GENETIC MODIFIERS OF HEMOSTASIS

by

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DEDICATION

To my family: My beloved wife Stacey, Dad, Mom, Jim, Bill, Nicole, Billy, Mike, Jamey, Mikey, Elisabeth, Grant, and recently Doug, Carol, Jody, Melissa, Helena, Olivia and (Sparty and Willie)

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FOREWARD

Since I have not followed the "traditional" route during my scientific career, the purpose of this foreward is to provide information about my career path and how the influence of my previous scientific experiences led to this thesis research. For this, it is necessary to briefly describe the beginning of my scientific career in 1992 and the subsequent decade I spent in science, culminating in 2002 when I began graduate school. I have also taken this opportunity to describe my involvement in published work since 2002 that are not included in the main body of this thesis. It is hoped that this information will provide background and the context in which I performed my thesis research.

I have worked on many different scientific questions during my career, which started as an undergraduate laboratory assistant in the Michigan State University (MSU) Pesticide Research Center. I worked on the life cycle of eriborus wasps. These wasps are natural predators of the European corn borer, an agricultural pest that damages millions of acres of corn per year. For this project, I solved a key technical problem of outdoor wasp containment, which allowed us to discover on which flowering plants the adult wasps fed. Weeds also cost countless billions in lost agricultural yields. Another project investigated the ability of aphids to parasitize weeds, thereby reducing their prolific seed production. The goal of this work was to develop novel methods of weed control. We characterized which weeds are preferred by aphids and to what extent a severe aphid infestation reduces their seed production. Concurrently, I was engaged in an independent study project in the MSU carcinogenesis laboratory, investigating the

mechanisms of ultraviolet hypermutability in xeroderma pigmentosum skin cells. This position was my first foray into biomedical research and provided me with basic laboratory skills such as culturing cells and molecular techniques.

After graduation, I worked in the MSU special coagulation laboratory, performing diagnostic blood analyses for patients on anticoagulation therapy. We also performed industrial contract research such as conducting quality control studies to normalize commercial thromboplastin reagent and optimizing the commercial production of blood products. As my "night" job, I worked concurrently in the Pharmacology/Toxicology department investigating the influence of cocaine and cocaine analogs on vascular smooth muscle contraction and platelet activation, using rat and rabbit models. The goal of this project was to understand the mechanisms by which cocaine and its analogs induce sudden vasoconstriction, leading to morbidity/mortality in drug abusers. These positions introduced me to the field of hemostasis. Although the cocaine studies were particularly interesting and had the potential for publication, the research was cut short by the departure of the principal investigator for an industrial job. From this, I learned that work not published is equivalent to work not performed.

I was soon lured to another lab in Pharmacology/Toxicology using rat models to elucidate the mechanisms by which endotoxin, the essential component of gram negative bacterial cell walls, can cause organ damage in bacteremia. My graduate-student supervisor in this large laboratory gave me much latitude in designing and implementing scientific studies. I was motivated and excited to be working independently. Although I was unfortunately not recognized with co-authorship of the several scientific papers that resulted from my efforts, I gained valuable experience with animal and cell-based

models, as well as accumulating scientific knowledge in the pathology of liver damage. The skills and knowledge I accumulated at MSU served me well in my future endeavors at the University of Michigan.

Based on the bittersweet experiences at MSU, I sought to obtain a staff position at the University of Michigan. My initial goal was to be able to contribute significantly to scientific research resulting in co-authorship on a publication(s) in order to strengthen my candidacy for medical school. I was fortunate that David Ginsburg was looking for a research technician. I was a good fit for the advertised position and was hired in August 1995, mainly to manage the mouse colonies. I was initially assigned to work simultaneously with four senior lab members. Though this was a challenge, it provided a stimulating learning environment. From working on these different projects, I learned how to solve scientific problems through a variety of approaches, from positional cloning of a von Willebrand factor modifier gene to developing models to study the pathophysiology of thrombosis and atherosclerosis. The massive workload encouraged me to develop ways to increase my efficiency in the routine, technical aspects of my job so I was able to concentrate on learning the greater goals of each project and making intellectual contributions to further their progress. The knowledge of these research areas served me well in future collaborations. Below, I first describe my research activities as a technician in the Ginsburg lab and how I eventually morphed into an independent scientist.

Working with Karen Mohlke, a Department of Human Genetics graduate student, I participated in mapping a mouse modifier of von Willebrand disease by generating and

phenotyping several thousand mice. I quickly became an expert at handling the extremely jumpy wild-derived hybrid mice used in these studies, saving enormous amounts of time and greatly accelerating the progression of this project. My efforts resulted in co-authorship of a series of papers reporting the mapping and identity of a modifier gene for plasma von Willebrand factor level[1-3].

I also simultaneously worked with a postdoctoral researcher named Kris Dougherty to characterize the plasminogen activator inhibitor 2 (PAI-2) deficient mice that she generated by gene targeting. We published the initial phenotypic characterization of mouse PAI-2 deficiency[4]. After this initial report, I made an interesting observation that spontaneous tumors arose in aged PAI-2 deficient mice. When I made this observation, Kris was no longer working in the lab. As a technician, my responsibilities precluded me from being the lead on a project, so I convinced another postdoc to work with me. Together, we demonstrated that host PAI-2 deficiency leads to more rapid primary tumor growth and ruled out bone marrow derived PAI-2 as the relevant source to prevent accelerated tumor growth. This manuscript is now in preparation (Payne-Rojkjaer et al).

Working concurrently with Dan Eitzman, who was a cardiology research fellow at that time, we investigated the potential contribution of fibrinolysis and coagulation on the progression of atherosclerosis using genetically altered mice. This involved crossing mice with genetic alterations in components of the fibrinolytic or coagulation cascade to atherosclerotic prone, hypercholesterolemic mice. When we studied the PAI-1 deficient mice on the atherosclerotic background, we initially saw no difference in the amount of

atherosclerosis at the aortic root, which was the primary vascular location analyzed at that time. This important negative result was published due to previous evidence that PAI-1 played a role in atherosclerotic disease[5]. However, in the process of developing a mouse thrombosis model to assess the role of PAI-1 in the context of a diseased artery, I observed that there was consistently less atherosclerotic plaque at the carotid bifurcation of PAI-1 deficient mice on the atherosclerosis prone background. This led to a follow up study demonstrating reduced atherosclerosis at sites of high shear stress, similar to the carotid bifurcation, in PAI-1 deficient atherosclerotic prone mice[6]. We identified a similar site-specific increase in atherosclerosis/thrombosis when deficiency of a potent coagulation inhibitor, tissue factor pathway inhibitor (TFPI), was bred onto the atherosclerotic background[7]. Also, in the process of characterizing the atherosclerosis models, we discovered the dramatic effects of acute high fat feeding in altering thrombosis and platelet reactivity[8].

We were also interested in developing *in vivo* mouse models of arterial and venous thrombosis to investigate the roles of fibrinolysis and coagulation in these processes. I made key contributions in developing a new, relatively non-invasive mouse model of carotid artery and jugular vein injury leading to thrombosis[9, 10] At the time this model was developed, I was an expert in its usage and this led to two collaborations. With the Nabel Laboratory, we investigated the role of heme oxygenase-1 in oxidative mediated thrombosis[11]. In collaboration with Doug Tollefsen's laboratory at Washington University, we established the importance of heparin cofactor II in *in vivo* thrombus formation[12]. Our work on fibrinolysis and thrombosis models eventually led to the preparation of review articles on murine thrombosis models[13] and the role of

PAI-1 in vascular thrombosis[14]. During this time, Dan Lawrence, an eminent PAI-1 researcher, became interested in the role of PAI-1 in angiogenesis. We collaborated to investigate angiogenesis in mouse models of PAI-1 deficiency and over-expression[15].

I began to focus my research on coagulation factor V when Jisong Cui, a postdoc who developed mouse models of FV deficiency and Factor V leiden (FVL), departed from the lab. Working on these mouse models was a major focus of the lab, so David convinced me to shift part of my efforts to working on the FVL mice. At this time, I started working quasi-independently in the Ginsburg lab while also collaborating on several projects with Dan Eitzman, as he left to set up his own lab in the Cardiology Department. Analysis of the FVL mice yielded several notable discoveries. We initially characterized the prothrombotic phenotype of the FVL mice[16]. In a follow-up paper, we characterized the FVL mice further by investigating the role of FVL in models of atherosclerosis and thrombosis[17]. In attempting to produce FVL mice for the above phenotypic experiments, I noted the existence of a deleterious 129 strain effect in FVL mice on a mixed genetic background that significantly reduced the survival of FVL homozygotes. These observations, along with *in vitro* data suggested the existence of modifier genes that could affect the penetrance of FVL. I crossed mouse models of FVL and tissue factor pathway inhibitor (TFPI) deficiency and identified a lethal perinatal thrombotic phenotype. This suggested that modest variation in TFPI expression could be an important genetic modifier for the thrombosis associated with factor V Leiden in humans[18].

In his book Greatness: Who Makes History and Why, Dean Keith Simonton describes some of the common characteristics of great biological scientists, one of which is a prolific publication record. The supposed correlation between this sort of productivity and greatness is that great scientists are constantly asking interesting questions and devising experiments to test them. Out of their many published studies come the few key discoveries for which they are remembered. Since I endeavored to be an excellent scientist, I tried to devise hypotheses on a daily basis. While reading an oncology journal, I wondered whether the thrombophilic FVL mutation could be contributing to the lung fibrosis seen in a subset of people treated with the chemotherapeutic drug bleomycin. In collaboration with the Eitzman lab, we performed experiments in the FVL mice to test this hypothesis and demonstrated that the presence of FVL is associated with increased fibrosis in a bleomycin lung injury model[19].

Meanwhile, the genesis of the project to search for modifiers of thrombosis occurred when I started crossbreeding FVL homozygotes to several strain backgrounds. This project was briefly assumed by an M.D./Ph.D. student who soon returned to the M.D. portion of the program. Since I remained involved, the project was briefly handed back to me upon her departure, then transferred to a postdoctoral researcher. He worked on the FVL strain modifiers until David realized that the FVL/TFPI synthetic lethal thrombosis could serve as the basis for a sensitized ENU screen for dominant modifiers of thrombosis. The postdoc then worked on setting up the sensitized screen until leaving the lab several months later. Since I was again waitlisted for medical school, David urged me to consider graduate school and offered me the ENU mutagenesis project as the basis of a thesis project. At this point, I thought I had a chance for a viable career in

science without an M.D. degree so I began graduate study in Fall 2002, working on this thesis project mainly focused on identifying thrombotic modifiers.

Although the search for thrombosis modifiers was a labor intensive and logistically demanding undertaking, I still had some time and energy to form a number of collaborations. Although a lot of additional work, I found these to be enjoyable because they provided an opportunity to increase the scope of my scientific knowledge and skills[*]. While some of them were not fruitful, several are worthy of mentioning, as described briefly below.

Obesity has emerged as an independent risk factor for complications of atherosclerotic vascular disease. Leptin, a hormone produced by the adipocyte, had an effect on platelet aggregation *in vitro*. In collaboration with the Eitzman lab, we demonstrated that leptin contributes to arterial thrombosis following vascular injury *in vivo* and these prothrombotic effects appear to be mediated through the platelet leptin receptor.[20]

Aihua Zhu joined the Ginsburg lab as a postdoc and was producing gene-targeted mice deficient in gamma-carboxylase. Gamma-carboxylase is an enzyme that adds carboxyl groups to glutamic acid residues in a number of proteins, including several involved in coagulation. Since I was by this time adept at phenotyping mice, I worked with her to phentoype the gamma-carboxylase deficient mice. This analysis revealed that half of the deficent mice died in mid-embryogenesis while half survived to term, with the latter animals dying uniformly at birth of massive intra-abdominal

[*] To Quote Thomas Edison: "Opportunity is missed by most people, because it is dressed in overalls and looks like work."

hemorrhage. These results demonstrated *in vivo* that gamma-carboxylation is an essential posttranslational modification required for the full activity of several hemostatic proteins[21].

The bacterium *Bacillus anthracis* triggers apoptosis in macrophages to evade the host defense system. They do this by inhibition of p38 MAP kinase activation in toll-like receptor-4 activated macrophages. In collaboration with JinMo Park, we described the role of PAI-2 as a downstream effector of p38 signaling, that critically participates in maintenance of macrophage survival[22].

Jill Johnsen, a hematology fellow, joined the Ginsburg lab and assumed the project to identify the precise genetic variation responsible for the previously reported tissue-specific expression change in N-acetylgalactosaminyltransferase expression[3]. Since I was very familiar with this project, we had many discussions about how to proceed. She decided to survey a number of inbred mouse strains for the presence of both the haplotype block and the VWF deficient phenotype. Through this analysis, we determined that several of the mouse strains contained the tissue-specific expression change leading to low VWF levels. This enabled us to define a 30 Kb haplotype block responsible for the switch[23].

The glycocalyx consists of membrane-bound macromolecules comprised of sulfated proteoglycans, hyaluronan, glycoproteins, and plasma proteins that coat the surface of several cell types, including red blood cells. Kumkum Ganguly was testing whether coupling tissue-type plasminogen activator (tPA) to red blood cells could

prolong its circulating half life. tPA is a potent activator of fibrinolysis that due to its inhibition by PAI-1, is only somewhat efficaceous for dissolving thrombi in acute coronary syndromes. Using mouse models, we found the glycocalyx of red blood cells protected tPA from inhibition by PAI-1 and still remained a potent activator of fibrinolysis, suggesting that this method of tPA delivery could be an effective fibrinolytic therapy[24].

Pregnancy failure has been demonstrated in female carriers of the Factor V Leiden mutation. Hartmut Weiler, a prominent blood coagulation researcher, was interested in placental thrombosis as a cause of pregnancy failure. We utilized my extensive mating data for many different permutations of FVL mouse crosses as a starting point to characterize fetal loss in factor V Leiden (FVL) mothers. This fetal loss was found to be triggered by fetomaternal synergy of prothrombotic states[25].

Lois Weisman, an investigator in the Life Sciences Institute was working on Vac14, an important regulatory protein in $PI(3,5)P_2$ synthesis. This signaling lipid functions in multiple signaling pathways and controls yeast vacuolization. To investigate the *in vivo* function of Vac14 her lab made a mouse model of Vac14 deficiency. Together we reported the characterization of a mouse mutant lacking Vac14 that exhibit massive neurodegeneration, demonstrating that $PI(3,5)P_2$ is critical for the survival of neural cells[26].

Similar to the way the search for thrombosis modifier genes evolved from our earlier studies, several of the above projects have prompted further investigations that comprise the main body of this thesis. As we were using the newly developed Factor V Leiden mouse model to investigate the *in vivo* effects of this variant on thrombosis, we

realized that we could use the FVL mouse in conjunction with FV deficiency and tissue specific FV transgenic mouse models to investigate the two proposed mechanisms by which FVL caused increased thrombosis susceptibility, namely resistance of FVL to degradation by APC, and the APC cofactor function of wild type FV in the APC-mediated degradation of FVIII. The results of these observations form the basis of Chapter II.

In the process of phenotyping of gene targeted PAI-2 deficient mice, we noticed a discordant *sml* phenotype in one of the three PAI-2 deficient lines. An interesting offshoot from the PAI-2 project forms the basis of Chapter III. As previously described, chapters IV and V report our attempts to identify thrombotic modifiers. The first chapter provides the theoretical background for the subsequent chapters and Chapter VI summarizes the work and provides speculation about some future directions.

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CHAPTER I

INTRODUCTION

Factors involved in thrombus formation

A recent event in vertebrate evolution is the development of a pressurized vascular system to effectively and efficiently circulate blood, thus supplying oxygen and nutrients to every cell[27, 28]. The reliance on this type of vascular system means that maintaining both its integrity and patency is essential for the viability of the organism. A surveillance system designed to protect the vasculature in the event of an injury is a necessity. Otherwise, the entire blood volume could be rapidly lost following a breach to the circulation. The surveillance system would ideally also protect the patency of the vessels to ensure that cells and organs continue to receive essential oxygen and nutrients. To fulfill both of these roles, vertebrates have evolved the hemostatic system[29]. The hemostatic system produces blood clots called thrombi at sites of vascular damage. Proteins that degrade existing thrombi or inhibit further thrombus formation have evolved to regulate this process. The hemostatic system requires the coordinated activity of both vessel wall and circulating components such as blood platelets and plasma factors to ensure the normal operation of the vascular system[30]. (Figure 1.1)

Thrombus formation

Under normal physiologic conditions, the most potent vascular initiators of hemostasis are separated from circulating downstream factors by the endothelial barrier[31]. Upon disruption of vascular integrity, circulating blood comes in contact with the underlying subendothelial tissue, which is rich in tissue factor (TF), collagen and von Willebrand factor (VWF). VWF (both subendothelial and circulating) and collagen recruit platelets to the injury site by binding to platelet receptors GP1b and GPVI[32]. Platelets aggregating at the injury site are activated by thrombin and ADP to release the contents stored in their dense granules, such as calcium and ADP, among others[33, 34]. ADP in turn stimulates additional platelet activation through the platelet surface ADP receptors $P2Y_1$ and $P2Y_{12}$[35, 36], while thrombin accomplishes this through the protease activated receptors on platelet surfaces[37]. Calcium, along with the platelet phospholipid membrane, is required for the assembly of the essential coagulation cascade enzyme/cofactor complexes, such as the prothombinase and tenase complexes, as described below. Simultaneously with the dense granules, the platelet alpha granules release a myriad of procoagulant molecules including Factor V (FV), thrombin, fibrinogen, VWF and GPIIb-IIIa complexes[38-41].

At the same time, the blood coagulation cascade is initiated when the circulating serine protease enzyme factor VII (FVII) contacts its receptor, tissue factor (TF), in the subendothelium and forms a complex[42]. This complex then activates the circulating serine proteases factor X (FX) to factor Xa (FXa) and, to a lesser extent, factor IX (FIX) to factor IXa (FIXa)[43]. FXa converts a small amount of the zymogen prothrombin to

thrombin. This thrombin in turn activates the essential nonenzymatic cofactors FV and Factor VIII (FVIII) to FVa and FVIIIa.

FXa, initially generated by the TF-VIIa complex or later the FVIII-FIXa complex, binds activated FVa on membrane surfaces. This complex, called prothrombinase, converts prothrombin to thrombin at least four logs more efficiently that FXa alone[44]. Meanwhile, FIXa binds to FVIIIa to form the tenase complex. This complex efficiently activates FX to form FXa[45]. The formation of the tenase and prothrombinase complexes leads to the amplification phase of coagulation. During this phase, the thrombin generated participates in a positive feedback loop, activating more of the factors V and VIII, which participate in additional tenase and prothrombinase complexes. These complexes efficiently generate a burst of thrombin[46]. In addition to its role in platelet activation, thrombin cleaves small peptides from the alpha and beta chains of fibrin, giving rise to fibrin monomers which then polymerize to form insoluble fibrin polymers. Platelets bind to the fibrin strands via their GPIIb-IIIa receptors thus forming the hemostatic plug[47]. Finally, thrombin activates factor XIII, an enzyme responsible for catalyzing the formation of covalent crosslinks between the fibrin strands, rendering them resistant to enzymatic dissolution[48]. This process is illustrated in Figure 1.2.

Coagulation inhibitors

Once blood loss has been contained, the coagulation cascade must be rapidly inactivated or the organism will suffer from local occlusive thrombosis or the more severe condition, disseminated intravascular coagulation[49]. There are three layers of inhibition designed to counteract blood coagulation: neutralization of the activated

coagulation enzymes by direct inhibition, degradation of the cofactors FVa and FVIIIa and dissolution of the fibrin clot. Two key inhibitors that directly inhibit the serine proteases of the coagulation cascade are tissue factor pathway inhibitor (TFPI) and antithrombin III (ATIII). (Figure 1.3) TFPI is a kunitz-type protease inhibitor that counteracts the earliest step in the coagulation cascade, thus inhibiting the subsequent cascade of events leading to the formation of a blood clot[50, 51]. TFPI is secreted mainly by the endothelium and inhibits the activity of TF/FVIIa by first binding to blood-borne Fxa, then forming a ternary complex with TF/FVIIa/FXa. By acting as a proximal mediator in the inhibition of coagulation, variations in endogenous TFPI may greatly influence the extent of thrombosis that occurs following vascular insult or as the result of a dysfunctional endothelium that produces TF[7]. The essential role of TFPI is further demonstrated by the fact that mice with total TFPI deficiency exhibit embryonic lethality.

ATIII is a potent multifunctional serine protease inhibitor. When bound to the therapeutic molecule heparin, ATIII provides a rapid inhibition mainly of thrombin, FIX and X, but it also inhibits several other active enzymes of the coagulation cascade[52, 53]. ATIII is a poor inhibitor until it binds to heparin, thus increasing its potency by inducing a conformational chance that enhances its affinity for thrombin. Heparin also bridges ATIII with its target serine protease. These mechanisms provide the basis for the therapeutic antithrombotic action of heparin that has been effectively used as an anticoagulant for over 70 years. Genetically, heterozygous ATIII deficiency in humans has been described as an important thrombophilic risk factor, further illustrating its importance in controlling coagulation[52, 54]. Total ATIII deficiency is extremely rare in humans and causes lethality in mice[55-57]. However, heterozygous ATIII deficiency

is present in approximately 1:2000 people and is associated with an approximately ten-fold increased risk of thrombosis. Other coagulation inhibitors such as alpha 2 macroglobulin, and heparin cofactor II[58] exist but will not be described in detail here.

Inactivation of coagulation

The protein C (PC) pathway results in the production of activated protein C (APC), which specifically degrades coagulation factors Va and VIIIa[30, 59]. (Figure 1.4) The first step in generating APC is the binding of thrombin to the endothelial cell receptor thrombomodulin (TM), which transforms thrombin into a potent activator of PC, transforming it to APC at the endothelial surface[60]. Binding of PC to the endothelial protein C receptor (EPCR) accelerates this reaction 20-fold[61]. APC subsequently downregulates coagulation by proteolytically inactivating FVa and FVIIIa, thus greatly reducing the generation of additional thrombin. Membrane associated protein S has been shown to be an essential cofactor for APC. Association of APC with protein S results in 20-fold increase in APC activity[62]. Moreover, protein S and partially or totally cleaved FV participate in synergy as a cofactor for the APC-mediated degradation of FVIIIa[63, 64], with FV enhancing the rate two-fold[65]. Cleavage of FV and Protein S by thrombin results in loss of this APC cofactor activity[63].

