Journal of Thrombosis and Haemostasis, **5**: 329–335

ORIGINAL ARTICLE

Genetic regulation of plasma von Willebrand factor levels: quantitative trait loci analysis in a mouse model

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To cite this article: Lemmerhirt HL, Broman KW, Shavit JA, Ginsburg D. Genetic regulation of plasma von Willebrand factor levels: quantitative trait loci analysis in a mouse model. *J Thromb Haemost* 2007; **5**: 329–35.

Summary. *Background:* The genetic factors responsible for the wide variation in plasma von Willebrand factor (VWF) levels observed among individuals are largely unknown, although these genes are also likely to contribute to variability in the severity of von Willebrand disease (VWD) and other bleeding and thrombotic disorders. We have previously mapped two genes contributing to the regulation of plasma VWF levels in mice (*Mvwf1* on chromosome 11 and *Mvwf2* on chromosome 6). *Objective:* To identify additional quantitative trait loci (QTL) contributing to the genetic regulation of murine plasma VWF levels. *Methods:* To map genetic loci contributing to the > 7-fold difference in plasma VWF levels between two mouse strains (A/J and CASA/RkJ), high-density individual genotyping and R/qtl analyses were applied to a previously generated set of ~200 F2 mice obtained from an intercross of these two inbred lines. *Results:* Genomic loci for two additional candidate VWF modifier genes were identified: *Mvwf3* on chromosome 4 and *Mvwf4* on chromosome 13. These loci demonstrate primarily epistatic effects when co-inherited with two CASA/RkJ *Vwf* alleles, although *Mvwf4* may also exert a small, independent, additive effect. *Conclusions: Mvwf3* and *Mvwf4*, combined with the effect of *Mvwf2*, explain ~45% of the genetic variation in plasma VWF level among the A/J and CASA/RkJ strains. *Mvwf3* and *Mvwf4* exhibit homology of synteny to three human chromosomal segments (on chromosomes 1, 5 and 6) previously reported by the Genetic Analysis of Idiopathic Thrombophilia (GAIT) study, suggesting that orthologs of *Mvwf3* and *Mvwf4* may also encode important VWF modifier genes in humans.

Keywords: quantitative trait loci, *Mvwf* modifier, von Willebrand factor.

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Received 24 July 2006, accepted 10 November 2006

Introduction

In plasma, von Willebrand factor (VWF) mediates platelet adhesion at sites of vascular injury and serves as the carrier protein for factor VIII (FVIII) [1]. Plasma VWF level regulation is critical for the maintenance of hemostatic balance. Deficiencies in this blood coagulation protein can lead to von Willebrand disease (VWD), the most common inherited bleeding disorder in humans [1]. In contrast, elevated levels of VWF and FVIII correlate with an increase in thrombotic risk [2–4].

Although the risk of bleeding or thrombosis segregates with phenotypic extremes, plasma VWF levels are highly variable and present as a broad continuum within the general population, typically ranging between 50% and 200% of the population average among phenotypically normal individuals [1]. The true impact of this variability on disease risk can be difficult to determine, particularly when distinguishing between individuals with VWF levels in the lower range of 'normal' and the upper range of those seen in type 1 VWD (20–50% of the mean) [1]. None the less, the VWF level is determined in large part by genetic factors, with heritability ranging from 25–32% from pedigree analysis [5,6] to as high as 66–75% from twin studies [7,8]. A number of environmental factors also contribute to VWF levels, including age, stress and hormonal status [9].

The majority of VWD cases caused by VWF quantitative deficiency are also associated with mutations in the *VWF* gene [10], especially in severe VWF deficiency (type 3) [1] and the most penetrant forms of mild VWF deficiency (type 1) [11–16]. Several association studies suggest a similar relationship between genotype and phenotype in the general population, where up to 20% of plasma VWF level variability can be traced to the *VWF* gene itself [17–19]. *VWF* alone, however, is not sufficient to explain the heritability of plasma VWF levels. Cases of non-linkage to VWF level in both VWD and non-VWD families [14–16,20–22] as well as the characteristic reduced penetrance and variable expressivity observed among type 1 VWD patients, suggest a significant role for modifier genes in VWF level determination [9].

