameliorate the disease phenotype. We also conducted the first clinical trial of AAV-mediated gene transfer to liver; here we demonstrated that we could achieve therapeutic levels of circulating Factor IX (~10% normal) but that expression lasted for only a period of weeks and was terminated by a $CD8^+$ T cell response to AAV capsid that destroyed transduced hepatocytes. We developed two strategies to address this issue, first, short-term immunosuppression to block the $CD8^+$ T cell response to capsid, and second, design and testing of a more efficient AAV vector, delivered at lower doses, to test the hypothesis that delivery of vector at a lower dose would avoid immune responses. The latter goal has recently been accomplished by investigators at University College London, and St. Judes Children's Research Hospital in a study in which we participated. In a very recent study, we have used zinc finger nucleases to edit the genomic sequence, and have successfully used AAV vectors to deliver ZFNs to mouse liver to correct a murine model of hemophilia B.

An additional line of investigation in the past 10 years was the development of a FVIIa gene-based approach for treating hemophilia complicated by inhibitory antibodies. Our lab pioneered this approach and established this mode of treatment, as evidenced by a dramatic improvement in the bleeding diathesis of hemophilic dog treated with AAV expressing canine FVIIa. Such methodology is currently the subject of ongoing optimization. More recently we have extended our studies to the conduct of clinical trials for other genetic diseases. The Center for Cellular and Molecular Therapeutics at CHOP, which I direct, sponsored a clinical trial of AAV-mediated gene transfer for the inherited retinal degenerative disorder Leber's congenital amaurosis due to RPE65 mutations. This led to improvements in visual/retinal function for all 12 subjects enrolled in the study; more recently we have successfully injected the other eye in three

of these twelve subjects, with plans underway to inject the second eye in the remaining eligible subjects. This trial was noteworthy for being the first gene therapy trial for a non-fatal disorder that was allowed to enroll pediatric subjects. Our group has had a longstanding interest in safety issues related to AAV vector administration, and we have published studies on germline transmission, risks related to immune response to vectors administered in vivo, and risk of vector integration in liver. Risks related to immune response are intimately tied to issues in vector manufacturing, which has been a strength of our research group.

**B. Positions and Honors**

Positions and employment

*1978-1979*, **Intern**, Medicine, North Carolina Memorial Hospital, Chapel Hill, NC; *1979-1981*; **Assistant Resident Physician**, Medicine, NC Memorial Hospital, Chapel Hill, NC; *1981-1984*, **Fellow in Hematology Section**, Medicine, Yale Univ. School of Med., New Haven, CT; *1984-1985*, **Instructor**, Hematology Section, Medicine, Yale Univ. School of Med., New Haven, CT; *1985-1992*, **Assistant and Associate Professor of Medicine and Pathology** and **faculty member**, Curriculum in Genetics, UNC School of Medicine, Chapel Hill, NC; *1985-1992*, **Director**, Clinical Coagulation Laboratory, and **Attending Physician**, University of North Carolina Hospitals, Chapel Hill, NC; *1992-present*, **Attending Physician**, Division of Hematology, The Children's Hospital of Phila., Phila., PA; *1992-1999*, **Associate Professor of Pediatrics and Pathology**, University of PA, Phila., PA; *1992-2000*, **Director**, Hematology/Coagulation Laboratories, The Children's Hospital of Philadelphia, *1992-2000*; *1992-2001*, **Member**, Graduate Group in Cell and Molecular Biology, University of PA, Phila., PA; *1995-3002*, **Director of Research**, Hematology Division, The Children's Hospital of Philadelphia, Phila., PA; *1999-present*, **William H. Bennett Professor of Pediatrics**, University of PA, Phila., PA; *2002-present*, Named as **Investigator, Howard Hughes Medical Institute**, Philadelphia, PA; *2004-present*, **Director**, Center for Cellular and Molecular Therapeutics, The Children's Hospital of Phila, PA

Honors, Awards and NIH Committees (selected):

*1968-1972*, **National Merit Scholar**; *1991*; **Elected to American Society for Clinical Investigation** (ASCI); *1994-1998*, **NIH, NHLBI, Member**, Hematology I Study Section; *1996*, **Elected to Association of American Physicians** (AAP); *2000*, **Scientific Co-chair**, American Society of Hematology, 42nd annual meeting; *2000*, **Elected as Fellow AAAS**, for "Distinguished contributions in the field of human gene therapy; *2000*, **National Hemophilia Foundation Researcher of the Yea**r Award; *2001-2005*, **Member**, FDA Cellular, Tissue and Gene Therapies Advisory Committee (CTGTAC); *2001-present*, **Member**, Editorial Board, Blood; *2001*, **Elected to American Clinical and Climatological Association**; *2002*, Named **Investigator, Howard Hughes Medical Institute**; *2004-2005*, **President**, American Society for Gene Therapy (ASGT); *2005-2008*, **Member**, NHLBI Advisory Council; 2005-2008, **Elected Councilor**, American Society of Hematology (ASH), member of Executive Committee; *2007*, **Elected to Institute of Medicine**; *2007-2009*, **Associate Editor**, Journal of Clinical Investigation; *2009-2011*, **Chair**, ASGCT Clinical Gene Transfer Comprehensive Review Course; *2010*, **Distinguished Career Award**, American Society of Gene and Cell Therapy; *2011*, **Elected to American Academy of Arts and Sciences**; *2011*, **Recipient**, TakeshiAbe Memorial Award and Lectureship, XXIII Congress of the ISTH; *2012*, **Recipient**, Leadership in Research Award, NHF; *2012*, **Recipient**, Martin Villar Haemostasis Prize Award, Grifols, S.A.