The physiological importance of the PC pathway in regulating hemostasis is highlighted by the fact that acquired or congenital PC deficiency leads to thrombotic disease[57, 66-68]. In addition, elements of the PC system have been identified as far back evolutionarily as bony fishes, suggesting this system is essential for the regulation of blood coagulation[29]. Not surprisingly, molecular defects in other components

associated with the PC pathway and coagulation cascade result in increased thrombosis risk. These include protein S deficiency, EPCR deficiency, the prothrombin 20210 variant and mutations in the FV gene. These defects range in prevalence from relatively rare[69-71], to relatively common in selected populations[72].

APC resistance

Three APC cleavage sites have been identified in both FVa and FVIIIa, at positions Arg506, Arg306 and Arg679 in FVa and Arg336, Arg562, and Arg740 in FVIIIa[59, 73]. (Figure 1.5) FVa is completely inactivated to FVi when it has been processed by APC at these three sites. Cleavage at Arg506 is known to be the most kinetically favored cleavage site and therefore rapid, while subsequent cleavage at Arg306 results in a nearly complete loss of activity in vitro[74]. In addition, cleavage of FV at Arg306 is dependent on the presence of negatively charged phospholipid and strongly enhanced by the presence of protein S, while Arg506 is less dependent on these molecular interactions[59]. Cleavage at Arg679 results in complete inactivation of FV.

Humans that carry FV variants at two of the three APC cleavage sites have been identified and been shown to have increased thrombosis risk[75-77], though mutations that alter Arg306 (FV Cambridge and FV Hong Kong) are rare and controversy exists about their increased thrombotic risk. Figure 1.5. A glutamine for arginine substitution at position 506 (R506Q) called Factor V Leiden (FVL), is a common polymorphism in Caucasians. This amino acid substitution confers resistance of FVL to degradation by APC. Thus, the active cofactor lasts ten times longer in the circulation than "normal" FV, resulting in a gain-of-function as a cofactor in the prothrombinase complex.

Subsequently, it has been discovered that the endogenous wild type FV serves as an essential cofactor for the PC mediated degradation of FVIIIa, increasing the rate of FVIIIa degradation by two-fold[65]. To express full APC cofactor activity, FV must retain part of its B domain and be cleaved by APC at Arg506[78-80]. Thus, partially cleaved wild type FV is an effective cofactor. FVL abolishes this cleavage site and confers resistance of this protein to degradation by APC, and also diminishes its capacity to act as APC cofactor. These two independent mechanisms are thought to contribute to the increased risk of thrombosis in FVL carriers. However, conflicting reports have been published about the ability of FVL to serve as an APC cofactor for the inactivation of Factor VIII[81]. It has been shown to be approximately ten-fold less efficient as a cofactor for APC-mediated *in vitro* thrombin generation assays, although a subsequent report suggests that FVL lacks cofactor activity entirely[78]. Since the anticoagulant cofactor function of FV is still poorly characterized, in Chapter II we use mouse models to investigate its role *in vivo*.

Clot dissolution by fibrinolysis

In addition to the inhibitory checks described above, the fate of a forming intravascular thrombus is also determined by a delicate balance between procoagulant factors and factors promoting degradation of the fibrin component of the thrombus (fibrinolysis). As described above, fibrin is an essential component of the thrombus and fibrinogen deficiencies have been described and exhibit a severe bleeding phenotype[82]. The fibrinolytic cascade is comprised of molecules whose function is to degrade the fibrin component of the blood clot and is itself under the exquisite control of activators

and inhibitors[83]. The degradation of fibrin is accomplished by plasmin, with disruption of the fibrin scaffold leading to dissolution of the clot. Thus, as fibrinolysis and thrombus formation occur simultaneously, fibrinolysis is a dynamic modulator of clot formation.

The proteolytic conversion of the zymogen plasminogen to the active serine protease plasmin is regulated by a complex system of proteins that includes urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), and is opposed by the plasminogen activator inhibitors. (Figure 1.6) The plasminogen activator inhibitors (PAI's), are PAI-1 and PAI-2. PAI-2 has also been demonstrated to inhibit uPA and tPA *in vitro.* However, its precise biological relevance to fibrinolysis is not known. For this reason, we created a knockout mouse model of PAI-2. We inadvertently discovered a linked phenotype first identified during the analysis of this PAI-2 deficient mouse model, which is the subject of Chapter III.

The clinical importance and phenotypic variability of thrombosis

Thrombosis occurring in the arteries or veins accounts for approximately 40 percent of deaths in western societies. Even sub-lethal thrombosis results in significant morbidity and contributes significantly to health care expenditures. Genetic and environmental risk factors contribute to both arterial and venous thrombosis. Although there are clearly shared environmental risk factors between them[84], there have been few genetic risk factors identified in common between arterial and venous thrombosis. This could be partly due to anatomical and rheological differences, as well as the fact that arterial thrombosis almost always occurs in the context of a pre-existing atherosclerotic

lesion. Due in part to the complicated etiology of arterial thrombosis, the genetics of this condition are ill-defined. However, as described above, mutations involving several genes in the coagulation cascade have been clearly implicated in venous thrombosis[85].

Venous thrombosis is common in western societies, occurring in approximately 1-2 per thousand individuals per year[86]. Venous thrombosis is a clinically heterogeneous disease. Deep venous thrombosis (DVT) is the most common presentation and is usually detected when an occlusive blood clot forms intravascularly in the great vessels of the legs. If left untreated, thrombi from a DVT can occasionally break away from the main clot and travel through the venous circulation to the lungs, where they can lodge and cause the life threatening condition called pulmonary embolism. Venous thrombosis and pulmonary embolism are associated with substantial morbidity and mortality[87]. If left untreated, pulmonary embolism is lethal in 25 percent of cases. Other less common forms of venous thrombosis occasionally manifest themselves at other sites, such as in superficial veins or the vessels of the arm or retina.

Phenotypic heterogeneity has been noted even in well known Mendelian disorders such as hemophilia and cystic fibrosis[88-90]. The phenotypic variability of venous thrombosis should not be surprising given the complexity of hemostasis, with many molecules acting in concert to determine the extent and duration of thrombus formation. There is often substantial variation in phenotype among FVL patients presenting with venous thrombosis, including age of onset, recurrence rate and emboli formation.

There are effective drug therapies for treating acute venous thrombosis. However, a low risk, effective long term preventative therapy is lacking at present.

Delineating the genetics of venous thrombosis may lead to improved risk assessment and treatment modalities.

Genetics of thrombosis risk and evidence for modifier genes

Predisposition to venous thrombosis can be heavily influenced by mutations in a single gene and/or combinations of genes. Although venous thrombosis clearly exhibits familial inheritance, in many cases no genetic risk factor can be found[91]. It has been hypothesized to be oligogenic[92] with a handful of genetic variants in various combinations contributing to the risk[86].

Quantitative increases or deficiencies in coagulation factors or their inhibitors can influence the thrombotic potential of the hemostatic system. The levels of coagulation factors clinically considered to be in the normal range vary from 50-150 percent of a mean based on values obtained form large numbers of the human population[93]. Reports have clearly demonstrated that a general deficiency of procoagulant factors decreases the ability of stable thrombi to form, thus lowering it into a range where bleeding becomes a risk[94, 95]. Variations in functionally related genes such as those in the PC pathway have been demonstrated to increase the susceptibility to venous thrombosis[96]. In addition to deficiencies in Protein C and ATIII, elevated levels of certain circulating factors, such as factor VIII, IX, XI and fibrinogen have been reported to increase susceptibility to thrombosis[97]. However the mechanisms responsible for these alterations are unknown[97] though it seems to make mechanistic sense in that they increase thrombin and/or fibrin generation.

In addition to the quantitative effects of hemostatic proteins on thrombus susceptibility, there are also qualitative effects, such as FVL. FVL is the most common genetic risk factor for venous thrombosis in European-derived populations, with a frequency of about 5 percent[53], and is found in 25-40 percent of all clinically significant thromboses[77]. APC resistance due to FVL is thought to account for up to 25 percent of the genetically attributable thrombosis risk according to family based population studies[98].

Despite being present in many thrombosis patients, FVL is incompletely penetrant, with only about ten percent of heterozygotes developing thrombosis in their lifetimes. The extensive clinical variability seen in venous thrombosis can be partially explained by genetic interactions between FVL and other known genetic risk factors for thrombosis. For example, patients co-inheriting both PC deficiency and FVL have an approximately 80 percent lifetime risk for thrombosis. These observations underscore the genetic complexity of hemostatic processes and it could be speculated that genetic risk factors may not be limited to genes in the coagulation or fibrinolytic pathways.

Presently, patients presenting with a thrombotic disorder can be screened for the FVL mutation, but due to the low penetrance, determining their FVL genetic status has little effect on disease treatment[99]. Several human candidate gene association studies have identified known coagulation components as risk factors but they are limited in their ability to pinpoint novel risk factors, especially those that may be outside the coagulation cascade[100]. Due to the genetic complexity of the human populations and the highly variable environment, identifying such genes in humans has proven difficult. For this reason, we turned to mouse models to find genes that contribute to venous thrombosis.

The historical role of genetics in hemostasis

Profound functional insights have resulted from the study of naturally occurring mutants of the blood coagulation cascade. Historically, these insights have been from human studies[52, 77, 99, 101-105]. Forward, also known as classical or phenotype driven genetics, coupled with modern biochemical or molecular approaches have been essential in discovering and describing many of the components of the coagulation cascade in humans. For example, the moderate to severe bleeding phenotype initially called hereditary pseudohemophilia in the residents of the Åland islands led Dr. von Willebrand to carefully study and describe a group of families[106]. Later work confirmed that defects in the protein called von Willebrand factor (VWF) were responsible, placing a functional role of this gene/protein firmly in blood coagulation[107-110]. The FVL mutation was identified by the development of a biochemical assay to assess APC function and subsequently discovering that a subset of thrombosis patients carried FV that was resistant to inactivation by APC[77, 111].

Using reverse genetics to develop mouse models of coagulation

In recent years, technology has enabled the identification of the entire genomic sequence of many species, which has facilitated the prediction and discovery of many genes in the genome without knowing their function[112, 113]. Advances in transgenic mouse technology have given rise to reverse (or gene driven) genetics by making it possible to systematically perturb the expression of a gene or genes of interest by deletion or over-expression in this model organism[114]. The phenotypic characterization of

these mice is then attempted by the generation of hypotheses built on prior information about the gene or pathway. To probe the *in vivo* relevance of coagulation factors, our lab has developed a number of transgenic mouse models. In addition, there are already transgenic mouse models for almost every known coagulation gene, with the exception of protein S[115]. Although there have been some surprises, analysis of these mouse hemostasis models has demonstrated that these known "coagulation" genes behave much as predicted.

Reverse genetics to discover interactions between known coagulation components

Previously, van't Veer et al. used a synthetic *in vitro* thrombin generation assay system to model the impact of various coagulation proteins on overall hemostatic balance, including FVL and moderate reductions in TFPI level[116]. These investigators found that in the presence of the PC pathway, a 50 percent reduction in TFPI level in the presence of homozygous FVL results in thrombin generation close to that observed in the absence of the PC pathway. Based on these data, van't Veer et al. suggested that mild to moderate decreases in TFPI in the genetic context of FVL homozygosity would result in a significant worsening of the thrombotic phenotype.

Given the importance of FVL in thrombosis risk, we have knocked in the FVL mutation into the endogenous FV locus[16]. Our aim was to further study this variant *in vivo*, specifically in the setting of vascular disease[17]. We reported FVL homozygous and heterozygous mice to have a mild prothrombotic tendency, with spontaneous fibrin deposition in several tissues[16]. We also utilized a knockout mouse model of TFPI deficiency to further explore the role of endogenous TFPI in thrombosis. Mice totally

deficient in TFPI, previously reported by Huang et al., result in an embryonic lethal phenotype. However, mice heterozygous for TFPI appear normal despite reduced plasma activity levels of TFPI[117].

Based on observations by van't Veer et al. and reports that TFPI deficiency has been associated with venous thrombosis in a small number of humans[118, 119], we hypothesized that mild TFPI deficiency would impact the phenotype of the FVL homozygous mice *in vivo*. Through breeding experiments, we found that when homozygosity for FVL and one disrupted copy of TFPI are combined in the same mouse (FVL/TFPI), the result is a severe, lethal perinatal thrombosis[18]. The mice survive *in utero* until at least day 18.5 *post coitus(pc)*. Death may be the result of birthing trauma that activates blood coagulation. This interesting result uncovered a novel *in vivo* interaction between FVL and TFPI. It is important to note that this synthetic lethal interaction was in the genetic context of the purebred C57BL/6J background[18].

We have exploited the FVL/TFPI synthetic lethal thrombotic phenotype derived from our reverse genetic approach to serve as the phenotype for forward genetic screening strategies aimed at finding coagulation modifier genes that lessen the severity of thrombosis in these mice, thereby restoring viability. We undertook a dual approach of using both inbred mouse strain variability and chemically induced mutagenesis in an attempt to identify strain-specific and ENU-induced thrombotic modifiers.

Searching for novel genetic modifiers using mouse models

Inbred mouse strains share much naturally occurring genotypic and phenotypic variation. Some of the most divergent mouse strains are more distantly related to each other than humans are to chimpanzees[120]. The many generations of divergent breeding means that distantly related inbred strains may possess more changes between them than can be observed from 10,000 G3 mice from a recessive ENU induced mutagenesis screen (see below)[121].

Among them, inbred mouse strains have been shown to differ markedly in almost every phenotype analyzed, including hematological parameters, such as susceptibility to bleeding and clinical assessments of blood coagulation such as the PT and APTT[1, 122, 123]. Given this evolutionary history and the evidence for genetic interactions contributing to thrombosis susceptibility in humans, it is not surprising that there are many examples where particular hemostatic mouse models display different phenotypes in the context of different genetic backgrounds[13, 124]. We and others have previously shown striking intra-strain differences in thrombosis related phenotypes[3, 125, 126]. For example, we have found that heterozygous PC deficient mice have reduced survival on a mixed 129S1/SvImJ- C57BL/6J compared to a pure C57BL/6J background[13]. Mouse genetic background also has potent effects on vascular response to injury. When tested using the Rose Bengal model, purebred C57BL/6J mice exhibit occlusion times that are significantly longer than purebred mice of the KK/HlJ strain[127]. Similar strain differences are also present between C57BL/6J, 129S1/SvImJ and other strains.

A modifying locus for a Mendelian disorder has been defined as an inherited genetic variation, distinct from the disease gene, which leads to a qualitative or

quantitative difference in any aspect of the disease phenotype[128]. There are continuing efforts by others to exploit the advances in mouse genetic models, genomic and bioinformatics tools to identify modifier genes responsible for the phenotypic variation observed in thrombotic phenotypes that vary on different inbred strain genetic backgrounds.

Rosen et al. have generated mice expressing a targeted, genetically engineered allele of the coagulation Factor VII gene that expresses less than one percent of FVII[124]. On the C57BL/6J genetic background this mouse displays a more severe bleeding phenotype than mice on a mixed C57BL/6J:129x1/SVJ background. Using a backcross approach, these investigators have identified 8 putative loci enriched for 129x1/SVJ alleles, suggesting that these loci might contain genes that modify the phenotype based on this genetic disorder.

Hoover-Plow et al. previously uncovered differences in bleeding time between the A/J and C57BL/6J mouse strains. In a recent study, this group utilized A/J:C57BL/6J chromosomal substitution strains to identify quantitative trait loci on chromosomes 5, 17 and 11 that regulate bleeding times in mice[122].

Most recently, in the process of backcrossing the FVL and TFPI to different inbred mouse strains in order to map mutations from our mutagenesis study (Chapter V), we observed complete viability of FVL/TFPI on the DBA/2J background. Chapter IV provides the details of our search for strain modifier genes using the strain background variability of the FVL/TFPI phenotype.

Genetic Screening

As in mouse genetics, early genetic investigations using model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* were focused on the mapping of spontaneous visible mutant phenotypes. However, in the 1960's, ethyl methane sulphonate (EMS) was determined to be a potent point mutagen in *D. melanogaster*[129], followed by the observation that this chemical also readily produced mutants in *C. elegans*[130]. Once the methodology for efficiently generating mutations using EMS was developed, investigators harnessed this approach in genetic screens to identify individuals who possess a particular phenotype of interest. Genetic screening strategies are attractive because they link phenotype with the genetic and molecular events responsible for a particular biological process. Sydney Brenner and the team of Christiane Nusslein-Volhard and Eric Wieschaus developed the first large scale EMS mutagenesis screens in *C. elegans* and *D. malanogaster*, respectively[130, 131]. In particular, the *D. malanogaster* screen searching for mutations that affect embryo patterning was revolutionary in that these investigators attempted to completely delineate embryo patterning. Although, the catalog of mutants they generated was not complete, this study opened up many new areas of investigation in the field of *D. melanogaster* genetics[129].

Studies in *C. elegans* and *D. melanogaster* resulted in these and many subsequent successes of genetic screening using EMS mutagenesis. The short generation times of *C. elegans* and *D. melanogaster* played a large part in the rapidity of the successful implementation of genetic screens compared to those in the mouse. Not surprisingly, advanced genetic screening strategies could be more quickly theorized and realized. Perhaps the most elegant specialized screening techniques that have been developed in

these organisms are modifier or enhancer/suppressor screens**.** Through modifier screens, it is possible to identify additional genes influencing the biological process of interest because dominant enhancers/suppressors can be identified due to the sensitized genetic background which were not identified in a more basic screen[132].

Modifier screens begin with a strain whose genetic composition or chemical sensitivity causes a defined phenotypic defect. This phenotype should be amenable to modification. For example, a hypomorphic allele of Sevenless was discovered that provides just enough signaling activity for most of the eye R7 cells to form in *D. melanogaster*. However, any further reduction in Sevenless expression causes all the R7 cells to convert to cone cells. Thus, the phenotype observed in a modifier screen conducted in this genetic background is sensitized to mild perturbations in Sevenless expression[129]. In this sensitized background new mutants that either enhance or suppress the phenotype were discovered as gain of function in Son of sevenless and Ras1[132].

ENU induced chemical mutagenesis in the mouse

The profound insights that have resulted from the study of naturally occurring mutants in almost every area of biological research, ranging from hematology to development[95, 102, 133] have driven researchers to turn to intentionally creating phenotypes by random mutagenesis rather than waiting for naturally occurring phenotypes to emerge from their mouse colonies.

Work performed at the Oak Ridge National Laboratory by the Russells and colleagues played an important role in the quest for finding suitable mutagenic agents in

mice[134]. These investigators experimented with the mutagenic effects of both ionizing radiation and chemical agents. To assess the mutagenic effects of these modalities, they used the specific locus method using the T-stock mouse. This method employs seven recessive, easily identifiable heterozygous viable mutations affecting easily recognizable traits such as coat color and ear size as a phenotype to observe mutations that result in loss of heterozygosity. While mouse spermatogonial germ cells were found to be relatively resistant to the mutagenic effects of radiation, the chemical agents N-ethyl-N-nitrosourea (ENU) and chlorambucil were found to be efficacious in inducing germline mutations as measured by visual inspection of mutagenized T-stock mice[135, 136]. Both chemicals can induce mutations at an average per locus frequency which is greater than one in a thousand, although the types of mutations that are induced are quite different. ENU causes discrete lesions which are often point mutations[134], whereas chlorambucil causes large lesions which are often multi-locus deletions[137]. Unfortunately, chlorambucil induced mutations were often associated with reciprocal translocations, which can have deleterious effects on fertility and strain propagation[137].

Of the two chemicals, ENU has become the chemical of choice for creating mouse mutants in forward genetic screens based largely on the disadvantages associated with chlorambucil along with concomitant early successes described by Bode[138], Su et al[139] and Vitaterna et al[140]. These studies identified visible mutants as well as novel genes for familial adenomatous polyposis and circadian rhythm and proved the viability of an ENU-based phenotype driven approach. These observations provided the incentive to initiate additional large-scale ENU mouse mutagenesis projects. Such forward genetic

screens have since led to critical new insights into the molecular pathways involved in coat color[141] hearing[142] and immunology[143], just to name a few.

ENU has also been used effectively in *Danio rerio* as a means of producing large numbers of novel, mainly point mutants[144]. Phenotypes resulting from ENU induced point mutations may also prove to more closely approximate the human condition than traditional mouse transgenics, which risk perturbing the genome due to insertion of the vector. Chapter III describes the discovery of a point mutant in the *Irs1* gene that we identified while analyzing gene targeted PAI-2 deficient mice. This nonsense point mutant phenotype differs in several important ways from the *Irs1* deficient mice produced by gene targeting, raising the possibility that regional genetic interference from the targeting construct could lead to imposter phenotypes attributed to *Irs1* deficiency. Some investigators have begun a reverse genetics approach screening for ENU mutants in a gene of interest to uncover an allelic series of point mutants[145, 146].

In mice, ENU has been well documented to generate random point mutations throughout the organism, both in somatic cells as well as in the 150 to 200 spermatogonial germ cells of male mice. ENU works by transferring its ethyl group to oxygen or nitrogen radicals at a number of reactive sites in each of the nucleotides: the N1, N3, N7 groups of adenine, the O6, N3 and N7 of guanine, the O2, O4 and N3 of thymine and the O2 and N3 of cytosine[147]. These ethyl groups can than cause the replication machinery to mistake the modified nucleotide, resulting in mispairing. Some sites are more reactive than others. The most common site for the occurrence of mutations is at A-T base pairs. Between 70 percent and 85 percent of all ENU-induced nucleotide substitutions are estimated to be either A-T to T-A transversions or A-T to G-

C transitions. When the mutation is located in an exon, these substitutions result in approximately 70 percent nonsynonymous changes of which approximately 65 percent are missense changes, and the remainder are nonsense or splice mutations[148].

ENU can also cause protein carbamoylation of amino acids, resulting in significant nonheritable effects[147]. The mutational effects on the spermatogonial germ cells are of interest for forward genetics studies because these cells give rise to sperm. Mutated mice can then be used in mating experiments to query the genome for the modulation of a phenotype of choice.

