Journal of Thrombosis and Haemostasis, **3**: 1561–1568

REVIEW ARTICLE

Identifying novel genetic determinants of hemostatic balance

D. GINSBURG

Departments of Internal Medicine and Human Genetics, Howard Hughes Medical Institute, University of Michigan, Life Sciences Institute, Ann Arbor, MI, USA

To cite this article: Ginsburg D. Identifying novel genetic determinants of hemostatic balance. *J Thromb Haemost* 2005; **3**: 1561–8.

Summary. Incomplete penetrance and variable expressivity confound the diagnosis and therapy of most inherited thrombotic and hemorrhagic disorders. For many of these diseases, some or most of this variability is determined by genetic modifiers distinct from the primary disease gene itself. Clues toward identifying such modifier genes may come from studying rare Mendelian disorders of hemostasis. Examples include identification of the cause of combined factor V and VIII deficiency as mutations in the ER Golgi intermediate compartment proteins LMAN1 and MCFD2. These proteins form a cargo receptor that facilitates the transport of factors V and VIII, and presumably other proteins, from the ER to the Golgi. A similar positional cloning approach identified *ADAMTS-13* as the gene responsible for familial TTP. Along with the work of many other groups, these findings identified VWF proteolysis by ADAMTS-13 as a key regulatory pathway for hemostasis. Recent advances in mouse genetics also provide powerful tools for the identification of novel genes contributing to hemostatic balance. Genetic studies of inbred mouse lines with unusually high and unusually low plasma VWF levels identified polymorphic variation in the expression of a glycosyltransferase gene, *Galgt2*, as an important determinant of plasma VWF levels in the mouse. Ongoing studies in mice genetically engineered to carry the factor V Leiden mutation may similarly identify novel genes contributing to thrombosis risk in humans.

Keywords: coagulation protein disorders, factor V, factor VIII, hemostasis, genetic model, von Willebrand factor.

Introduction

Hemostasis is a relatively recent evolutionary innovation, first arising in vertebrates coincident with the appearance of a high pressure, closed circulatory system [1,2]. The latter introduced the need for a rapidly responding and tightly regulated hemostatic system. In an invertebrate, sublethal injury to the exoskeleton is probably uncommon, and can be repaired with a slow time course. In contrast, in the setting of the vertebrate circulatory system, even a minor injury could result in exsanguination in a matter of minutes, requiring a rapid and directed response. However, if this response is too robust, the outcome would be lethal occlusion of the vascular tree. The end result of these complex evolutionary forces is the highly integrated hemostatic system, involving many genes and interconnected regulatory pathways. A tip of this delicate balance in the wrong direction can result in pathologic bleeding, whereas a tilt in the other direction may lead to pathologic thrombosis.

Perhaps more than any other biologic system, our current understanding of hemostasis is derived in large part from the study of human genetic diseases. Genetic deficiencies in nearly every component of the clotting system have been identified either in humans, or recently in mice, or both [3,4]. Although we have a fairly complete catalog of such genetic defects on the bleeding side of human disease, there are likely to be a number of genes contributing to thrombotic disorders that remain to be identified.

Even among the well defined, single gene disorders of hemostasis and thrombosis, considerable variability remains to be explained. Patients with such an inherited bleeding or thrombotic disease presumably carry a mutation in a major known disease gene. Although this mutation may be the primary cause of the disease, the clinical manifestations are often still highly variable. For many genetic diseases, not everyone inheriting the disease-causing mutation manifests clinical symptoms, a phenomenon referred to by geneticists as *incomplete penetrance*. For example, in families with typical autosomal dominant type 1 von Willebrand disease (VWD), penetrance is generally incomplete (50% or lower) [5]. In addition, among those individuals in the VWD family who do express bleeding symptoms, the severity of this bleeding can also be highly variable, a phenomenon referred to as *variable expressivity*.

Correspondence: David Ginsburg, Howard Hughes Medical Institute, Departments of Internal Medicine and Human Genetics, University of Michigan, Life Sciences Institute, Room 5028, 210 Washtenaw Avenue, Ann Arbor, MI 48109-2216, USA.
Tel.: +1 734 647 4808; fax: +1 734 936 2888; e-mail: ginsburg@umich.edu

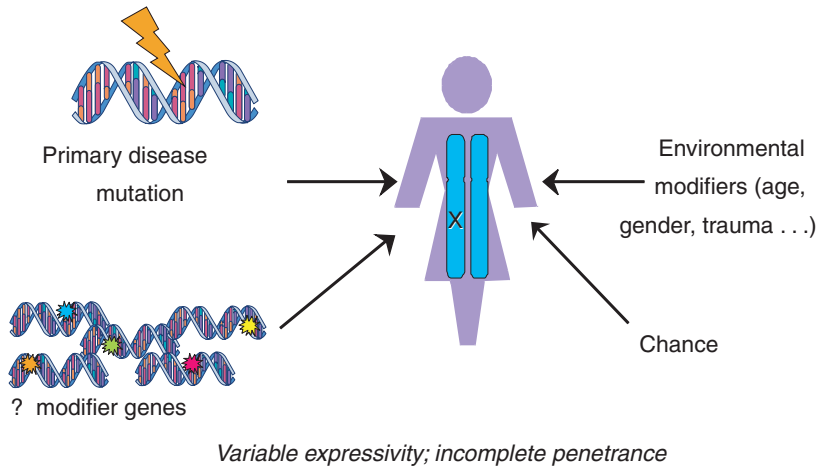

Fig. 1. Factors affecting severity of inherited bleeding and thrombotic diseases.

