Children's Hospital of Philadelphia

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Children's Hospital of Philadelphia received $3,640,981 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Role of Crk and CrkL in Normal and Neoplastic Growth - Normal growth requires a delicate balance among the processes that grow new cells and those that eliminate superfluous cells. Cancer arises because of a breakdown in this balance between cell division and cell death. Cancer cells continue to grow as they fail to respond to signals that tell them to stop. The investigator proposes to study the role of two growth control genes, Crk and Crk-like (CrkL), in normal growth and development as well as in cancer cells. The strategy involves the use of genetic techniques to remove Crk and CrkL from different kinds of cells, in culture and in mice, and then to examine the consequences for cell growth. In addition, the genes will be removed from tumor cells to determine if they function in similar or different ways in cancer.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

Crk and CrkL are adaptor proteins that function in signal transduction processes during normal and neoplastic growth. Understanding the specific biological functions of Crk and CrkL has been challenging because deletion of either gene from the mouse germline results in embryonic or early postnatal lethality, and because both proteins function very similarly in biochemical and molecular assays. Previously, the Curran laboratory generated mutant mice carrying floxed alleles of *Crk* and *CrkL* allowing conditional mutation using the CRE system. Ablation of both Crk and CrkL in neuronal precursor cells demonstrated that they provide specific overlapping functions downstream of Reelin in the control of radial neuronal migration during brain development. The present project represents an expansion and new direction of this study to investigate the role of Crk and CrkL in a range of cell types in vitro and in vivo. Establishment of the $Crk^{fl/fl}/CrkL^{fl/fl}$ strain of mice as well as $Crk^{fl/fl}$ and $CrkL^{fl/fl}$ strains provides a unique and powerful experimental tool that enables the studies proposed in specific aims 1-3. The overall hypothesis to be addressed is that Crk and CrkL function in signal transduction processes by

linking tyrosine phosphorylation signals to alterations in cell growth, adhesion and migration properties. Three specific aims will be addressed that focus on the role of Crk and CrkL in fibroblast growth (Aim 1), in other cell types in vitro and in vivo (Aim 2) and in cell transformation and tumor growth (Aim 3). Comparison of phenotypes obtained from $Crk^{fl/fl}/CrkL^{fl/fl}$ cells and tissues with those from $Crk^{fl/fl}$ and $CrkL^{fl/fl}$ mice will provide detailed understanding of how Crk and CrkL play both overlapping and individually unique functions in various cells and tissues. The initial study of Crk and CrkL functions in fibroblast cells will elucidate their general role in cell biological processes. Understanding both conserved and specific functions of Crk and CrkL in various cell types will enable us to address fundamental questions in birth defects and cancer.

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Expected Research Outcomes and Benefits

This research will reveal the basic mechanisms whereby certain oncogenes (*Crk* and *CrkL*) affect the growth of normal cells as well as tumor cells. Insights from the basic observations concerning the growth of normal cells lacking Crk and CrkL will provide clues about the way in which cancer cells grow abnormally. Studies in mice will reveal the biological functions of Crk and CrkL in different tissues. Deleting Crk and CrkL in cancer cells will reveal whether these proteins are required for the formation and continued growth of tumors. This information will be used to predict new approaches for intervention in the treatment of cancer.

Summary of Research Completed

Changes in protein expression in the absence of Crk and CrkL in fibroblast cells
In order to understand the underlying mechanism of cytoskeletal collapse in the absence of Crk and CrkL, the researchers attempted to identify proteins that contribute to Crk/CrkL-dependent cytoskeletal integrity. The researchers reasoned that such proteins would rapidly disappear or their phosphorylation levels would dramatically change once cells lose Crk and CrkL. Therefore, the researchers monitored levels of protein expression and phosphorylation for candidate proteins downstream of Crk and CrkL in many known signaling pathways to see if there are any proteins which closely follow the footsteps of Crk and CrkL. In double floxed cells expressing both GFP and Cre, there was a minor decrease in protein quantity for Rho GTPases such as Rac, cdc42 and RhoA. Whereas protein levels of FAK, Akt, and Src were not decreased, there were

