Genetic Architecture Underlying Variation in Extent and Remodeling of the Collateral Circulation

Shiliang Wang,* Hua Zhang,* Xuming Dai, Robert Sealock, James E. Faber

Rationale: Collaterals are arteriole-to-arteriole anastomoses that connect adjacent arterial trees. They lessen ischemic tissue injury by serving as endogenous bypass vessels when the trunk of 1 tree becomes narrowed by vascular disease. The number and diameter ("extent") of native (preexisting) collaterals, plus their amount of lumen enlargement (growth/remodeling) in occlusive disease, show remarkably wide variation among inbred mouse strains (eg, C57BL/6 and BALB/c), resulting in large differences in tissue injury in models of occlusive disease. Evidence suggests similar large differences exist among healthy humans.

Objective: To identify candidate loci responsible for genetic-dependent collateral variation.

Methods and Results: Cerebral collateral number and diameter were determined in 221 C57BL/6×BALB/c F2 progeny, followed by linkage analysis to identify quantitative trait loci (QTL) for collateral number and diameter. Four QTL were obtained for collateral number, including epistasis between 2 loci. A QTL that was identical to the strongest QTL for collateral number on chromosome 7 (logarithm of the odds [LOD]=29, effect size=37%) was also mapped for collateral diameter (LOD=17, effect size=30%). Chromosome substitution strain analysis confirmed this locus. We also obtained a unique QTL on chromosome 11 for collateral remodeling after middle cerebral artery occlusion. Association mapping within the chromosome 7 QTL interval using collateral traits measured for 15 inbred strains delineated 172-kbp (P=0.00002) and 290-kbp (P=0.0004) regions on chromosome 7 containing 2 and 7 candidate genes, respectively.

Conclusions: We conclude that collateral extent and remodeling are unique, highly heritable complex traits, with 1 QTL predominantly affecting native collateral number and diameter. (Circ Res. 2010;107:558-568.)

Key Words: collateral vessels ■ genetics ■ quantitative trait loci ■ cerebral circulation ■ arteriogenesis

Atherosclerotic, atherothrombotic, and thromboembolic occlusive vascular diseases constitute the primary cause of morbidity and mortality in developed countries. Many physiological systems are concomitantly recruited, albeit with significant interindividual variation, which lessen the accompanying ischemic tissue injury. Among these, 3 vascular protective mechanisms are paramount: (1) the number and diameter of arteriole-to-arteriole anastomoses present in the tissue before the onset of disease that cross-connect occasional distal-most arterioles of adjacent trees (ie, the "native collateral extent"); (2) an anatomic increase in lumen diameter and wall thickness of these vessels caused by obstruction of flow to one of the trees, a process termed collateral remodeling, collateral growth, or arteriogenesis; and (3) ischemic angiogenesis, ie, the sprouting of additional capillaries.1–6 Arteriogenesis, which requires days to weeks to achieve up to an approximately 10-fold increase in diameter depending on tissue and species, occurs when perfusion of an adjacent tree is chronically reduced below a critical level. This increases flow-dependent shear stress on collateral endothelial cells, which in turn initiates an inflammatory-like remodeling process. It is becoming recognized that the extent of the native collateral circulation and collateral remodeling have significantly greater impact on restoring blood flow to ischemic tissue than does angiogenesis, which can only increase dispersion of whatever flow is provided by the collateral network.1–5,6

The severity of the clinical manifestations of occlusive vascular diseases (ie, myocardial infarction, stroke, and chronic ischemic disease of the heart, brain, and lower extremities) have long been known to vary widely among individuals. This is presumably attributable to variation in environmental influences and risk factors, as well as genetic mutations and polymorphisms affecting the extent pathological and physiological processes. Although much effort has focused on identifying the sources of this variation, the possibility that differences in extent of the native collateral circulation exist among healthy individuals and contribute

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significantly when disease strikes has, until recently, received little attention. Meier et al found, using an index primarily dependent on collateral density and diameter, that coronary collateral conductance varied by \(\approx 10\)-fold in healthy human subjects. Because these subjects lacked coronary artery disease, the variation could not be affected by differences in stenosis-induced collateral remodeling. Studies using dynamic angiography in patients with acute stroke from middle cerebral artery obstruction, where differences in remodeling can again be ruled out because of the acute nature, suggest that wide variation in native collateral conductance is also present in the cerebral circulation. Likewise, evidence exists for significant variation in collateral extent in the peripheral limbs of healthy individuals. Reduced collateral density and diameter evidenced large differences in multiple tissues in adult C57BL/6 and BALB/c mouse strains. In fact, C57BL/6 and BALB/c mice have the largest extremes in collateral extent in the cerebral circulation among 15 strains, for example exhibiting a greater than 30-fold difference in collateral density. Importantly, these differences in collateral extent in the cerebral circulation are shared qualitatively by other tissues, including skeletal muscle and intestine. Moreover, the rank-order of cerebral collateral extent among 15 mouse strains closely predicts the rank-order of severity of stroke (infarct volume) after permanent middle cerebral artery occlusion. This finding establishes a major role for genetic variation in the extent of the collateral circulation in determining variation in severity of ischemic tissue injury. Interestingly, collateral remodeling during stroke also evidenced significant variation, but with a strain-specific pattern significantly different from the pattern associating infarct volume with collateral extent. This suggests that different pathways are responsible for collateral formation versus collateral remodeling, and that genetic variation in them is likely to be mediated by different loci.

Nothing is known about the genetic loci responsible for the remarkably wide variation in native collateral extent and collateral remodeling described above. However, studies using gene targeting methods have identified 2 genes, Vegfa and Clic4, whose expression positively regulates collateral formation in the embryo and thus collateral extent in the adult; one of these (Vegfa) also participates in collateral remodeling. Hence, polymorphisms in these genes are a priori candidates for causing variation in collateral circulatory function. Identification of the alleles underlying individual variation in collateral extent and remodeling is important not only to help define the responsible signaling pathways for these 2 processes, but also to allow assessment of risk-sensitivity if occlusive vascular disease develops and to permit stratification of individuals enrolled in clinical trials testing new collaterorgenic therapies. Therefore, in the present study we sought to identify genetic loci governing variation in collateral extent and remodeling. To achieve this goal we created 243 F2 mice intercrossed between the C57BL/6 and BALB/c strains and performed linkage analysis to identify quantitative trait loci (QTLs). We then used association mapping among 15 inbred strains to identify candidate genes responsible for variation in collateral function.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Animals**

F1 progeny obtained from reciprocal matings of C57BL/6J (B6) and BALB/cByJ (Bc) were mated to produce an F2 population. Chromosome substitution strains were C57BL/6J-Chr7A/JNaJ (chromosome substitution strain [CSS]7) and C57BL/6J-Chr17A/JNaJ (CSS17).

