Children's Hospital of Philadelphia

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

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.

Principal Investigator

Tom Curran, PhD
Deputy Scientific Director
The Children's Hospital of Philadelphia
Colket Translational Research Building, Room 4060
3501 Civic Center Boulevard
Philadelphia, PA 19104-4318

Other Participating Researchers

Tae-Ju Park, PhD, Joseph Briggs, PhD - employed by The Children's Hospital of Philadelphia

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

Decreased motility of fibroblast cells in the absence of Crk and CrkL

To examine whether Crk and CrkL play essential roles in growth of fibroblast cells, the researcher infected wild-type or Crk/CrkL double floxed fibroblast cells with lentivirus expressing green fluorescent protein (GFP) and Cre recombinase. Infected cells were identified with green fluorescence under the Eclipse TE2000 inverted microscope. Cells started to exhibit green fluorescence a day after infection. Green fluorescence became very strong three and five days after infection. Crk was expressed mainly in the nucleus, as its staining coincided with nuclear staining with DAPI . In wild-type fibroblast cells, Crk expression was not affected by GFP expression. In contrast, Crk expression was significantly reduced in double floxed cells that

highly expressed GFP. The results suggest that Crk is efficiently removed from cells by infection with lentivirus expressing GFP and Cre.

Then the researcher examined whether there is any change in motility of fibroblast cells in the absence of Crk and CrkL. Three days after infection with lentivirus expressing GFP and Cre, time-lapse images of GFP-positive cells were taken for 48 hours with one-hour interval. For quantitative analysis of cell motility, grid lines were put on the images and crossing of GFP-positive cells over the grid lines was monitored. GFP-positive wild-type cells were actively moving around three and five days after infection, although a few cells exhibited decreased motility five days after infection. On the other hand, many GFP-positive double floxed cells did not change their positions five days after infection. Although some GFP-positive double floxed cells were moving three days after infection, the number of nonmoving cells increased two days later. Assessment of forty cells for their movement across the grid lines per hour suggests that GFP-positive double floxed cells had a gradual decrease in their motility as time elapsed three days after infection. In contrast, GFP-positive wild-type cells maintained their motility throughout the infection. The results suggest that fibroblast cells lose their motility in the absence of Crk and CrkL.

Morphological alteration of fibroblast cells in the absence of Crk and CrkL

While the researchers were assessing the motility change in GFP-positive double floxed cells, the researchers also observed a morphological change in the cells. Thus, the researchers examined further to see whether loss of Crk and CrkL causes morphological change in fibroblast cells. One day after infection, few cells were GFP-positive, and most of the wild-type and double floxed cells had complex processes. However, three days after infection many of the GFP-positive double floxed cells lost their processes and became isolated from their neighboring cells. Six days after infection most of the GFP-positive double floxed cells became smaller and round-shaped without processes. On the other hand, GFP-positive wild-type cells were well spread and interacting with neighboring cells with their long processes even six days after infection. Then the researchers examined whether simultaneous loss of two proteins, Crk and CrkL, is required for the morphological alteration. Both Crk floxed and CrkL floxed cells, unlike double floxed cells, retained their processes when they were GFP-positive. Therefore, the results suggest that fibroblast cells undergo morphological alteration in the absence of both Crk and CrkL.

Then the researchers looked at whether the morphological alteration is accompanied by changes in cytoskeletal structures. Polymerized actin, stained with phalloidin, reflected well-spread cytoplasm of double floxed cells, before they strongly expressed GFP. Three days after infection, GFP-positive double floxed cells started to shrink their cytoplasm, resulting in dramatic reduction of cytoplasm to the periphery of the nucleus a day later. Microtubule, another essential component of the cytoskeleton, was also examined using alpha-tubulin antibody. Well-dispersed distribution of microtubules, except for the nucleus, were observed in double floxed cells negative for GFP. However, in GFP-positive double floxed cells, microtubules collapsed, leaving a sac-like disorganized microtubule structure that surrounded the nucleus. In addition, focal adhesion, stained with vinculin antibody, disappeared from the GFP-positive double floxed cells as the cytoplasm of the cells shrank. These results together suggest that loss of Crk and CrkL

triggers a dramatic shrinkage of the cytoplasm through the loss of focal contact to the substrate and collapse of actin filaments and microtubules.