modest decreases in their phosphorylation levels. On the other hand, protein quantity and phosphorylation of mTor were not affected by the loss of Crk and CrkL. In contrast, the levels of total and phosphorylated proteins except for S6 kinase were increased in double floxed cells expressing GFP alone, probably due to increased density of cells as the culture continued. Interestingly the protein level of actin decreased dramatically over the course of infection while tubulin, GAPDH and vinculin showed either a modest decrease or an insignificant change. Among the proteins tested, actin showed the most rapid decline in its protein level closely following decrease of Crk and CrkL proteins. The results suggest that the actin level rapidly decreases in the absence of Crk and CrkL, which might be responsible for disintegration of actin network and subsequent collapse of cytoskeletal structures.

Phosphorylated Dok-7 peptides purify Crk/CrkL from muscle

The release of Agrin by motor neurons stimulates tyrosine phosphorylation of MuSK, a receptor tyrosine kinase expressed in skeletal muscle, to promote formation of the neuromuscular synapse. Phosphorylated MuSK recruits Dok-7, an adaptor protein that is expressed selectively in muscle and concentrated at neuromuscular synapses. Neuromuscular synapses fail to form in the absence of Dok-7, and mutations in human *Dok-7* are a major cause of congenital myasthenia, a group of neuromuscular disorders characterized by muscle weakness and fatigue. How Dok-7 stimulates synaptic differentiation is poorly understood. The researchers in collaboration with researchers at NYU Medical School investigated roles of Crk and CrkL in neuromuscular synapse formation.

The researchers identified the proteins that are recruited to pY396 and pY406 in Dok-7, using a biochemical assay to isolate proteins that bind these phosphorylation sites. The researchers synthesized two biotinylated 15mer phosphopeptides, one centered on pY396 and a second centered on pY406, as well as two control biotinylated peptides, centered on Y396F or Y406F to isolate binding proteins in lysates from C2 myotubes and 293 cells. The phosphopeptides were attached to streptavidin-agarose beads, which were incubated with cell lysates from cultured myotubes. The bound proteins were resolved by SDS-PAGE and detected by silver staining. A doublet of proteins, which migrated at ~37 kD, bound specifically to the pY406 and pY396 peptides but neither control peptide. The proteins contained within this doublet were excised and analyzed by Q-TOF MS, which identified the proteins as CrkL and CrkII. In addition, the researchers probed Western blots of the proteins that bound the phosphopeptide-affinity columns with antibodies specific for CrkI or CrkII as well as with antibodies against the related gene, CrkL. CrkI, CrkII and CrkL bound to pY396 and pY406 peptides but not to non-phosphorylated control peptides. These data confirm that CrkI, CrkII and CrkL bind to both phosphopeptides. Thus, Crk and CrkL bind directly to Dok-7 phosphorylated at Y396 or Y406.

Agrin stimulates recruitment of CrkL and Crk to Dok-7

The researchers next asked whether Crk, CrkL are recruited to Dok-7 following Agrin stimulation. To determine whether Agrin stimulates association of CrkL with Dok-7, the researchers stimulated muscle cells with Agrin, isolated CrkL by immunoprecipitation and probed Western blots for Dok-7. CrkL associated with Dok-7 following Agrin-stimulation. To determine whether CrkI and/or CrkII associates with Dok-7 in muscle, the researchers treated muscle cells with Agrin, isolated Dok-7 by immunoprecipitation and probed Western blots with antibodies that recognize both forms of Crk. CrkII associated with Dok-7 in an Agrin-dependent

manner. Substantially lower levels of CrkI co-isolated with Dok-7, likely due to the lower expression level of CrkI in muscle cells. Thus, Agrin stimulation leads to the association of CrkL and Crk with Dok-7 in skeletal muscle.