**Phenotyping**

Mice were subjected to right-side middle cerebral artery occlusion (MCAO) and collateral number and diameter measured 6 days later. To image the pial circulation, the rostral vasculature was maximally dilated, filled with yellow Microfil with viscosity adjusted to prevent capillary filling, and fixed (paraformaldehyde). Collaterals connecting the anterior cerebral (ACA) and middle cerebral (MCA) artery territories were imaged. Cortical territories supplied by the MCA, ACA and PCA were determined. ANOVA or \(t\) tests were used together with Bonferroni correction where appropriate.

**DNA Isolation and Genotyping**

Tail genomic DNA was genotyped using the 377-SNP GoldenGate genotyping array (Illumina, San Diego, Calif). Manual genotyping was done for one marker (rs32420445). SNP positions were obtained from Build 37.1 of the NCBI SNP database.

**Linkage Analysis**

Collateral traits are defined as collateral number, diameter and “conductance” (number \times diameter) measured in the left (nonocluded) hemisphere. The remodeling trait is the average fold change in collateral diameter between right and left hemisphere 6 days after MCAO. These traits in the F2 population were subjected to linkage analysis using single and multiple QTL models in the R statistical software.
program. Thresholds for significant and suggestive QTL were defined as \( P = 0.05 \) and \( P = 0.63 \), respectively.

**Association Mapping**

The efficient mixed model association algorithm (EMMA) was applied in R to collateral number measurements on 15 inbred strains, 8 to 10 individuals each, obtained previously (see Online Figure I).18

**Results**

**Native Collateral Trait Differences Between B6 and Bc Strains Are Heritable**

We previously showed in multiple tissues that Bc mice have smaller collateral number and diameters than B6 mice.16 This is striking in the pial circulation, where Bc mice average less than one collateral per hemisphere, compared to 9 in B6. We further showed that among 15 strains Bc had the lowest pial collateral number and near-lowest diameter (13 \( \mu m \)).18 Among those strains, B6 has the largest collateral diameter (23 \( \mu m \)), and nearly the largest number of pial collaterals. Therefore, B6 and Bc were chosen for identification of QTL for native pial collateral number and diameter, for a quasindice of total collateral “conductance” (defined as number×diameter) and for increase in collateral diameter (remodeling) induced by MCAO. MCAO in one hemisphere does not alter collateral number or diameter in the other, nonoperated hemisphere.18 After exclusion of 22 individuals for poor filling or other technical problems, 221 F2 mice were analyzed for native collateral properties and 190 for remodeling. The mice were genotyped at 228 informative SNPs across the genome (see Methods).

Cerebral arteriograms from nonoperated B6 and Bc mice are shown in Figure 1A. The entire population of MCA-ACA collaterals is confined to the pial surface, aiding direct quantification.18 The Bc brain shown, with no pial collaterals, is typical of most Bc mice. Previously reported values for collateral diameter and number in B6 and Bc16,18 were confirmed here: 9 and 22.5 \( \mu m \) in B6, 0.5 and 12.5 \( \mu m \) in Bc) (Figure 1B).

All 3 collateral parameters were intermediate in the F1 population between those for B6 and Bc (Figure 1B) although closer to those for B6. Collateral diameter and conductance in F1 mice were significantly lower than in B6 (\( P < 0.05 \)) but were still larger than the average of B6 and Bc values. For collateral number, the trend toward fewer than in B6 was not significant. Thus, collateral traits in B6 are semidominant over Bc.

**Genome-Wide Single QTL Mapping Detects a Major Effect on Chromosome 7 (Canq1) for Native Collateral Traits**

In the F2 population, all 3 collateral traits were approximately normally distributed, indicative of polygenic traits, with the distributions containing suggestions that they may be sums of
multiple normal distributions (Figure 1C). These traits were independent of sex, parental origin, or body weight (Online Figures II and III). Variation in collateral traits was also independent of body weight among 15 inbred strains.\textsuperscript{18} Collateral number and diameter were minimally correlated ($r^2=0.13$), confirming previous results\textsuperscript{18} and suggesting that number and diameter are largely independent traits (Online Figure IV).

The phenotype-genotype data were subjected to genome-wide LOD score profiling using the single QTL model in R/QTL. This identified a single, highly significant locus on distal chromosome 7 for all 3 collateral traits (LOD scores of 21, 17, and 27 for number, diameter, and conductance, respectively; genetic [heritable] effect sizes $\geq30\%$ for all three) (Figure 2A; Online Table I). The peak is located 0.5 cM telomeric to marker rs13479513 (134.25 Mb; Figure 2A, inset). The 95% confidence level is 62 to 67 cM, which is estimated to correspond to 132.4 to 135.8 Mb in physical location. The single QTL model also identified a significant QTL (genome-wide 0.05 significance level) for collateral number on chromosome 1, 2 suggestive QTL (genome-wide 0.63 significance level) on proximal chromosome 6 and chromosome 10 for number, and 2 suggestive QTL on chromosomes 1 and 10 for conductance (Figure 2A).

Additional QTL and Epistasis Identified by Genome-Wide Multiple QTL Mapping

Experiments using haploinsufficient and null gene targeted mice have shown that Vegfa, Flt1 (vascular endothelial growth factor [VEGF] receptor 1) and Clic4 expression levels are strong positive determinants of collateral number (J. Lucitti and J.E.F., unpublished data, 2010).\textsuperscript{19,20} However, our single QTL analysis revealed no QTL in the regions of these genes (Figure 2A), possibly because of the predominant major effect on chromosome 7. Moreover, the multiple suggestive QTL identified using the single QTL model suggest a complex genetic architecture may underlie variation in native collateral extent. Also, a single QTL model cannot detect epistasis. To address these issues, we subjected the data to genome-wide multiple QTL profiling with increased statistical power using the Stepwiseqtl function in R/QTL.