The methods of ENU mutagenesis have been well documented in many different mouse strains[149, 150]. ENU is administered intraperitoneally in male mice (called the G0 generation) and circulates to the testes where it exerts its mutational effects on the spermatogonial germ cells. Careful dosing is necessary because ENU can kill the entire cohort of spermatogonial germ cells, or leave just a few mutated live cells to re-populate the testes. This obviously reduces the number of novel independent mutations that can be queried from each G0 mouse. The G0 mice undergo a sterile period of about 10 to 20 weeks while the spermatogonial cells recover and repopulate, after which time the mice can be bred. Depending on the mouse strain chosen for the G0 generation and the dose of ENU used, it is possible to attain an average mutation frequency of up to 1.5×10^{-3} per locus, so approximately 1 in every 1000 gametes from a mutagenized male might be expected to carry a mutation in any gene of interest[151, 152]. Thus, ENU induces a mutation rate 10 to 15 fold higher than that of the endogenous background mutation rate seen in mice[121].

The general approach of whole-genome dominant ENU mutagenesis is illustrated in Figure 1.7. First, G0 male mice are injected with ENU. Since each uniquely mutated spermatogonial germ cell will contribute to total sperm production, one ENU treated male can pass on many different sets of random mutations to its offspring. Thus, each offspring of these ENU mutagenized males, called the G1 generation, will carry one inactive copy for a different set of approximately 20 to 30 genes in each mouse. Dominant screens are relatively simple in that the offspring of the mutagenized male can be screened for the phenotype of interest. Information from large scale dominant genome wide screens indicates that up to two percent of progeny can carry a heritable mutant phenotype[153].

Screening a large number of progeny from an ENU screen can theoretically cover at least one mutant in every gene in the genome[154]. The choice of phenotype is essential for the success of a mutagenesis screen and requires careful forethought at the outset to maximize the likelihood of success. Most publications to date have relied on easily recognizable phenotypes, such as coat color, skeletal defects, and behavioral abnormalities[141, 142, 154, 155] although some screens have employed more complex phenotypes[156, 157].

ENU screens can also be designed to discover recessive mutations. Recessive screens are more intensive and require an additional breeding step for analysis of potential recessive mice in the G3 generation. (Figure 1.8) Recessive screens are very laborious and logistically demanding[153], and several large centers have been formed to undertake comprehensive and systematic dominant and recessive ENU mutagenesis programs to assemble a functional map of the mammalian genome. Ongoing

mutagenesis programs have been recently tabulated in a recent review[153, 158]. These programs are screening for as many phenotypes as they can devise, though obviously limited by specific interests as well as resources[151]. Hematological parameters such as susceptibility to bleeding and clinical assessments of blood coagulation, including the prothrombin time and activated partial thromboplastin time[159] are being undertaken at some of the leading mutagenesis centers.

Because of the logistical difficulties in conducting large scale ENU mutagenesis screens, more elaborate schemes have been developed to investigate a genomic region or regions of interest. Such screens may employ mice that are hemizygous for a genomic region of interest, which can be accomplished through deletion or a balancer chromosome containing a dominant visible marker. Recessive mutations are recovered as a result of loss of heterozygosity[160].

Recently, sensitized genetic screens have been devised and successfully implemented in the mouse. An example of a sensitized screen was recently reported regarding the control of platelet production. Thrombopoeitin specifically regulates platelet production by binding through the thrombopoetin (MPL) receptor on megakaryocytes. Recently, Carpinelli et al. used MPL deficiency as a sensitized screen to uncover a mutation in the c-Myb gene as a dominant modifier of platelet number in this model of congenital thrombocytopenia[161, 162]. Thus, genetic modifier screens, which have proven so effective in lower organisms[163, 164] are also feasible in the mouse.

As described in Chapter V, we took advantage of the FVL/TFPI lethal genotype combination as a phenotype to set up a sensitized ENU mutagenesis screen. Heritable

mutant phenotypes can then be mapped to the mutated region of the genome by standard mouse positional cloning techniques[1, 3].

Mapping ENU induced mutants

The successful mapping and identification of the mutation is predicated upon having the mutant be relatively impervious to the effects of strain background. Once a relevant phenotype has been identified and its heritability proven, genetic crosses are required to map the mutant phenotypes to a specific region of the genome by standard mouse positional cloning techniques[1, 3]. (Figure 1.9) The mutation can then be identified by fine mapping down to a resolution acceptable for evaluation of candidate genes, ideally less than 1Mb in size[143]. Logical candidates are afforded priority, but it is essential to keep in mind that the mutation may be in a novel gene whose function is unknown. While nearly 80 percent of mutants have been identified in coding regions in some studies, this may be an ascertainment bias and it is important to consider the possibility that the causative mutation could be anywhere in the region[121].

Insights from mouse models of the hemostatic system

In this thesis, the above mentioned genetic techniques were utilized to investigate transgenic mice of the hemostatic system. Chapter II explores the APC cofactor activity of wildtype Factor V as an endogenous anticoagulant mechanism. Chapter III identifies the mutation responsible for an interesting phenotype first observed in gene targeted PAI-2 deficient mice and chapters IV and V report our attempts to identify thrombotic modifiers using mouse inbred strain variation and a sensitized suppressor screen.

Figure 1.1 Thrombotic potential of the coagulation system

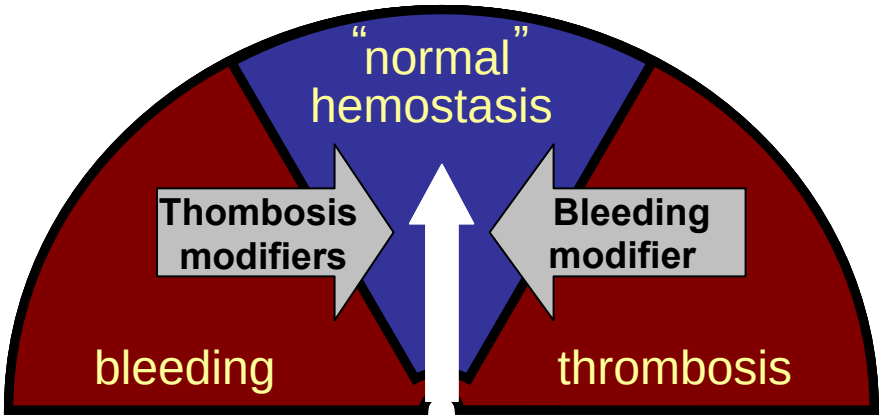


Figure 1.1 Thrombotic potential of the coagulation system. With minor fluctuations, the system is designed to operate in the normal (blue) range, as indicated by the gauge arrow. Genetic modifiers either reducing or increasing (light gray arrows) the thrombotic potential of the system will cause systemic malfunction (red zones), where there is a heightened susceptibility to either bleeding or thrombosis.

Figure 1.2 Thrombosis at a site of vascular injury

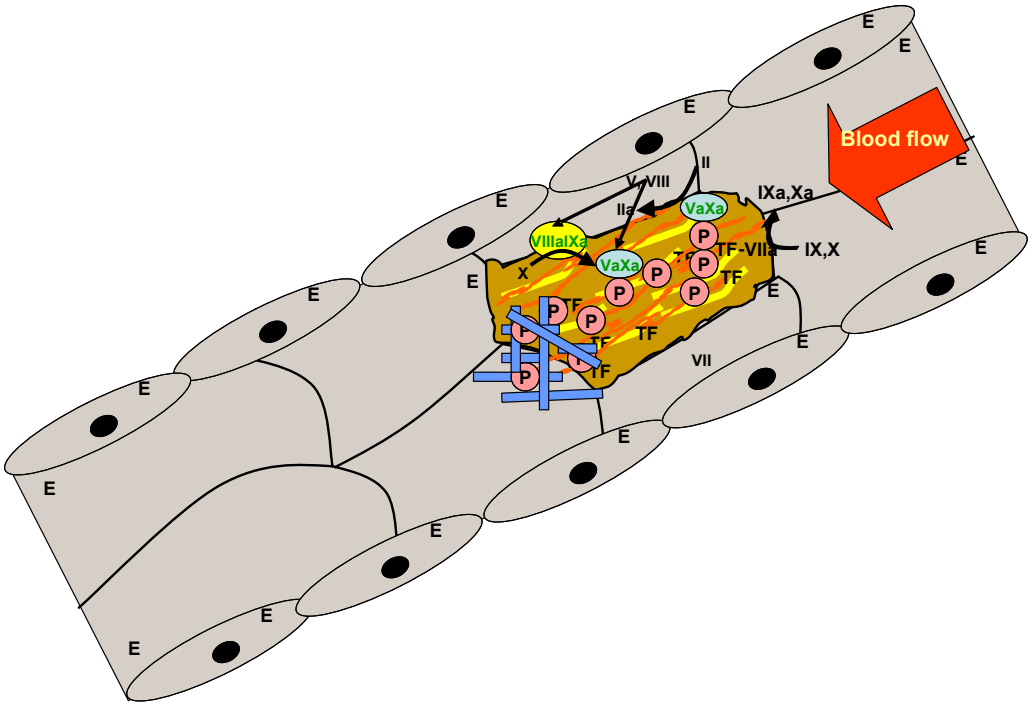


Figure 1.2 Thrombosis at a site of vascular injury. Endothelium, denoted by the "E" lines a blood vessel with blood flowing in the direction denoted by the red arrow. Following vascular disruption shown in gold, subendothelial molecules are exposed: collagen (yellow), VWF (red) and tissue factor (TF). Platelets encounter and bind to the collagen and VWF through their surface receptors, GP VI and GP1B. Platelets release the contents of their granules, which in turn activates and recruits additional platelets. Circulating FVII binds and is activated by TF, FVIIa in turn activates FIX and FX to FIXa and FXa. These molecules activate prothrombin to thrombin, denoted as II to IIa. IIa then activates FV and FVIII, resulting in the formation of the tenase (yellow oval) and prothrombinase (light blue oval) complexes that result in a burst of IIa production. IIa further activates platelets and cleaves fibrinogen into fibrin, which along with platelets functions as the hemostatic plug.

Figure 1.3 Coagulation Inhibitors

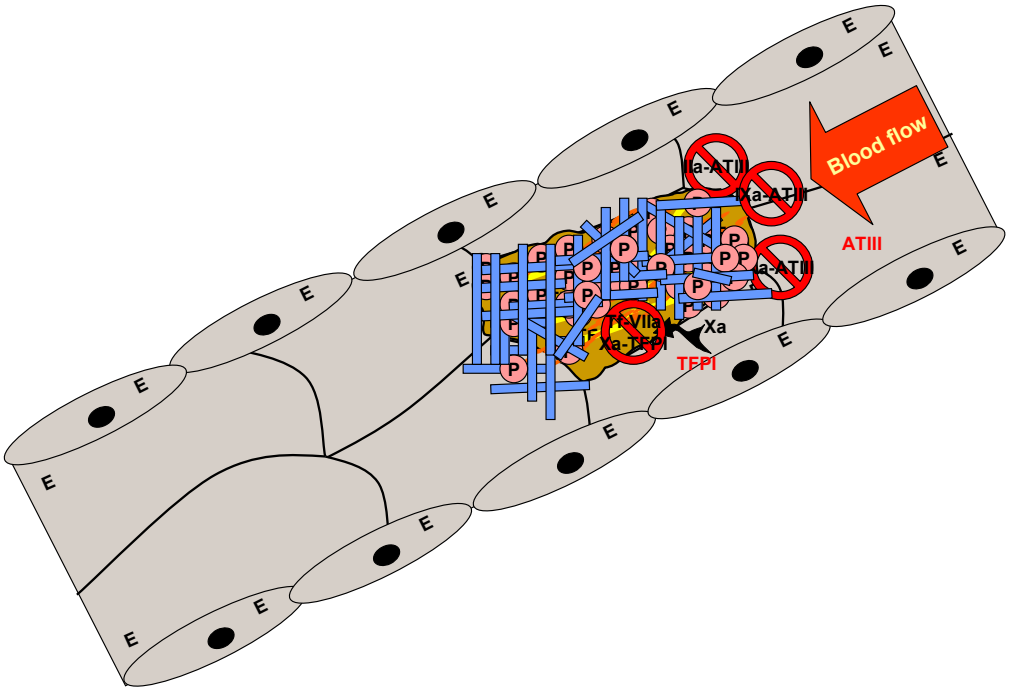


Figure 1.3 Coagulation Inhibitors. To prevent pathologic thrombosis, the coagulation cascade has a series of inhibitors. Tissue factor pathway inhibitor (TFPI) is membrane localized kunitz-type protease inhibitor secreted mainly by the endothelium. TFPI binds first to circulating Xa then forms a ternary complex involving TF/FVIIa, thus inhibiting the subsequent cascade of events leading to the formation of a blood clot, as shown with the red prohibition sign. Antithrombin III (ATIII) is a potent multifunctional serine protease inhibitor. When bound to heparin, ATIII provides a rapid and robust inhibition mainly of thrombin, FIX and X, as well as the other active enzymes of the coagulation cascade, also shown with the red prohibition sign.

Figure 1.4 Protein C pathway to downregulate coagulation

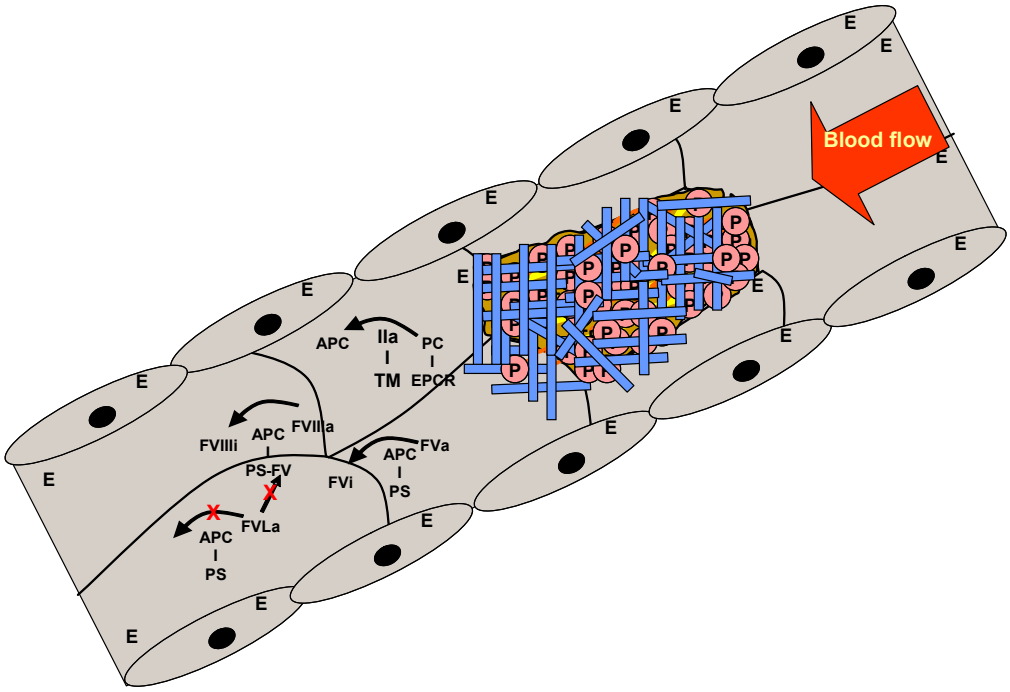


Figure 1.4 Protein C pathway to downregulate coagulation. APC is generated by the binding of the the endothelial cell receptor thrombomodulin (TM) to thrombin (IIa), transforming it into a potent activator of PC. Binding of PC to the endothelial protein C receptor (EPCR) accelerates this reaction. Once APC is formed, it downregulates coagulation by inactivating the non-enzymatic cofactors FVa and FVIIIa to FVi and FVIIIi, thus greatly reducing the generation of additional thrombin. Protein S (PS) has been shown to be an essential cofactor for APC-mediated inactivation of FV. Protein S and FV participate as cofactors for the APC-mediated degradation of FVIIIa. Factor V Leiden, FVL is resistant to degradation by APC and also acts as a poor APC cofactor.

Figure 1.5 Mechanism of APC action and APC resistance due to Factor V Leiden

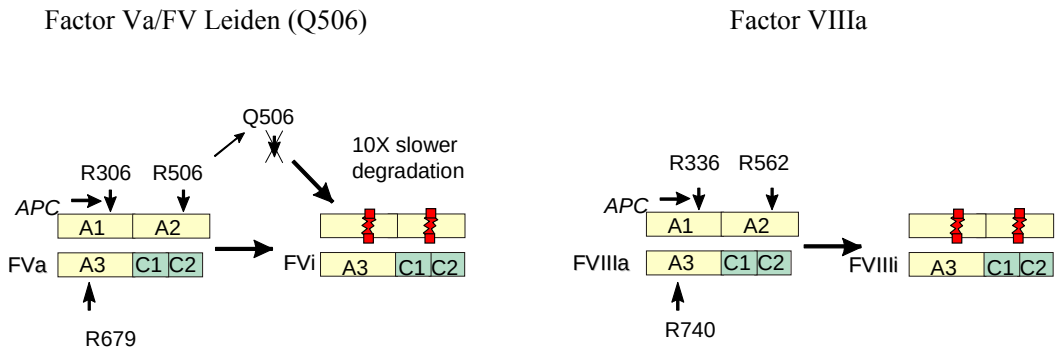


Figure 1.5 Mechanism of APC action and APC resistance due to FVL.
Three APC cleavage sites have been identified in both FVa and FVIIIa, at positions Arg306, Arg506 and Arg679 in FVa and Arg336, Arg562, and Arg740 in FVIIIa. FVa is completely inactivated to FVi when it has been processed by APC at these three sites. FV mutations at Arg306 and 506 have been described that confer resistance to APC. In particular, FVL Arg506Glu has been described to result in a 10-fold slower inactivation than "wild-type" FV.

Figure 1.6 Fibrinolysis after thrombosis initiation

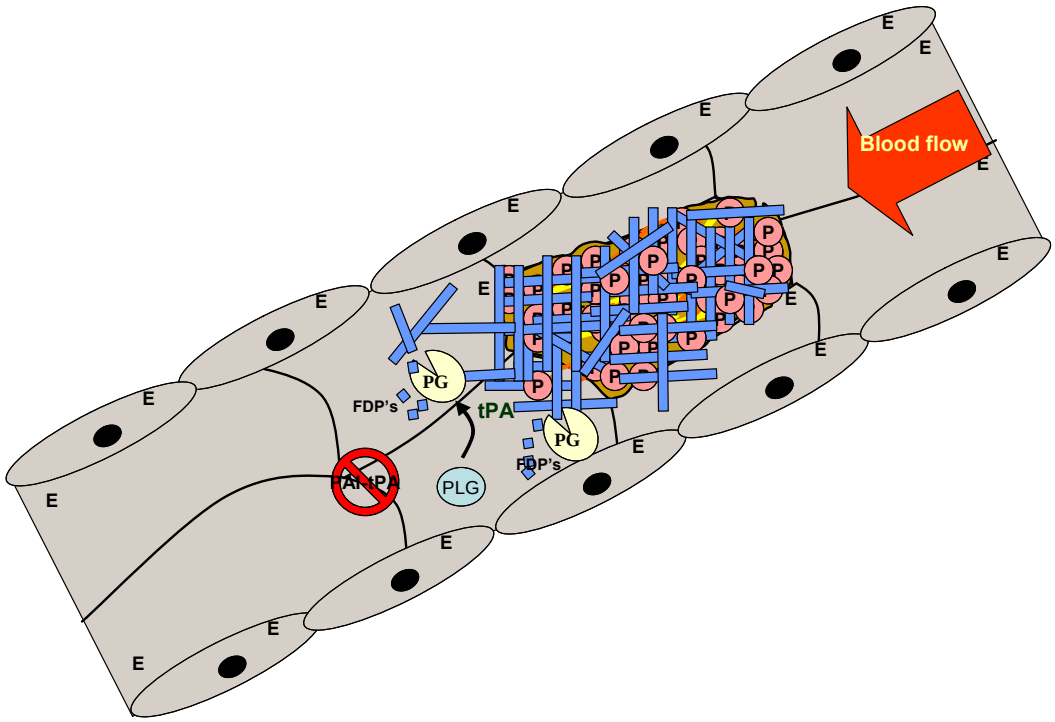


Figure 1.6 Fibrinolysis after thrombosis initiation. The coagulation cascade and platelet activation leads to formation of a platelet (P, pink circles)-fibrin (blue bars) thrombus. Tissue-type plasminogen activator (tPA) is produced by endothelial and smooth muscle cells, co-localizes to fibrin, and cleaves plasminogen (PLG) to form the active protease, plasmin (PG, yellow pacman). Plasmin cleaves fibrin causing lysis of the thrombus with liberation of fibrin degradation products (FDP's). To control plasminogen activation plasminogen activator inhibitors (PAI) rapidly inhibit tPA (red prohibition sign), preventing formation of PG from plasminogen.

Figure 1.7 ENU Whole Genome Dominant Mutagenesis Screen

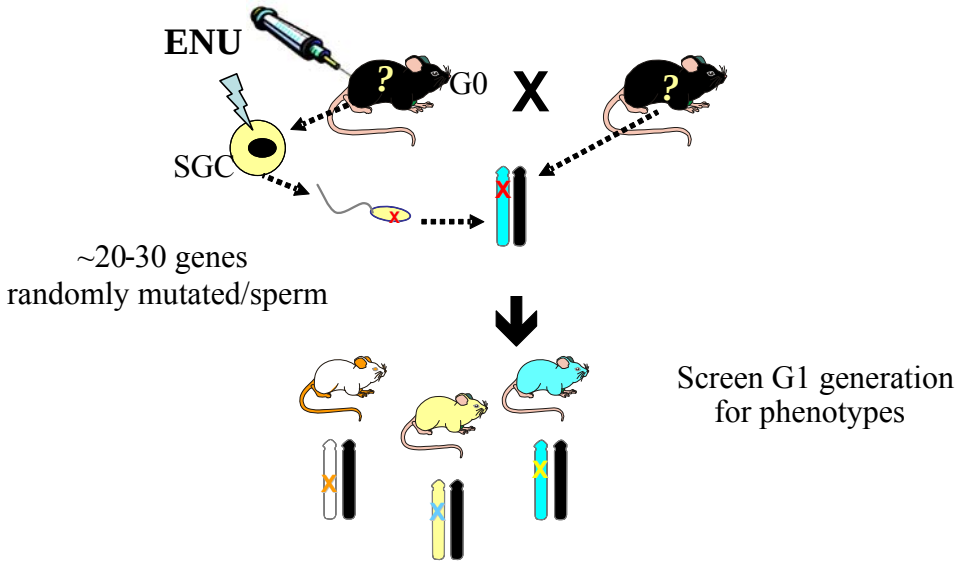


Figure 1.7 ENU whole genome dominant mutagenesis screen. Male mice are intraperitoneally injected with ENU (Syringe), which causes random mutations in spermatogonial germ cells (SGC). Since each uniquely mutated spermatogonial cell will contribute to total sperm production, one ENU treated male can pass on many different sets of ~30 random mutations to its offspring. G1 progeny are screened for dominant phenotypes, in this case, the easily identifiable coat color changes. Once a phenotype of interest is identified, steps can be taken to positionally clone the responsible genetic lesion.