Crk and CrkL play critical roles in neuromuscular synapse formation

To determine the role of Crk and CrkL at neuromuscular synapses, the researchers inactivated Crk and CrkL selectively in skeletal muscle by crossing myf5^{cre} mice, which express Cre recombinase in myoblasts and myotubes, with mice carrying floxed alleles of CrkL and Crk. The researchers failed to recover Crk$^{fl/fl}$;CrkL$^{fl/fl}$;myf5^{cre} mice postnatally, although all other allele combinations were recovered at the expected Mendelian frequency. Because neuromuscular synapses are dispensable during embryonic development but required for survival at birth, these data raised the possibility that a loss of skeletal muscle Crk and CrkL led to neonatal lethality. Indeed, at E18.5, one day prior to birth, the researchers recovered Crk$^{fl/fl}$;CrkL$^{fl/fl}$; myf5^{cre} mice at the frequency expected for Mendelian inheritance. Therefore, the researchers analyzed neuromuscular synapses in E18.5 mice by staining whole mounts of muscle with probes that label motor axons, nerve terminals and AChRs. In wild-type mice, motor axons branch and terminate in a band of synapses, adjacent to the main intramuscular nerve, in the middle of the muscle. Each muscle fiber is innervated at a single synaptic site, marked by an accumulation of synaptic vesicles in the nerve terminal and a high density of AChRs in the postsynaptic membrane. Mice deficient in skeletal muscle Crk and CrkL displayed severe defects in presynaptic and postsynaptic differentiation. First, motor axons were not confined to a narrow endplate band but were distributed in a broader region of Crk/CrkL deficient muscle. Second, the number and size of synapses were reduced by two-fold. Third, the density of synaptic AChRs was reduced by 25%. Thus, Crk/CrkL mutant synapses contain ~three-fold fewer AChRs than normal synapses. Because the safety factor for synaptic transmission at mouse neuromuscular synapses is 2.5 to 4, this reduction in AChR number may compromise the reliability of synaptic transmission and result in neonatal lethality of mice deficient in muscle Crk and CrkL. Finally, motor axons often failed to terminate at synapses and instead continued to grow beyond AChR clusters, resembling terminal sprouts found in adult muscle after inhibiting synaptic transmission or following reinnervation. Thus, muscle-derived Crk/CrkL fulfills a critical role in the clustering of AChRs at synapses, the regulation of synaptic size and the control of motor axon growth.

Taken together, the results obtained during the period confirm that Crk and CrkL play critical overlapping roles in maintaining cytoskeletal integrity in fibroblast cells. Analysis of time-dependent changes in protein expression in the absence of Crk and CrkL suggested that loss of actin possibly contribute to the cytoskeletal collapse. In addition, Crk and CrkL are recruited to tyrosine phosphorylated Dok-7 upon Agrin stimulation in skeletal muscle cells. Furthermore, inactivation of Crk and CrkL in skeletal muscle leads to severe defects in presynaptic and postsynaptic differentiation at neuromuscular synapses in vivo, revealing a critical role for Crk and CrkL in synapse formation.

<u>Publications</u>

Park TJ, Curran T: Alternative Splicing Disabled by Nova2. Neuron Page: 66(6): 811-3, June 2010.

Hallock Peter T, Xu Chong-Feng, Park Tae-Ju, Neubert Thomas A, Curran Tom, Burden Steven J: Dok-7 regulates neuromuscular synapse formation by recruiting Crk and Crk-L. Genes &

development 24(21): 2451-61, Nov 2010.