What are the causes for incomplete penetrance and variable expressivity? These factors are illustrated in Fig. 1. For most inherited bleeding and thrombotic disorders, there are obvious environmental factors that can alter disease severity. For example, age, gender, prior surgery and trauma are all major risk factors for thrombosis and bleeding. Finally, we must consider the actions of other genes that may modify the effect of the primary disease causing mutation [6]. It is the identity of these modifier genes that is the major focus of this review and a primary goal of our laboratory's research program. This problem resembles the prominent challenge to the field of human genetics of uncovering the genes contributing to complex, multi-gene disorders such as hypertension, diabetes, and cardiovascular disease [7].

The most direct approach to identify modifier genes is to study genetic linkage or association in large collections of human families or populations. These strategies have been applied to a number of complex human disorders with only limited success to date [7]. This manuscript will discuss two other approaches. The first is based on the hypothesis that clues toward identifying common modifier genes may come from studying rare Mendelian disorders of hemostasis. The second group of studies takes advantage of the power of mouse genetics. It is our hope that genes identified through genetic studies in the mouse, as well as studies of rare Mendelian disorders, may provide candidates to later be tested directly in humans through whole genome linkage and association studies.

Clues from rare Mendelian hemostatic disorders

The power of human genetics, particularly bolstered by the dramatic recent advances provided by the human genome project, now make it possible in principle to directly identify the underlying gene for nearly any simple, single gene disorder. The rate-limiting step is usually the identification of sufficient human genetic material in the form of well characterized families and pedigrees.

Combined deficiency of factor V and factor VIII

An example of this approach comes from the study of a rare inherited bleeding disorder, combined deficiency of factor V and factor VIII (F5F8D). These patients exhibit a remarkable coordinate reduction in the plasma level of clotting factors V and VIII. Although originally suspected to be due to deficiency of protein C inhibitor [8], this explanation was later disproved [9] and the cause of this disorder remained a mystery until a direct genetic approach became possible, as a result of the human genome project. It is remarkable to note that F5F8D patients exhibit very similar reductions in both factor V and factor VIII, generally to the range of approximately 10% of normal, despite expression of these two proteins in different tissues and to markedly different levels (plasma levels of factor V are approximately 100-fold higher than those of factor VIII). The key to this puzzle that made a genetic approach possible was the pioneering work of Seligsohn *et al.* [10], who, along with other groups, had carefully characterized these families. Using a genetic approach called homozygosity mapping to study these pedigrees, we were able to map the gene responsible for F5F8D in most families to the long arm of chromosome 9 [11], subsequently confirmed by another group [12]. Further genetic studies eventually identified the responsible gene as *ERGIC53*, now referred to as *LMAN1* [13]. This protein was previously identified as a marker of the intermediate compartment between the ER and Golgi, although of unknown function. The identification of mutations in *LMAN1* as a cause of F5F8D suggested that this gene plays a unique role in the transport of proteins between the ER and Golgi.

However, mutations in *LMAN1* only explain about three-fourth of patients with F5F8D, with the remaining subgroup demonstrating normal levels of LMAN1 and no mutations in the *LMAN1* gene [14,15]. This observation led to further genetic analysis in the remaining subgroup of patients, with identification of the responsible gene as *MCFD2* [16]. Mutations in *LMAN*1 or *MCFD2* appear to explain all or

nearly all patients with F5F8D. MCFD2 is an approximately 16 kDa protein that is retained within the intermediate compartment between the ER and Golgi via its interaction with LMAN1. These two proteins form a complex that appears to serve as cargo-receptor for the selective facilitated transport of a specific subset of secreted proteins, including factor V and factor VIII. These findings are a striking example of how studies of human disease can provide basic insight into fundamental biologic problems. In addition, this work identifies a novel biologic pathway that impacts on hemostatic balance and is a potential source of modifier gene effects for hemostatic diseases. One could imagine that polymorphic variation within components of this system could affect factor V and factor VIII levels and thereby alter bleeding or thrombotic risk. This hypothesis remains to be tested.

ADAMTS-13 and TTP

Using a similar positional cloning strategy, we recently identified *ADAMTS-13* as the gene responsible for familial TTP [17]. This approach was again based on the study of rare patients with this inherited disorder. Independently, three other groups identified the same protein as the von Willebrand factor (VWF) cleaving protease by purification of this activity from plasma, followed by protein sequence analysis [18–20]. These findings identified a new pathway for the regulation of VWF and platelet function and a new candidate for a genetic modifier potentially affecting overall hemostatic balance and the risk of bleeding and/or thrombosis. A number of common polymorphisms in the ADAMTS-13 gene have been identified [17], including a very common mutation in the Japanese population resulting in partial loss of function [21]. These polymorphisms are thus candidates to modify the risk for a variety of thrombotic and vascular diseases, although this hypothesis also remains to be tested.