Figure 1.8 Recessive ENU Mutagenesis Screen

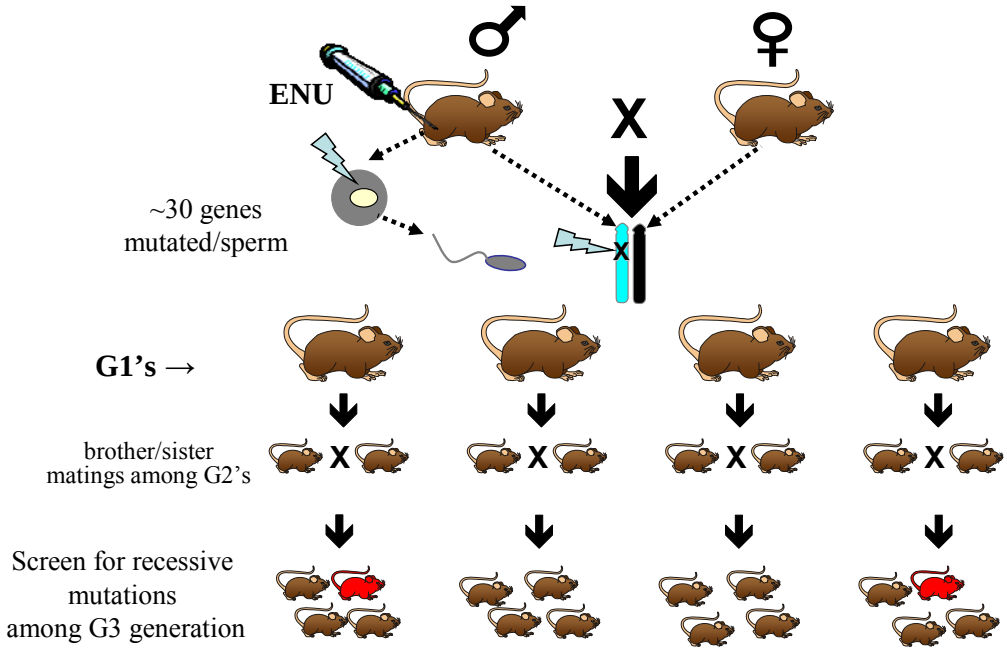


Figure 1.8 Recessive ENU mutagenesis screen. As in figure 1.7, mice are treated with ENU and bred to produce G1 progeny. G1 male-female breeding pairs from the same mating are set up to produce the G3 generation. This generation is screened for recessive phenotypes, as shown by the mice in red. Recessive screens involve an extra breeding step and screening many more progeny than dominant screens to achieve genome saturation.

Figure 1.9 Mapping putative modifiers from a dominant ENU mutagenesis screen

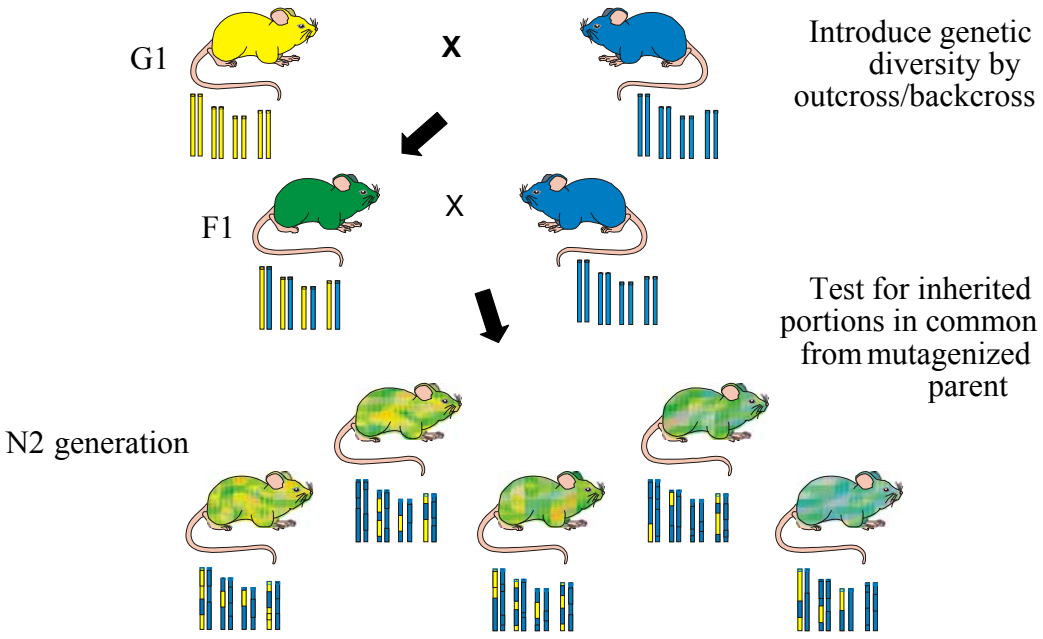


Figure 1.9 Mapping putative modifiers from a dominant ENU mutagenesis screen. Each mouse exhibiting a phenotype of interest is outcrossed to a mouse strain different than the one in which the original mutagenesis was performed, preferably a polymorphic strain, producing an F1 generation. The resulting F1s are then backcrossed back to the non-mutagenized parental strain to produce an N2 generation. These progeny are then analyzed for inherited portions in common from the mutagenized parent, thus localizing the mutation to a genomic location.

CHAPTER II

FACTOR V EXERTS A NATURAL ANTICOAGULANT FUNCTION *IN VIVO* WHICH IS DECREASED BY THE FACTOR V LEIDEN MUTATION*

Abstract

The Factor V Leiden (FVL) mutation (R506Q) renders FVL resistant to degradation by Activated Protein C (APC), resulting in prolonged factor V (FV) prothrombinase cofactor activity and increased thrombin generation. Heterozygotes for FVL have an approximately 10 percent lifetime incidence of venous thrombosis, compared to 80 percent in homozygotes. One likely explanation for the increased penetrance in homozygotes is the increased amount of APC resistant FV. However, recent *in vitro* studies on patients co-inheriting an inactive FV allele along with FVL (hemizygotes, $FV^{Q/-}$) suggest that these patients have a similar coagulation profile as homozygotes, $FV^{Q/Q}$). In addition, *in vitro* studies suggest that FV can also serve as a cofactor for APC in the inactivation of Factor VIIIa, with FVL demonstrating ten-fold reduced cofactor activity relative to wild type. Thus, inheriting FVL would result in partial loss of this APC cofactor activity. To address the *in vivo* relevance of these two potential mechanisms to the increased thrombosis penetrance associated with FVL, we bred $FV^{Q/-}$ with $FV^{Q/+}$ $TFPI^{+/-}$ (reduced tissue factor pathway inhibitor) mice. Analysis of mice from this cross demonstrated a similar uniform perinatal lethality for the $FV^{Q/-}$ $TFPI^{+/-}$ and $FV^{Q/Q}$ $TFPI^{+/-}$ genotypes, in contrast to the normal survival of $FV^{Q/+}$ $TFPI^{+/-}$

mice. To directly compare the thrombotic lethality of $FV^{Q/-}$ with $FV^{Q/+}$, we crossed $FV^{+/-}$ with $FV^{Q/Q}$ and observed a significant reduction of $FV^{Q/-}$ compared to $FV^{Q/+}$ mice at weaning ($p<0.001$). Analysis of offspring from a subsequent intercross between $FV^{Q/-}$ and $FV^{Q/Q}$ mice demonstrated a highly significant reduction in the survival of $FV^{Q/-}$ mice compared to $FV^{Q/Q}$. These results were confirmed using additional crosses. Next, liver specific FV^{Malb} $FV^{-/-}$ or platelet specific FV^{Pf4} $FV^{-/-}$ transgenics crossed with $FV^{Q/+}$ $TFPI^{+/-}$ mice resulted in partial or complete rescue of the lethal $FV^{Q/-}$ $TFPI^{+/-}$ phenotype. Taken together, these data confirm the association of $FV^{Q/Q}$ with a more severe thrombotic phenotype than $FV^{Q/+}$, in mice as well as humans. In addition, these findings identify the critical mechanism for enhanced thrombosis as loss of a wild-type-specific activity, rather than the increased level of APC resistance. Furthermore, this critical wild type FV function (possibly as the previously reported APC cofactor), can be provided by either the platelet or plasma pool of FV. These findings have important implications for the role of FV in the regulation of hemostatic balance and suggest novel targets for therapeutic intervention.

Introduction

Factor V (FV) is a central regulator of the coagulation cascade, serving as a nonenzymatic cofactor for activated Factor Xa in the prothrombinase complex[165, 166]. The prothrombinase complex amplifies coagulation by producing a burst of thrombin generation. FV is localized in two distinct compartments. Approximately 80 percent circulates in the plasma as a single-chain procofactor and is thought to be primarily synthesized in the liver, while approximately 20 percent of total blood FV is concentrated

within the platelet alpha-granules. Platelet FV is either endocytosed, processed and packaged into the alpha granules (humans) or synthesized directly by megakaryocytes (mice)[166-170]. Platelet FV is believed to be stored in a partially cleaved and active form which is released upon platelet activation to provide high local concentrations of FV at sites of platelet activation that contribute to effective hemostasis.

The natural anticoagulant protein C (PC) pathway provides a negative feedback loop to downregulate further thrombin generation[30]. Thrombin bound to thrombomodulin on the surface of endothelial cells changes its substrate specificity to cleave protein C to activated protein C(APC)[59]. APC downregulates coagulation by proteolytically inactivating FVa and FVIIIa, thus greatly reducing the generation of additional thrombin. Membrane-associated protein S has been shown to be an essential cofactor for APC, resulting in ~20-fold increased activity[62].

APC specifically and sequentially cleaves FVa Arg506, Arg306, and Arg679[59, 73]. Initial cleavage is at Arg506, markedly accelerating subsequent events, with cleavage at Arg306 results in a nearly complete loss of activity[74]. An arginine-506-glutamine substitution at amino acid 506, Factor V Leiden (FVL) confers partial resistance to degradation by APC. The FVL polymorphism exhibits a prevalence of ~2-10 percent in most European populations, and is the most common known genetic risk factor for venous thrombosis[86]. Although homozygous individuals demonstrate an approximately 80 percent lifetime penetrance of thrombosis compared to ten percent in heterozygotes, the mechanism responsible for this variable penetrance is unclear.

Subsequently, it has been discovered that the endogenous wild type FV serves as an essential cofactor for the PC mediated degradation of FVIIIa, increasing the rate of

FVIIIa degradation by two-fold[65]. APC resistance and reduced APC cofactor activity are thought to contribute to the increased risk of thrombosis in FVL carriers. However, the relative contributions to each of these mechanisms has not been characterized *in vivo*.

Mice carrying the ortholog of the human FVL mutation exhibit a mild to moderate prothrombotic phenotype, closely resembling the human disorder, with a similarly more severe thrombosis in homozygotes[16]. FVL heterozygous mice are also resistant to endotoxin, leading to the identification of similarly improved survival in human sepsis patients[171]. FV deficient mice ($FV^{-/-}$) display a bleeding phenotype similar to humans[172]. However, unlike humans, in which FV is thought to be synthesized primarily by the liver and subsequently sequestered by the platelets, mouse FV is synthesized in two distinct tissues, the megakaryocyte and the liver, thus allowing the analysis of each of these sources *in vivo*.

We now report a critical role for FV APC cofactor activity to control thombosis susceptibility *in vivo*. This wild type FV function can be provided by either platelet or liver expression of FV.

Methods

Mice

$FV^{Q/+}$ mice carrying a knock-in of the R504Q mutation (orthologous to the human FVL mutation) were previously reported ($F5^{tm2Dgi}$/J stock # 004080, The Jackson Laboratory)[16], as were the generation of $FV^{+/-}$($F5^{tm1Dgi}$ stock # 004078, The Jackson Laboratory)[172] and platelet and liver-specific FV transgenes[166]. TFPI-deficient mice were a kind gift of George Broze[117]. All mice used in this study were

backcrossed to C57BL/6J mice (stock # 000664, The Jackson Laboratory) for at least 5 generations (N5) before intercrossing. Timed matings were performed as previously described to analyze *in utero* progeny from gestational day 18.5[16].

Genotyping for FV^{Q}, $TFPI^{-}$, FV Tg^{Malb}, FV^{-} ($Tg(Alb\text{-}F5)^{2Dgi/J}$ stock # 007244, The Jackson Laboratory) and FV Tg^{Pf4} $FV^{-/-}$ were performed by PCR analysis of tail DNA using primers as previously described[18, 166]. Mice were housed and cared for in SPF conditions, under compliance with the University of Michigan Committee on Use and Care of Animals.

Adult mice were perfusion-fixed with 4% paraformaldehyde as previously described[7] and multiple organs were sectioned and stained with H&E.

The significance of survival differences between groups was determined using the X^2 test. A $p<0.05$ was considered significant.

Results

Effect of FVL hemizygosity on the TFPI deficient background

We previously established synthetic lethality of the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype in mice[18] due to widespread thrombosis in the immediate perinatal period. To examine the impact of haploinsufficiency for TFPI in the setting of FV hemizygosity ($FV^{Q/-}$), $FV^{Q/-}$ mice were mated with $FV^{Q/+}$ $TFPI^{+/-}$ double heterozygotes. The FV locus is on chromosome 1 and the TFPI locus is on chromosome 2, so they are independently assorting. The offspring of this cross and the expected distribution between the eight possible genotypes is shown in Figure 2.1. Of the 203 progeny analyzed, comparison of the $FV^{Q/-}$ and $FV^{Q/Q}$ mice revealed a non-significant reduction in $FV^{Q/-}$ compared to

$FV^{Q/Q}$. However, the reduction of $FV^{Q/-}$ compared to $FV^{Q/+}$ was significant ($p<0.001$). Only one live $FV^{Q/-}$ $TFPI^{+/-}$ mouse was observed at weaning, compared with three $FV^{Q/Q}$ $TFPI^{+/-}$ mice. The reduction of $FV^{Q/-}$ progeny in this cross suggests that $FV^{Q/-}$ phenotype is more severe than either $FV^{Q/Q}$ or $FV^{Q/+}$, both in the presence or absence of reduced TFPI.

Effect of FVL hemizygosity on survival

We analyzed a series of crosses designed to directly compare the numbers of viable weaning age $FV^{Q/-}$ mice observed, compared to one or more of the following sibling groups shown in Table 2.1. In the first cross ($FV^{+/-}$ with $FV^{Q/Q}$), although equal numbers of $FV^{Q/-}$ and $FV^{Q/+}$ progeny were expected, only 5 of 38 mice carried the $FV^{Q/-}$ genotype, compared to 33 of 38 $FV^{Q/+}$ ($X=20.6$, $p<0.001$, 1 degree of freedom). These data suggest that loss of the wildtype allele results in a severe thrombotic phenotype.

To directly address the effect of FVL dosage on viability, we mated $FV^{Q/Q}$ with $FV^{Q/-}$ mice. As can be seen in the second cross in Table 2.1, only 53 of 184 mice carried the $FV^{Q/-}$ genotype, compared to 131 of 184 $FV^{Q/Q}$ ($X=20.6$, $p<0.001$, 1 degree of freedom). These data indicate that decreasing the dose of FVL has adverse effects on viability.

To provide a direct comparison of $FV^{Q/+}$, $FV^{Q/Q}$ and $FV^{Q/-}$ in the same genetic context, we crossed $FV^{Q/-}$ with $FV^{Q/+}$. As seen in the third cross in Table 2.1, pairwise comparison of $FV^{Q/-}$ to both $FV^{Q/Q}$ and $FV^{Q/+}$ revealed significant reduction of $FV^{Q/-}$ ($X=4.3$, $p<0.05$, 1 degree of freedom and $X=11.6$, $p<0.001$ respectively, 1 degree of freedom).

To investigate the mechanism of $FV^{Q/-}$ associated mortality, timed matings were performed to analyze progeny from gestational day 18.5, just prior to birth. Genotypes for 171 progeny at embryonic day 18.5 from a $FV^{Q/+}$ with $FV^{+/-}$ cross are shown in the fourth row of Table 2.1. The distribution is not significantly different than the expected 1:1:1:1, demonstrating that $FV^{Q/-}$ mice are lost primarily between birth and weaning.

Histology on aged $FV^{Q/-}$ mice showed evidence of intravascular thrombosis in the lung with accompanying inflammation. Figure 2.4. This suggests that even viable $FV^{Q/-}$ have an increased prothrombotic state.

Either platelet or plasma derived FV provides FV anticoagulant function

To assess the role of small amounts of wildtype FV to restore viability to the $FV^{Q/-}$ $TFPI^{+/-}$ genotype, FV^{Malb} or FV^{Pf4} transgenics were employed. These mice were mated onto the $FV^{-/-}$ deficient background, yielding FV^{Malb} $FV^{-/-}$ and FV^{Pf4} $FV^{-/-}$ mice, which were then crossed with $FV^{Q/+}$ $TFPI^{+/-}$ mice. The eight possible genotype combinations of progeny from these crosses are shown in Figures 2.2 and 2.3. The genotypes of interest are $FV^{Q/-}$ $TFPI^{+/-}$ with and without FV^{Malb} or FV^{Pf4}. As shown previously, there is an almost complete absence of the $FV^{Q/-}$ $TFPI^{+/-}$ genotype, while mice positive for either transgene exhibited between 50-100% rescue. Thus, even small amounts of FV derived from either the plasma or platelets are capable of restoring viability to this severe thrombotic phenotype.

Discussion

Our data demonstrate more severe thrombosis in $FV^{Q/-}$ mice than in either $FV^{Q/+}$ or $FV^{Q/Q}$ mice. These findings imply a natural anticoagulant function for the wildtype FV allele protecting the $FV^{Q/+}$ from the more severe thrombosis observed in $FV^{Q/-}$. The improved survival of $FV^{Q/Q}$ compared to $FV^{Q/-}$ suggests that the FV^{Q} allele may also exert a modest anticoagulant function but at a much reduced level compared to the wildtype allele. Moreover, the deleterious effects of $FV^{Q/-}$ were abrogated by small amounts of either liver or platelet derived wildtype FV.

The primary prothrombotic activity of FVL has been thought to result from increased thrombin generation due to prolonged FVa activity as a consequence of delayed FVL inactivation by APC. However, Lu et al. demonstrated that wildtype FV serves as an essential cofactor for the APC-protein S mediated degradation of FVIIIa, increasing the rate of FVIIIa degradation by approximately two-fold[65]. To express this APC cofactor activity, FV must retain part of its B domain and be cleaved by APC at Arg506[59, 78-80]. Thus, partially cleaved wildtype FV is an effective cofactor and FVL has been subsequently shown to be approximately ten fold less efficient as a cofactor for the APC mediated inactivation of Factor VIII[81], using *in vitro* thrombin generation assays[78].

The studies described here have important implications for the genetics of thrombotic risk associated with FVL in humans. Since the carrier frequency of FV deficiency is estimated at ~1 per 1000, one in 25 patients identified as FVL homozygotes by the *in vitro* APC resistance test are actually hemizygotes. A small number of hemizygous humans have been identified among venous thrombosis patients found to carry FVL. Since their biochemical APC resistance profiles are similar to that of FVL

homozygotes, these patients have been referred to as pseudohomozygotes. Due to the small number of hemizygous patients identified, it has been difficult to ascertain the effects of hemizygosity on thrombosis risk, although it has been suggested that these patients are as prone to developing thrombosis as FVL homozygotes[78].

In addition, a number of FV alleles have been described which result in the quantitative and/or qualitative deficiency of FV[173]. Factor V levels are widely divergent in the population, ranging from 50-150 percent of the mean[46, 174]. Although a study by Kamphuisen et al. suggested that FV total antigen levels did not contribute to thrombosis susceptibility in FVL carriers, factors that reduce the expression of the "wildtype" factor V allele may increase the penetrance of developing thrombosis in FVL

Variation in disease susceptibility by affecting the expression of a wildtype allele opposing a mutant one is not without precedent. For example, phenotypic variation of the "wildtype" allele has been demonstrated in incomplete penetrance at RP11, a dominant model of retina retinitis pigmentosa. Permutations in the ubiquitously expressed splicing factor PRPF31 were shown to cause disease but only in the individuals carrying a high expressing "wildtype" allele[175]. In addition, a common low expressing variant of the ferrochelatase gene is inherited with a loss of function mutation to influence the penetrance of the autosomal dominant form of erythropoetic protoporphyria[176, 177].

Although an undefined function for wildtype FV cannot be ruled out, we believe that the FV-APC cofactor activity exerts a potent antithrombotic activity. The most compelling evidence for the importance of FV-APC cofactor activity comes from adding small amounts of wildtype FV back to the *in vivo* mouse system. Either liver or

platelet-derived wildtype FV restored viability to the FV hemizygotes. These results provide compelling evidence that the critical role of wild type FV as APC cofactor can be provided by either the platelet or plasma pools of FV. These data also suggest that the phenotypic differences between $FV^{Q/Q}$ and $FV^{Q/-}$ are not solely due to the increased kinetics of FVL inactivation at higher FVL concentrations[74].