Research Project 2: Project Title and Purpose

Gene Transfer of Activated FVII for the Treatment of Inherited Coagulation and Platelet Disorders - The treatment of hemophilia (a bleeding disorder) is complicated by the development of antibodies (inhibitors) against the therapeutic factor (Factor VIII or Factor IX) infused in response to bleeds. Clinical data demonstrate the successful but costly treatment of such patients with infusion of high-dose FVIIa. To address this with gene transfer, an engineered FVIIa transgene has been developed and delivered, the expression of which resulted in a substantial improvement in the bleeding diathesis of hemophilic mice and dogs. The experiments here aim to further demonstrate the feasibility of this approach in animals with inhibitors as well as other inherited coagulation defects, and the further optimization of the gene delivery vehicle and transgene. These are critical steps towards a clinical application that can result in a single, simplified treatment for bleeding disorders.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

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 hemophilia cases. Over the past 15 years, such patients, as well as those with congenital FVII deficiency or inherited platelet disorders, have been treated successfully with recombinant human Factor VIIa (rhFVIIa)**.** Based on the current understanding of coagulation, rhFVIIa can simplify patient management to a single product, but the short half-life, multiple dosing and high cost have prevented such an approach. Gene transfer has been proposed as an alternative to protein infusion in the management of bleeding in hemophilia. Specifically, gene transfer with FVIIa can potentially bypass the shortcomings of rhFVIIa protein therapy by conferring long-term expression and offer an immunological (since all hemophilia patients are tolerant to FVIIa) as well as a therapeutic advantage. The researcher has previously engineered a FVII transgene that is secreted in its active form (FVIIa) and demonstrated improvement of the bleeding diathesis in hemophilic mice and dogs, by viral-vector mediated, liver-directed, FVIIa gene transfer. Moreover, the researcher's data from hemophilic dogs suggest that efficacy is proportional to the vector dose utilized and thus disorders that are currently treated with low-dose rhFVIIa (e.g. FVII deficiency) will be appealing targets for such gene transfer. However, further development of this therapeutic relies on aspects that are currently unknown and thus the researcher is pursuing experiments to address them, as follows: (1) What is the efficacy of this gene transfer approach in hemophilia with inhibitors or platelet disorders? For this, the researcher will use hemophilic mouse models to generate inhibitors against FVIII or FIX, or use mouse models of platelet disorders and subsequently deliver mouse FVIIa via gene transfer in order to study efficacy in vitro and in vivo. (2) Can the vector be optimized to allow for easier scalability and avoiding adverse events from high viral loads? For this, the researcher will perform an

optimization of vector and transgene and compare efficacy in mice and hemophilic dogs. (3) Can this approach be utilized in congenital FVII deficiency? For this, the researcher will use a newly established dog model of FVII deficiency to evaluate the effects on bleeding diathesis following cFVIIa gene transfer. These answers will greatly impact this gene-based approach that can potentially provide a unified, simpler alternative to hemophilia treatment.

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Expected Research Outcomes and Benefits

A major complication in the treatment of hemophilia (a bleeding disorder) with protein infusion is the development of antibodies against the infused factor (Factor VIII [FVIII] or Factor IX [FIX]) occurring in a considerable number of severe cases of the disease. Infusion of high doses of a purified factor (activated FVII [FVIIa], that these patients already have), has demonstrated excellent efficacy in such patients but its short circulating life in blood results in a substantial financial burden. Alternatively, gene therapy may offer long-term correction of the bleeding diathesis via stable expression of the appropriate gene(s). The researcher has developed such an approach with a gene coding for FVIIa and has shown correction of the bleeding diathesis in both hemophilic mice and dogs. However, certain key questions of using this strategy remain unanswered. Specifically, the first aim is to investigate whether FVIIa gene transfer can correct the bleeding phenotype in a mouse model of hemophilia in the *presence* of inhibitory antibodies to FVIII or FIX or in mice with platelet disorder, as has been shown with FVIIa infusion. These animal models will provide the experimental confirmation for the researcher's proposed gene transfer approach. Since high doses were necessary to correct the bleeding diathesis in hemophilic dogs, the researcher also wants to improve the vector/transgene that can lower the effective dose. This is important since high vector doses impose scalability issues for human application and may have adverse events resulting from high viral loads. The last aim is to investigate whether gene transfer with FVIIa can correct the bleeding diathesis in a newly described dog model of FVII deficiency. The researcher's data in hemophilic dogs suggest that efficacy is proportional to the infused vector dose for gene transfer; since FVII deficiency is currently treated with low-dose FVIIa protein infusion, it would thus be an ideal and feasible target for FVIIa gene transfer. Overall, the experiments aim to further preclinical development of this approach that may lead to a single, simplified treatment for bleeding disorders.