Aside from *VWF*, the ABO blood group is the most clearly established genetic modifier of circulating VWF levels in

humans, accounting for approximately 30% of heritable VWF level variation [7]. Even with the combined effects of *VWF* and ABO blood group, however, a considerable amount of genetic variation remains to be explained. One effort to dissect this variation by whole-genome linkage and quantitative trait loci (QTL) analysis identified *ABO* and several additional small-effect candidate loci, but failed to identify *VWF* [20], illustrating the difficulty of complex trait dissection and locus validation in human studies.

Plasma VWF levels among inbred mouse strains are also highly variable, making mice a potentially useful model system in which to study and identify candidate *VWF* modifier genes [23,24]. We have previously reported on the identification of two VWF modifier genes from two different intraspecific mouse genetic crosses. Analysis of an (RIIIS/J × CASA/RkJ) F2 population identified *Mvwf1* (for modifier of VWF 1) as a dominant RIIIS/J-specific regulatory variant of *B4galnt2* (previously referred to as *Galgt2*) [25]. A recent analysis of an (A/J × CASA/RkJ) F2 population linked a portion of VWF level variation to a CASA/RkJ-specific coding sequence alteration in *Vwf* (*Mvwf2*) [24]. We now report on a further analysis of this latter F2 population using high-density genotyping and QTL analysis to identify additional genetic modifiers, which may explain some of the previously uncharacterized VWF level variation in the (A/J × CASA/RkJ) F2 population.

Materials and methods

Plasma sampling and VWF level measurement

A previously described (A/J × CASA/RkJ) F2 population [24], generated by intercrossing F1 offspring of A/J females and CASA/RkJ males, was used as the test population. Blood samples from parental strains, F1 and F2 mice were obtained as previously described [24]. Briefly, plasma samples from 1–3 retro-orbital bleeds and/or a terminal inferior vena cava bleed were used to determine mean VWF levels for each mouse. Except for two animals in which only three samples were collected, four plasma samples were obtained for each of the F2 animals. A sandwich ELISA method was used to capture and detect the presence of VWF in plasma samples as previously described. ELISA values are represented as a percentage of the pooled CASA/RkJ plasma VWF level (arbitrarily defined as 100%). The VWF levels used here are identical to the data previously reported in Lemmerhirt *et al*. [24].

Purification of liver DNA and F2 genotyping

Purified DNA was obtained by phenol/chloroform extraction of ~25 mg of proteinase K digested liver samples. Following precipitation with isopropanol, pellets were washed with 70% EtOH, air dried, and resuspended in 500 μL TE, pH 8.0. Samples were quantified by spectrophotometer reading at 260/280 (DU530; Beckman Coulter, Fullerton, CA, USA), and diluted to a final concentration of 15 ng μL^{-1} before genotyping.

Mice comprising the F2 population were genotyped at 174 polymorphic microsatellite markers and one single nucleotide polymorphism (SNP) across the mouse genome, covering all 19 autosomes and the X chromosome (Fig. S1). The genotyping success rate was approximately 98%, and the average distance between markers was 8.7 cM. Genotyping was conducted at three different locations as follows: 140/175 markers at the Marshfield Clinic (NHLBI genotyping service); 24/175 at the University of Michigan genotyping core using a Model 3730XL DNA Analyzer and primers from Applied Biosystems, Inc. (Foster City, CA, USA); and 11/175 markers were amplified using unlabeled MIT primers (Invitrogen, Carlsbad, CA, USA) and separated by agarose gel electrophoresis or genotyped by a SNP fluorescence polarization assay (primers HR192–194, A/G SNP) [26]. A complete list of the genotyping primers used is provided in Table S1.