Genetic approaches in the mouse

Our laboratory is taking two complementary approaches in the laboratory mouse in an effort to identify novel genetic loci, which may serve as modifiers of hemostatic balance in humans and thus, as modifier genes for inherited bleeding and clotting diseases. Recent advances in mouse and human genetics have provided powerful new tools to address complex genetic disorders and to identify modifier genes [6]. Our first approach focuses on a mouse model for the most common inherited bleeding disorder in humans, VWD. The second approach centers on a mouse model for the most common inherited genetic risk factor for thrombosis in human, factor V Leiden.

It is important to note that these two approaches could potentially be quite complementary. That is genes that modify plasma VWF levels, in addition to altering risk for bleeding in VWD patients and hemophilics, might also alter thrombotic risk in patients with thrombophilia mutations. Similarly, mutations identified in the factor V Leiden screen could also alter the balance in a clinically significant way in patients with inherited bleeding disorders. Indeed, factor V Leiden has already been identified as a potential modifier of bleeding severity in hemophilia [22,23].

Identifying modifier genes for VWF in the mouse

To study genetic factors that could potentially modify the penetrance and expressivity of VWD, we are focusing on the control of plasma VWF levels in the mouse. VWF level is known to have a large genetic component in humans, with heritability estimates ranging from ~60–70% from twin studies [24] to as low as 25% from linkage studies in large pedigrees [25,26]. ABO blood group is known to be a major genetic modifier of plasma VWF level and is perhaps one of the best defined examples of a human modifier gene. Individuals with blood type O, generally have VWF levels that are 25%–30% lower than individuals with any of the non-O blood types [27]. ABO blood group appears to account for approximately 30% of the genetic variation in VWF level [24,28] with the modifier genes responsible for the remaining 70% still to be identified. In addition to being important determinants of incomplete penetrance and variable expressivity in VWD and hemophilia, variation in VWF level, with its secondary effect on FVIII, may also represent an important risk factor for thrombosis. Recent studies suggest that elevated VWF levels may account for as much as 16% of the attributable risk for thrombosis (compared to 25% for factor V Leiden) [29].

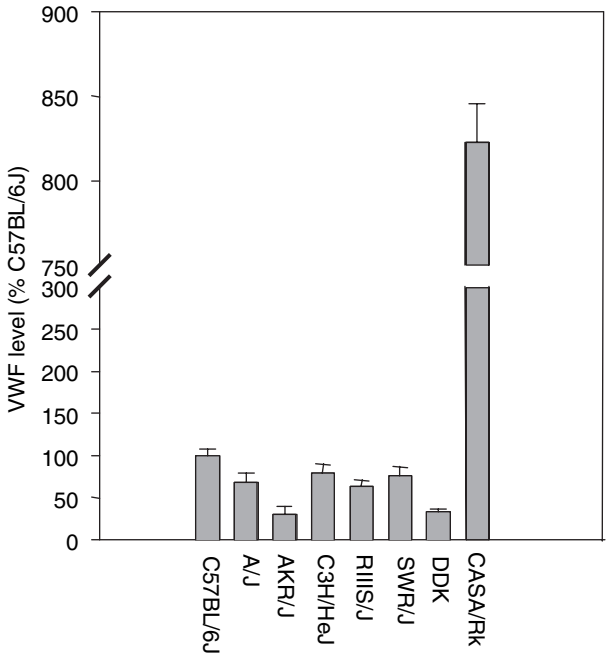

Fig. 2. Variation in plasma VWF level among inbred mouse strains. VWF levels were determined by ELISA as previously described [30,31,33].

A survey of a number of different inbred mouse strains demonstrates a wide variation in the level of plasma VWF, as shown in Fig. 2. This variation is at least as large as that observed in human populations, where VWF levels can vary over as much as fivefold (50%–250%). In previous work, we have taken advantage of the relatively high levels of plasma VWF in several wild-derived mouse strains, including CASA/Rk and PWK/Ph [30,31]. The levels of VWF in these mice are five- to 10-fold higher than in C57BL/6J and up to 20-fold higher than in the mouse strain RIIIS/J. The latter strain had been originally reported by Sweeney *et al*. [32] as a mouse model for type 1 VWD, with a VWF level of approximately 50% compared with C57BL/6J. We previously mapped the major gene responsible for low plasma VWF in the RIIIS/J strain to mouse chromosome 11 [30], defining the corresponding locus as *Mvwf* (for modifier of VWF). Subsequent positional cloning efforts [33] identified *Mvwf* as a mutation in the glycosyltransferase gene, *Galgt2*. The remarkable molecular mechanism underlying *Mvwf* was shown to be a tissue-specific switch in *Galgt2* expression pattern. In the C57BL/6J strain and most other inbred mice, VWF expression is restricted to the GI epithelial cell. However, in RIIIS/J and related strains, *Galgt2* expression is turned off in the epithelium and turned on in the entire endothelial cell vascular bed. As the endothelial cell is also the site of VWF protein expression, the result of GALGT2 and VWF expression in the same cell is a subtle alteration in the carbohydrate structure on VWF, which leads to rapidly accelerated clearance [33].