Table 2.1 Genotype distribution of weaning age pups obtained from experiments designed to assess lethality of $FV^{Q/-}$ compared to $FV^{Q/+}$ or $FV^{Q/Q}$

Number of offspring/Genotype		**$FV^{+/+}$**	**$FV^{+/-}$**	**$FV^{Q/Q}$**	**$FV^{Q/-}$**	**$FV^{Q/+}$**	**Total number of pups analyzed**
Parental Genotypes							
$FV^{+/-}$	**$FV^{Q/Q}$**	**-**	**-**		**5***	**33**	**38**
$FV^{Q/Q}$	**$FV^{Q/-}$**	**-**	**-**	**131**	**53***	**-**	**184**
$FV^{Q/-}$	**$FV^{Q/+}$**	**-**	**18**	**14**	**5**	**23**	**60**
Embryo mating $FV^{Q/+}$	**Embryo mating $FV^{+/-}$**	**36**	**49**	**-**	**36**	**50**	**171**

Figure 2.1 Genotype distribution of weanling mice obtained from $FV^{Q/-}$ mated with $FV^{Q/+}$ $TFPI^{+/-}$ mice

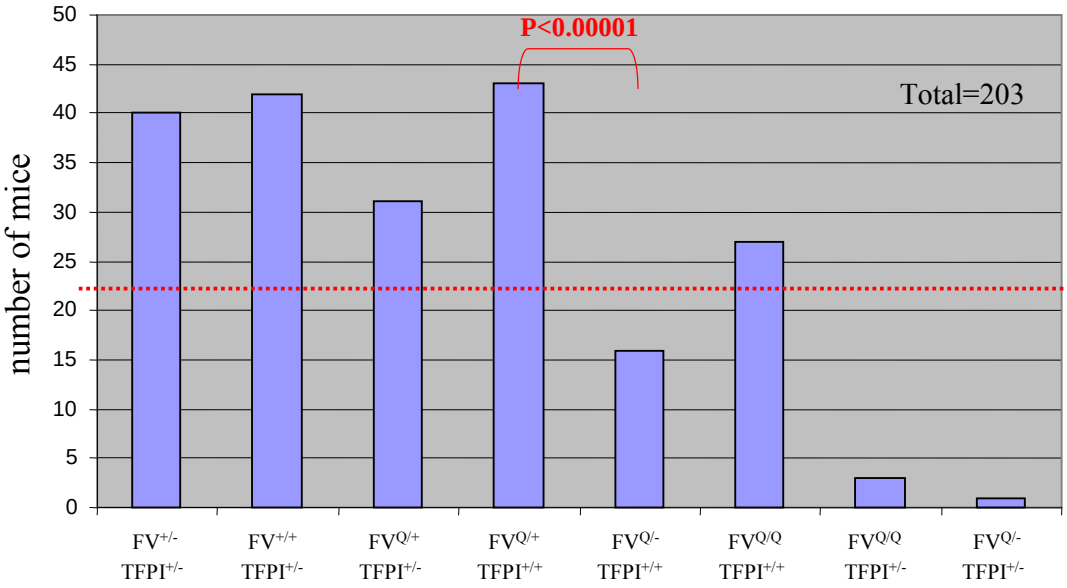


Figure 2.1 Genoypte distribution of weanling mice obtained from $FV^{Q/-}$ mated with $FV^{Q/+}$ $TFPI^{+/-}$ mice. Of the eight possible genotypes expected to be equally distributed among the progeny genotypes of this cross, pairwise comparison reveals significant reduction in $FV^{Q/-}$ versus $FV^{Q/+}$ and $FV^{Q/Q}$ ($X=29.5$, $P<0.00001$, 1 degree of freedom). Nonsignificant reductions are also noted in $FV^{Q/-}$ versus $FV^{Q/Q}$ in the context of TFPI deficiency. The red line denotes the expected numbers of each genotype.

Figure 2.2 Genotype distribution of weaning age pups obtained from liver specific transgenic rescue experiments

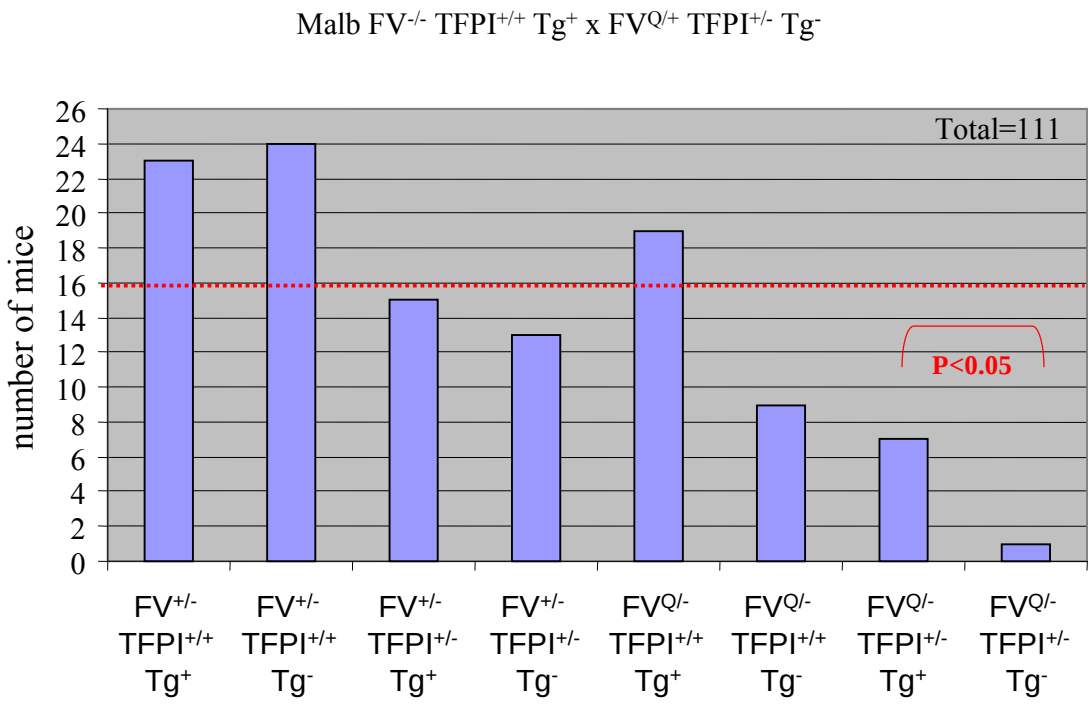


Figure 2.2 Genotype distribution of weaning age pups obtained from liver specific transgenic rescue experiments. Pairwise comparison reveals significant increases in numbers of $FV^{Q/-}$ $TFPI^{+/-}$ weanlings also carrying the liver specific wild type Factor V transgene (Malb), compared to nontransgenics ($X=4.5$, $P<0.05$, 1 degree of freedom). The cross is illustrated at the top of the slide and the red line denotes the expected numbers of each genotype.

Figure 2.3 Genotype distribution of weaning age pups obtained from platelet specific transgenic rescue experiments

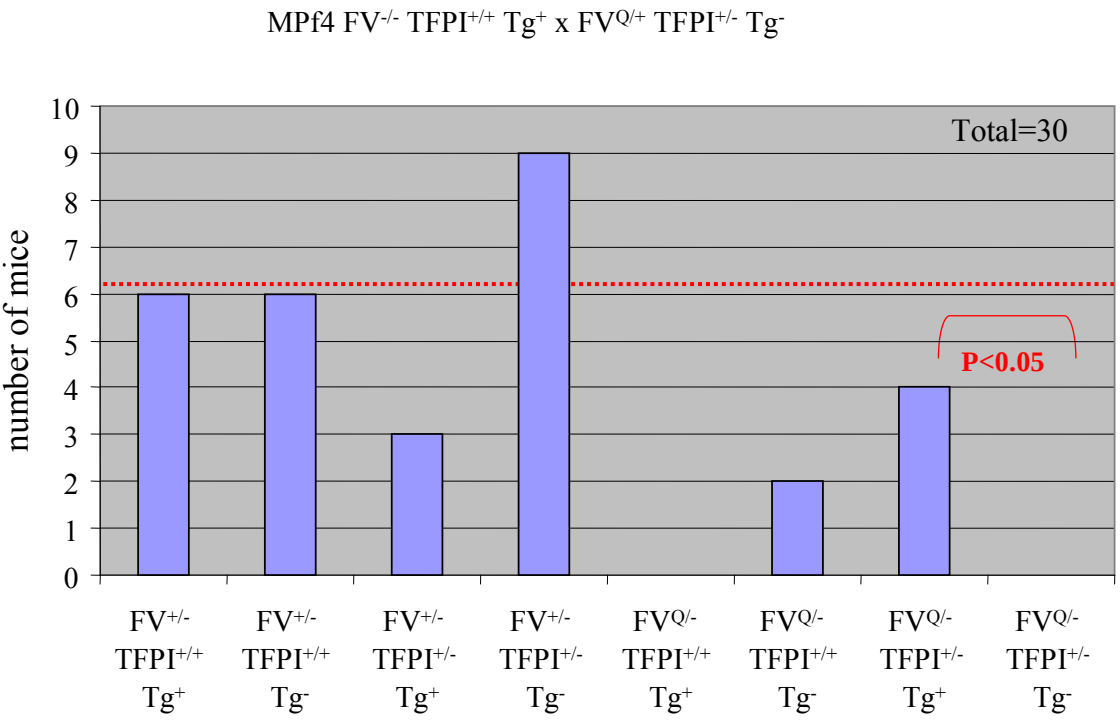
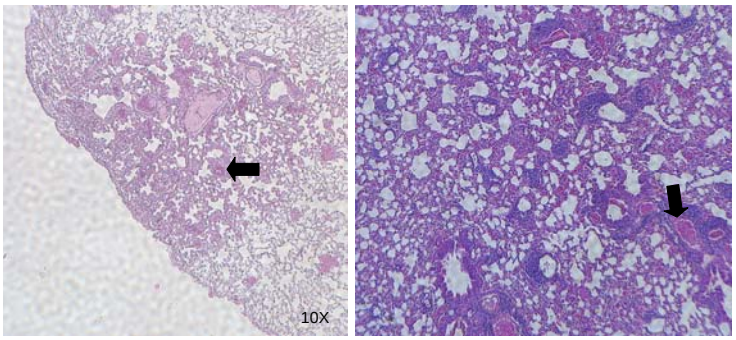


Figure 2.3 Genotype distribution of weanling mice obtained from $FV^{Q/-}$ mated with $FV^{Q/+}$ $TFPI^{+/-}$ mice. Pairwise comparison reveals significant increases in numbers of $FV^{Q/-}$ $TFPI^{+/-}$ weanlings also carrying the platelet specific wild type Factor V transgene (MPf4), ($X=4$, $P<0.05$, 1 degree of freedom). The cross is illustrated at the top of the slide and the red line denotes the expected numbers of each genotype.

Figure 2.4 FV Leiden hemizygous lungs from
two aged hemizygous mice



(Mouse # 74518) (Mouse # 57731)

Figure 2.4 Microscopic findings in aged (1 year old) hemizygous $FV^{Q/-}$ mice. Lung thrombosis (arrows) with accompanying inflammation. Sections are stained with hematoxylin and eosin (H&E).

Notes

*This chapter is in preparation for submission as a manuscript entitled "Factor V Exerts a natural anticoagulant function *in vivo* which is decreased by the Factor V Leiden mutation" by Randal J. Westrick, Hongmin Sun, Sara L. Manning, Angela Y. Yang, Abigail L. Peterson, David R. Siemieniak, Daniel T. Eitzman and David Ginsburg.

CHAPTER III

PASSENGER GENE POINT MUTATION: DISCOVERY OF A SPONTANEOUS *IRS1* MUTATION IN LINKAGE DISEQUILIBRIUM WITH *SERPINB2* DEFIENCY*

Abstract

In characterizing gene targeted mice deficient in *SerpinB2*, we observed mice which were small at birth, exhibited delayed growth and had decreased lifespan and fecundity. We initially attributed this growth abnormality to deficiency of *SerpinB2*, an intracellular serpin hypothesized to play roles in apoptosis and tumor growth. However, analysis of *SerpinB2* deficient mice derived from two additional independent ES cell clones revealed no growth abnormalities. Upon re-examination of the original *SerpinB2* deficient mouse line, we observed recombination between the small phenotype, which we designated *sml,* and the *SerpinB2* locus in approximately 10% of the offspring from a *SerpinB2* heterozygote intercross. An intercross with CASA/RK mice mapped the locus responsible for the small phenotype to a 2.78 Mb interval ~30 Mb proximal to *SerpinB2* on mouse chromosome 1, bounded by markers D1Mit44 and D1Mit216. Inspection of genes in the candidate interval revealed that gene targeted mice deficient in *Irs1* have been reported to display delayed growth. Sequencing of *Irs1* from *sml* mice, one of the 28 known genes in the candidate interval, revealed a nonsense mutation at serine 57, which results in complete loss of *Irs1* mRNA, protein and function. Analysis of ES cell DNA demonstrated that the S57X *Irs1* mutation arose spontaneously in the original

embryonic stem cells. Although the *sml* phenotype is very similar to previously reported *Irs1* deficient mice, there are several notable distinctions. This is likely due to complete deficiency in the S57X mutants or to the minimal genetic interference of this point mutant. New mutations arising during ES cell culture are likely to be a frequent but underappreciated occurrence. When linked to the targeted allele, such mutations could result in incorrect assignment of phenotype and may account for a subset of markedly discordant results for experiments independently targeting the same gene.

Introduction

Gene targeting in ES cells has revolutionized the use of the mouse as a model organism for studying gene function *in vivo*, providing powerful insights into the functions of individual genes and their relationships to complex phenotypes[178]. However, this methodology is technically demanding, expensive and time-consuming, discouraging the production of multiple engineered mouse lines from independently targeted ES cell clones for confirmation. As a result, many "knockout" mouse reports are based on the analysis of animals derived from a single targeted ES cell clone. In certain cases the desired genetic manipulation is not obtained or well characterized but results in a functional knockout[179].

In cases where the same locus has been independently targeted by more than one strategy, important and surprising differences have sometimes been observed. The explanation for such discrepancies include genetic strain background of the gene targeted mice and structural differences in the targeted alleles[180-182]. Genetic differences between the ES cell line used for targeting and the strain chosen to propagate the gene

targeted mice in linkage with the targeted allele, called passenger genes, can be particularly confounding[183]. Gene targeting may also unexpectedly interfere with the expression of a nearby gene, as was observed with retained selectable marker cassettes[184] and was likely observed for three of five *Myf-5/MRF4* targeting constructs[182, 185]. It is likely that there are other artifacts resulting from the process of gene targeting that remain to be discovered[186].

The protein product of the *SerpinB2* gene, plasminogen activator inhibitor-2, (PAI-2), is a member of the serine protease inhibitor family. Its main physiological function is thought to be regulation of plasminogen activation in the extravascular compartment through urokinase and tissue plasminogen activator inactivation[187-189]. PAI-2 may also play a role in other processes including apoptosis, tumor metastasis, embryo implantation, macrophage survival and fibrinolysis[22, 190]. The high plasma levels observed during pregnancy also suggest that PAI-2 could be important for placental maintenance or during development[191]. PAI-2 deficiency has not yet been identified in humans. We previously reported the generation of PAI-2 deficient mice by gene targeting. Surprisingly, PAI-2 deficient mice exhibited no overt phenotype, even when combined with PAI-1 deficiency. To investigate the *in vivo* function of PAI-2, we generated three independent *SerpinB2* knockout mouse lines derived from three independent ES cell clones.

In our initial analysis, we unexpectedly observed two conflicting phenotypes in the *SerpinB2* deficient mice. Two lines exhibited a normal phenotype, with littermates of each genotype grossly indistinguishable from one another[4]. Correct targeting and deletion of PAI-2 was confirmed in all three lines. All subsequent studies and reports

from other groups[22, 190, 192, 193] were confined to the first two lines. However, the third line we analyzed exhibited a phenotype which we named *sml* due to the runted and scruffy appearance of the animals. *Sml* was assumed to be due to another as yet uncharacterized genetic defect.

We now report the identification of a specific molecular defect responsible for the *sml* phenotype as a novel nonsense mutation (S57X) in a nearby gene, insulin receptor substrate 1 (*Irs1*). This gene resides on mouse chromosome 1 in linkage with the targeted allele. Thus, initially *SerpinB2* deficiency appeared to be concordant with the *sml* phenotype. The S57X *Irs1* mutation originated in embryonic stem (ES) cells during the isolation of *SerpinB2* targeted ES clones. Additionally, in the process of discovering the genetic lesion responsible for *sml*, we uncovered a lethal strain specific phenotype when our mice were backcrossed to the C57BL/6J genetic background.

Materials and Methods

Generation of *SerpinB2* deficient *sml* and normal mouse lines

A total of three independent *SerpinB2* deficient lines were generated, two (*SerpinB2*^{15B11} and *SerpinB2*^{13B5}) from the 129Sv cell line D3 (obtained from T. Doetschman, University of Cincinnati) and one (*SerpinB2*^{10G3}) from cell line CJ7[194]. Clone 15B11 from the D3 cell line was used to generate six chimeric male founder mice, who were then mated to (C57BL/6J × DBA/2J) F_1 females. The resulting F_1 pups were then interbred to generate F_2 progeny. DNA prepared from tail biopsies was genotyped at the *SerpinB2* locus as previously described [4]. *SerpinB2*^{15B11} mice exhibited the distinctive *sml* phenotype and were subjected to further genetic and phenotypic analysis.

The *SerpinB2*^{13B5} and *SerpinB2*^{10G3} lines were analyzed as previously reported[4] and have been distributed to other investigators and deposited at The Jackson labs www.jax.org/.

Initial phenotypic analysis of *sml SerpinB2*^{15B11} deficient mice

280 F_2 progeny of the initial F_1 intercross were used for weight analyses. Pups were weighed at weaning. To reduce the effect on weight of litter size, age at weaning, and maternal care, weights were normalized to the mean weight of *SerpinB2* wildtype mice within each litter. For the comparison of weight stratified by *SerpinB2* genotype, litters lacking *SerpinB2* wildtype mice were not included. To analyze relative growth, eleven mice from two litters were weighed every two to three days from birth through six weeks of age. Prior to tagging, mice were distinguished by colored non-toxic marker. Kaplan-Meyer analysis was performed using age and death dates from 100 mice. Sera collected from *SerpinB2*-deficient mice of the initial intercross were tested at Charles River Laboratories (Wilmington, MA) for the following common infectious agents: SEN, PVM, MHV, MVM, GD-VII, REO-3, MPUL, MPV, EDIM, LCMV, MAD, ECTRO, K, POLY, MTLV, MCMV, HANT, ECUN and CARB. The effect of maternal care was evaluated by fostering pups to wildtype mothers[195].

DNA analysis for aberrant targeting

DNA was prepared as previously described[4] from ES cells of the three targeted *SerpinB2* deficient lines as well as control nontargeted ES cells. DNA was also prepared from mice generated from the three *SerpinB2* deficient clones. Probes included a 1 kb

*Sca*I/*Hind*III fragment of the *SerpinB2* gene located 5' to the targeting vector (probe A) and an *Xba*I/*EcoR*I fragment located within the 3' arm of the targeting construct (probe B) as previously described[4]. A *SerpinB2* cDNA probe was generated as a 1.7 kb *Pst*I-*Cla*I fragment, and the *neo* probe consisted of a 0.5 kb PCR-amplified fragment, generated with primers described previously[4]. Standard Southern blot analysis was performed on DNA digested with *Bgl*I for analysis with probe A or *Sca*I for analysis with probes B, *SerpinB2* cDNA or *neo*. Further Southern blot analysis was performed with probe B on mouse DNA digested with *Apa*I, *Nhe*I, or *Stu*I.

Outcross-intercross genetic mapping strategy

For genetic mapping, an intersubspecific outcross was performed between an inbred strain of *Mus musculus castaneus* (CASA/Rk) and three *SerpinB2* deficient *sml* mice on a mixed *Mus musculus domesticus* background (129S1/SvIMJ, C57BL/6J and DBA/2J). The (*sml* × CASA/Rk) F_1 mice were intercrossed to produce the F_2 generation. At weaning, pups were euthanized and weighed, and tail biopsies were collected. Weights of pups were normalized to the weight of wildtype pups in the same litter, as described above. For the initial stage of mapping, *sml* mice were defined as those with normalized weight ratios lower than 0.7.

Phenotypic analysis of *sml* mice

For further analysis of the phenotype, 283 *sml* progeny from a (CASA/Rk × *sml*) F_1 intercross were studied. All mice were genotyped at markers flanking the

nonrecombinant *sml* interval described below, D1Mit216 and D1Mit8. Autopsies were performed on two *sml* and one normal-sized three-month old mice.

Statistics

Data from mouse crosses was statistically analyzed using Chi square. Differences were evaluated by t-test and corrected for multiple comparisons by Bonferroni where necessary.

***Irs1* sequence analysis**

A series of primer pairs were designed using the primer3 program to generate overlapping PCR amplicons approximately 500 base pairs in length that span the entire coding region of *Irs1*[196]. PCR was performed on DNA isolated from a mouse with the *SerpinB2*^{15B11} *sml* phenotype. PCR amplicons were purified using the Qiaquick PCR gel extraction kit (Qiagen), sequenced and assembled into a contig using DNAStar (Lasergene), then compared to the publicly available *mus musculus domesticus* sequence (Genbank accesssion number NM_010570).

Genotyping the *Irs1* point mutant

Irs1 C170A point mutants were genotyped using a PCR based RFLP assay employing the *Taq*1 enzyme, since the C170A mutation abolishes a *Taq*1 restriction site (TCGA to TAGA). Heterozygotes were backcrossed to C57BL/6J for 6 generations. An intercross of the N6 backcrossed heterozygous mice was analyzed for surviving mice of each genotype at weaning.

Western blotting:

To assess the deficiency of IRS1 Protein, an IRS1 western blot was performed. The quadriceps muscles of wild type, heterozygous and IRS1 Ser57X homozygous mutants were removed, snap frozen and homogenized. Homogenates were then run on a 4-12% SDS-PAGE gel and probed with a rabbit anti-rat polyclonal antibody[197, 198]. This antibody was generated against a peptide corresponding to the sequence of COOH-terminal 14 amino acids of rat IRS1 (Millipore Inc. 290 Concord Rd. Billerica, MA 01821 USA) http://www.millipore.com/catalogue/item/06-248.

Results

sml phenotype associated with *SerpinB2* deficiency

To generate *SerpinB2*-deficient mice, three targeted ES cell clones (15B11, 10G3 and 13B5) were injected into C57BL/6J blastocysts[4]. Six chimeric males obtained from clone 15B11 were used to generate mice heterozygous for *SerpinB2,* and multiple F_1 heterozygous matings were initiated. Analysis of F_2 progeny alive at weaning revealed a divergence from the expected Mendelian ratios, with a decrease in $SerpinB2^{15B11}$ null pups ($P<0.05$), most notably for female offspring ($P<0.01$). $SerpinB2^{15B11}$ mice also exhibited decreased lifespan, as shown by Kaplan-Meyer analysis. Figure 3.1B.