Summary of Research Completed

In this reporting period the researcher's lab performed experiments as described in Aims 1-3, according to the milestones set in the original project description.

AIM 1

Inhibitor development profile in hemophilia A and B mice

Using methodology described in the previous period (7/1/2009 – 6/30/2010), 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). 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 are shown in the Figure 1. The hemophilia A mice shown in Figure 1 show 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 (reported in the previous period [7/1/2009 – 6/30/2010]). Based on this result, despite using methodology with a strong adjuvant, it was concluded that the researcher was not able to generate mice with a robust immune response to human recombinant FVIII.

The researcher also used the methodology described in this Aim to generate hemophilia B mice with inhibitors that were subsequently injected with an adeno-associated viral vector encoding mFVIIa (AAV-mFVIIa, as presented in the previous report [7/1/2009 – 6/30/2010]) as well as a variant of mFVIIa with enhanced activity (injected at a 40-fold lower vector doses vs. AAV-mFVIIa). Continuous expression of either transgenes resulted in normalization of the aPTT. These data were published in the journal Blood:

Margaritis P, Roy E, Faella A, Downey HD, Ivanciu L, Pavani G, Zhou S, Bunte R, High KA. Catalytic domain modification and viral gene delivery of activated Factor VII confers hemostasis at reduced expression levels and vector doses in vivo. Blood April 2011; 117:3974-82.

Milestones: (1) Efficacy of AAV-mFVIIa in hemophilia A mice with inhibitors: the researcher was unable to generate hemophilia A mice with inhibitors.

Bernard-Soulier (BSS) mouse experiments

The researcher injected mice with BSS phenotype with an AAV serotype 8 vector encoding for mFVIIa at a 1.2E12 vector genomes/mouse and followed the hemostatic and functional effects of mFVIIa expression long-term. The researcher used the prothrombin time (PT) as a measure of expression of mFVIIa. Since continuous expression mFVIIa in hemophilic as well as hemostatically normal mice can result in premature mortality, the researcher used two assays to detect aberrant systemic activation of coagulation (platelet counts and levels of thrombin-antithrombin [TAT] complex). To test the hemostatic capacity of the expressed mFVIIa in vivo, the researcher used a tail clip assay. In this assay, following tail transection, the amount of blood

loss is quantified and hemostatic improvement results in reduced amount of blood lost, compared to untreated controls. The results of these assays are shown in Figure 2. Despite the improvement in the tail clip assay, BSS mice expressing mFVIIa failed to show an improvement in $FeCl_3$ carotid injury model, relative to untreated controls.

Milestones: (1) Establish in vivo baseline hemostatic parameters for BSS mice (tail-clip assay, $FeCl_3$) and determine the effect of AAV-mFVIIa gene transfer in such mice in vitro/in vivo: accomplished.

Glanzmann thrombasthenia (GT) mouse experiments

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) was administered intravenously via the tail vein (1.2E12 vector genomes/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 3. Despite the evident expression of mFVIIa in GT mice, there was no statistical difference in the amount of blood loss between AAV-mFVIIa treated and untreated GT mice (tail clip assay). The researcher's previous experience with the $FeCl_3$ carotid artery model in hemophilic as well as BSS mice expressing mFVIIa, suggest that despite improvements in a tail clip assay in those mice (vs. untreated mice), carotid artery occlusion is much more difficult to occur. Taking this into consideration, since GT mice expressing mFVIIa failed to show any effects in the tail clip assay, the researcher did not perform the $FeCl_3$ carotid artery injury model.

Milestones: (1) Establish in vivo baseline hemostatic parameters for GT mice (tail-clip assay, $FeCl_3$) and determine the effect of AAV-mFVIIa gene transfer in such mice in ccomplished.