These findings suggest that polymorphic variation in carbohydrate processing could be an important general mechanism for the genetic modification of plasma levels for a whole host of plasma proteins, including a number of clotting factors. Genes involved in the synthesis or processing of glycoconjugates are estimated to comprise up to 1% of the human genome and many are highly polymorphic [34]. One obvious example of such polymorphic variation can be found in the glycosyltransferases responsible for the ABO blood group polymorphism in humans. Indeed, the ABO structure is known to be present on plasma VWF [35] and it is likely that the effect of ABO blood type on plasma VWF level results from a very similar mechanism to that identified for the *Mvwf* mutation in mice. Specifically, there is evidence to suggest that the type ABO sugar structures on VWF may subtly alter VWF clearance, accounting for the reduced levels in individuals with blood type O [36]. Recent studies in our laboratory demonstrate that a number of other inbred mouse strains carry this same *Mvwf* mutation, presumably derived from a common founder allele in the mouse population. Current efforts are also focused on identifying the specific DNA sequence change on the *Mvwf* allele responsible for this remarkable switch in tissue-specific gene transcription program.

Using a similar approach, we have now performed genetic crosses for several other mouse strains and in preliminary studies have mapped at least three other genes within the mouse genome that appear to modify plasma VWF levels. It is our hope that the identification of these genes may uncover other important candidates as potential modifiers of plasma VWF level in humans.

Whole genome ENU mutagenesis to screen for modifiers of factor V Leiden

Factor V Leiden was discovered through direct genetic studies in humans [37], beginning with the pioneering work of Dahlbäck and co-workers [38,39], who identified APC resistance and demonstrated that it is an autosomal dominant trait. Subsequent work by a number of groups succeeded in identifying a single nucleotide change resulting in the amino acid substitution R506Q in factor V as the most common known genetic predisposition to thrombosis in humans [40]. The factor V Leiden mutation appears to have arisen as a single mutation event in human evolutionary history, dated approximately 20 000–30 000 years ago [41]. This mutation is generally only found in Europe and the USA, where the population prevalence ranges from 2% to 10%. Despite several studies suggesting detrimental effects of factor V Leiden, including the obvious known increase in thrombosis predisposition, its high allele frequency suggests at least some form of balancing positive selection. A recent report from Kerlin *et al*. [42] found a surprising resistance of factor V Leiden mice (described below) to endotoxin-induced mortality. Extrapolating these results to humans, they also demonstrated a remarkable protection from sepsis-related mortality in humans carrying factor V Leiden. These findings suggest that the positive selection for factor V Leiden in humans may lie in protection from a specific infectious pathogen.

In order to study genetic factors modifying factor V Leiden thrombosis predisposition, we generated a mouse model by a 'knock-in' of the factor V Leiden mutation into the endogenous factor V gene at the orthologous amino acid position (R504Q). The resulting mice exhibit a mild prothrombotic predisposition, very similar to the human phenotype [43]. A rare, spontaneous thrombus was observed in heterozygous mice, while homozygous mice showed a more severe thrombosis, which was variably affected by mouse genetic background. The factor V Leiden mouse also provides a valuable tool for testing interactions with other clotting factor mutations. Genetic cross of the factor V Leiden mice with mice carrying a knockout in the protein Z gene showed a surprising synthetic lethal interaction [44]. Protein Z-deficient mice exhibit normal survival, as do factor V Leiden mice. However, mice carrying both defects showed a uniformly lethal thrombotic phenotype early in life. These findings provided the first direct evidence for the function of protein Z as a regulator of hemostasis.

Similarly, a dramatic synthetic lethal interaction was observed in mice carrying a knockout of the tissue factor pathway inhibitor gene (TFPI) [45]. Although complete deficiency of TFPI in the mouse results in an early embryonic lethality [46], mice heterozygous for the TFPI knockout allele that are expressing 50% of the normal level of TFPI, exhibit

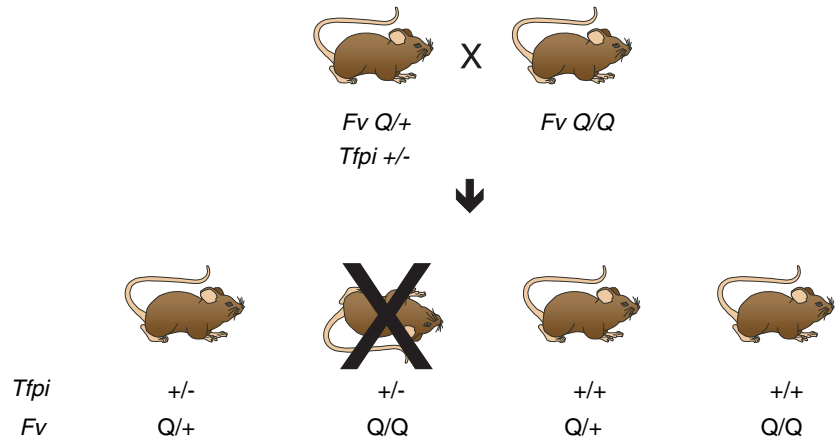

Fig. 3. A cross between mice carrying the factor V Leiden mutation and a knockout in the Tfpi gene (adapted from data in Ref. [45]).