Increased cell death in the absence of Crk and CrkL
Next the researchers wondered whether the loss of motility and dramatic shrinkage of the cytoplasm led to cell death in GFP-positive double floxed cells. Apoptotic cells were stained using the TUNEL assay. Double floxed cells infected with GFP and Cre showed increased apoptotic cells, compared to double floxed cells infected with GFP alone or wild-type cells. The TUNEL-positive cells were smaller with higher refraction. Quantitative analysis of the TUNEL staining indicates that double floxed cells had more TUNEL-positive cells than wild-type cells when they were infected with GFP and Cre and that apoptosis was accelerated five days after infection.

Taken together, the results obtained during the period clearly indicate that Crk and CrkL are essential for cell growth and maintenance of cytoskeletal structures in fibroblast cells and that simultaneous loss of Crk and CrkL leads to reduced motility, collapse of cytoskeletal structures, and cytoplasm shrinkage, resulting in cell death. Further detailed analyses of the sequence of molecular events triggered by the absence of Crk and CrkL may shed light on the mechanism whereby Crk and CrkL contribute to cellular growth and survival.

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.

Principal Investigator

Katherine A. High, MD
Professor of Pediatrics/Investigator
The Children's Hospital of Philadelphia
Room 302, Abramson Pediatric Research Center
3516 Civic Center Blvd.
Philadelphia, PA 19104

Other Participating Researchers

Paris Margaritis, PhD, Guang Qu, PhD - employed by The Children's Hospital of Philadelphia

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 (7/1/2009 – 6/30/2010) the researcher's lab performed experiments as described in Aims 1-3, according to the milestones set in the original project description. These experiments and the obtained results are described in detail below:

AIM 1

Inhibitor development profile in hemophilia A and B mice.

Since the researcher had encountered difficulties in generating hemophilia A mice with sustained inhibitory antibodies (see previous report), in this period the researcher utilized hemophilia B mice instead, in order to develop inhibitory antibodies to human Factor IX (hFIX). Such mice would be subsequently used to receive an adeno associated virus (AAV) encoding murine FVIIa (mFVIIa) to determine the hemostatic effect of continuous expression of mFVIIa as supplied by the AAV vector. Since recombinant FVIIa enhances hemostasis in either type of inhibitor patients (hemophilia A or B), it is expected that utilizing either type of mouse (hemophilia A or B) would be informative in the studies proposed here. The researcher initially obtained baseline plasma samples several days prior to the start of the experiment. Mice were subsequently injected subcutaneously with 200 µl of an emulsion consisting of complete Freund's adjuvant (CFA) and 20 µg of hFIX (Mononine®, CSL Behring). Four dorsal sites were utilized, 50 µl per site. Three weeks later, the procedure was repeated substituting CFA with incomplete Freund's adjuvant (IFA) but injecting four different dorsal sites. Plasma samples were obtained following tail bleeding at different timepoints (as shown in Figure 1A) for determination of antibody level (total IgG) as well as inhibitor titer (Bethesda assay), as previously described (Fields PA et al., Mol. Ther 2001). The protocol used resulted in progressively increasing inhibitor titer (A to C, Figure 1B) that was confirmed by a similar observation in total anti hFIX IgG (A to C, Figure 1C). After timepoint C, the researcher administered 1.2E12 vector genomes of AAV serotype 8 encoding mFVIIa via the tail vein. Since expression of mFVIIa would make determination of