As shown in Figure 2B, this analysis confirmed and strengthened the QTL on chromosome 7 for collateral number, the LOD score improving from 21 to 29 and the effect size increasing from 32% to 37%. It also gave a strong QTL (LOD score 13) at 45 cM (90 Mb) on chromosome 1, which presumably represents the same genetic influence as the weaker QTL on chromosome 1 (LOD score 3.8, 49 cM) in the single QTL analysis. Multiple QTL mapping also identified significant loci on chromosomes 3 and 8 for collateral number (LOD scores of 7 and 5, resp.), and interaction between the QTL on chromosomes 1 and 3 (LOD score 7). Suggestive QTL on chromosomes 6 and 10 were not confirmed. These results are summarized in Figure 2B; the inset shows the final model for collateral number. We have named the QTL according to convention by descending order of LOD score as Canq1–4 (collateral artery number QTL), Cadq1 (diameter), and Cacq1–2 (“conductance”). Table 1 summarizes effect sizes, the modes of inheritance (additive or dominant) and their degree, and the statistical significance. We also detected slight segregation distortion between proximal chromosome 10 and distal chromosome 6 ($P<0.03$ by $\chi^2$ test). One possible explanation for these findings is that linkage disequilibrium exists between the loci on chromosomes 6 and 10 and the major effect on chromosome 7, a condition controlled for by multiple QTL analysis.

Like the single QTL analysis, multiple QTL analysis failed to find any effect of Vegfa, Flt1, or Clic4. To search exhaustively for potential epistases with these genes, we fixed all main effects and the interaction, then searched for interaction between Canq1 and any other locus. No significant interaction was found (Online Figure V).

![Figure 2. Genome-wide mapping of collateral traits in C57 F2 mice. A, LOD profiling using the single QTL model. Locations of genotyping SNPs are shown as ticks on the abscissa. Ninety-five percent confidence level (dashed line) was estimated using 1000 permutations. Inset, Higher resolution genetic map of the peak on chromosome 7. The location of the marker nearest the peak is indicated (triangle). B, LOD profiling using the multiple QTL model. Significant QTLs were found on chromosomes 1, 3, 7, and 8 for number (black line), on chromosome 7 for diameter (dashed line), and on chromosomes 1 and 7 for conductance (dotted dashed line). Inset, Schematic of the multiple QTL model for collateral number having the highest LOD. The solid line denotes interaction between the QTLs on chromosomes 1 and 3 (LOD score 7).]
To evaluate the genetic effects of the QTL on chromosome 7 for the 3 collateral traits, the average phenotype values were plotted against genotype for mice grouped by genotype at the marker nearest the peak, rs13479513. Mice homozygous for the B6 allele or heterozygous (B6-Bc) did not differ significantly from B6 or the F1 population, respectively, in any of the traits (Figure 3A and 3B). The Bc-Bc group also did not differ from the Bc parental in diameter. Thus, these phenotypes are largely determined by the single B6 allele when present. For collateral number, however, Bc-Bc F2 mice had significantly more pial collaterals than the parent Bc (3.9 versus 4.5, P < 0.001). The same is true of the derived parameter, “conductance” (56 conductance units versus 12 for Bc, P < 0.05). In addition, in our study of 15 inbred strains (18), Bc was significantly lower in pial collateral number than the 2 closest strains (Bc, 0.2 collaterals per hemisphere; SWR, 1.3, P = 0.0004 versus Bc; AKR, 1.7, P < 0.0001 versus Bc; Online Figure I). These results suggest that additional loci, possibly unique to Bc among these strains, are responsible for the very low numbers of collaterals in Bc mice and that additional loci must act with the chromosome 7 QTL to cause the very low collateral number in Bc mice.

The Major Effect of Chromosome 7 Is Confirmed With Chromosome Substitution Strains

The inbred strain A/J is similar to Bc in haplotype structure under Cacq1. It is also similar in hindlimb necrosis and recovery of hindlimb perfusion following femoral artery ligation22 and infarct volume after MCAO23 (see Discussion). These phenotypic characteristics, which gave a QTL in the same position as Cacq1, were recapitulated in both of these studies in the strain C57BL/6J-Chr 7A/J/NaJ (CSS7), in which chromosome 7 in the B6 stain has been replaced by chromosome 7 from A/J. We therefore measured native collateral traits for CSS7. New data on A/J mice (n = 8) in this study with collateral number but not diameter.

Table 1. Chromosome Location, LOD Score, Confidence Interval, and Genetic Descriptors of Collateral QTL Identified Using Multiple QTL Model

<table>
<thead>
<tr>
<th>Trait</th>
<th>QTL</th>
<th>Chr Location, cM (Mb)</th>
<th>LOD</th>
<th>95% Confidence Interval (cM)</th>
<th>Effect Size*</th>
<th>Additive ± SE†</th>
<th>Dominance ± SE†</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Canq2</td>
<td>1</td>
<td>45.0 (90)</td>
<td>12.6</td>
<td>38–49</td>
<td>13.3</td>
<td>−1.09±0.22</td>
<td>0.34±0.32</td>
</tr>
<tr>
<td></td>
<td>Canq3</td>
<td>3</td>
<td>5.0 (18)</td>
<td>6.6</td>
<td>0–28</td>
<td>6.3</td>
<td>0.09±0.22</td>
<td>−0.15±0.31</td>
</tr>
<tr>
<td></td>
<td>Canq1</td>
<td>7</td>
<td>63.6 (134)</td>
<td>28.7</td>
<td>62–65</td>
<td>37.2</td>
<td>−2.67±0.22</td>
<td>1.42±0.30</td>
</tr>
<tr>
<td></td>
<td>Canq4</td>
<td>8</td>
<td>16.8 (47)</td>
<td>5.0</td>
<td>6–24</td>
<td>4.9</td>
<td>0.85±0.21</td>
<td>−0.67±0.31</td>
</tr>
<tr>
<td></td>
<td>CADq1</td>
<td>7</td>
<td>64.0 (134)</td>
<td>30.5</td>
<td>62–66</td>
<td>44.0</td>
<td>0.85±0.21</td>
<td>−0.67±0.31</td>
</tr>
<tr>
<td></td>
<td>Cacq2</td>
<td>1</td>
<td>47.3 (106)</td>
<td>6.0</td>
<td>31–81</td>
<td>6.6</td>
<td>−27.27±5.05</td>
<td>0.18±7.07</td>
</tr>
<tr>
<td></td>
<td>Cacq1</td>
<td>7</td>
<td>64.0 (134)</td>
<td>30.5</td>
<td>62–66</td>
<td>44.0</td>
<td>−67.90±5.06</td>
<td>28.31±7.07</td>
</tr>
<tr>
<td>Remodeling</td>
<td>Carq1</td>
<td>11</td>
<td>55.6 (93)</td>
<td>3.5</td>
<td>41–72</td>
<td>8.1</td>
<td>0.15±0.01</td>
<td>−0.03±0.01</td>
</tr>
</tbody>
</table>

*Percentage of the total phenotypic variation explained by locus. †Additive or dominance coefficients.