normal survival. Again, a remarkable synthetic lethal phenotype was observed between mice carrying the factor V Leiden mutation and those carrying the TFPI mutation [45]. The key genetic cross is shown in Fig. 3. Mice homozygous for the factor V Leiden mutation are essentially normal, as are mice that are doubly heterozygous for the factor V Leiden mutation (*FVQ*/+) and the TFPI mutation (*Tfpi*+/−). In the bottom part of the figure, the four possible genotypes in the offspring from this cross are seen, with the expected Mendelian frequencies of 25% for each genotype. To our surprise, only a rare mouse survived to weaning carrying a combination of *FVQ*/*Q* and *Tfpi*+/−, whereas close to expected numbers were observed for all other genotypes. These data demonstrate a synthetic lethal interaction between these two genetic defects. This result was elegantly predicted *in vitro* by van't Veer *et al.* [47], whose work provides a biochemical explanation for the *in vivo* interaction between factor V Leiden and TFPI. These authors observed in an *in vitro* clotting assay a marked increase in the dependence of thrombin generation on TFPI concentration when factor V Leiden was substituted for wild-type factor V. Thus, our findings in genetically engineered mice represent a satisfying *in vivo* confirmation of these *in vitro* predictions.

We are currently using this observation as the basis for a large-scale genetic screening experiment in the mouse, as depicted in Fig. 4. For this study, the same genetic cross as shown in Fig. 3 is performed, with the introduction of random mutations by the chemical agent ENU (*N*-ethyl-*N*-nitrosourea). Random chemical mutagenesis has been used widely as an experimental tool in lower organisms and is now being

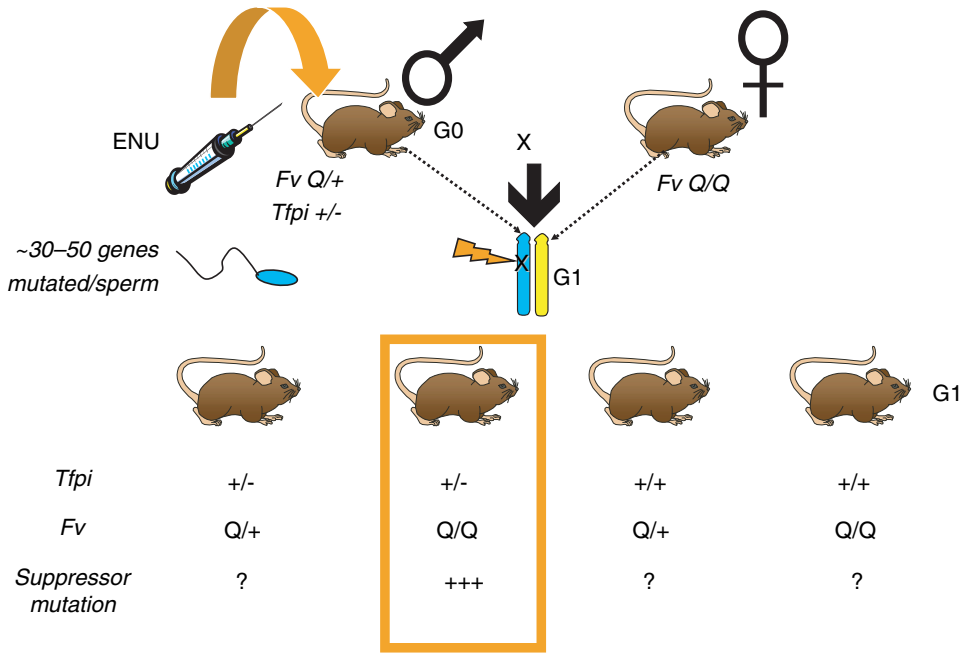

Fig. 4. ENU mutagenesis strategy for sensitized suppressor screen.

applied by a number of groups to screen for specific mutations in the experimental mouse. Such genetic screens can theoretically detect a mutation in any gene, limited only by the ingenuity of the screening approach. One of the early successes of this method was the isolation of the first mammalian clock gene by screening of mutagenized mice for alterations in circadian rhythm [48].