PCR amplification of a previously characterized marker linked to *Mvwf2* (*D6Mit12*) detected two genotyping discrepancies between the original characterization of the F2 population at *D6Mit12* and the replicate DNA samples prepared for high-density genotyping. These two samples were eliminated from subsequent analyses, resulting in an F2 population where $n = 198$ (108 females, 90 males).

Statistical analyses

Statistical analyses were performed with R/qtl version 1.02–2 [27], an add-on package to the general statistical software R [28]. We considered $\log_{10}$ transformed VWF levels, as a result of a slight skew in the F2 phenotype distribution. The influence of sex on the phenotype was established via a *t*-test.

QTL analysis was initially performed by interval mapping [29], whereby each locus is considered, one at a time, as a putative quantitative trait locus, and LOD scores are calculated to measure the evidence for linkage at each position. Because of the clear sex difference in the VWF level phenotype, sex was included as an additive covariate (and this adjustment for the sex-specific effect was used in all subsequent analyses). In this model, the effect of a quantitative trait locus is assumed to be the same in males and females. Separate analyses of the males and females, and combined analyses with sex included as a covariate interacting with the putative quantitative trait locus, indicated no evidence for a sex-specific difference in the QTL effects.

The strong effect of the *Vwf* locus on the phenotype led us to include the marker *D6Mit12* (near *Vwf*) as an additive covariate. In this model, we assume that the effect of a putative quantitative trait locus is not dependent on the *D6Mit12* genotype. In order to identify loci exhibiting possible interactions with *Vwf*, interval mapping was also performed with *D6Mit12* included as an interactive covariate, in which case the effect of a quantitative trait locus is allowed to be dependent on the genotype at *D6Mit12*. As the flexibility of this model weakens our ability to detect QTL, and as no evidence of linkage was found by this approach, we then split the F2 population into three groups, according to the individual

genotype at *D6Mit12*, and performed interval mapping separately within each group. Two-dimensional genome scans, with two-QTL models, were also performed, but no evidence for linkage to additional loci was obtained.

The statistical significance of the results was evaluated by permutation tests [30]; 100 000 permutation replicates were used. Approximate confidence intervals for the locations of the identified QTL were obtained via 1.5-logarithm of odds (LOD) support intervals: the intervals in which the LOD score did not fall below 1.5 of its maximum on the chromosome [29]. Estimates of the percentage of the phenotypic variance explained by sex and *D6Mit12* genotype were obtained via the change in the residual sum of squares in the fit of a linear model with and without each factor. The percentage of the phenotypic variance explained by all identified QTL was estimated by the change in the residual sum of squares in the fit of a linear model including sex and all QTL.

Results and discussion

Plasma VWF levels in the inbred mouse strains A/J and CASA/RkJ differ by approximately 8-fold [24]. When plasma VWF levels were examined in a population of (A/J × CASA/RkJ) F2 progeny ($n = 198$), a broad range of VWF levels were observed (11–83% of CASA/RkJ; Fig. 1). This distribution is reminiscent of the wide range of plasma VWF levels observed in humans and suggests the involvement of multiple genetic and/or environmental factors. Previous heritability estimates suggest that the majority (approximately 65%) of VWF level variability between the A/J and CASA/RkJ strains is genetically regulated [24]. We noted that approximately 5% of the total F2 phenotypic variance was attributable to sex differences; the average (± SE) of VWF in males and females was 42.5% (± 1.5) and 36.3% (± 1.2), respectively. While we have not reported significant sex-specific differences in VWF levels among mouse populations previously, sex-specific effects are well documented in humans [1,31] and sex-specific variants