To see phenotypic effects, the genotype at a hypothetical marker located exactly at the peak of each of the QTL on chromosomes 1, 3 and 8 was imputed and the mice grouped according to these genotypes (Figure 3B). This shows that the QTL on chromosome 1 acts in the same direction as that on chromosome 7 (ie, to favor greater number and conductance in B6; first and third panels in Figure 3B), whereas those on chromosomes 3 and 8 act in the other direction, albeit weakly (first panel).

Figure 3. Influence of identified QTL on collateral traits. A, Phenotypic effects of the chromosome 7 QTL. Collateral number, diameter, and conductance of parental, F1, and F2 mice grouped according to genotype at marker rs13479513 (see Figure 1, inset). F2-B6/B6 mice and F2-B6/Bc mice are similar to parental B6 and Bc, respectively, in all 3 traits. F2-Bc/Bc mice are similar to Bc in diameter (middle). These suggest that the chromosome 7 QTL accounts for the major difference in collateral number between B6 and Bc. However, F2-Bc/Bc mice are significantly different from Bc in collateral number and conductance, suggesting that additional loci must act with the chromosome 7 QTL to cause the very low collateral number in Bc mice. B, Phenotypic effects of the QTL identified by multiple QTL mapping. For each trait and QTL, F2 mice were grouped by genotype at the imputed peak SNP (see Online Methods) and the average for each group plotted versus genotype (see text).
confirmed our previous results\textsuperscript{18}; A/J has 65% fewer pial collaterals than B6, and CSS7 is not statistically different from A/J (Figure 4A). CSS7 has slightly larger collateral diameter than Bc (12.6 versus 8.6 μm for A/J, \(P<0.003\)), but diameter is substantially smaller than in B6 (12.6 versus 24.6 μm for B6, \(P<0.001\); Figure 4B). Similarly, CSS7 has 70% lower conductance than B6, compared to 86% lower for A/J and 98% lower for Bc (Figure 4C). Hence, CSS7 largely mimics Bc in all 3 native collateral traits, as it does for necrosis in hindlimb ischemia and cerebral infarct volume following MCAO\textsuperscript{22,23}.

However, the significant difference between CSS7 and A/J for diameter and conductance and trend toward significant difference for number suggest that loci on additional A/J chromosomes influence collateral traits. Failure to find such evidence in previous studies using CSS7 analysis for recovery of hindlimb perfusion and necrosis score\textsuperscript{22} and infarct volume\textsuperscript{23} may arise because these downstream traits are removed from the underlying physiological mechanisms that are dominated by native collateral extent and collateral remodeling.

Different amounts of cortical territory supplied by each of the main arteries (MCA, ACA, and PCA) are determinants of infarct volume after MCAO\textsuperscript{18} and could, in principle, be determinants of pial collateral extent. And A/J mice have a larger percentage MCA territory that could potentially confound interpretation of the A/J and CSS7 data.\textsuperscript{18} Therefore, we also examined tree territories. The percentage MCA territory in CSS7 mice was not different from that of B6 and Bc mice (Figure 4D). In contrast, chromosome 17 (see below) transferred a significant part of the enlarged MCA territory phenotype of A/J to B6, consistent with the notion that loci on chromosomes other than 7 are responsible for the enlarged MCA tree in A/J mice. These data are consistent with our previous finding that collateral number and diameter show essentially no correlation with tree territory (\(r^2=0.05\) and 0.01, resp.)\textsuperscript{18}

Because no QTL was found on chromosome 17 for collateral traits, cerebral infarct volume\textsuperscript{23}, or necrosis score and recovery of perfusion in a hindlimb ischemia model,\textsuperscript{22} CSS17 was also examined for pial collateral traits as a negative “control” to validate the CSS approach. CSS17 was not different from B6 in collateral number and conductance (Figure 4A and 4C). However, collateral diameter was significantly lower in CSS17 than in B6 (20 versus 24.6 μm, \(P<0.0002\)), but it was still larger than in A/J by a substantial amount (20 versus 8.6 μm, \(P<0.0001\)). These data suggest that the preparation of the CSS strains did not, per se, cause the Bc-like low values for native collateral traits found in CSS7, thereby reinforcing the conclusion that the main effect for low collateral number, diameter, and conductance resides on chromosome 7. The significant reduction in collateral diameter in CSS17 indicates that chromosome 17 of A/J mice harbors a genetic factor(s) that negatively affects this trait when introgressed onto the B6 background.

Importantly, the above findings offer a physiological basis for the previously identified QTL on chromosome 7 linked to hindlimb ischemia and cerebral infarct volume,\textsuperscript{22,23} ie, a gene variant(s) that confers variation in native collateral extent.

**Cell–Cell Signaling and Immune Response**

**Pathway Genes Are Enriched in Canq1**

We subjected all genes in the Entrez database within the 95% confidence interval of Canq1 (132.3 to 135.8) to Ingenuity pathway enrichment analysis (Ingenuity Systems, Redwood, CA). After adjustment for multiple-testing errors (Benjamini–Hochberg adjusted probability value <0.05), enrichment was found for cell–cell signaling and immune response genes (Online Figure VI). These findings are supported by potential candidate genes responsible for variation in collateral extent revealed by association mapping (below).