Unfortunately, no highly sensitive and specific and yet rapid and inexpensive test is currently available to screen for thrombosis predisposition or bleeding in the mouse (or in humans for that matter). The key innovation in our approach is to use a specific 'sensitized' genetic background for the mutagenesis study, rather than performing it in wild-type mice. A similar approach was recently used for genes affecting thrombopoiesis [49]. Our 'sensitized' genetic background takes advantage of the synthetic lethal phenotype described in Fig. 3. In this screen, the same genetic cross is performed, but now with the introduction of random mutations throughout the genome, looking for mice that survive the lethal genetic combination as a result of one of these introduced mutations. Thus, this sensitized suppressor screen looks for gene mutations that suppress the lethal synthetic phenotype.

ENU treatment using standard protocols is estimated to produce multiple mutations, resulting in the disruption of, on average, 30–50 different genes in each sperm produced by the ENU-treated male [50]. The G1 offspring mice from this cross will thus carry an inactivated copy for 30–50 genes, with a normal copy from the mother. The screen is designed as a dominant screen and will only detect those mutations where a loss of only one of two copies of the gene results in a sufficient tip in the hemostatic balance to rescue the lethal thrombotic phenotype. It is these very mutations that we hypothesize will identify the genes of greatest interest as potential candidates for thrombosis modifiers in humans. Common polymorphisms within these genes in the human population may by themselves have little effect, but when combined with a primary prothrombotic or hemorrhagic disease mutation, could result in a major shift in overall hemostatic balance. In preliminary studies, a number of such candidate suppressor gene mutations have been identified and current efforts are focused on mapping and eventually positionally cloning these genes. The mutations identified in this way may provide important insight into the overall regulation of hemostasis balance, as well as identifying key candidates to test as modifier genes in humans.

What classes and types of genes might we anticipate to identify in this way? First, we expect that we may identify known clotting factors for which the overall level of expression is particularly critical, such as variation in TFPI level in the setting of factor V Leiden, as described above. Again, these genes would be key candidates to explore as potential modifier genes in humans. A second class of genes might involve regulators of pathways entirely outside of our conventional view of 'hemostasis'. Examples here would include the ER to Golgi transport pathway identified through the studies of F5F8D or the metalloprotease ADAMTS-13 identified through genetic studies of TTP. Any such novel pathways would provide new areas for biologic investigation, as well as novel potential targets for anticoagulant drug development and new candidates for genetic modifier genes in humans.

Acknowledgements

Supported by National Institutes of Health Grants No. Po1HL57346 and HL39639. DG is a Howard Hughes Medical Institute Investigator.

References

1 Jiang Y, Doolittle RF. The evolution of vertebrate blood coagulation as viewed from a comparison of puffer fish and sea squirt genomes. *Proc Natl Acad Sci USA* 2003; **100**: 7527–32.
2 Davidson CJ, Tuddenham EG, McVey JH. 450 million years of hemostasis. *J Thromb Haemost* 2003; **1**: 1487–94.
3 Ginsburg D. Hemophilia and other disorders of hemostasis. In: Rimoin DL, Connor JM, Pyeritz RE, eds. *Emery and Rimoin's Principles and Practice of Medical Genetics*, Vol. II., 4th edn. Churchill Livingstone, New York, 2002: 1926–58.
4 Ginsburg D. Disorders of the fibrinolytic system. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn. McGraw Hill, New York, 2001: 4505–16.
5 Nichols WC, Cooney KA, Ginsburg D, Ruggeri ZM. von Willebrand disease. In: Loscalzo J, Schafer AI, eds. *Thrombosis and Hemorrhage*, 3th edn. Lippincott Williams & Wilkins, Philadelphia, 2003: 539–59.
6 Nadeau JH. Modifier genes in mice and humans. *Nat Rev Genet* 2001; **2**: 165–74.
7 Carlson CS, Eberle MA, Kruglyak L, Nickerson DA. Mapping complex disease loci in whole-genome association studies. *Nature* 2004; **429**: 446–52.
8 Marlar RA, Griffin JH. Deficiency of protein C inhibitor in combined factor V/VIII deficiency disease. *J Clin Invest* 1980; **66**: 1186–9.
9 Gardiner JE, Griffin JH. Studies on human protein C inhibitor in normal and Factor V/VIII deficient plasmas. *Thromb Res* 1984; **36**: 197–203.
10 Seligsohn U, Zivelin A, Zwang E. Combined factor V and factor VIII deficiency among non-Ashkenazi Jews. *N Engl J Med* 1982; **307**: 1191–5.
11 Nichols WC, Seligsohn U, Zivelin A, Terry VH, Arnold ND, Siemieniak DR, Kaufman RJ, Ginsburg D. Linkage of combined factors V and VIII deficiency to chromosome 18q by homozygosity mapping. *J Clin Invest* 1997; **99**: 596–601.
12 Neerman-Arbez M, Antonarakis SE, Blouin J-L, Akhtari M, Afshar Y, Tuddenham EGD. The locus for combined factor V-factor VIII deficiency (F5F8D) maps to 18q21, between D18S849 and D18S1103. *Am J Hum Genet* 1997; **61**: 143–50.
13 Nichols WC, Seligsohn U, Zivelin A, Terry VH, Hertel CE, Wheatley MA, Moussalli MJ, Hauri H-P, Ciavarella N, Kaufman RJ, Ginsburg D. Mutations in the ER-Golgi intermediate compartment protein ERGIC-53 cause combined deficiency of coagulation factors V and VIII. *Cell* 1998; **93**: 61–70.
14 Neerman-Arbez M, Johnson KM, Morris MA, McVey JH, Peyvandi F, Nichols WC, Ginsburg D, Rossier C, Antonarakis SE, Tuddenham EGD. Molecular analysis of the ERGIC-53 gene in 35 families with combined factor V-factor VIII deficiency. *Blood* 1999; **93**: 2253–60.
15 Nichols WC, Terry VH, Wheatley MA, Yang A, Zivelin A, Ciavarella N, Stefanile C, Matsushita T, Saito H, de Bosch NB, Ruiz-Saez A, Torres A, Thompson AR, Feinstein DI, White GC, Negrier C, Vinciguerra C, Aktan M, Kaufman RJ, Ginsburg D, Seligsohn U. ERGIC-53 gene structure and mutation analysis in 19 combined factors V and VIII deficiency families. *Blood* 1999; **93**: 2261–6.