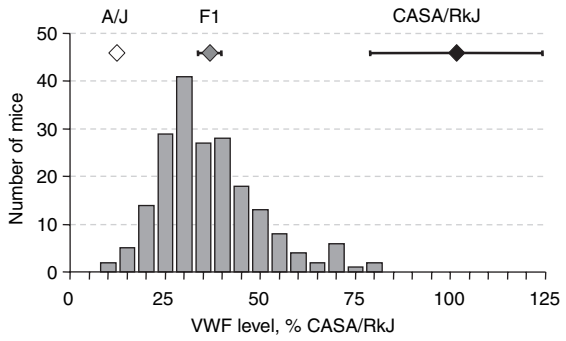

Fig. 1. Original distribution of plasma von Willebrand factor (VWF) levels in 200 (A/J × CASA/RkJ) F2 mice. VWF levels were calculated as a percentage of the CASA/RkJ parental strain. The latter is arbitrarily defined as 100%. F2 values are summarized by the bar graph. Averages for the parental and F1 populations (± SD) are shown: A/J (open diamond), CASA/RkJ (black diamond) and F1 (gray diamond). This figure is adapted from Figure 1 of Lemmerhirt *et al.* (*Blood* 2006; 108: 3061).

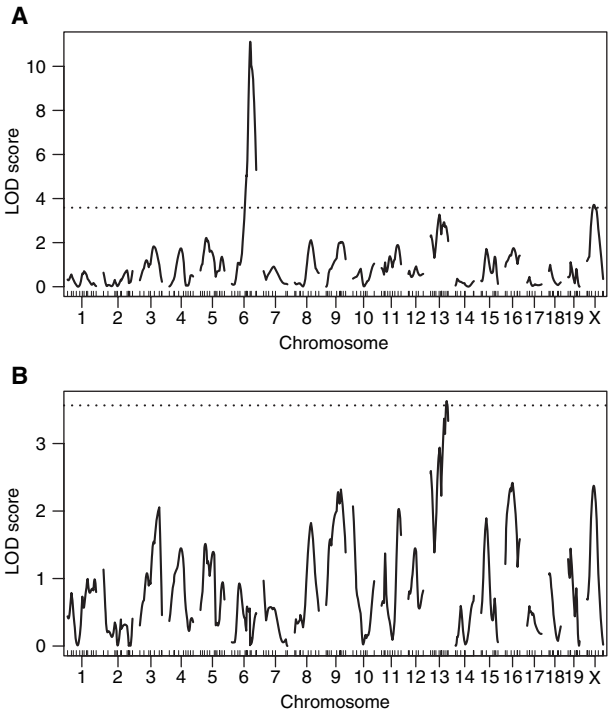

Fig. 2. Logarithm of odds (LOD) curves from a single quantitative trait locus analysis. (A) Inclusion of sex as an additive covariate. (B) Inclusion of both sex and the genotype at *D6Mit12* as additive covariates. The dashed horizontal lines indicate the 95% LOD thresholds.

in measures of partial thromboplastin time (potentially related to VWF and/or FVIII) have also been observed in mice [32].

Following genotyping with 175 polymorphic markers (Fig. S1), QTL analysis employing a single-locus model of inheritance (with adjustment for the effect of sex on VWF phenotype) identified two markers with significant linkage: *D6Mit12* and *DXMit19* with LOD scores of 11.12 and 3.72, respectively (Fig. 2A, Table 1). A third marker, *D13Mit24*, was considered strongly suggestive (LOD = 3.27), but fell below the 95% LOD threshold of 3.59. *D6Mit12* was by far the strongest genetic influence identified, accounting for approximately 18% of the total phenotypic variance, or about 28% of the genetic variance, confirming our previous estimate of

Table 1 Summary of suggestive and significant LOD scores from R/qtl analysis

Covariates	Chromosome	Position (cM)	Marker	LOD	*P*-value
Sex	6	63.7	*D6Mit12*	11.12	< 0.0001
	13	40.0	*D13Mit24*	3.27	0.094
	X	33.3	*DXMit19*	3.72	0.038
Sex and *D6Mit12*	13	65.0	*D13Mit196*	3.63	0.049
	X	32.3	*DXMit19*	2.37	0.500
Sex, in group CC at *D6Mit12*	4	40.2	*D4Mit132*	4.45	0.016
	13	26.0	*D13Mit248*	4.23	0.024