**Association Mapping Links Pial Collateral Traits to a Narrow Region Within Canq1**

Given the strength of the QTL on chromosome 7, one can speculate that inbred strains that share haplotype identity with B6 will have a B6 collateral phenotype, and similarly for Bc. Thus, by comparing the structure within the region of the QTL across multiple inbred strains, it should be possible to...
narrow the region harboring the genetic element(s) responsible for the QTL. However, as extensively discussed by Kang et al., the population structure and relatedness among inbred strains lead to high false-positive rates and low statistical power. To circumvent these problems, we applied the efficient mixed model association (EMMA) algorithm designed by Kang et al to the 134 individuals (8 to 10 per strain) of the 15 inbred strain set we studied previously (Online Figure I). Herein, we examined remodeling 6 days after MCAO because at this time remodeling had reached a maximum in B6 mice but was considerably less in Bc. A genome-wide scan using the single-QTL model for fold change revealed a significant QTL identical to Canq1 peak (127 to 143 Mb; cM shown in the inset). The most significant group (155 SNPs, \(P=4.17 \times 10^{-14}\); group B) spans a 290-kbp region. The genes in these groups are shown in Table 2. The vertical dashed lines in the inset delimit the 95% confidence interval of Canq1.

### LOD Score Profiling for Collateral Remodeling Identifies a QTL on Chromosome 11

Obstruction of the trunk of a main artery tree cross-connected to an adjacent tree by collaterals, as in MCAO, induces sustained unidirectional flow and shear stress in the collaterals. This induces the collateral vessels to increase their lumen diameter and wall thickness, a process called outward remodeling or arteriogenesis that can be quantified as a fold change in diameter in the operated hemisphere relative to the nonoperated hemisphere. We showed that in mice this process occurs during the first few days after MCAO, and that the amount of remodeling is subject to strong genetic influence. Herein, we examined remodeling 6 days after MCAO because at this time remodeling had reached a maximum in B6 mice but was considerably less in Bc. A genome-wide scan using the single-QTL model for fold change revealed a significant QTL identical to Canq1 and a weaker QTL on chromosome 10 (Figure 6). The relationship between velocity in cross-sectional area proscribes that larger diameter collaterals will have lower fluid shear stress after ligation (eg, MCAO), leading to less shear-induced remodeling. In support of this, a highly significant inverse correlation was obtained for collateral remodeling and baseline diameter (Figure 6), in agreement with previous results. Hence, diameter is a covariate for remodeling. When set as such in the statistical

### Table 2. Candidate Genes Resulting From EMMA Analysis of Chromosome 7 QTL Locus

<table>
<thead>
<tr>
<th>Start (bp)</th>
<th>Symbol</th>
<th>Orient</th>
<th>E</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>132362957</td>
<td>LOC670828</td>
<td>−</td>
<td>Protein</td>
<td>Similar to 40S ribosomal protein S7 (S8)</td>
</tr>
<tr>
<td>132476076</td>
<td>4933440M02Rik</td>
<td>−</td>
<td>mRNA</td>
<td>RIKEN cDNA 4933440M02 gene</td>
</tr>
<tr>
<td>132554205</td>
<td>LOC100043014</td>
<td>−</td>
<td>Protein</td>
<td>Predicted gene 417I</td>
</tr>
<tr>
<td>132588190</td>
<td>Jmdj5</td>
<td>+</td>
<td>Best RefSeq</td>
<td>Jumonji domain containing 5</td>
</tr>
<tr>
<td>132611162</td>
<td>Nsmce1</td>
<td>−</td>
<td>Best RefSeq</td>
<td>Non-SMC element 1 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>132690783</td>
<td>EG244214</td>
<td>+</td>
<td>mRNA</td>
<td>Predicted gene, EG244214</td>
</tr>
<tr>
<td>132695796</td>
<td>Il4ra</td>
<td>+</td>
<td>Best RefSeq</td>
<td>Interleukin 4 receptor, alpha</td>
</tr>
<tr>
<td>132746991</td>
<td>II21r</td>
<td>+</td>
<td>mRNA</td>
<td>Interleukin 21 receptor</td>
</tr>
<tr>
<td>132784468</td>
<td>Gtf3c1</td>
<td>−</td>
<td>Best RefSeq</td>
<td>General transcription factor III C 1</td>
</tr>
</tbody>
</table>

Genes in bolded rows have \(P<2.2 \times 10^{-5}\); genes in nonbolded rows have \(P<4.17 \times 10^{-4}\). Orient indicates orientation of transcription.
Correlates with infarct volume. In the present study, we being at the 2 extremes, and that this variation strongly background, with C57BL/6 (B6) and BALB/c (Bc) mice that these traits in the brain vary widely with genetic modeling after arterial occlusion. We recently reported mice6,14–20 and potentially in humans, and collateral re-

Discussion

This study sought to identify the genetic determinants responsible for the wide variation in the number and diameter of native (preexisting) collaterals in healthy tissue of inbred mice6,14–20 and potentially in humans, and collateral re-

model, the QTL on chromosomes 7 and 10 were lost, and a significant QTL on chromosome 11 (LOD score 3.5) was obtained (Figure 6, blue curve; Table 1). These results are consistent with the expectation that genes regulating collateral formation (ie, native number and diameter) in the embryo14 are likely to differ from those regulating remodeling in the adult.6

Figure 6. Collateral remodeling after MCA occlusion: genome-wide mapping identifies a QTL on chromosome 11. Diameter measurements made in 190 F2 mice 6 days after right-side MCA occlusion were subjected to single QTL mapping. Black Curve, Native collateral diameter (non-occluded side); the LOD score is lower than in Figure 1 because there were fewer mice (see Online Methods). Red Curve, Fold increase in collateral diameter after remodeling (occluded over nonoccluded). Blue Curve, Fold increase in diameter after rescanning with native collateral diameter as a covariate (see inset for close correlation between fold increase in diameter in the right hemisphere and native collateral diameter). The chromosome 7 QTL for remodeling (red curve) was lost (blue curve), but a QTL for remodeling on chromosome 11 achieved significance (Carq1, Table 1; P<0.05).

obstruction in the brain, heart and peripheral limbs may be a person’s genetic predisposition influencing the extent of the collateral circulation.