inhibitory antibody titer problematic (mFVIIa would reduce the clotting time and the Bethesda assay used to determine inhibitory antibody titer is clotting-based), the researcher monitored the persistence of anti-hFIX antibody by measurting total anti-hFIX IgG. The levels of such IgG were not statistically different from those prior to AAV-mFVIIa administration (Figure 1C) at week 4 or 9 post AV-mFVIIa administration. This suggested that the antibody response to hFIX was maintained after AAV-mFVIIa administration. Despite the presence of anti-hFIX antibody, expression of mFVIIa from the administered AAV8 vector resulted in near-normalization of the activated partial thromboplastin time (aPTT, Figure 1D). The researcher has previously shown that hemophilia B mice that have near-normalization of the aPTT following AAV-mFVIIa administration also show a significant reduction in their blood loss, following a tail clip injury (Margaritis P et al, J Clin Invest 2004; Aljamali MA et al., J Clin Invest 2008). Collectively, the data suggest that expression of mFVIIa via AAV gene transfer exerts a hemostatic effect even in the presence of inhibitory antibodies to hFIX in hemophilia B mice. Part of the data reported here were presented as a poster at the American Society of Hematology Annual Meeting (New Orleans, Dec 5-8, 2009; Abstract #2466).

Since the above methodology resulted in a robust antibody response to hFIX, the researcher is currently investigating its use to generate inhibitory antibodies to human FVIII in hemophilia A mice. It is expected however that mice with such inhibitors will behave similarly to hemophilia B mice with hFIX inhibitors following AAV-mFVIIa gene transfer, as the researcher showed above.

Milestones: (1) generation of AAV-mFVIIa: fully accomplished
(2) Efficacy of AAV-mFVIIa in hemophilia mice with inhibitors: partially accomplished (used hemophilia B mice instead of A and demonstrated in vitro correction of hemostasis)

Bernard-Soulier (BSS) mouse experiments

During this reporting period, the researcher's laboratory was moved to a new location and animal facility. As a result, mice in the new facility had to be re-derived from frozen embryos, and the resulting heterozygote mice bred to generate mice with the BSS phenotype that would be used in experiments proposed in AIM 1. This process is currently ongoing.

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: partially accomplished.

Glanzmann thrombasthenia mouse experiments

Mice with the Glanzmann's thrombasthenia (GT) phenotype were obtained from the Jackson Labs as heterozygotes. The researcher initiated breedings between heterozygotes and obtained mice with all expected genotypes. Although the current number of homozygous GT mice is small for the researcher to perform any experiments administering AAV-mFVIIa and determining the phenotypic effects on their hemostasis, the researcher has obtained basic

hemostatic parameters (complete blood counts [CBC] and prothromin time, Table 2) from all three genotypes from heterozygote matings (wild-type, heterozygote and homozygote GT). Three parameters from CBC analysis are shown in Table 2, since these are significantly lower in GT mice (Hodivala-Dilke KM et al., J Clin Invest, 1999). Although the observed values are slightly higher than those previously published by Hodivala-Dilke et al., the overall trend is in agreement, and GT mice have reduced values for all three parameters shown in Table 2. The small numbers in each cohort could account for those differences not being statistically significant. The prothombin time (PT) from each cohort of mice was not statistically different (Table 2). The researcher is currently expanding the breedings in order to obtain sufficient number of mice for experiments with AAV-mFVIIa.