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 4. 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. Plasma was collected and assayed at 24 and 48h post plasmid administration and expression of cFVIIa was monitored by PT. As shown in Figure 5A, the scAAV-cFVIIa-CO plasmid expresses a functional cFVIIa, confirming the functionality of the expression cassette in that construct. Subsequently, the researcher tested the ability of the scAAV-cFVIIa-CO construct to package into AAV. A serotype 8 vector was generated with the scAAV-cFVIIa-CO construct and its ability to express cFVIIa was monitored by PT following IV administration in C57BL/6 mice. The researcher administered 3.52E11 vector genomes/mouse of the scAAV8-cFVIIa-CO.

As a comparative control, the researcher used a serotype 8 AAV-cFVIIa (AAV8-cFVIIa). The researcher injected AAV8-cFVIIa at 3.52E11 vector genomes/mouse (similar dose for scAAV8-cFVIIa-CO) as well as 1.2E12 vector genomes/mouse). A week post AAV administration, transgene expression was monitored by PT. As shown in Figure 5B, the scAAV8-cFVIIa-CO vector was able to generate functional cFVIIa, reducing the PT similarly to either dose of AAV8-cFVIIa.

Milestones: (1) Test the improved vector preparations (expressing cFVIIa) for their hemostatic effects in HA mice (Aim 2): partially accomplished (used C57BL/6 mice)
(2) Administer the improved vectors (expressing cFVIIa) in hemophilia A or B dogs, depending on the availability (Dr. Timothy C. Nichols, UNC Chapel Hill colony). Begin monitoring of hemostatic efficacy in infused dogs (Aim 2):not accomplished.

AIM 3

Expression of cFVII or cFVIIa in dog with FVII deficiency

The researcher has generated an AAV vector plasmid construct encoding for the zymogen cFVII. The researcher is in the process of generating the AAV vector preparation for the construct encoding cFVIIa.

Milestones: (1) Generate the AAV vector plasmid and preparation for expression of cFVII zymogen (for Aim 3) based on results from comparative analysis of the current AAV and the improved vectors, as described in Aim 2. Test the vector preparations (expressing cFVII or cFVIIa) for their hemostatic effects in HA or HB mice (Aim 3): partially accomplished
(2) Administer the vectors expressing cFVII zymogen or cFVIIa in dogs with FVII deficiency (Dr. Timothy C. Nichols, UNC Chapel Hill colony). Begin monitoring of hemostatic efficacy in infused dogs (Aim 3). Not accomplished.

Figure 1. Total IgG against hFVIII assayed 2 weeks post IFA-human FVIII administration in hemophilia A mice.

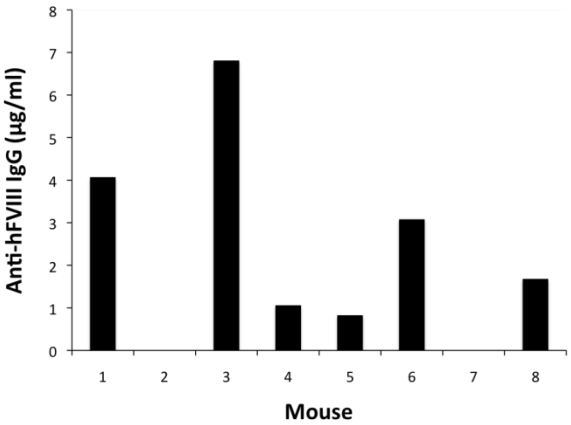

Figure 2. BSS male mice were injected with 1.2E12 vector genomes/mouse of AAV-mFVIIa (serotype 8) via the tail vein (arrow). Untreated mice were used as controls (BSS). Expression of mFVIIa in AAV-mFVIIa injected mice (BSS-mFVIIa) was stable as shown by sustained reduction of the PT. Despite its procoagulant 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 (these mice have reduced platelet counts). However, expression of mFVIIa resulted in statistically significant reduction in blood loss following tail clip, performed at 11 weeks post AAV administration**.**