In the study by Dokun et al.,22 the magnitudes of necrosis score and recovery of perfusion assessed 21 days after femoral artery ligation depended in large part on both collateral extent and collateral remodeling, although additional mechanisms could contribute.1–6,16–20 However, infarct volume assessed 24 hours after MCAO,23 when only minimal remodeling of pial collaterals could have occurred, depends to a larger degree on native collateral extent. Given the dependence of shear stress-induced remodeling on collateral diameter, our results argue strongly that variation in native collateral extent exerted by the chromosome 7 locus is the major biological substrate underlying both previously re-

infarct volume assessed 24 hours after MCAO. The latter effect is opposite to what is expected, suggesting that secondary changes in vascular structure, smooth muscle tone or pressure-independent genetic factors also linked to the rat locus might instead be involved.24 In addition, the location of the telomeric end of the introgressed piece corresponds to 128 Mb in mouse chromosome 7, which is well upstream of the peaks suggested by EMMA and the multiple QTL analysis. Finally,
arterial pressures in Bc and B6 mice are virtually identical, whether measured in the anesthetized or conscious state.\textsuperscript{25–28} Thus, these findings do not support arterial pressure differences as a physiological mechanism underlying the common QTL identified on chromosome 7 in the present or previous studies.\textsuperscript{22,23}

Majid et al\textsuperscript{20} found no significant difference in blood flow velocity between the B6 and Bc strains measured with a laser Doppler probe in an estimated 1 mm\textsuperscript{3} of tissue in the “core of the MCA ischemic territory” 10 minutes after MCAO, and concluded that nonhemodynamic variables (ie, including no differences in collateral extent) are responsible for the different infarct volumes in the 2 strains. However, several considerations call this conclusion into question: Values trended lower for the Bc mice. The Doppler probe that was used measures velocity rather than flow; velocity can remain unchanged despite reduced flow if diameter also declines (eg, by passive collapse after MCAO), measurement of overall cerebral blood flow velocity in the MCA territory from a single-point probe is unreliable (eg, 24). Lastly, the Doppler signal includes blood flow in the dura mater, which is not supplied by the MCA.

Canq1, LSq-1,\textsuperscript{22} and Civq1\textsuperscript{23} are broad loci that contain many genes. The gene list under LSq-1 was reduced by interval-specific haplotype association mapping based on the criterion that haplotypes under the QTL would be identical in Bc and A/J mice and different from B6.\textsuperscript{22} For cerebral infarct volume (Civq1), the criterion was expanded to include strain SWR, further reducing the list of candidate genes to only 12 in a 10 Mb region from 132.47 (49334400M02RIK) to 141.000 known, high quality SNPs in the 15 Mb region. Low statistical power and high false-positive rate can arise in haplotype mapping in mice because of genome-wide application, limited sample size, and lack of control for population structure.\textsuperscript{21} Therefore, we confined our association mapping to 127 to 142 Mb (Canq1) and native collateral number from 15 inbred strains (n = 8 to 10 of each strain; 18). We also used dense markers to infer the kinship matrix for the 15 strains. Finally, we applied the resultant kinship matrix to the EMMA algorithm to model population structure as a random effect to account for the population structure problem. This analysis pointed to a narrow region encompassing 9 genes, of which 7 failed to satisfy the haplotype criterion applied by Dokun et al and by Keum and Marchuk. Thus, only one gene (49334400M02RIK, a predicted gene with EST support but no annotation) is common to the list of Keum and Marchuk and the list suggested by EMMA. There are no known microRNAs in the high-probability 9-gene EMMA region, nor is it known whether other classes of regulatory DNA or RNA elements (eg, long noncoding RNAs) are present. Future work will be needed to identify the genetic element(s) underlying the major QTL on chromosome 7 and to define the molecular pathways controlling collateral formation in the embryo and thus the wide variation in the adult.

Several genes with known influences on native collateral extent or collateral remodeling were not detected in our analysis. Experiments using mice haploinsufficient or null for Vegfa, Flt1 (VEGF receptor 1) and Clic4, have shown that these molecules are strong positive determinants of perinatal collateral formation and maturation and thus native collateral number and diameter in the adult (J. Lucitti and J.E.F., unpublished data, 2010).\textsuperscript{19,20} Furthermore, induction of VEGF-A expression is significantly lower in Bc compared to B6 (expression of Flt1 and Clic4 have not been examined in Bc versus B6).\textsuperscript{14,16,19,30,31} However, we found no QTL in the regions of these genes in either single or multiple QTL analysis. In addition, no interaction between Canq1 and any other locus was found when all main effects and the epistasis between Canq2 and Canq3 were fixed. This outcome was expected for Vegfa and Clic4, because these genes and their flanking sequences are essentially identical in B6 and Bc. The Flt1 gene and flanking sequences are different between B6 and Bc, and are identical in Bc and A/J. However, whether this has functional consequences in the context of the B6 and Bc backgrounds is not known. One or more of the genetic elements underlying Canq1-Canq4 may act in a weak trans manner to influence expression of VEGF-A, Flt1, or Clic4, or other molecules involved in collateral formation may compensate for deficiencies in this pathway in Bc mice. Additional studies will be required to determine the role(s) of variants underlying the QTL identified in the present study.

Ingenuity pathway analysis within the 95% confidence interval of Canq1 found enrichment for immune response genes, and interleukin (IL)4 receptor α and IL21 receptor were among the 9 genes in the high significance region identified by EMMA association mapping. Whereas collateral remodeling is known to be highly dependent on the immune system,\textsuperscript{1–5} no studies have examined whether collateral formation and maturation during the perinatal period, which determine native collateral number in the adult, involve immune cell function. Van Weel et al\textsuperscript{32} found evidence suggesting involvement of the known difference in natural killer cell (NKC) function in B6 versus Bc mice, associated with a different haplotype on distal chromosome 6 involving the NKC gene complex locus,\textsuperscript{23} in the difference in recovery of blood flow after femoral artery ligation in these strains (which is dependent, in large part, on native collateral extent and collateral remodeling). However, this group recently reported, using congenic mice with this locus introgressed into the opposite strain, that the difference in native collateral number does not depend on the NKC locus.\textsuperscript{34} This is consistent with our finding of no QTL on distal chromosome 6. It also is consistent with lack of agreement of the aforementioned QTL on chromosome 6 with the NKC gene complex locus,\textsuperscript{33} in the difference in recovery of blood flow. Our results linking variation in remodeling of pial collaterals to its dependence on initial collateral diameter and thus the chromosome 7 locus, plus the diameter-independent locus on chromosome 11, are not congruent with a role for chromosome 6 in collateral remodeling. Van Weel et al did not examine if mice congenic for the chromosome 6 NKC locus show transference of differences in the remodeling phenotype.