$SerpinB2^{15B11}$ null mice appeared noticeably smaller than their wild-type and heterozygous littermates. Figure 3.1A. We weighed and normalized the weights of F2 progeny and stratified by *SerpinB2* genotype as shown in Figure 3.2A. The normalized weights of *SerpinB2* null mice are significantly smaller (0.62 ± 0.22) and mice

heterozygous for *SerpinB2* trend toward being slightly smaller (0.94 ± 0.18) than wild-type controls (1.0 ± 0.09).

Progeny from two litters were weighed every 2-3 days from birth through six weeks of age. Figure 3.2B. Decreased size was detectable at birth and easily distinguishable at two weeks of age. Despite their small stature, healthy adult male and female *SerpinB2*^{15B11} mice were fertile. Females were able to carry null pups to term, although they displayed reduced litter size. Autopsy examination of *sml* mice identified no significant gross or microscopic abnormalities aside from the proportionally decreased stature and muscle wasting in a subset of mice. As previously reported, a *sml* phenotype was not observed in the *SerpinB2*^{10G3} and *SerpinB2*^{13B5} lines[4].

Correct targeting at the *SerpinB2* locus

SerpinB2 was identified as a homologous recombinant by Southern blot analysis, as previously described[4]. Probes were derived from 5' sequence located outside the targeting vector and a 3' fragment from within the targeting vector. No second site of integration was detected[4] (and data not shown). In addition, a *neo* probe detected a fragment of the identical size from all three targeted clones without additional fragments that would indicate an additional insertion and a cDNA probe demonstrated complete deletion of *SerpinB2*.

Fostering experiments excluding a maternal effect as the cause of the *sml* phenotype (data not shown). Sera collected from *sml* mice were found to be negative for a number of common infectious agents. Complementation testing between the *SerpinB2*^{15B11} and *SerpinB2*^{10G3}/*SerpinB2*^{13B5} alleles failed to generate any progeny with

the *sml* phenotype, excluding a vertically transmitted infection or other maternally transmitted factor(s) (data not shown).

Segregation of the *sml* phenotype from the *SerpinB2* deficiency

With the continued analysis of additional progeny from F1 intercrosses of the *sml* line, occasional normal sized *SerpinB2* null and small-sized *SerpinB2* heterozygotes were detected. Figure 3.2A. Genotypes in animals with discordant phenotypes were confirmed by Southern blot analysis. The number of discordant animals was consistent with a second locus for *sml* located ~12cM from the *SerpinB2* gene, with apparent discordance between the *sml* phenotype and *SerpineB2* genotype due to recombination between *sml* and the *SerpinB2* gene.

The *sml* gene is localized to a 30 Megabase region proximal to *SerpinB2*

To map this second locus, *sml* mice were mated to the *Mus castaneus* strain CASA/Rk. Mating pairs were progeny tested to select those in which both parents carried the *sml* allele. Only mating pairs producing two or more *sml* pups that were nonrecombinant across the candidate interval were used for subsequent mapping. Initially, sixteen *sml* mice were genotyped for thirteen markers from mouse chromosome 1 (D1Mit124, 178, 332, 216, 8, 415, 10, 26, 218, 263, 498, 199, 266). Genotyping was performed as previously described[1], and *Mus musculus domesticus* alleles were scored identically, disregarding strain. *Sml* was localized between markers D1Mit216 and D1Mit8, approximately 30 Megabases proximal to the *SerpinB2* locus on chromosome 1. Figure 3.3. 265 additional F_2 progeny, both small and normal-sized, were genotyped at

these two markers. To further narrow the location of *sml*, recombinant animals were typed at an additional six markers located between D1Mit216 and D1Mit8, including D1Mit44, 183, 254, 382, 383 and 439. Subsequent fine mapping in an additional 265 progeny localized *sml* to a 2.78 Mb interval between D1Mit216 (79,802,769bp) and D1Mit44/D1Mit382 (82,581,512bp).

A C170A (C57X) mutation in *Irs1* is responsible for *sml*

There are 14 known genes within the 2.78 Mb candidate interval, including *Cul-3* (cullin-3)[199], *Serpine2* (protease nexin 1)[200], *Dock10*(27), *Irs1* (insulin receptor substrate 1), *Rhbdd1* (rhomboid domain containing protein 1) and *Col4a4* (procollagen, type IV, alpha 4). IRS1 is a 1231 amino acid intracellular adaptor molecule first identified as a downstream mediator of insulin receptor signaling, but also linked to several signal transduction pathways that are essential for cell growth and proliferation[201, 202]. Targeted deletions of *Irs1* have been previously reported[203-205], with the main phenotype across all these reports consistent for significant embryonic and postnatal growth retardation, with birth weights reported at 40-60% compared to heterozygous or wild-type littermates[203, 204].

The single coding exon of *Irs1* was PCR amplified from genomic DNA obtained from a *sml* mouse, with sequence analysis identifying a single C to A transversion at nucleotide 170 resulting in a serine to nonsense mutation (S57X) at codon 57. Figure 3.4A. This mutation precedes all identified functional domains of *Irs1* and thus would be predicted to result in a complete loss of function. To confirm that the *Irs1* mutation was inherited in accordance with the *sml* phenotype, genotyping of 23 mice with the *sml*

phenotype for the C170A point mutation demonstrated a complete concordance between the *sml* phenotype and homozygosity for the C170A mutation (data not shown). The C170A mutation was absent from the two other *SerpinB2* targeted null lines. In addition, the mouse strains C57BL/6J and 129S1/SvIMJ, as well as another gene targeted strain utilizing the D3 ES cell line[16], did not carry this mutation.

The C170A *sml* mutation results in loss of *Irs1* protein expression

Western blot analysis of quadriceps muscle tissue total cell extract demonstrated the expected ~145 kDa protein in tissue from wildtype and heterozygous C170A mice, with complete absence in tissue from mice homozygous for the C170A mutation. Figure 3.4B

The *sml* phenotype is independent of *SerpinB2* genotype

To demonstrate that the *SerpinB2* deficiency did not contribute to the phenotypic abnormalities observed in our study, we segregated the C170A mutation away from the *SerpinB2* null mutation[206]. We observed no gross phenotypic differences in *Irs1* deficient mice based on *SerpinB2* status.

Strain background leads to phenotypic differences in *Irs1* deficient mice

Irs1 deficient mice show significant embryonic and postnatal growth retardation suggesting that *Irs1* plays a key role in relaying the growth stimulating effects of insulin and insulin like growth factor[201, 205]. However, no significant embryonic or postnatal lethality was observed in the previous reports of targeted *Irs1* deficient mice[203]. In

contrast, *Irs1* C170A homozygous mice on a mixed 129/C57BL/6J genetic background demonstrate decreased longterm survival, runting persistent up to a year and reduced fat mass. With further backcrossing (greater than six generations) into the C57BL/6J background, a marked increase in lethality was observed, with less than two percent of *Irs1* C170A homozygotes surviving to weaning. (Figure 3.5A) An outcross of *Irs1* C170A heterozygotes to the 129S1/SvIMJ strain followed by F_1 intercross resulted in improved survival at weaning, with ~25 percent of the F2 weanlings observed to be C170A homozygotes. (Figure 3.5B)

Discussion

Analysis of three *SerpinB2*-deficient knockouts mouse lines revealed discordant phenotypes. Two lines revealed the authentic phenotype of *SerpinB2* deficient mice and are the subject of a previous report[4]. The third line, *SerpinB2*^{15B11} exhibited a delayed growth phenotype. Analysis of the *SerpinB2*^{15B11} homozygotes revealed that *sml* recombined from *SerpinB2* approximately 12 percent of the time, suggesting that this locus was a genetic distance of approximately 12 cM away from the SerpinB2 locus. By positional cloning, we delineated a 2.78 Mb nonrecombinant interval that localized the region 30 Mb proximal to *SerpinB2* on chromosome 1. Of the 14 genes in the candidate region, only *Irs1* deficiency was reported by multiple groups to have a postnatal growth defect[203-205, 207]. Sequencing the single coding exon revealed a C170A stop mutation, truncating the protein at amino acid 57 that would be predicted to result in complete loss of function. Thus, the *sml* phenotype is directly attributable to IRS1 deficiency.

The *sml* mutation in the *SerpinB2*^{15B11} clone is unlikely a distant effect mechanistically related to the injection or targeting event. For a targeting event to delete *SerpinB2* and also cause the *sml* phenotype, the event would have acted at a distance of approximately 30 megabases. In addition, we have observed no evidence for nonhomologous recombination. It is more likely that this point mutant arose in the embryonic stem cell line that was used for the gene targeting of *SerpinB2*^{15B11}. *Sml* mice homozygous for the C170A mutation were observed among the progeny of all six chimeric founders from injection of the 15B11 clone. An additional *SerpinB2* targeted clone also derived from the D3 ES cell line did not generate mice with the *sml* phenotype, suggesting that the *sml* mutation is not present in the D3 cell line or the C170A mutation exists as a minor allele in the D3 cell line. Other targeted mice generated using the D3 line are also wildtype at the *sml* locus by C170A genotyping[172]. Taken together, these data demonstrate that the *sml* mutation most likely arose as a spontaneous event either in the individual cell or subpopulation of ES cells giving rise to the 15B11 ES cell clone, or concurrently with the targeting.

While we and others attempt to limit the number of passages of an ES cell line, cells containing new mutations are expected at low levels during the introduction of the targeting construct and could be randomly selected as a homologous recombinant clone. ES cells exhibit spontaneous mutation frequencies that approach 1×10^{-6} per division[208, 209]. ES cells are hypersensitive to DNA damage and readily undergo apoptosis or differentiation to remove the damaged cells from the totipotent pool[209, 210]. Since ES cells have been demonstrated to have enhanced mutation repair mechanisms for certain classes of mutations, such as double strand break repair, the

classes of mutations that would be propagated and observed are also expected to be restricted to certain classes, such as point mutations that have no deleterious growth effects on the ES cell[209, 210]. Spontaneous mutations at second sites are likely to be a common event in gene targeting experiments; however to our knowledge no other cases have been documented. A spontaneous mutation unlinked to the targeted locus would usually be lost during breeding to establish the line, particularly by a backcross to a standard laboratory strain. However, a subtle mutation could confound analysis of early passage animals similar to strain-specific differences that are being documented with increasing frequency[183]. A spontaneous mutation that is linked to the targeted locus, as we describe here, would be maintained and could affect characterization of targeted animals, particularly if the conclusions are based on a single clone. While many linked mutations would become evident because of recombination, as observed here, a mutation that is more tightly linked would only be detected by the analysis of multiple targeted clones[183, 211].

Many published reports describing analysis of a single clone are subject to potential error. While some examples of conflicting knockout phenotypes have been traced to strain-specific modifier genes and the anatomy of the targeting constructs, some could result from inadvertent mutation of a nearby gene, as observed here[186]. Mutations at second sites may also explain the basis for some examples of differing phenotypes obtained from independent knockouts of the same gene. Mutations arising during targeting would be an important issue for approaches to saturate the mouse genome with a library of knockout alleles[212]. Together, these possibilities suggest

that observation of any phenotype, particularly one that is not expected, be confirmed by analysis of multiple independent clones.

Genetic interactions have been previously reported in the insulin receptor pathway[205, 207, 213]. Genetic strain specific modifiers have also been reported. Mice that are double heterozygous for insulin receptor and *Irs1* mutations have been assessed on three different genetic backgrounds, 129Sv, C57BL/6 and DBA/2. These mice showed dramatically different strain specific phenotypes, with the C57BL/6J mice displaying marked hyperglycemia and hyperinsulinemia[214], whereas total deficiency of the insulin receptor was more severe on the mixed C57BL/6J 129SvEvTac genetic background. This demonstrates particularly potent strain modifier effects between the C57BL/6J and 129Sv and DBA/2 strains[183, 211]. However, these studies are unclear as to the details of the actual strains used in their crosses. Strain backgrounds can have a critical effect on phenotype[13]. Even mice of the same strain, such as C57 from different vendors are effectively substrains that are separated by hundreds of generations[195].

Recently, Selman et al. reported that *Irs1* deficient female mice have increased lifespan whereas *Irs1* deficient males showed no significant effect on lifespan on the "C57" genetic background[215]. The precise "C57" strains used in this study are not detailed. These results dramatically differ from our observation of ~100 percent lethality in *Irs1* null mice on the C57BL/6J background, with normal survival to weaning on the mixed C57BL/6J-129S1Sv/IMJ background. It is possible that further studies of *Irs1* deficiency in the context of different mouse strains would be able to uncover one or more susceptibility loci. Recently Watkins-Chow et al. discovered a

copy number variant of the insulin degrading enzyme in the inbred C57BL/6J mouse population from Jackson labs. This copy number variant was present in over 75% of the mice[155, 183, 216, 217]. It is possible that mutations such as this are responsible for the discordant phenotypes observed in *Irs1* deficient mice on the "C57" background.

The C170A *Irs1*null phenotype differs in several important ways from the *Irs1* deficient mice produced by gene targeting, raising the possibility that regional genetic interference from the targeting construct could lead to imposter phenotypes attributed to *Irs1* deficiency. The C170A *Irs1*null mice reported here are free from the possibility of genetic perturbations seen in some targeted mice[182]. In this respect, these mice should prove to be a valuable addition to the genetic tools used in the study of IRS1 signaling.

Figure 3.1 Gross appearance and reduced survival of homozygous *SerpinB2*^{15B11} deficient mice

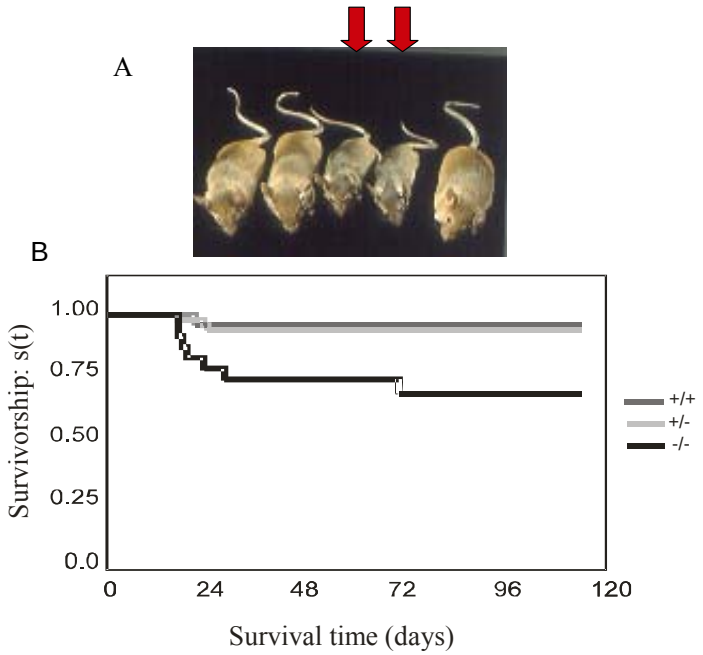


Figure 3.1 Gross appearance and reduced survival of homozygous *SerpinB2* deficient mice.
3.1A shows 5 progeny littermates from an F1 intercross. The *sml* phenotype, characterized by a grossly smaller appearance as shown by the red arrows, compared to littermates.
3.1B Kaplan-Meier survival analysis of wildtype *SerpinB2* homozygous knockout (-/-) mice (n = 24) heterozygous (+/-) (n = 51) and wild-type (+/+ (n = 25) littermates. As can be seen from these survival curves, there is a significant loss of homzygotes beginning at postnatal day 21.

Figure 3.2 Relative weights and growth curves of progeny from an F1 SerpinB2 heterozygous intercross

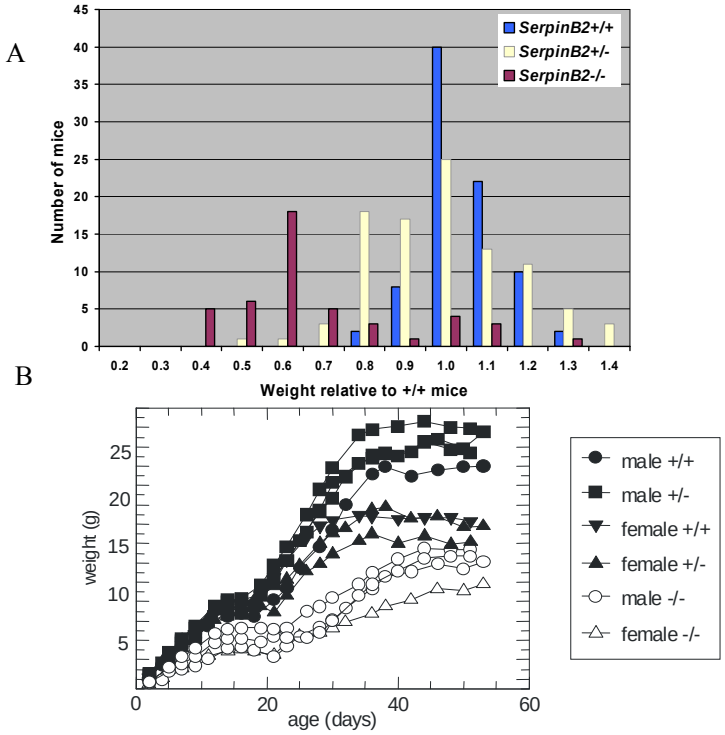


Figure 3.2 Relative weights and growth curves of progeny from an F1 *SerpinB2* heterozygous intercross.
3.2A Shows a histogram of normalized weights of F2 mice from an F1 intercross of *SerpinB2* +/- mice. Only mice from litters containing at least one *SerpinB2* wild type mouse were included N=219. A bimodal weight distribution of SerpinB2 deficient mice suggests the presence of recombinants between *SerpinB2* and the *sml* phenotype.
3.2B Growth curves of two litters from an intercross of *SerpinB2* +/- mice N=10. Progeny from two litters were weighed every 2-3 days from birth through six weeks of age. Key shows *SerpinB2* genetic status. Growth delay is recognizable by 2 days after birth. The weight of *SerpinB2* deficient males approaches that of *SerpinB2* +/- females by six weeks of age.

Figure 3.3. Genetic mapping of *sml*

mouse#	phenotype	D1Mit124	Mit178	Mit332	Mit216	Mit144	Mit382	Mit383	Mit438	Mit8	Mit80	Mit415	Mit10	SerpinB2	Mit26
Mb			34.8	43.1	49.7	50.3	50.4	50.4	51.8	51.8	51.8	52	56.6	61.1	62.1
2A7	small	C/d	C/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
2A8	small	C/d	C/d	d/d	d/d	d/d	d/d	d/d	d/d	C/d	C/d	C/d	C/d	+/-	C/d
2B5	small	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
2B6	small	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
3E6	small	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
4A6	small	C/d	C/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
4C11	small	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	C/d	+/-	C/d
4D7	small	C/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
5C6	small	C/d	C/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
5C7	small	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
7B7	small	C/d	C/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
8C4	small	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
8C5	small	C/d	C/d	C/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
8E7	small	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	d/d	-/-	d/d
8C5				small	C/d	d/d	d/d	d/d		d/d					
2A8				small	d/d	d/d	d/d	d/d		C/d					

Figure 3.3. Genetic mapping of *sml* using normalized weights as phenotype. Genetic map of the *sml* region on distal mouse chromosome 1. Mice inheriting the *sml* region are shown in blue and yellow regions correspond to inheritance of *Mus castaneus.* S*ml* is localized between markers D1Mit216 and D1Mit8.

Figure 3.4 Sequencing and western blotting of Irs1 from *sml* mice

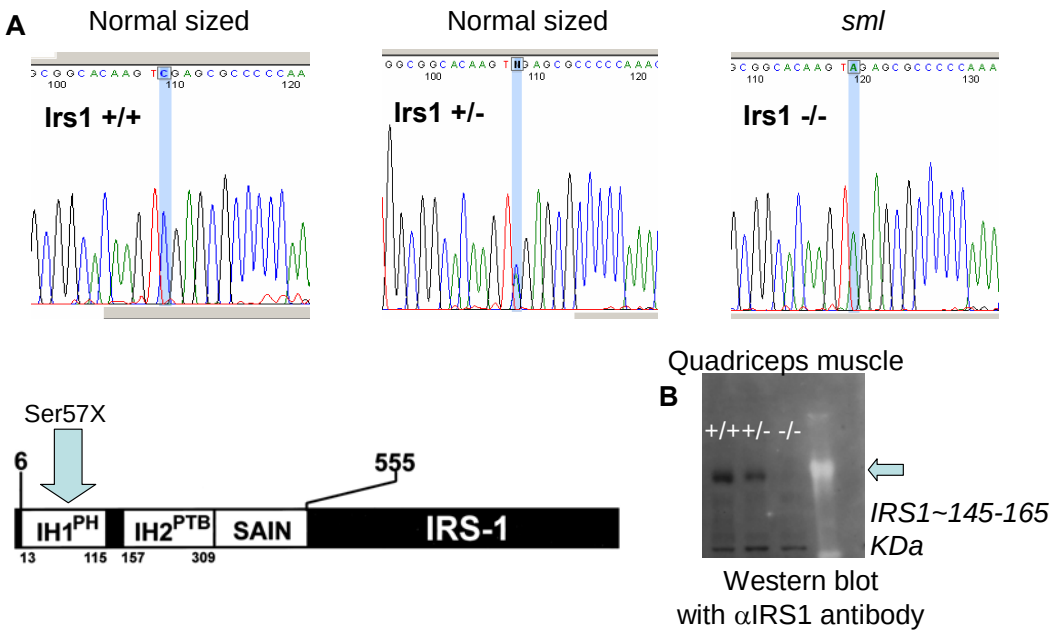


Figure 3.4 Sequencing and western blotting of *Irs1* from *sml* mice.
3.4A shows the *Irs1* sequence tracings from the vicinity of nucleotide 170. As shown in light blue, there is an "C" in the wildtypes (*Irs1* +/+), an "A" and "C" peak nearly superimposed in the heterozygotes (*Irs1* +/-), and a "A" in the homozygous deficients (*Irs1* -/-). This mutation results in a serine to stop mutation at codon 57 (Ser57X) of the IRS1 protein.
3.4B IRS1 western blot on muscle tissue. There is complete absence in *IRS1* -/-, and a strong signal corresponding to *IRS1* protein in the *IRS1* +/+. *IRS1* +/- show an intermediate signal, consistent with one functional allele.