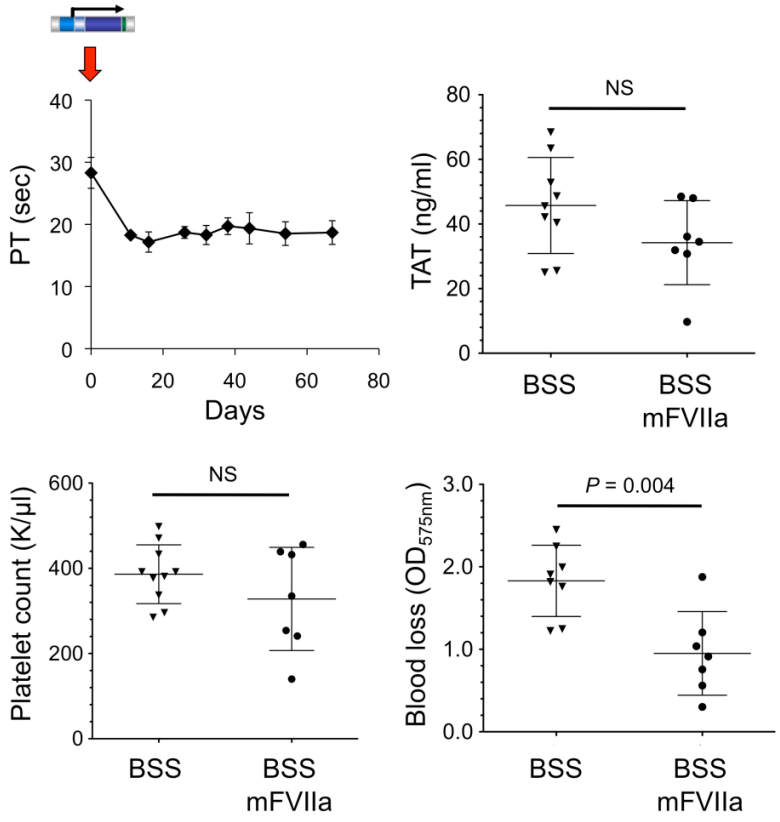

Figure 3. AAV-mFVIIa was administered IV in mice with GT (1.2E12 vector genomes/mouse; GT-AAV). Wild type littermates or untreated GT mice were used as controls. Murine FVIIa expression following AAV administration resulted in supraphysiological reduction in the PT. Despite this, GT-AAV mice did not 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.

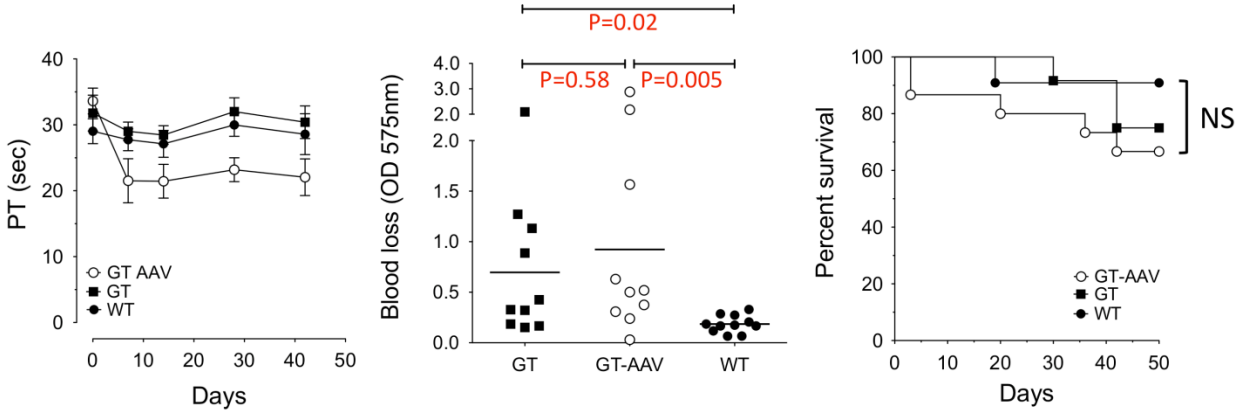

Figure 4. Schematic representation of the single stranded (AAV-cFVIIa) and double stranded AAV vector (scAAV-cFVIIa-CO) expressing canine FVIIa (cFVIIa). This vector contains the human α1 antitrypsin promoter/apolipoprotein E enhancer (hAAT/ApoE), a hybrid intron, a polyadenylation signal (pA) from human 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.