We identified different genetic architectures for variation in collateral number, diameter and collateral remodeling.
Native collateral number and diameter in young adult mice (eg, 10 week-old as studied herein) are determined by 2 processes: collateral formation, which occurs late embryonically after the general circulation has formed; and collateral maturation, which occurs during the first 3 postnatal weeks.1,4 The former involves sprouting of a unique plexus of arteriole-fated endothelial cell tubes, whereas the latter involves pruning away of a portion of these nascent collaterals, followed by lumen enlargement and smooth muscle cell investment of those that are retained.4,14 Collateral remodeling, which occurs in obstructive disease in the adult, involves a complex signaling and restructuring process.1–5 Although much is known about the molecular signaling pathways directing collateral remodeling, almost nothing is known about those directing formation and maturation.6 The very different mechanisms and times of occurrence of the 3 processes suggest they are guided, at least in part, by genes unique to each. In support of this, collateral number and diameter shared a common QTL (Cagq1), whereas number and diameter, and amount of remodeling have unique rank-orders among 15 inbred strains.18 Importantly, all 3 traits are critical determinants of the severity of stroke and ischemic disease of the heart and other tissues.6

In conclusion, our findings show that the extent and remodeling of the native collateral circulation is subject to wide variation that is attributable primarily to a remarkably strong polymorphism on chromosome 7, with smaller contributions from several additional QTL. These findings provide the underlying physiological basis for a recently reported QTL at the same locus on chromosome 7 linked to severity of hindlimb ischemia and cerebrovascular stroke.22,23 Variation in collateral traits reflect yet-to-be identified genetic and environmental factors that impact formation and maturation of collaterals, maintenance of them during natural growth to adulthood and subsequent aging, and collateral remodeling in obstructive disease. Our findings establish a foundation for future studies to identify the alleles and molecular pathways that direct these processes and account for their wide variability among healthy individuals, with obvious implications for patient evaluation and management.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- Most tissues have a collateral circulation composed of a small number of native preexisting "endogenous bypass vessels" interconnecting adjacent arterial trees, whose extent (density and diameter) is a critical determinant of the severity of tissue injury in obstructive diseases such as stroke, coronary artery disease, and peripheral artery disease.

- The extent of native preexisting collaterals (ie, conductance of the collateral circulation), plus their amount of lumen enlargement (growth/remodeling) in obstructive disease, exhibits remarkably wide variation dependent, in part, on differences in genetic background.

- The genetic loci responsible for variation in native collateral extent or collateral remodeling induced by arterial obstruction have not been identified.

**What New Information Does This Article Contribute?**

- The cerebral collateral circulation and its response to middle cerebral artery occlusion in in-bred mouse strains was used to identify 4 quantitative trait loci (QTLs) that together comprise the majority of the heritable difference in native collateral extent; a different QTL was identified for variation in collateral remodeling.

- Association mapping narrowed the potential candidate genes from ~300 to 9 within the main locus on chromosome 7.

The native density and diameter of collaterals, as well as their outward remodeling in obstructive disease (arteriogenesis), vary widely, in part because of unknown genetic factors. We show that in mice, this genetic variation maps to a remarkably strong polymorphism on chromosome 7. Moreover, our findings provide the underlying physiological basis for a recently reported QTL at the same locus linked to differences in severity of limb ischemia and stroke. Variation in collateral extent in tissues reflects yet-to-be identified genetic and environmental factors that impact the formation and the maintenance of these vessels. Our findings provide a foundation for future studies to identify the alleles and molecular pathways that direct these processes and account for their wide variability among even healthy individuals. Such knowledge may permit stratification of patients according to collateral extent for treatment with arteriogenic therapies, in clinical trials to assess new arteriogenic therapies, and in assessment of risk severity for stroke, myocardial infarction, and peripheral artery disease. This new understanding may also lead to novel therapies to promote formation of new collaterals in patients with collateral circulatory insufficiency.
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Supplemental Materials

Expanded Materials and Methods
Supplemental References
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Online Figure II
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Expanded Materials and Methods

Animals
Mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). For linkage analysis, F1 progeny were obtained from reciprocal matings of C57BL/6J (B6) and BALB/cByJ (Bc) mice (B6 female X Bc male and the reverse), then mated to produce a total 243 of F2 mice (128 males and 115 females). Association mapping was done using the data of Zhang et al. (1); see Supplemental Figure 1, obtained from 10-12 week old individuals (n = 8 -10) of the following strains: C57BLKS/J (BLKS), FVB/NJ (FVB/N), CBA/J (CBA), DBA/2J (DBA/2), NOD/ShiLtJ (NOD), SJL/J (SJL), 129S1/SvImJ (129S11), NZW/LacJ (NZW), KK/HiJ (KK), C3H/HeJ (C3H), A/J (A), AKR/J (AKR), SWR/J (SWR), B6 and Bc. The importance of chromosome 7 was confirmed using C57BL/6J-Chr7A/J/NaJ (chromosome substitution strain-7, CSS7) and C57BL/6J-Chr17A/J/NaJ (CSS17). Mice were housed in a temperature- and humidity-controlled environment with a 14-h:10-h light-dark cycle. After weaning, mice were maintained on a chow diet (Old Guilford 234A; Guilford) until 10-12 wk of age. Surgical procedures were conducted following the protocol approved by the IACUC of the University of North Carolina at Chapel Hill.