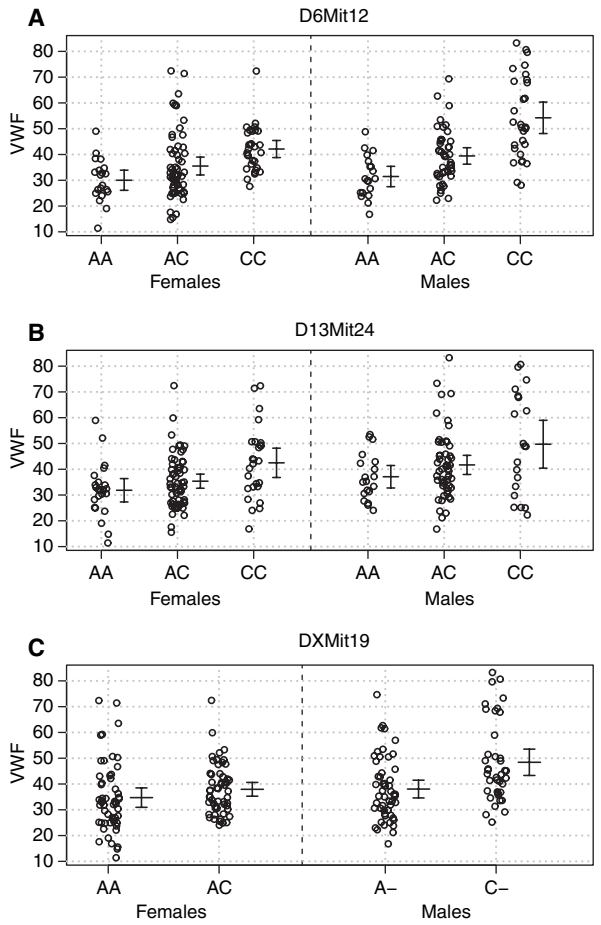

Fig. 3. Genotype and phenotype correlation at three candidate additive von Willebrand factor (VWF) modifier loci. Individual VWF levels and marker genotypes, partitioned by sex, at three candidate murine modifier loci identified via a single quantitative trait locus model. VWF levels are represented as a percentage of CASA/RkJ parental values. Error bars represent 95% confidence intervals.

linkage to *D6Mit12* [24]. These loci were also examined for allele-specific effects on phenotype, as depicted in Fig. 3. Consistent with phenotypes observed in the parental strains, the presence of CASA/RkJ alleles at each candidate locus correlated with an increase in VWF levels. Intermediate phenotypes were noted in heterozygote animals, consistent with additive effects of alleles at each locus.

The molecular mechanism underlying the strong linkage to *D6Mit12* has previously been characterized as a CASA/RkJ-specific coding sequence variant (R2657Q) in the murine *Vwf* gene (*Mvwf2*), which results in higher circulating VWF levels in plasma [24]. The linkage identified on the X chromosome and the potential linkage to chromosome 13 were of particular interest, as they were not detected in our previous analysis of the same sample population and neither has been previously associated with VWF level regulation [24]. The previously identified murine modifier (*Mvwf1*), which is a result of a mutation at the *B4galnt2* locus, is not present in either of the strains studied here [24]. As expected, therefore, no significant evidence for linkage is observed at this locus (maximum LOD score on murine chromosome 11 is 2.0). Although the ABO blood group is an important VWF modifier in humans, previous studies indicate that the ortholog of the ABO locus is not polymorphic in mice [33,34], which is consistent with the absence of significant linkage at this locus in our study (maximum LOD score on murine chromosome 2 is 1.1).