Figure 3.5 Evidence of modifier genes in the 129S1/SvImJ and or C57BL/6J strains

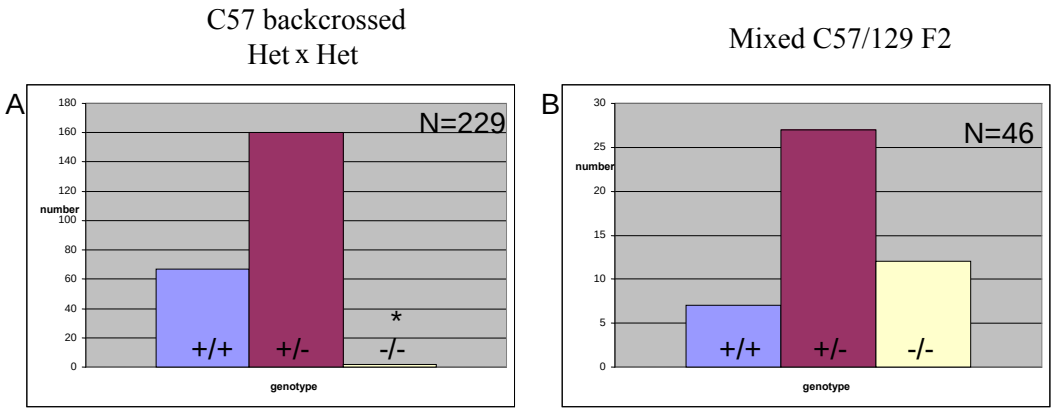


Figure 3.5 Evidence of modifier genes in the 129S1Sv/ImJ and/or C57BL/6J strains responsible for variable lethality in the *Irs1* -/- mice. C170A mutants backcrossed 6 generations onto the C57BL/6J background display nearly complete lethality. (X=72 *$p<1x10^{-15}$, 2 degrees of freedom). Normal survival is restored upon reintroduction of 129S1Sv/ImJ by outcross/intercross of *Irs1* +/-.

Notes

*This chapter is in preparation for submission as a manuscript entitled "Passenger gene point mutation: discovery of a spontaneous *Irs1* mutation in linkage with *SerpinB2* deficiency" by Randal J. Westrick, Karen L. Mohlke, Lindsey M. Korepta, Angela Y. Yang, Sara L. Manning, Mary E. Winn, Goujing Zhu, Kristiann M. Dougherty, and David Ginsburg

CHAPTER IV

MAJOR THROMBOSIS RESISTANCE LOCI IDENTIFIED BY GENETIC DISSECTION OF AN OLIGOGENIC TRAIT

Abstract

Incomplete penetrance observed in carriers of Factor V Leiden (FVL) is modulated by genetic interactions both in humans and mice. We have previously reported a striking synthetic lethal genetic interaction between FVL homozygosity ($FV^{Q/Q}$) and heterozygous TFPI deficiency ($TFPI^{+/-}$). However, after backcrossing the FV^{Q} and $TFPI^{-}$ alleles six generations onto the DBA/2J genetic background and subsequent intercrossing of $FV^{Q/+}$ $TFPI^{+/-}$ mice, complete viability of the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype combination was observed at weaning. Subsequent crosses back to $FV^{Q/Q}$ on the C57BL/6J lethal strain background suggested dominantly acting genes in the DBA genetic background were responsible for restoration of viability. An extensive $FV^{Q/Q}$ $TFPI^{+/-}$ pedigree was developed from this backcross approach. 161 pedigree members were subjected to SNP genotyping followed by linkage analysis. LOD scores ranging between 1.52 and 5.04 were observed. Taken together, these results suggest that there are genetically isolatable modifiers of the $FV^{Q/Q}$ $TFPI^{+/-}$ phenotype in the B6-DBA mouse strain combination. Any modifiers isolated from this study could be candidates for modifying thrombosis in humans.

Introduction

Venous thrombosis is a prevalent multifactorial disorder resulting from a number of genetic and environmental risk factors. Factor V Leiden (FVL) is a common polymorphism in European populations that renders human FVL heterozygotes susceptible to thrombosis. Ten percent of FVL heterozygotes will present with clinical thrombosis in their lifetimes, suggesting the presence of interacting genetic and/or environmental factors. Co-inheritance of FVL with another genetic risk factor such as antithrombin III, protein C and S deficiencies, and the prothrombin 20210 mutation, increases penetrance[218-221]. However, other, as yet undiscovered genes are likely to exist that can interact with FVL to increase or decrease susceptibility to thrombosis. Mice engineered by gene targeting to carry the ortholog of FVL exhibit a phenotype similar to humans. FVL in the mouse also exhibits a pronounced intereaction with other genes, including protein Z and tissue factor pathway inhibitor (TFPI). In particular, homozygosity for FVL($FV^{Q/Q}$) exhibits a striking synthetic lethal interaction with heterozygous TFPI deficiency[18] ($TFPI^{+/-}$) in C57BL/6J (B6).

Strain background has been demonstrated to cause variable phenotypes in mouse models of thrombosis and bleeding[13, 122, 124, 222, 223]. In order to screen for strain modifiers affecting $FV^{Q/Q}$ $TFPI^{+/-}$, we crossed $FV^{Q/+}$ $TFPI^{+/-}$ into six mouse strain backgrounds. In contrast to $FV^{Q/Q}$ $TFPI^{+/-}$ lethality observed on the C57BL/6J background, we observed complete viability of these mice on the DBA/2J background at weaning. This report outlines our preliminary findings.

Materials and methods

Mice

Parental strains: C57BL/6J (B6, stock number 000664) and DBA/2J (DBA, stock number 000671) mice were purchased from Jackson labs.

Transgenic mice: FV^{Q} mice were previously generated[16]. TFPI-deficient mice were a generous gift of Dr. George Broze[117]. Perinatal lethal thrombosis in the combined $FV^{Q/Q}$ $TFPI^{+/-}$ mice was previously described[18].

All mice were maintained on normal chow in specific pathogen-free (SPF) facilities. All animal care and experimental procedures complied with the principles of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use and Care of Animals.

DNA

DNA was isolated from tail biopsies as previously described[18]. For SNP analysis, DNA from tail biopsy was isolated with the Puregene system for DNA isolation (Qiagen Corp www.qiagen.com). Genomic DNA concentration was quantitated in duplicate using Picogreen (Molecular Probes, Inc., Eugene, Oregon http://www.probes.com) and fluorometrically quantitated with a Wallac Victor2 machine (www.PerkinElmer.com). When sufficient genomic DNA was not available, whole genome amplification was performed using the Illustra GenomiPhi V2 DNA Amplification Kit following manufacturer's instructions (www.gelifesciences.com).

Following whole genome amplification, amplified samples were purified using the Marligen DNA purification kit (www.marligen.com).

Genotyping and phenotyping

Mice were genotyped for TFPI and FVL as previously described[18]. SNP genotyping was performed either at the Harvard Medical School Mutation Mapping and Developmental Analysis Project with a customized 768 SNP panel, (555 are informative between B6 and DBA, average marker density of ~1 in 5.4Mb)[224] or in the University of Michigan Sequencing core using the Illumina Golden Gate assay with the mouse medium density SNP array (http://www.illumina.com/pages.ilmn?ID=163) containing 1449 SNP markers (834 of which are informative between B6 and DBA average marker density is ~1 in 3.5Mb).

Statistical Analysis

The significance of survival differences between groups was determined using the X^2 test. Prior to parametric linkage analysis, all genotypic data were evaluated for Mendelian inheritance of marker alleles with the program Pedcheck[225]. Since the initial F1 cross and all subsequent backcrosses were created using inbred lines, genotypic information was known for all founders in the pedigree. All individuals involved in a Mendelian inconsistency were coded as missing for that particular marker. LOD scores were computed using an autosomal dominant model for disease inheritance, as implemented in the computer program Mendel v8.0 (http://www.genetics.ucla.edu/software/mendel). Since all founders have complete genotypic data, marker allele frequencies do not affect the LOD statistics and were fixed

at 0.5 for both alleles. The disease allele frequency was fixed at 0.01. Two penetrance models were used to search for genes. The first used an autosomal dominant model with reduced penetrance (80%) and allowed for locus heterogeneity (phenocopy rate of 3%). The other was a strict dominant model (100% penetrance and no locus heterogeneity).

Results

A strain-dependent $FV^{Q/Q}$ $TFPI^{+/-}$ modifier locus

The FVL and TFPI knockout alleles were backcrossed onto several genetic backgrounds, including six generations onto the DBA strain. A cross between N6 DBA $FV^{Q/+}$ $TFPI^{+/-}$ and $FV^{Q/Q}$ was performed and the progeny analyzed, as shown in Table 4.1. Compared to our previous data of the same cross on the B6 background, with a significant loss of $FV^{Q/Q}$ $TFPI^{+/-}$ mice ($p<0.001$), this cross unexpectedly yielded a normal Mendelian distribution of the four possible genotypes at weaning. The live $FV^{Q/Q}$ $TFPI^{+/-}$ mice on the DBA background appeared phenotypically normal, although we occasionally observed grossly runted $FV^{Q/Q}$ $TFPI^{+/-}$ mice compared to their littermates. These data suggest that either the DBA strain background contains 1 or more gene(s) that permit survival of mice carrying the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype or that there are one or more genes in the B6 background that result in lethality.

The DBA strain-specific modifier of $FV^{Q/Q}$ $TFPI^{+/-}$ is autosomal dominant

$FV^{Q/Q}$ $TFPI^{+/-}$ mice on the DBA background were bred to $FV^{Q/Q}$ B6 mice, producing F1 mice that had the genotype distribution as shown in Table 4.2 and 4.3. Compared to the expected Mendelian frequencies, the $FV^{Q/Q}$ $TFPI^{+/-}$ mice in this cross are

present at weaning at percentages consistent with a dominant inheritance pattern of a DBA strain modifier. Backcrossing again to the $FV^{Q/Q}$ B6 mice yielded N2 generation $FV^{Q/Q}$ $TFPI^{+/-}$ mice born at the expected Mendelian frequency. This result suggests that there are either multiple modifiers in the DBA strain or alternatively, that a dominant modifier is linked to the $TFPI^{-}$ allele on Chromosome 2.

Whole pedigree genome analysis identifies candidate modifier loci

Four $FV^{Q/Q}$ $TFPI^{+/-}$ mice from the N2 backcross generation were chosen at random for further backcrossing to $FV^{Q/Q}$ B6 to produce an N3 generation. Six mice were then chosen at random for the generation of backcross generations N4-N7. Progeny from each generation were screened at weaning for $FV^{Q/Q}$ $TFPI^{+/-}$. The entire pedigree consists of 234 surviving $FV^{Q/Q}$ $TFPI^{+/-}$ offspring. These are broken down by generation as follows: 61 N2's, 63 N3's, 34 N4's, 24 N5's, 21 N6's and 31 N7's. The penetrance at each generation ranges from 80 to 100 percent. DNA from 161 $FV^{Q/Q}$ $TFPI^{+/-}$ mice from generations N2-N7 was subjected to whole genome SNP genotyping analysis. We then performed linkage analysis, shown in Figure 4.1, and identified six suggestive (LOD >1.9) loci[226], with LOD scores as follows: chromosome 18, 47 megabase (Mb):1.92; chromosome 7, 68 Mb:2.13; chromosome 3, 92 Mb:2.23; chromosome 10, 119 Mb:2.51; chromosome 12, 86 Mb:5.04; and chromosome 2, 155Mb: 5.43 Mb. The overall maximum LOD is on chromosome 2 (5.43). However, this locus is 71 Mb distal to to TFPI, which corresponds to a recombinational distance of roughly 35 centimorgans. Thus, this locus is partially linked with TFPI and may result from a passenger gene effect[183]. The next highest LOD is on chromosome 12 (5.04). These preliminary data

are consistent with one or more major modifier loci on chromosome 12 and/or 2, with additional smaller contributions from a number of loci.

Discussion

We previously described a synthetic lethal genotype combination of $FV^{Q/Q}$ $TFPI^{+/-}$ on the B6 genetic background[18] due to perinatal thrombosis. To our surprise, complete viability of this genotype combination was observed in the context of the DBA strain background. This result suggested that the DBA mouse genome contained a dominant or recessive gene(s) that can modify the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype from lethal to viable.

Through a series of breeding experiments we determined that there was a DBA autosomal dominant inheritance pattern. However, it cannot be ruled out that there is a DBA modifier gene linked to the TFPI knockout allele, which could account for the high penetrance observed in this cross.

As part of our strategy to create congenics in order to segregate modifiers that by themselves are sufficient to rescue the lethal phenotype, a more extensive pedigree spanning seven generations and containing multiple mice from each generation was produced. From 161 mice of this cohort, seven genomic regions of suggestive linkage were identified, with one major effect modifier potentially mapped to chromosome 12. These region(s) are now candidates for our ongoing studies aimed at isolating DBA strain-specific alleles that can modify the $FV^{Q/Q}$ $TFPI^{+/-}$ thrombotic phenotype.

In addition to the putative loci thought to modify thrombosis susceptibility in this study, we have surveyed other inbred mouse strains and have identified ones which clearly fall into the two phenotypic classes: lethal (129S1/SvIMJ) and viable (A/J, SJL/J,

BALB/cJ). It is likely that there will be both shared and unique modifiers among these strains and B6-DBA.

Other groups have undertaken the search for thrombosis modifier genes in mice. Rosen et al. have utilized a model of Factor VII deficiency to isolate bleeding modifiers[124]. On the B6 genetic background this mouse displays a more severe bleeding phenotype than mice on a mixed B6:129x1/SVJ background. Using a backcross approach, these investigators have identified 8 putative 129x1/SVJ modifier loci. It appears that two of the loci they identified in this study may overlap with our suggestive regions on 8 and 10. Using bleeding/rebleeding time, the Hoover-Plow group previously uncovered differences between the A/J and B6 mouse strains. They went on to identify quantitative trait loci on chromosomes 5, 11 and 17 that regulate this phenotype in mice[122].

Taken together, these findings highlight the sensitivity of the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype for screening inbred strains for thrombotic modifier genetic interactions. Our observations reinforce the notion that variation in genetic content between inbred strains can be used to uncover important genetic thrombosis modifiers. Advantages of the forward genetics approach described here are the detection of phenotype altering genetic variants that may enable us to identify novel genes or novel functions associated with known genes that contribute to thrombosis susceptibility. The genetic localization of putative modifier loci in this study represents an early attempt to ascertain the identity of these modifiers. Conceivably, this will further our understanding of the complexity involved in regulating thrombus formation. Any genes identified in the study will enable

the directed study of human orthologous genes and their investigation for variants that increase thrombosis susceptibility.

Table 4.1 Establishing the genetic basis of the DBA strain dependent $FV^{Q/Q}$ $TFPI^{+/-}$ phenotypic switch

Number of offspring/Genotype		**$FV^{Q/+}$ $TFPI^{+/-}$**	**$FV^{Q/Q}$ $TFPI^{+/-}$**	**$FV^{Q/+}$ $TFPI^{+/+}$**	**$FV^{Q/Q}$ $TFPI^{+/+}$**	**Total number of pups analyzed**
Parental Genotypes/Expected		**25%**	**25%**	**25%**	**25%**	
$FV^{Q/Q}$ (C57)	**$FV^{Q/+}$ $TFPI^{+/-}$ (C57)**	28% (26)	2% (2*)	37% (35)	33% (31)	**94**
$FV^{Q/Q}$ (DBA)	**$FV^{Q/+}$ $TFPI^{+/-}$ (DBA)**	22.5% (14)	24.2% (15)	29% (18)	24.2% (15)	**62**

Table 4.2 Establishing inheritance pattern for DBA strain modifier

Parental Genotypes		**# of offspring**		**Total number of pups analyzed**
$FV^{Q/Q}$ $TFPI^{+/-}$ (DBA)	**$FV^{Q/Q}$ $TFPI^{+/+}$ (B6)**	**$FV^{Q/Q}$ $TFPI^{+/+}$**	**$FV^{Q/Q}$ $TFPI^{+/-}$**	
Expected %		**50%**	**50%**	
Expected		**(21)**	**(20)**	
Observed%		**50%**	**50%**	**41**
Observed		**(25)**	**(16)**	

Table 4.3 Establishing dominant inheritance pattern for DBA strain modifier

Parental Genotypes		**# of offspring**		**Total number of pups analyzed**
$FV^{Q/Q}$ $TFPI^{+/-}$	**$FV^{Q/Q}$ $TFPI^{+/+}$ (B6)**	**$FV^{Q/Q}$ $TFPI^{+/+}$**	**$FV^{Q/Q}$ $TFPI^{+/-}$**	
Single Dominant				
Expected %		**67%**	**33%**	
Expected		(87)	(43)	
Observed %		52%	48%	**130**
Observed		(67)	(63)	

Figure 4.1 Linkage analysis of DBA:B6 FVQ/Q TFPI+/- Pedigree

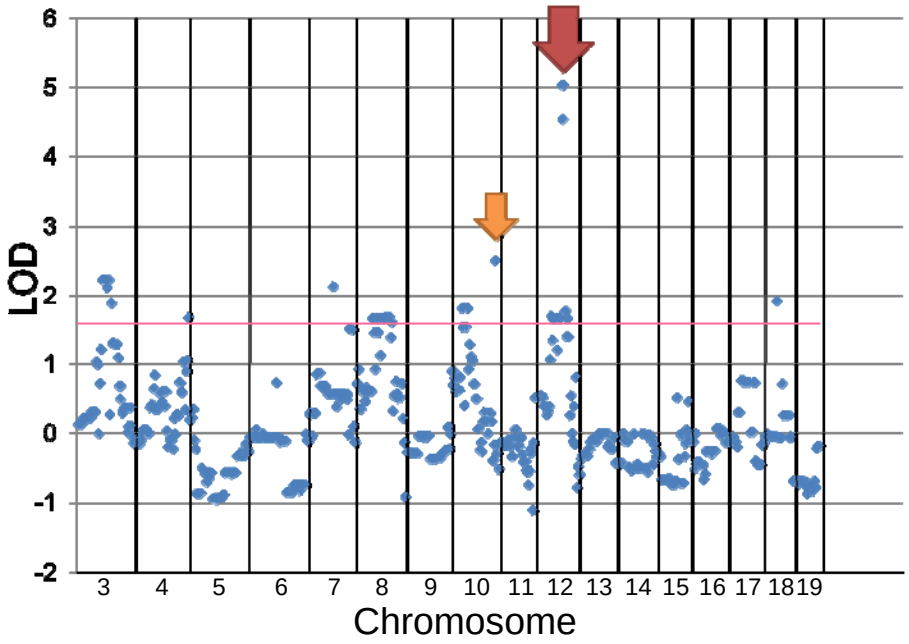


Figure 4.1. Two point linkage analysis of DBA:B6 $FV^{Q/Q}$ $TFPI^{+/-}$ Pedigree. Whole genome SNP genotyping analysis was performed on 161 of $FV^{Q/Q}$ $TFPI^{+/-}$ individuals from the pedigree. Linkage analysis identified six suggestive loci (above the rose colored bar, representing a 1.9 LOD score, suggestive for linkage in a mouse backcross). The chromosome 12 region has a maximum significant LOD score of 5.04(red arrow). The orange arrow denotes the third highest LOD score of 2.5 on chromosome 10. Not shown are chromosomes 1 and 2.

CHAPTER V

A SENSITIZED SUPPRESSOR SCREEN TO IDENTIFY MODIFIER GENES FOR FACTOR V LEIDEN DEPENDENT THROMBOSIS IN THE MOUSE

Abstract

Venous thrombosis affects ~300,000 individuals per year in the USA. A gain-of-function mutation in the factor V gene, Factor V Leiden (FVL) is the most common known inherited risk factor for venous thrombosis. However, penetrance is incomplete, with only ~10% of FVL heterozygotes experiencing clinically significant thrombosis in their lifetimes. Previously, we demonstrated synthetic lethality in mice between homozygosity for the FVL mutation ($FV^{Q/Q}$) and heterozygous deficiency for another key coagulation component, tissue factor pathway inhibitor ($TFPI^{+/-}$). In order to identify potential modifier genes contributing to FVL penetrance, we have utilized this lethal genetic interaction as a phenotyping tool for a sensitized ENU mutagenesis screen in mice. As proof of concept, we proposed that loss of one tissue factor ($TF^{+/-}$) allele would compensate for reduced TFPI, thus suppressing the lethal $FV^{Q/Q}$ $TFPI^{+/-}$. To test this hypothesis, we mated $FV^{Q/Q}$ x $FV^{Q/+}$ $TFPI^{+/-}$ $TF^{+/-}$ and observed $FV^{Q/Q}$ $TFPI^{+/-}$ $TF^{+/-}$ survivors at one half the expected frequency. Thus, $TF^{+/-}$ exhibits an ~50% penetrant suppression of the $FV^{Q/Q}$ $TFPI^{+/-}$ lethal phenotype. This suggests that mutations in TF are one of a subset of mutants that should emerge from our screen. We next applied this synthetic lethal interaction for a sensitized, genome-wide mutagenesis screen for modifiers of FVL and overall hemostatic balance. Male $FV^{Q/Q}$ mice were exposed to

mutagenic doses of ENU and bred to $FV^{Q/+}$ $TFPI^{+/-}$ double heterozygous females. Surviving G1 offspring were analyzed to identify surviving mice with the otherwise lethal $FV^{Q/Q}$ $TFPI^{+/-}$ genotype. Analysis of 6677 G1offspring (corresponding to ~2X genome coverage for dominant, loss of function mutations) identified 92 $FV^{Q/Q}$ $TFPI^{+/-}$ mice that survived to weaning. While 71 of the mice exhibited early lethality or sterility, 21 surviving $FV^{Q/Q}$ $TFPI^{+/-}$ G1 mice were progeny tested, with 13 exhibiting successful transmission of a putative suppressor mutation to one or more subsequent $FV^{Q/Q}$ $TFPI^{+/-}$ G2 offspring. Our preliminary findings demonstrate the feasibility of our sensitized approach in the identification of dominant suppressors of the $FV^{Q/Q}$ $TFPI^{+/-}$ lethal phenotype and suggests that there are ~10-20 thrombosis modifier loci for the $FV^{Q/Q}$ $TFPI^{+/-}$ phenotype. Dominant mutants isolated from these studies will become candidate modifier genes for contributing to human thrombosis and bleeding risk.

Introduction

Factor V Leiden (FVL) is the most common inherited risk factor for venous thrombosis with an allele heterozygote frequency of approximately 2.5 percent in European-derived populations[53, 111, 227, 228]. FVL is estimated to account for up to 25 percent of the genetically-attributable thrombosis risk in humans[98]. However, penetrance is low, with only ten percent of FVL heterozygotes and 80 percent of homozygotes developing thrombosis in their lifetime. The severity of thrombosis also varies widely among affected individuals[86].