Milestones: (1) obtain mice, establish baseline blood count and hemostatic measurements: fully accomplished

AIM 2

The researcher previously constructed a plasmid that, following AAV vector manufacture, would encode a self-complementary AAV vector (scAAV-cFVIIa-co), expressing a codon-optimized canine FVIIa (cFVIIa) transgene as well as a version that would be packaged as a single strand AAV (ss-AAV-cFVIIa-co). Concurrent work in the laboratory using the same backbone with a different transgene suggested that expression from such constructs was lower than expected from the published report describing these backbones (Nathwani AC et al., Blood 2006). Based on this, the researcher decided to generate a new expression cassette that can be packaged in either self-complementary AAV or single stranded AAV. This cassette contains the following elements: hepatic control region (HCR) from the apolipoprotein E gene, a human alpha-1 antitrypsin promoter (hAAT), optimized Kozak sequence, codon-optimized cFVIIa and a human growth hormone polyadenylation signal. To keep the size to a minimum, the cFVIIa transgene also contains the native intron 3 from the canine *F7* gene (~ 80 base pairs). This expression cassette was cloned in a plasmid and was flanked by the native AAV2 5' inverted terminal repeat (ITR) as well as a mutated 3' ITR from AAV4 [constituting the deleted 3' terminal resolution site (Δ*trs*)]. In this backbone, during AAV manufacturing, a self-complementary AAV vector would be produced. The researcher has also used the same strategy but incorporated a codon-optimized canine FVII zymogen transgene that may be used in AIM 3. The researcher is currently generating the backbone that will result in a single-stranded AAV vector but contains the same expression cassette.

Milestones: (1) generation of vector backbones for self-complementary and single strand AAV: partially accomplished

AIM 3

Dr. Timothy Nichols at the University of North Carolina (Chapel Hill) has established a colony of dogs with canine FVII deficiency due to a missense mutation (G96->E; Callan MB et al. J Thromb Haemost 2006), resulting in <2% FVII activity. Dr. Nichols obtained a litter of 6 dogs from breeding such homozygous FVII deficient dogs, shown in Table 3. Plasma from the original founders (Cleatus, Otis, Pearl and Dot) was used in a prothrombin time assay (dependent

on FVII antigen/activity), and was found abnormally prolonged compared to normal (~27 sec), as expected (Table 3). An additional assay (rotational thromboelastography, an in vitro assay of global hemostasis using whole blood and initiating coagulation with tissue factor) was used in two dogs (Pearl and Otis). The researcher observed increased time to initial fibrin formation (18.7 and 21.5 min, respectively) compared to hemostatically normal dogs (12.8 ± 4.6 min). This is expected since these dogs have <2% FVII activity, hence prolonged initiation of coagulation.

Milestones: (1) establish breeding capacity of the FVII deficient dogs: fully accomplished.

Figure 1. Development of inhibitory antibodies to human Factor IX in hemophilia B mice and administration of AAV-mFVIIa. (A) Timeline of experimental procedure. (B) Bethesda units of inhibitory antibodies to hFIX in HB mice. (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 in the grey box.