The very strong effect of the *Vwf* locus led us to repeat the genome scan, including the marker *D6Mit12* as an additive covariate; the intent was to clarify evidence for linkage on chromosomes 13 and X, and to identify additional loci. As shown in Table 1, the inclusion of *D6Mit12* as an additive covariate improved the evidence for chromosome 13, which now achieved significance (*D13Mit196*, LOD = 3.63). However, there was a loss of linkage to the X chromosome, and no other significant linkage was detected (Fig. 2B, Table 1). Together, these data suggest that in an additive model, *Vwf* is the predominant genetic influence on VWF levels between these strains. Chromosome 13 genotype may also influence VWF levels independently, although future validation using a larger sample size is necessary to confirm this effect. Although the evidence for X chromosome linkage in the initial scan appears to result from a chance association in the genotypes between the X chromosome and the *Vwf* locus on chromosome 6, we cannot rule out the possibility that a locus on the X chromosome also contributes to plasma VWF level.

In order to identify additional loci that may interact with *Vwf*, we performed a genome scan with *D6Mit12* included as an interactive covariate; however, no significant interactions between *Vwf* and other loci were detected. To characterize possible interactions between *D6Mit12* and other loci more fully, we partitioned the (A/J × CASA/RkJ) F2 population into three groups according to the genotype of each individual at *D6Mit12* and performed genome scans separately within each group. While no significant linkage was detected within the AA or AC subgroups, two significant linkages were detected in the CC group: a marker on chromosome 13 nearly 40 cM away from the previously identified additive marker *D13Mit196* (*D13Mit248*, LOD = 4.23) and a previously unidentified locus on chromosome 4 (*D4Mit132*, LOD = 4.45; Fig. 4, Table 1). The putative modifier genes underlying these linkage groups were termed *Mvwf3* (chromosome 4) and *Mvwf4* (chromosome 13). These results suggest that loci on chromosomes 13 and 4 may interact with *Vwf* to impact overall VWF levels in mice when the *Vwf* genotype is homozygous for CASA/RkJ alleles (Fig. 5). Mechanistically, these epistatic interactions could impact strain-specific VWF protein production, post-translational modification, intracellular processing, secretion, or clearance from the plasma circulation. We have previously demonstrated that there are not strain-specific transcriptional differences at the *Vwf* allele in (A/J × CASA/RkJ) F1 animals, making an epistatic interaction impacting transcription unlikely [24]. Although we are suggesting an epistatic relationship between *D6Mit12* and these putative loci, we also acknowledge that the power to detect and characterize epistasis in QTL analysis is limited [35]. Further analysis of

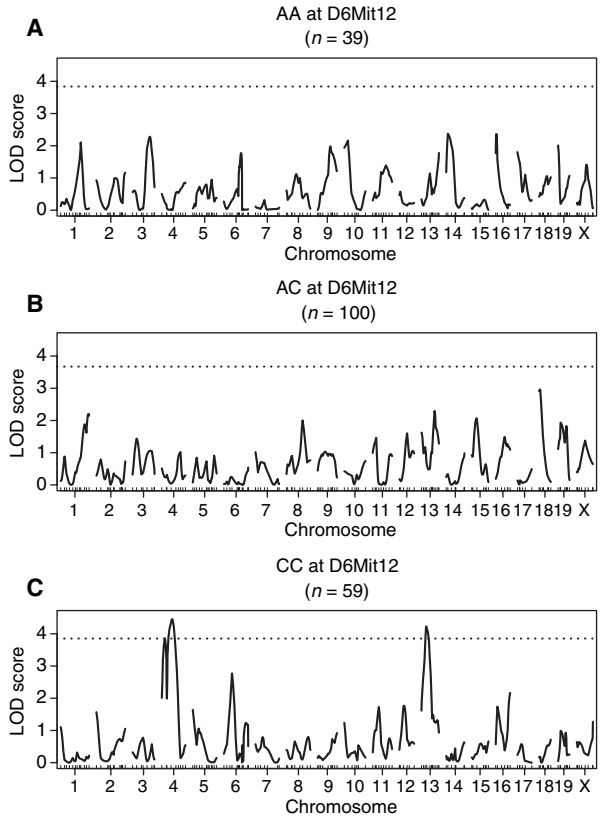

Fig. 4. Logarithm of odds (LOD) scores, partitioned by *D6Mit12* genotype. LOD scores obtained by single quantitative trait locus analyses, performed separately in the three groups defined by the genotype at *D6Mit12*, with sex included as an additive covariate. The dashed horizontal lines indicate the 95% LOD thresholds.