The incomplete penetrance and highly variable expressivity of clinical thrombosis among FVL patients can at least partially be explained by genetic interactions between

FVL and other known thrombotic risk factors[218-221], such as heterozygous deficiency for antithrombin III, protein C or protein S, as well as the common prothrombin 20210 polymorphism[218-221]. However, <2 percent of FVL heterozygotes would be expected to co-inherit one or more of these risk factors, suggesting that a large number of additional genetic variants interacting with FVL to increase or decrease susceptibility to thrombosis remain to be identified. Recent genetic studies in human thrombosis patients (with or without FVL) have failed to identify novel thrombosis susceptibility genes or genetic modifiers of FVL[92, 98].

Mice carrying the ortholog of the human FVL mutation exhibit a mild to moderate prothrombotic phenotype, closely resembling the human disorder, with a similarly more severe thrombosis in homozygotes[16]. We previously reported a synthetic lethal interaction between FVL homozygous ($FV^{Q/Q}$) and heterozygous tissue factor pathway inhibitor ($TFPI^{+/-}$) deficient mice. Nearly all mice with this lethal genotype combination ($FV^{Q/Q}$ $TFPI^{+/-}$) succumb to widespread, systemic thrombosis in the immediate perinatal period[18].

Mutagenesis screens provide a powerful tool for the dissection of molecular pathways *in vivo*, and have long been used as a standard tool in model organisms including *C. elegans*[229], *D. melanogaster*[230] and *D. rerio[231]*. Similar approaches have also been successfully applied in mice to explore genetic networks regulating a variety of processes, including coat color[141], hearing[142], circadian rhythm[140], and the innate immune response[143]. A variation of this approach employs a genetic, chemical, or environmental sensitizer to focus on a specific phenotype of interest. For example, a recent mutagenesis screen in thrombopoietin receptor deficient mice

uncovered a previously unappreciated role of the c-Myb gene as a dominant modifier of circulating platelet number[161]. ENU is generally the mutagenic agent of choice, inducing germline mutations at a rate ten to fifteen fold greater than the endogenous background in untreated mice[121].

We now report a dominant, sensitized, ENU mutagenesis screen for thrombosis modifier genes, based on the synthetic lethal $FV^{Q/Q}$ $TFPI^{+/-}$ interaction. Introduction of heterozygous deficiency for tissue factor ($TF^{+/-}$) effectively rescues $FV^{Q/Q}$ $TFPI^{+/-}$ lethality, with $FV^{Q/Q}$ $TFPI^{+/-}TF^{+/-}$ demonstrating normal fertility and survival to adulthood, without evidence for thrombosis. Extension of this strategy to a whole genome ENU mutagenesis screen of 6677 G1 offspring identified 92 $FV^{Q/Q}$ $TFPI^{+/-}$ mice that survived to weaning, with progeny testing of 21 suggesting that approximately half carry an authentic, dominant thrombosis suppressor mutation. Given that we should have achieved approximately twofold genome coverage, these data suggest the presence of approximately 10-20 such thrombosis suppressor loci in the mammalian genome.

Materials and methods

Mice

Parental strains: C57BL/6J (B6, stock number 000664) mice were purchased from Jackson labs.

Transgenic mice: FV^{Q} mice were previously generated[16]. TF and TFPI-deficient mice were a generous gift of Dr. George Broze[117]. All mice were backcrossed greater than 8 generations to B6. Perinatal lethal thrombosis in the combined $FV^{Q/Q}$ $TFPI^{+/-}$ mice was previously described[18].

All mice were maintained on normal chow in specific pathogen-free (SPF) facilities. All animal care and experimental procedures complied with the principles of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use and Care of Animals.

Genotyping and phenotyping

DNA was isolated from tail biopsies as previously described[18]. Mice were genotyped for TFPI and FVL as previously described[18].

The significance of survival differences between groups was determined using the X^2 test.

ENU mutagenesis

ENU was purchased from Sigma chemical company, St. Louis MO in ISOPAC vials, and prepared according to protocol http://pga.jax.org/enu_protocol.html. Initially, a single dose of 150 mg/kg was administered interperitoneal into 159 $FV^{Q/Q}$ B6 male mice (called generation 0 or G0 mice). Subsequently, ENU was administered in three weekly doses of 90 mg/kg or 100 mg/kg.

Results

Haploinsufficiency for tissue factor rescues the $FV^{Q/Q}$ $TFPI^{+/-}$ synthetic lethality

We proposed to use the early lethal thrombotic phenotype previously reported in $FV^{Q/Q}$ $TFPI^{+/-}$ mice as the basis for a whole genome ENU mutagenesis screen for

dominant thrombosis suppressor genes. The nearly complete lethality of the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype (>98%[18]) suggested that few false positive mutations should be encountered. However, this observation also raised the concern that no genes might be identified for which a haploinsufficient loss-of-function mutation could sufficiently alter the hemostatic balance to permit survival of $FV^{Q/Q}$ $TFPI^{+/-}$ mice.

As a potential "proof of concept", we hypothesized that a 50% reduction in TF might effectively compensate for the similar reduction in TFPI level in $FV^{Q/Q}$ $TFPI^{+/-}$ mice. To test this hypothesis, $TF^{+/-}$ mice were crossed with $FV^{Q/+}$ $TFPI^{+/-}$ mice to generate triple heterozygous $FV^{Q/+}$ $TFPI^{+/-}$ $TF^{+/-}$ mice. The latter mice were then crossed back to $FV^{Q/Q}$ B6 mice and a total of 272 progeny genotyped at weaning. Table 5.1. One quarter of the progeny from this cross are expected to carry the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype, with one half of these mice $TF^{+/-}$. As shown in Table 5.1, 14 $FV^{Q/Q}$ $TFPI^{+/-}$ $TF^{+/-}$ but no $FV^{Q/Q}$ $TFPI^{+/-}$ $TF^{+/+}$ mice were observed ($X=14$, $p < 0.0001$ 1 degree of freedom). Thus, haploinsufficiency for TF rescues the $FV^{Q/Q}$ $TFPI^{+/-}$ lethality *in vivo*. However, the 14 observed $FV^{Q/Q}$ $TFPI^{+/-}$ $TF^{+/-}$ progeny is ~one half of the expected, suggesting that this rescue is incompletely penetrant. These results demonstrate the feasibility of the sensitized $FV^{Q/Q}$ $TFPI^{+/-}$ suppressor screen and suggest that mutations at the TF locus should be among the set of suppressor mutations identified in a whole genome saturating analysis.

Visible mutants seen in ENU mutagenesis screen

As proof that the mutagenesis procedures employed in this study are effective, we have observed a number of previously described visible dominant mutants[154], ranging

from belly spotting to skeletal abnormalities in approximately 5.9 percent of our G1 mice. Figure 5.1. Previous studies observed a 4.2 percent rate of observable mutants[154]. This suggests that our mutagenesis procedures are working to effectively produce mutants.

ENU mutagenesis generates viable G1 $FV^{Q/Q}$ $TFPI^{+/-}$ progeny

ENU mutagenized G0 $FV^{Q/Q}$ males were crossed to $FV^{Q/+}$ $TFPI^{+/-}$ females and the resulting G1 progeny screened by genotyping at weaning for FV^{Q} and $TFPI^{-}$. A total of 6,677 G1 mice were genotyped, and 92 live mice were identified at weaning with the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype.

~1/2 of surviving $FV^{Q/Q}$ $TFPI^{+/-}$ carry a heritable thrombosis suppressor mutation

First, the heritability of each of the 92 G1 putative suppressor mutants was ascertained by progeny testing. Each mutant was crossed back to B6 $FV^{Q/Q}$. The observation of one or more $FV^{Q/Q}$ $TFPI^{+/-}$ mice among the progeny suggests that a particular mutant carries a transmissable modifier mutation. 59 of the 92 surviving $FV^{Q/+}$ $TFPI^{+/-}$ G1 mice were unable to produce offspring, either due to early lethality or infertility. Indeed, ~40 percent of these mice (24/59) exhibited a grossly runted and/or scruffy appearance. Nine $FV^{Q/Q}$ $TFPI^{+/-}$ G1 mice produced one or more G2 progeny surviving to weaning, but none of the $FV^{Q/Q}$ $TFPI^{+/-}$ genotype. Four of the nine produced eight or more offspring, suggesting that they were negative for a suppressor. However, the remaining five G1's produced six or less $FV^{Q/Q}$ $TFPI^{+/-}$ mice, thus it cannot be determined that these mice truly lacked a suppressor.

Thirteen G1 mice successfully progeny tested in the B6 background by producing one or more $FV^{Q/Q}$ $TFPI^{+/-}$ progeny. These progeny tested G1's comprise a set of mutants that will be subjected to positional cloning.

Discussion

Our aim was to identify dominant mutations with a major effect on hemostatic balance. One potential pitfall in this mutagenesis approach is the possibility that there may be no mutations that will result in the desired phenotypic change, i.e. the phenotype is so severe that viability cannot be restored.

The major function of TFPI is to oppose the activation of coagulation by tissue factor. Our data demonstrate that reduction of TF levels by ~50% restored viability to the $FV^{Q/Q}$ $TFPI^{+/-}$ mice, presumably by compensating for the similar reduction in TFPI. These results serve as a "proof of principal" for the feasibility of a suppressor screen based on the lethal $FV^{Q/Q}$ $TFPI^{+/-}$ phenotype, and suggest that inactivating mutations in TF should be among the spectrum of mutations identified by this approach. These data also suggest that the quantitative TFPI/TF balance is critical for hemostatic balance, particularly in the setting of FVL and that even modest variations in expression of either gene could be an important modifier of thrombosis susceptibility in humans.

Our sensitized mutagenesis screen has identified 92 independent $FV^{Q/Q}$ $TFPI^{+/-}$ suppressed mutants. This represents 4 percent of the total $FV^{Q/Q}$ $TFPI^{+/-}$ conceptions surveyed. Based on our previous data, roughly half of the $FV^{Q/Q}$ $TFPI^{+/-}$ newborns isolated in this study could represent stochastic background survivors[18]. Indeed, 59 $FV^{Q/Q}$ $TFPI^{+/-}$ G1's (~2.6 percent of the total observed) appeared sickly, died or were not

able to produce due to other physical problems. This rate of stochastic survival would indicate that there are ~20 authentic G1 mice generated in our study, which is in line with the 13 heritable G1 mice that we have observed. Non-heritability of phenotype is commonly seen in ENU mutagenesis screens and is possibly due to multiple ENU mutations necessary for the phenotype or other as yet undescribed mechanisms[153, 232].

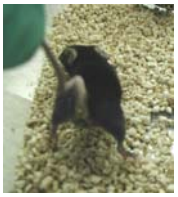
Since many ENU mutants result in a loss of function, an approximately 50 percent reduction in expression levels in mutants isolated in this dominant screen should identify strong candidates for human studies, since variation of this magnitude is likely a common occurrence in humans.

Table 5.1 $TF^{+/-}$ rescues $FV^{Q/Q}TFPI^{+/-}$ lethality with 50% penetrance

Number of offspring/ Genotype		$FV^{Q/+}$ $TFPI^{+/-}$ $TF^{+/-}$	$FV^{Q/+}$ $TFPI^{+/-}$ $TF^{+/+}$	$FV^{Q/+}$ $TFPI^{+/+}$ $TF^{+/-}$	$FV^{Q/+}$ $TFPI^{+/+}$ $TF^{+/+}$	$FV^{Q/Q}$ $TFPI^{+/-}$ $TF^{+/-}$	$FV^{Q/Q}$ $TFPI^{+/+}$ $TF^{+/-}$	$FV^{Q/Q}$ $TFPI^{+/+}$ $TF^{+/+}$	$FV^{Q/Q}$ $TFPI^{+/-}$ $TF^{+/+}$	**Total number of pups analyzed**
Expected		38.9	38.9	38.9	38.9	38.9	38.9	38.9	0	
Parental genotypes										
$FV^{Q/Q}$	$FV^{Q/+}$ $TFPI^{+/-}$ $TF^{+/-}$	39	58	38	53	14	27	44	0	272

Figure 5.1 ENU Mutagenesis: Observed dominant mutations

Total analyzed	6,677
Small size	248
Coat color	51
Hydrocephalus	24
Skeletal abnormalities	29
Eye/ear defect	14
Other	31



Curly tail

Belly spot

Figure 5.1 ENU Mutagenesis: Observed dominant mutations.
Of the 6,677 mice analyzed, the numbers of visible mutants in each category are tabulated. The curly tail and belly spot mutant are representations of the skeletal abnormality and coat color class of mutations, respectively.

Notes

*This chapter is in preparation for submission as a manuscript entitled “A sensitized suppressor screen to identify modifier genes for Factor V Leiden dependent thrombosis in the mouse” by Randal J. Westrick, Sara L. Manning, Goujing Zhu, Mary E. Winn, Sarah L. Dobies, Angela Y. Yang, Abigail L. Peterson, David R. Siemieniak, Ethan Sanford, Catherine Lee-Mills, Jesse Plummer, Grace M. Stotz, Lindsey M. Korepta, and David Ginsburg.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Venous thromboembolic disease is a common, clinically heterogeneous disease, with deep venous thrombosis as its most common presentation[86]. A handful of genetic variants are hypothesized to contribute to thrombosis risk. Thus, venous thrombosis has been termed an oligogenic disease[86, 92]. FVL is the most common inherited risk factor for venous thrombosis and is thought to account for approximately 25 percent of the total genetic risk. Despite being inherited by many thrombosis patients, FVL is incompletely penetrant, with only about ten percent of heterozygotes developing thrombosis in their lifetimes. The extensive clinical variability seen in venous thrombosis can be partially explained by genetic interactions between FVL and other known genetic risk factors. I have made use of our previously described model of the synthetic lethal $FV^{Q/Q}$ $TFPI^{+/-}$ genotype[18] as a sensitized screen to search for strain specific and ENU induced thrombosis modifiers.

In Chapter IV, I identified a potent DBA strain suppressor of the $FV^{Q/Q}$ $TFPI^{+/-}$ phenotype. Subsequent crosses back to $FV^{Q/Q}$ on the B6 lethal strain suggested that dominantly acting genes were responsible for this suppression. Whole genome SNP genotyping followed by linkage analysis on 161 members of an extended pedigree of $FV^{Q/Q}$ $TFPI^{+/-}$ mice identified six genomic regions suggestive of linkage, with potential major effect modifiers located on chromosomes 2 and 12. These regions are now

candidates for our ongoing studies aimed at isolating DBA strain modifiers. Taken together, these results suggest that there are genetically isolatable modifiers of the $FV^{Q/Q}$ $TFPI^{+/-}$ phenotype in the B6-DBA mouse strain combination. In addition, the data in Chapter IV also provides proof of principle that the mutagenesis strategy based on the $FV^{Q/Q}$ $TFPI^{+/-}$ phenotype as outlined in Chapter V is a valid approach for identifying thrombotic modifier loci.

Additional studies are required to define and describe the DBA strain modifiers involved in suppressing the lethal thrombosis in our mouse model. The numbers of informative mice are being expanded in order to provide additional statistical power. We recently initiated a collaboration with statistical geneticists in order to more thoroughly analyze our existing data. In addition, we are continuing to create congenic mouse lines. We have very recently generated 27 N7 animals. Since these mice are expected to inherit few regions of DBA DNA, we should be one step closer toward eliminating the complex contributions of the other suppressor loci and reducing this problem to a simple Mendelian trait. Analysis of the genomes of the N7 $FV^{Q/Q}$ $TFPI^{+/-}$ and those of subsequent generations will hopefully provide the power to identify at least one DBA modifier.

It is possible that a modifier gene linked to the $TFPI^{-}$ allele could be responsible for the suppressor phenotype. Initiating a cross of N7 DBA:B6 $FV^{Q/Q}$ $TFPI^{+/-}$ x $FV^{Q/+}$ $TFPI^{+/-}$ on the B6 background and analyzing $FV^{Q/Q}$ $TFPI^{+/-}$ progeny for parent of origin of the $TFPI^{-}$ allele should provide evidence to support or refute this hypothesis.

In Chapter V, I described a genome-wide mutagenesis screen based on the $FV^{Q/Q}$ $TFPI^{+/-}$ lethal phenotype. As proof of principle, we demonstrated that a 50 percent

reduction of $TF^{+/-}$ is capable of suppressing the $FV^{Q/Q}$ $TFPI^{+/-}$ lethal phenotype. This suggests that mutations in TF are one of a subset of mutants that should emerge from our screen. We screened nearly 7000 G1 mice and ultimately identified 13 of 92 G1's that were able to transmit a putative suppressor mutation to one or more $FV^{Q/Q}$ $TFPI^{+/-}$ G2 offspring. This study demonstrates the feasibility of our sensitized approach in the identification of dominant suppressors of the $FV^{Q/Q}$ $TFPI^{+/-}$ lethal phenotype and suggests that there are ~10-20 thrombosis modifier loci for this phenotype. Since many ENU mutants result in a loss of function, an approximately 50 percent reduction in expression levels in mutants isolated in this dominant screen should identify strong candidates for human studies, since variation of this magnitude is likely a common occurrence in humans.

Much additional work is required to elucidate the ENU suppressor mutants in this study. Since mutations in the TF gene are one class of modifiers that should emerge from this screen, I have been sequencing the entire TF coding region for each of the 92 live G1 progeny identified in this screen. Thus far, no TF mutants have been identified. However, I have not yet rigorously analyzed sequence data from the majority of the mice.

We have initiated an outcross/backcross in order to identify the genetic location of each of the 13 suppressor mutations. Difficulties have arisen during positional cloning. We have been using $FV^{Q/Q}$ backcrossed onto 129S1/SVIMJ as the mapping strain. These mice are difficult to obtain, owing to an approximately 30 percent strain background lethality[16]. In some instances, genetically mixed 129S1/SVIMJ:B6 mapping progeny were bred back to B6 $FV^{Q/Q}$ mice to generate additional informative mice for mapping. We have sought to utilize additional backgrounds for mapping, however the strains

chosen thus far are $FV^{Q/Q}$ $TFPI^{+/-}$ viable. Chapter IV. Despite these problems, pedigrees of informative mice from seven of the 13 progeny tested G1 mice have been generated. These pedigrees will be subjected to whole genome scanning and analysis. These analyses could be facilitated by the recent technological advances in genome sequencing and the identification of millions of SNPs from inbred mouse strains[120].

Additional project difficulties arose upon moving our mouse colony from the Medical Science Research Building to the Life Sciences Institute. We noticed a sharp decline in the fecundity of our mouse colony during the months of October/November that persisted until April. When the same scenario developed the following year, we carefully investigated the internal environment of our ventilated cages and found the humidity to be as low as ten percent. The recommended humidity for mice is approximately 50 percent. This was in direct contrast to the 50 percent reading as measured in the exit air of the vivarium system. Once alerted, the building engineers found a faulty valve in the vivarium system that was improperly regulating the humidity. This malfunctioning valve was a major setback for the project as it affected breeding in the winter months of nearly three years.

In chapter II, I examined the mechanisms by which inheritance of FVL exerts its pro-thrombotic effects using *in vivo* mouse models. APC resistance was originally thought to be the major mechanism by which FVL exerts its prothrombotic effect[233]. Since the discovery that wildtype FV serves as an essential cofactor for the APC-mediated degradation of FVIIIa[65], the relative contributions of each of these mechanisms to thrombosis risk has been unclear. The studies I performed demonstrated a critical role for the APC cofactor function of wildtype FV *in vivo*. In the absence

ofwildtype FV cofactor activity, mice hemizygous for FVL display a more severe thrombotic phenotype when compared to FVL heterozygotes. The cofactor activity is greatly diminished but not absent in the factor V Leiden molecule. This was demonstrated by the increased thrombotic severity observed in FVL hemizygotes compared to homozygotes. This wild type FV function can be provided by small amounts of FV derived from either the platelet or liver. Taken together, these findings identify the critical mechanism for this enhanced thrombosis as loss of a wild-type-specific activity, rather than the increased level of APC resistance and suggest an increased thrombotic risk of FVL carriers with reduced wild type FV levels.

These results have important implications for the role of FV in the regulation of hemostatic balance and suggest novel targets for therapeutic intervention. Recently, in collaboration with Alnylam Pharmaceuticals, we developed a panel of RNAi's specifically targeting either the wildtype or the $FV^{Q/Q}$ allele. I am planning to use these RNAi molecules in $FV^{Q/+}$, $FV^{Q/Q}$ and $FV^{Q/-}$ mice to observe the thrombotic effects of these compounds in an animal model. We hypothesize that targeting the wildtype FV mRNA would lead to an increase in thrombosis, as would targeting FVL in either the hemi or homozygous state, while targeting FVL in FVL heterozygotes would lead to reduced thrombosis.

Chapter III describes the genetic characterization of a runted phenotype named *sml* that we observed while phenotyping gene targeted mice deficient in *SerpinB2*. We initially attributed this growth abnormality to deficiency of *SerpinB2*. However, analysis of *SerpinB2* deficient mice derived from two additional independent ES cell clones revealed no growth abnormalities. The observation of recombination between *sml* and

SerpinB2 deficiency led us to positionally clone the responsible locus. By candidate gene sequencing we discovered a nonsense mutation in *Irs1*. From this study, we concluded that new mutations arising during ES cell culture are likely to be a frequent but underappreciated occurrence. It is important to consider that mutations linked to a targeted allele could result in incorrect assignment of phenotype and may account for a subset of markedly discordant results for experiments independently targeting the same gene[13, 183, 234].

Interesting questions have arisen from this study which may be addressed in the future. Analysis of the *Irs1* deficient mice demonstrated deviations from previously reported targeted *Irs1* knockouts. We observed a marked increase in lethality in our *Irs1* deficient mice backcrossed greater than 6 generations into the C57BL/6J background, with less than two percent of *Irs1* deficient mice surviving to weaning. Recently, Selman et al. reported that *Irs1* deficient female mice have *increased* lifespan, on the "C57" genetic background[215]. These dramatically disparate phenotypes could be attributed to background strain differences as the precise "C57" strains used in the Selmon study are not detailed[215]. Alternatively, the *Irs1* targeting construct could have altered the expression of neighboring genes in the mice used in the Selmon study. Identifying either of these mechanisms as the cause of the discrepant phenotypes between our *Irs1* point mutants and those used in the Selmon study could result in an interesting scientific contribution.

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