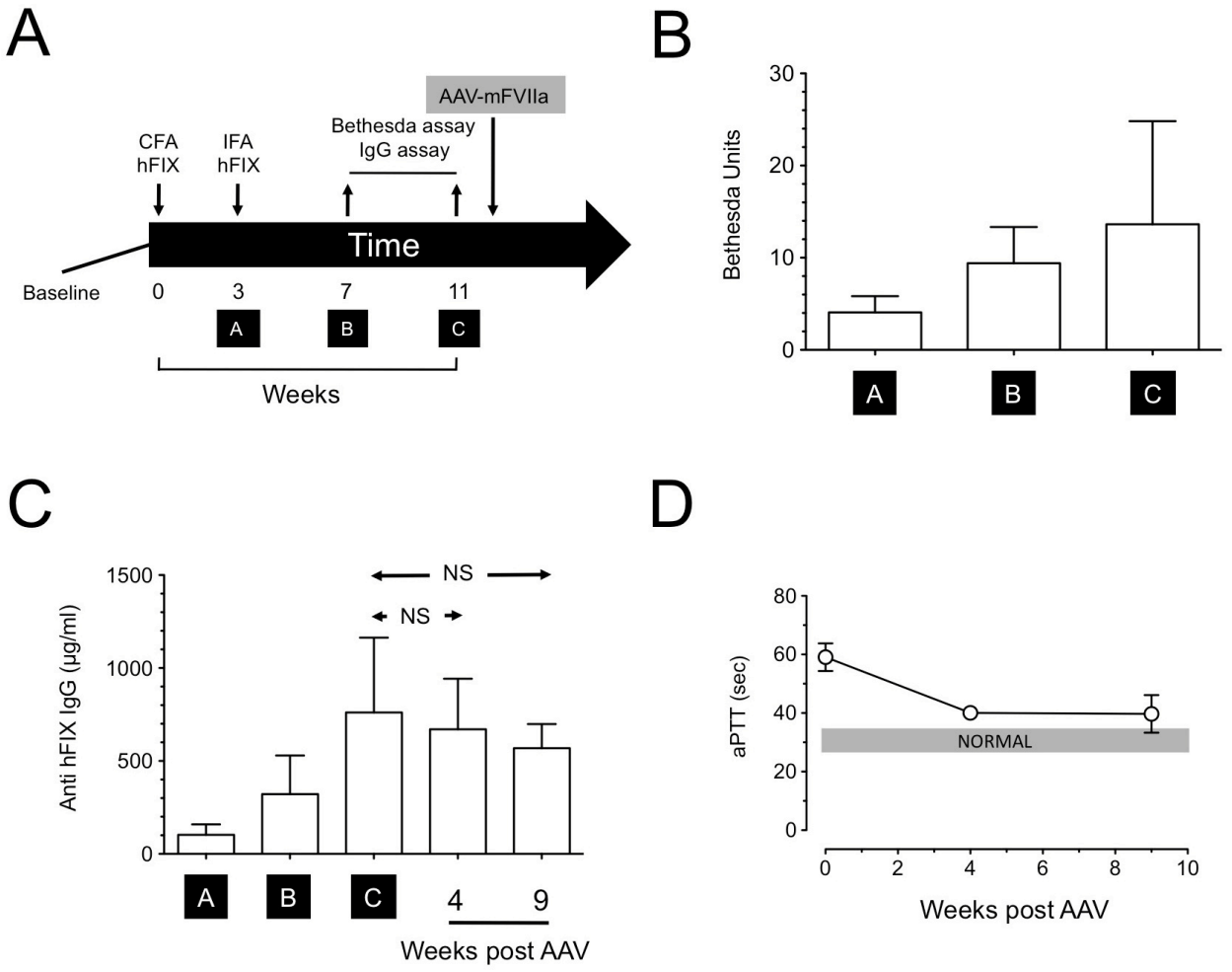

Table 2. Prothrombin time and three blood parameters in wild type (WT), heterozygotes (Het) and Glanzmann's thrombasthenia mice (GT), obtained from crosses between heterozygote animals. Abbreviations used: red blood cells (RBC); hemoglobin (Hb); hematocrit (HCT). At least 4 mice were used per cohort)

	WT	HET	GT
PT (sec)	29.4±3.7	29.0±1.3	28.6±1.0
RBC ($10^6/mm^3$)	14.9±2.0	18.0±3.4	11.8±2.3
Hb (g/dL)	20.5±3.2	20.9±1.2	18.5±1.7
HCT (%)	65.7±9.4	64.4±6.0	58.4±5.0

Table 3. Dogs with FVII deficiency identified at the colony of Dr. Timothy Nichols (Univ. North Carolina, Chapel Hill). Dogs with "N" in their identifier are offspring from a breeding of Cleatus and Pearl. ND, not determined.

Name	Gender	Date of Birth	Prothrombin time (sec)
Cleatus	Male	3/3/08	>200
Otis	Male	6/28/07	175.4
Pearl	Female	11/1/07	>200
Dot	Female	5/5/06	164.8
N22	Male	7/2/09	ND
N23	Male	7/2/09	ND
N24	Male	7/2/09	ND
N25	Male	7/2/09	ND
N26	Female	7/2/09	ND