16 Zhang B, Cunningham MA, Nichols WC, Bernat JA, Seligsohn U, Pipe SW, McVey JH, Schulte-Overberg U, de Bosch N, Ruiz-Saez A, White GC, Tuddenham EGD, Kaufman RJ, Ginsburg D. Bleeding due to disruption of a cargo-specific ER to Golgi transport complex. *Nat Genet* 2003; **34**: 220–5.
17 Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, Yang AY, Siemieniak DR, Stark KR, Gruppo R, Sarode R, Shurin SB, Chandrasekaran V, Stabler SP, Sabio H, Bouhassira EE, Upshaw JD Jr, Ginsburg D, Tsai H-M. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature* 2001; **413**: 488–94.
18 Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood* 2001; **98**: 1662–6.
19 Gerritsen HE, Robles R, Lammle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. *Blood* 2001; **98**: 1654–61.
20 Soejima K, Mimura N, Hirashima M, Maeda H, Hamamoto T, Nakagaki T, Nozaki C. A novel human metalloprotease synthesized in the liver and secreted into the blood: Possibly, the von Willebrand factor-cleaving protease? *J Biochem (Tokyo)* 2001; **130**: 475–80.
21 Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, Tamai H, Konno M, Kamide K, Kawano Y, Miyata T, Fujimura Y. Mutations and common polymorphisms in ADAMTS-13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci USA* 2002; **99**: 11902–7.
22 Nichols WC, Amano K, Cacheris PM, Figueiredo MS, Michaelides K, Schwaab R, Hoyer LW, Kaufman RJ, Ginsburg D. Moderation of hemophilia A phenotype by the factor V R506Q mutation. *Blood* 1996; **88**: 1183–7.
23 Lee DH, Walker IR, Teitel J, Poon MC, Ritchie B, Akabutu J, Sinclair GD, Pai M, Wu JWY, Reddy S, Carter C, Growe G, Lillicrap D, Lam M, Blajchman MA. Effect of the factor V Leiden mutation on the clinical expression of severe hemophilia A. *Thrombosis & Haemostasis* 2000; **83**: 387–91.
24 Orstavik KH, Magnus P, Reisner H, Berg K, Graham JB, Nance W. Factor VIII and factor IX in a twin population. Evidence for a major effect of ABO locus on factor VIII level. *Am J Hum Genet* 1985; **37**: 89–101.
25 Vossen CY, Hasstedt SJ, Rosendaal FR, Callas PW, Bauer KA, Broze GJ Jr, Hoogendoorn H, Long GL, Scott BT, Bovill EG. Heritability of plasma concentrations of clotting factors and measures of a prethrombotic state in a protein C-deficient family. *J Thromb Haemost* 2004; **2**: 242–7.
26 Souto JC, Almasy L, Borrell M, Gari M, Martinez E, Mateo J, Stone WH, Blangero J, Fontcuberta J. Genetic determinants of hemostasis phenotypes in Spanish families. *Circulation* 2000; **101**: 1546–51.
27 Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ, Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand Disease. *Blood* 1987; **69**: 1691–5.
28 Souto JC, Almasy L, Soria JM, Buil A, Stone W, Lathrop M, Blangero J, Fontcuberta J. Genome-wide linkage analysis of von Willebrand factor plasma levels: results from the GAIT project. *Thromb Haemost* 2003; **89**: 468–74.
29 Kamphuisen PW, Eikenboom JC, Bertina RM. Elevated factor VIII levels and the risk of thrombosis. *Arterioscler Thromb Vasc Biol* 2001; **21**: 731–8.
30 Mohlke KL, Nichols WC, Westrick RJ, Novak EK, Cooney KA, Swank RT, Ginsburg D. A novel modifier gene for plasma von Willebrand factor level maps to distal mouse chromosome 11. *Proc Natl Acad Sci USA* 1996; **93**: 15352–7.
31 Nichols WC, Cooney KA, Mohlke KL, Ballew JD, Yang A, Bruck ME, Reddington M, Novak EK, Swank RT, Ginsburg D. Willebrand disease in the RIIIS/J mouse is caused by a defect outside of the von Willebrand factor gene. *Blood* 1994; **83**: 3225–31.
32 Sweeney JD, Novak EK, Reddington M, Takeuchi KH, Swank RT. The RIIIS/J inbred mouse strain as a model for von Willebrand disease. *Blood* 1990; **76**: 2258–65.