# BIOGRAPHICAL SKETCH

| NAME (LAST, First)<br>**MARGARITIS, Paris** | POSITION TITLE<br>Research Assistant Professor of Pediatrics |
|---|---|
| eRA COMMONS USER NAME<br>**MARGARITIS** | |

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| The University of Newcastle-upon-Tyne, U.K. | BSc | 1995 | Genetics |
| The University of Oxford, U.K. | DPhil (PhD) | 2000 | Molecular Biology/Gene Transfer |
| The Children's Hospital of Philadelphia | Post-doctoral | 2000-2005 | Gene Transfer for Coagulation disorders |

**A. Personal Statement**

My research uses basic biochemical, molecular as well as complex in vivo methodology for translational research for the treatment of coagulation defects. This pivots on a unique model that we have developed, whereby we can enhance the extrinsic pathway of coagulation by raising the circulating levels of *activated* FVII (FVIIa) in animal models, thereby effecting hemostasis. This is achieved by gene delivery of the FVIIa transgene. The benefit of using FVIIa as a therapeutic transgene is immunological (recipient is tolerant to FVIIa, compared to FIX or FVIII gene transfer strategies for hemophilia) but it also enables the broader application spectrum of FVIIa gene transfer that includes inherited bleeding disorders where no protein replacement therapy exists (e.g. platelet disorders), that are currently treated with recombinant FVIIa. Therefore, FVIIa gene transfer can potentially unify the treatment of several coagulopathies to a single product. Indeed, over the past decade, in a series of novel experiments in both mice and dogs with hemophilia, we were the first to show that expression of FVIIa via gene transfer results in dramatic phenotypic improvements or complete correction. In addition, we developed murine- and canine-specific reagents, assays and endpoints that allow us to monitor in vivo effects in hemostasis resulting from the action of FVIIa. Current work is focused on reducing the overall vector dose required to achieve hemostatic efficacy for FVIIa-based gene therapy. This can be achieved by further optimization of both the transgene (DNA and/or protein level modifications) and delivery vehicle (structure and expression cassette of AAV). For example, using modifications in murine FVIIa to enhance its function, we were the first to show hemostatic efficacy at much reduced AAV vector doses and transgene expression levels than gene transfer of unmodified murine FVIIa. In addition, modifications in the AAV structure and expression cassette using canine FVIIa, have demonstrated a several fold advantage over existing AAV vectors used for canine FVIIa gene transfer. These studies are part of an ongoing research program to provide further support for a FVIIa gene-based approach for inherited bleeding disorders currently treated with recombinant FVIIa.

**B. Honors and Training**

Positions and employment

*2005-2008*: **Research Associate**, The Children's Hospital of Philadelphia, Dept. of Pediatrics, Division of Hematology, Laboratory of Katherine A. High, M.D.; *2008-2009*: **Senior Research Associate**, The Children's Hospital of Philadelphia, Dept. of Pediatrics, Division of Hematology, Laboratory of Katherine A. High, M.D.; *2010-present*: **Research Assistant Professor** of Pediatrics, University of Pennsylvania Perelman School of Medicine/The Children's Hospital of Philadelphia, Division of Hematology

Professional memberships

*2001-2003*: **Associate Member**, American Society of Gene and Cell Therapy; *2009-present*: **Member**, American Society of Hematology; *2009-present*: **Member**, American Society of Gene and Cell Therapy; *2009-present*: **Associate Faculty Member**, Faculty of 1000 Medicine; *2009-present*: **Member**, International Society of Thrombosis and Haemostasis

Editorial positions

*2008-present*: **Ad-hoc reviewer** for Blood, Journal of Thrombosis and Haemostasis, Thrombosis and Haemostasis, Thrombosis Research, PLoS One, Molecular Therapy, Journal of Genetic Syndrome and Gene Therapy, The Veterinary Journal; *2009-present*: **Reviewer** for Journal of Blood Medicine

Honors

*1994*: **Summer sponsored internship** by the Wellcome Trust at the Human Molecular Genetics Unit of the University of Newcastle-upon-Tyne (U.K.); *1995*: **"John Corran" Book Prize** for the most outstanding Genetics final-year student, University of Newcastle-upon-Tyne (U.K.); *1996-1999*: **Graduate Project scholarship** from the Greek "Alexander S. Onassis" Public Benefit Foundation

Organizing roles and other professional activities

*2011*: **Abstract Reviewer** (Blood coagulation and fibrinolytic factors), 53rd American Society of Hematology Annual Conference, Dec 10-13, San Diego (CA), U.S.A.; *2011-present*: **Member** of the Hematologic and Immunologic Gene and Cell Therapy Committee for the American Society of Gene and Cell Therapy (ASGCT); *2012*: **Coordinating Reviewer** (Gene Therapy and Transfer), 54th American Society of Hematology Annual Conference, Dec 8-11, Atlanta (GA), U.S.A.