Phenotyping
Middle cerebral artery occlusion (MCAO) was conducted as described previously (1). Briefly, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) ip. A 4-5mm incision was made between the right eye and ear, the temporal muscle was separated at its midpoint, and a ~2mm burr-hole over the trunk of the MCA in the right hemisphere was drilled with a handheld drill. The MCA was cauterized and transected, and the incision was closed. Mice were prepared for collateral number and diameter measurement 6 days later as described previously (1). Briefly, under deep anesthesia, the circulation was flushed with PBS-heparin via retrograde cannulation of the abdominal artery, then maximally dilated with adenosine and papaverine. The dorsal calvarium and adherent dura mater were removed to allow visual control. The thoracic aorta was cannulated retrograde and yellow MicrofilR (FlowTech, Inc., Carver, MA) with viscosity sufficient to prevent capillary filling was infused under a stereo microscope. The tissue was then fixed with topical paraformaldehyde (4%). On digital images (Leica MZ16FA, Bannockburn, IL) of the pial circulation, collaterals connecting the anterior cerebral (ACA) and middle cerebral (MCA) artery trees in both hemispheres were counted and measured for lumen diameter, defined as the average of diameter measures at 3-4 positions along the mid-zone of the collateral. After rejection of certain specimens for incomplete fills, damage to the pial surface, absence of a single central MCA trunk, etc., 221 mice were studied for native collateral number and diameter, and 190 mice were studied for fold-change in collateral diameter following MCAO. Cortical territories supplied by the MCA, ACA and PCA trees were determined morphometrically (1). ANOVA or two-sample t-tests were used to compare the collateral traits between different strains. P values were adjusted by the Bonferroni method when appropriate.
DNA isolation and genotyping

A standard high-salt protein precipitation procedure followed by isopropanol precipitation was used to isolate genomic DNA from proteinase K-digested tails. Genotyping was carried out in the UNC Genotyping Core Facility using the 377-SNP GoldenGate genotyping array (Illumina, San Diego, CA). Manual genotyping (PCR + BsrGI digestion) was done at an additional marker on chromosome 7 (rs32420445). SNP positions were obtained from Build 37.1 of the NCBI SNP database. A total of 228 SNPs were used for QTL analysis.

Linkage analysis

Native collateral traits are defined as collateral number, diameter and "conductance" (number X diameter) measured in the left (non-occluded) hemisphere. The remodeling trait is the average fold change of collateral diameter between left and right hemisphere 6 days after MCAO. These traits were subjected to linkage analysis using R/QTL in R 2.9.1 (downloads at website: http://www.r-project.org). Because no relationship between collateral number and diameter, and no effects of parental origin, gender, or body weight, were found (Supplemental figures 2-4), we first did single QTL analysis with no covariate (7). The native collateral and remodeling traits were then examined for multiple QTL and possible interactions (ie, subjected to model selection) in a stepwise QTL analysis according to Manichaikul et al. (3). For this, we, first, imputed genotype data at every 1 cM using a Monte Carlo algorithm (8). Then, we defined the penalty LOD score according to the formula given in Manichaikul's paper as the criterion of model comparison. Here, we used default empirical penalty score for main effects, heavy and light penalties for interaction (3.52, 4.28, 2.69). Finally, we fitted the linear model with the imputed SNP data using the forward/backward model selection to identify the best model, which was defined as the model having the maximum penalized LOD (pLOD) score (Zeng et al. 1999). In this step, we fixed the strongest QTL identified in single QTL model and then, searched for additional QTL and interacting QTL. We repeated this forward modeling until the model reached 7 QTL. We, then, back eliminated the QTL to the null model (no QTL). We calculated the pLOD score for each resultant model and compared these pLODs. The model with maximum pLOD was defined as the best model. To further identify loci interacting with the major effect, we fixed all main effects and interactions identified in the stepwise model selection, and then used likelihood ratio tests to scan the whole genome. The function "fitqtl" in R/qtl was used to calculate additive, dominance, their standard errors and percent variance estimates. This was done by fitting imputed genotypes of a hypothetical marker at each QTL peak into a multiple regression model (6).

Genetic distances and confidence intervals calculated for our F2 population were converted to physical positions, yielding the initial candidate gene list, by plotting the genetic positions of the markers against their known physical positions and extrapolating between marker positions.
Association analysis

Association mapping was done by applying the efficient mixed model association algorithm (EMMA; 2) in R to collateral number from 15 inbred strains (see Animals). The several SNP data sources were downloaded from the link - http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=snps/download and pooled together. We, first, calculated the kinship matrix among 15 inbred strains using high-confidence imputed SNP data within QTL region (9). The kinship matrix represents the pair-wise relatedness among 15 strains. To account for the relatedness among the strains, the kinship matrix was modeled as random effects. Each SNP within the Canq1 was modeled as fixed effect. After the mixed model was fitted, an F test was conducted and p value was obtained at each SNP location.

Supplemental References


Online Figure Legends

Online Figure I. Pial collateral number and diameter and cortical areas for 15 inbred strains used in the EMMA analysis (reprinted from ref 1). Variation in collateral number (per hemisphere) and diameter is unrelated to small variations in cortex area. Number of animals is given at the base of each column. Dashed lines on the dorsal cortex area panel delimit the 21% range of variation among the 15 strains.

Online Figure II. Gender and parental origin have no effect on native collateral dimensions. A, Native collateral number (left panel) and diameter of female and male F2 individuals (n = 221) were not different. B. Average collateral number (left panel) and diameter for the 221 F2 mice grouped according to parental origins in reciprocal crosses. In the open bar group (CxB, CxB; F1 female, F1 male), both F1 parents resulted from mating of a BALB/c dam (C) and a C57BL/6 sire (B). Corresponding notation holds for the other 3 groups.

Online Figure III. Native collateral traits have no relationship to body weight.

Online Figure IV. Collateral number and diameter in the F2 population are largely distinct traits, based on the low $r^2$ from linear regression of the two traits.

Online Figure V. Genome-wide scan finds no significant interactions between chromosome 7 QTL and other loci known to influence collateral traits. Interactions were sought using likelihood ratio tests between full and additive models, with all identified QTL fixed in the linear model (3). From left to right, arrows indicate approximate positions of Clic4, Flt1, and Vegfa, genes whose expression levels are positive determinants of native collateral number and diameter (4; J Lucitti and JE Faber, unpublished results; 5). Empirical p-value threshold for significant interaction is LOD 6.3 (6).

Online Figure VI. Cell-cell signaling and immune response pathways are highly enriched in chromosome 7 QTL region. Pathway enrichment analysis was conducted for all genes between chromosome 7 127 Mb (rs32467297) and 142 Mb (rs32420445) for all known pathways in the Ingenuity database. P values were adjusted for multiple comparisons (Benjamini-Hochberg; B-H p-value) and negative-logarithm-transformed.
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*Location (cM); †Confidence interval (cM); ‡Percent of the total phenotypic variation explained by locus.
§Additive or dominance coefficients; SE, standard error of the mean.
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