33 Mohlke KL, Purkayastha AA, Westrick RJ, Smith PL, Petryniak B, Lowe JB, Ginsburg D. *Mvwf*, a dominant modifier of murine von Willebrand factor, results from altered lineage-specific expression of a glycosyltransferase. *Cell* 1999; **96**: 111–20.
34 Schachter H. The clinical relevance of glycobiology. *J Clin Invest* 2001; **108**: 1579–82.
35 Matsui T, Shimoyama T, Matsumoto M, Fujimura Y, Takemoto Y, Sako M, Hamako J, Titani K. ABO blood group antigens on human plasma von Willebrand factor after ABO-mismatched bone marrow transplantation. *Blood* 1999; **94**: 2895–900.
36 Vlot AJ, Mauser-Bunschoten EP, Zarkova AG, Haan E, Kruitwagen CLJJ, Sixma JJ, van den Berg HM. The half-life of infused factor VIII is shorter in hemophiliac patients with blood group O than in those with blood group A. *Thrombosis & Haemostasis* 2000; **83**: 65–9.
37 Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64–7.
38 Svensson PJ, Dahlbäck B. Resistance to activated protein C as a basis for venous thrombosis. *N Engl J Med* 1994; **330**: 517–22.
39 Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: prediction of a cofactor to activated protein C. *Proc Natl Acad Sci USA* 1993; **90**: 1004–8.
40 Price DT, Ridker PM. Factor V Leiden mutation and the risks for thromboembolic disease: a clinical perspective. *Ann Intern Med* 1997; **127**: 895–903.
41 Zivelin A, Griffin JH, Xu X, Pabinger I, Samama M, Conard J, Brenner B, Eldor A, Seligsohn U. A single genetic origin for a common Caucasian risk factor for venous thrombosis. *Blood* 1997; **89**: 397–402.
42 Kerlin BA, Yan SB, Isermann BH, Brandt JT, Sood R, Basson BR, Joyce DE, Weiler H, Dhainaut JF. Survival advantage associated with heterozygous factor V Leiden mutation in patients with severe sepsis and in mouse endotoxemia. *Blood* 2003; **102**: 3085–92.
43 Cui J, Eitzman DT, Westrick RJ, Christie PD, Xu ZJ, Yang AY, Purkayastha AA, Yang TL, Metz AL, Gallagher KP, Tyson JA, Rosenberg RD, Ginsburg D. Spontaneous thrombosis in mice carrying the factor V Leiden mutation. *Blood* 2000; **96**: 4222–6.
44 Yin Z-F, Huang Z-F, Cui J, Fiehler R, Lasky N, Ginsburg D, Broze GJ Jr. Prothrombotic phenotype of protein Z deficiency. *Proc Natl Acad Sci USA* 2000; **97**: 6734–8.
45 Eitzman DT, Westrick RJ, Bi X, Manning SL, Wilkinson JE, Broze GJ Jr, Ginsburg D. Lethal perinatal thrombosis in mice resulting from the interaction of tissue factor pathway inhibitor deficiency and factor V Leiden. *Circulation* 2002; **105**: 2139–42.
46 Huang Z-F, Higuchi D, Lasky N, Broze GJ Jr. Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. *Blood* 1997; **90**: 944–51.
47 van't Veer C, Kalafatis M, Bertina RM, Simioni P, Mann KG. Increased tissue factor-initiated prothrombin activation as a result of the Arg506 –> Gln mutation in factor VLEIDEN. *J Biol Chem* 1997; **272**: 20721–9.
48 Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science* 1994; **264**: 719–25.
49 Carpinelli MR, Hilton DJ, Metcalf D, Antonchuk JL, Hyland CD, Mifsud SL, Di Rago L, Hilton AA, Willson TA, Roberts AW, Ramsay RG, Nicola NA, Alexander WS. Suppressor screen in Mpl-/- mice: c-Myb mutation causes supraphysiological production of platelets in the absence of thrombopoietin signaling. *Proc Natl Acad Sci USA* 2004; **101**: 6553–8.

50 Hrabe de Angelis MH, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D, Marschall S, Heffner S, Pargent W, Wuensch K, Jung M, Reis A, Richter T, Alessandrini F, Jakob T, Fuchs E, Kolb H, Kremmer E, Schaeble K, Rollinski B, Roscher A, Peters C, Meitinger T, Strom T, Steckler T, Holsboer F, Klopstock T, Gekeler F, Schindewolf C, Jung T, Avraham K, Behrendt H, Ring J, Zimmer A, Schughart K, Pfeffer K, Wolf E, Balling R. Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat Genet* 2000; **25**: 444–7.