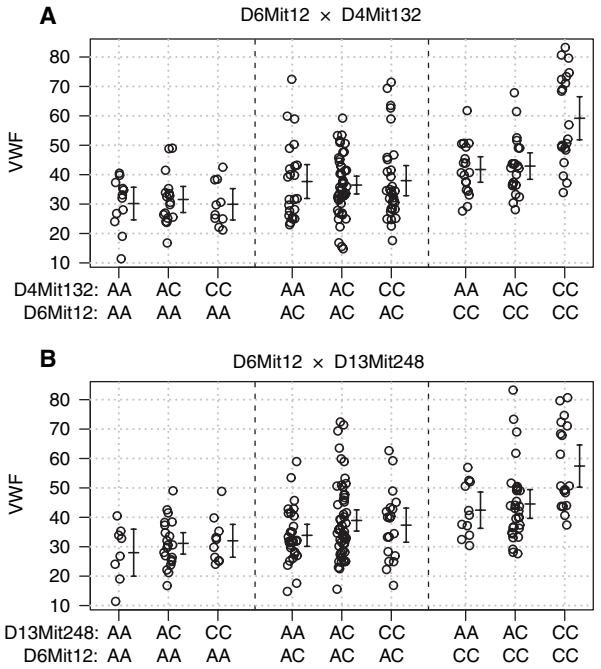

Fig. 5. Phenotype vs. genotype in a two-locus model of epistasis with *D6Mit12*. Individual von Willebrand factor (VWF) levels for individuals partitioned by their two-locus marker genotypes, for markers showing evidence for an epistatic interaction with *D6Mit12*. VWF levels are represented as a percentage of CASA/RkJ parental values. Error bars represent 95% confidence intervals.

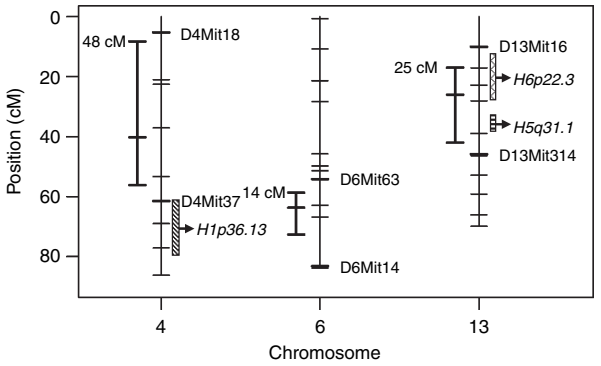

Fig. 6. 1.5-Logarithm of odds (LOD) support intervals for regions of significant linkage and relevant human homology of synteny. The 1.5-LOD support intervals are shown to the left of the chromosomes. Shown to the right (patterned boxes) are relevant regions of human homology of synteny containing quantitative trait loci associated with von Willebrand factor (VWF) level variation in the Genetic Analysis of Idiopathic Thrombophilia (GAIT) study, as reported by Souto *et al.* (*Thromb Haemost* 2003; 89: 468). Additionally, the LOD support interval on murine chromosome 6 shares homology of synteny with the *VWF* locus on human chromosome 12.

additional F2 offspring will be necessary to confirm the suggested epistasis.

Linkage to chromosomes 4 and 13 was not detected in our previous analysis of the same sample population using a low-density genome scan (45 polymorphic markers) on pooled DNA samples from the phenotypic outliers [24]. Although the use of pooling has been successfully employed as an initial mapping strategy to identify even minor QTL in multiple species [36–39], the power to detect loci that require epistatic interactions for significance is limited. Although the methods employed in our current approach strengthen our power to detect small additive effects and epistatic interactions, failure to detect some of these loci in our original pooling analysis may reflect a combination of insufficient marker density, inconclusive genotyping in some regions of linkage, and/or a masking effect of the *D6Mit12* genotype within the original pools [24]. Even with these two linkage approaches, we have not accounted for the total VWF variance observed between A/J and CASA/RkJ strains. Given the marker density employed, it is unlikely that we have missed any remaining genes of large effect. Further dissection of the genetic variation will require much larger data sets to detect any remaining small-effect modifiers.