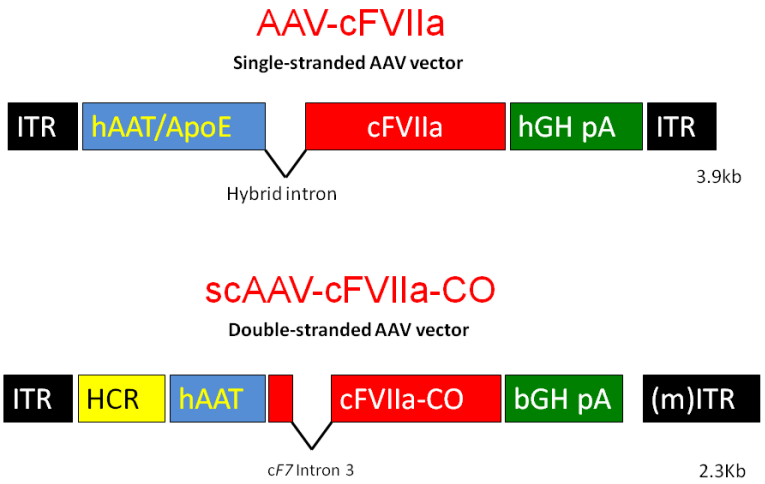
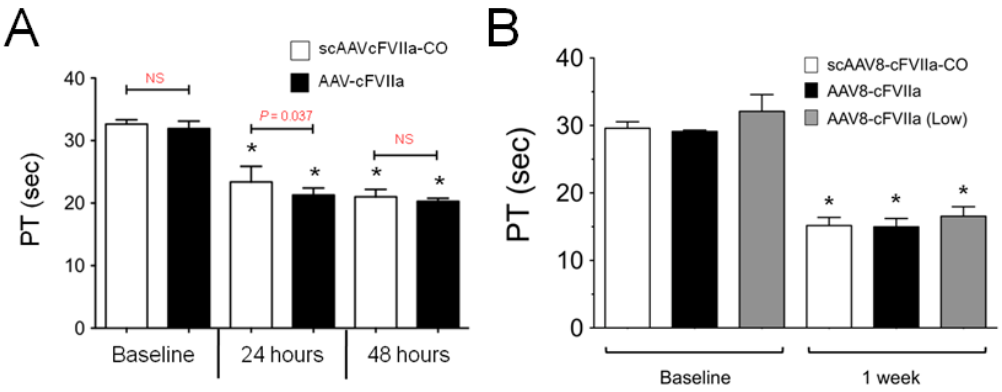
Figure 5. (A) C57BL/6 mice were administered a hydrodynamic injection of the scAAV-cFVIIa-CO and AAC-cFVIIa (control plasmid). Plasma after 24h and 48h was collected and expression was measured by a PT clotting assay. P values from a Student's t-test between the two cohorts of mice are shown. Asterisk denotes P<0.05 vs baseline for each plasmid tested. NS denotes non-significant differences. (B) C57BL/6 mice were injected IV with 3.52E11 vector genomes/mouse of scAAV8-cFVIIa-CO and either 1.2E12 vector genomes/mouse of AAV8-cFVIIa or 3.52E11 vector genomes/mouse of AAV8-cFVIIa (AAV8-cFVIIa-Low). PT following 1 week after AAV administration was used to monitor functional cFVIIa expression. Asterisk denotes P<0.001 vs baseline for each vector tested. Experimental data in (A) and (B) are derived from at least 3 mice per cohort.