The identification of biologically relevant modifier genes in mice can have a significant impact on our understanding of human disease [40,41]. As one example, *Nramp1*, first identified as a murine modifier of tuberculosis susceptibility, has shown direct cross-species relevance for understanding tuberculosis

infection in humans [42,43]. In our investigation of murine VWF level modifiers, we are ultimately interested in the genes which underlie these candidate modifiers, the mechanism by which they may be contributing to VWF level variation in the mouse, and their potential application for understanding of VWF level variation in humans. After correcting for linkage to *ABO*, genome-wide linkage analysis of 21 Spanish families participating in the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project identified significant linkage to five additional candidate VWF modifier loci, located on human chromosomes 1, 2, 5, 6 and 22 [20]. To assess the possible relationship between these human loci and the murine modifiers we have identified, we calculated the 1.5-LOD thresholds for our regions of significant linkage (Fig. 6). Alignment of these intervals with the human database [44] is consistent with a possible homology of synteny between three of the five candidate human VWF modifier loci identified in the GAIT study [20] and the corresponding regions in the mouse genome (Fig. 6). Interestingly, the murine linkage group on chromosome 13 aligns with orthologous linkages on both human chromosomes 5 and 6 (Fig. 6). The *Mvwf4* linkage group could represent two distinct murine VWF modifier loci, perhaps accounting for the distinct additive and epistatic effects we mapped to murine chromosome 13. Taken together, these data suggest that the genetic factors determining VWF levels in the mouse may also be biologically relevant to human VWF level regulation.

In summary, we have used high-density genotyping and QTL analysis both to confirm the influence of *Vwf* (*Mvwf2*) on murine VWF levels and to identify two new regions of the mouse genome which co-segregate with VWF level variation in the A/J and CASA/RkJ strains. The influence of candidate loci on chromosomes 4 and 13 is most likely through epistatic interactions with the *Vwf* CASA/RkJ allele, although chromosome 13 may also exert a small independent, additive effect. We have designated the underlying modifier loci as *Mvwf3* and *Mvwf4*, respectively. Initial linkage to the X chromosome was not confirmed after the inclusion of *D6Mit12* as a covariate, making it difficult to discern how much effect, if any, is attributable to an X chromosome locus. Collectively, these putative modifier loci and *Mvwf2* (*Vwf*) account for approximately 29% of the total VWF level variation (or ~45% of the genetic variation) observed between these populations. Identification of the specific genes underlying *Mvwf3* and *Mvwf4* may provide novel insight into the mechanisms by which VWF levels are regulated in the mouse and may also have direct relevance as modifiers of VWF plasma level and VWD penetrance and expressivity in humans.

Acknowledgements

Support from the National Institute of Health to D. Ginsburg (R37-HL39693) and K. W. Broman (GM074244), the NHLBI Mammalian Genotyping Service (Contract Number HV48141), and the University of Michigan Genotyping Core (with support from the University of Michigan Endowment for the Basic Sciences) is gratefully acknowledged. H. L. Lemmerhirt was also supported by a National Science Foundation (NSF) Graduate Fellowship Award. D. Ginsburg is an investigator of the Howard Hughes Medical Institute.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supplementary material

The following supplementary material is available for this article:

Table S1. High-density microsatellite panel for (A/J × CASA/RkJ) F2 genotyping.

Fig. S1. High-density microsatellite panel for F2 genotyping. A total of 175 polymorphic markers (indicated by the hash marks) were used in a secondary screen for linkage in the F2 population. The average distance between markers was 8.7 cM.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1538-7836.2006.02325.x

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