High-Resolution Genetic Mapping in the Diversity Outbred Mouse Population Identifies Apobec1 as a Candidate Gene for Atherosclerosis

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ABSTRACT

Inbred mice exhibit strain-specific variation in susceptibility to atherosclerosis and dyslipidemia that renders them useful in dissecting the genetic architecture of these complex diseases. Traditional quantitative trait locus (QTL) mapping studies using inbred strains often identify large genomic regions, containing many genes, due to limited recombination and/or sample size. This hampers candidate gene identification and translation of these results into possible risk factors and therapeutic targets. An alternative approach is the use of multiparental outbred lines for genetic mapping, such as the Diversity Outbred (DO) mouse panel, which can be more informative than traditional two-parent crosses and can aid in the identification of causal genes and variants associated with QTL. We fed 292 female DO mice either a high-fat, cholesterol-containing (HFCA) diet, to induce atherosclerosis, or a low-fat, high-protein diet for 18 wk and measured plasma lipid levels before and after diet treatment. We measured markers of atherosclerosis in the mice fed the HFCA diet. The mice were genotyped on a medium-density single-nucleotide polymorphism array and founder haplotypes were reconstructed using a hidden Markov model. The reconstructed haplotypes were then used to perform linkage mapping of atherosclerotic lesion size as well as plasma total cholesterol, triglycerides, insulin, and glucose. Among our highly significant QTL we detected a ~100 kb QTL interval for atherosclerosis on Chromosome 6, as well as a 1.4 Mb QTL interval on Chromosome 9 for triglyceride levels at baseline and a coincident 22.2 Mb QTL interval on Chromosome 9 for total cholesterol after dietary treatment. One candidate gene within the Chromosome 6 peak region associated with atherosclerosis is Apobec1, the apolipoprotein B (ApoB) mRNA-editing enzyme, which plays a role in the regulation of ApoB, a critical component of low-density lipoprotein, by editing ApoB mRNA. This study demonstrates the value of the DO population to improve mapping resolution and to aid in the identification of potential therapeutic targets for cardiovascular disease. Using a DO mouse population fed an HFCA diet, we were able to identify an A/J-specific isofrom of Apobec1 that contributes to atherosclerosis.

KEYWORDS
quantitative trait loci
multiparental models
lipoproteins
atherosclerosis
MPP
multiparental populations
Multiparent Advanced Generation Inter-Cross (MAGIC)

Identifying novel gene(s) and pathways that regulate susceptibility to atherosclerosis and its underlying risk factors is critically important for biomedical science. Genome-wide association studies (GWAS) have aided in the identification of more than 50 genomic loci associated with atherosclerosis (Holdt and Teupser 2013) and plasma lipid levels (Johansen et al. 2011; Swerdlow et al. 2012). However, elucidation of the mechanisms underlying novel loci resulting from GWAS studies is challenging, and in most cases such loci only explain a fraction of the genetic variance related to the trait (Hardy and Singleton 2009; Lucas et al. 2012; Musameh et al. 2014). For example, GWAS have been successfully used to identify more than 40 loci for coronary artery
disease, but these loci in aggregate explain less than 10% of the phenotypic variance associated with coronary artery disease (Sayols-Baxeras et al. 2014).

Mouse models are well suited for studies of complex traits such as atherosclerosis because environmental conditions and other confounding cofactors can be carefully controlled. This reduces the effects of environmental variation on clinical traits and thus increases the portion of the variance that can be explained by genetics. Recently, a “Multiparent Advanced Generation Inter-Cross” (i.e., MAGIC) population was developed from eight inbred strains of mice and is referred to as the Diversity Outbred (DO) mouse population (Churchill et al. 2012). The DO mice are mosaics of C57BL6/J, A/J, NOD/ShiLtJ, NZO/HiLtJ, WSB/EiJ, CAST/EiJ, PWK/PhJ, and 129S1/SvImJ, and these mice complement another large endeavor called the Collaborative Cross (Aylor et al. 2011). Both the DO and Collaborative Cross populations have tremendous genetic diversity, containing approximately 45 million segregating single-nucleotide polymorphisms (SNPs) (Yang et al. 2011). The DO population is maintained by a randomized outbreeding strategy and thus is an ideal resource for high-resolution genetic mapping due to the high allelic diversity and increased number of recombinations represented by this population compared with typical inbred mouse strains (Svenson et al. 2012).

In the current study, we use DO mice to study plasma lipids and atherosclerosis. We identify a significant quantitative trait locus (QTL) for atherosclerosis with a submegabase resolution within a locus previously identified as associated with atherosclerosis in mice. Using publically available expression data and expression quantitative trait loci (eQTL) analysis, we identify Apobec1 as a high-probability candidate gene. We confirm the eQTL for Apobec1 in the current study using quantitative real-time polymerase chain reaction and quantitate possible coregulation of circulating apolipoprotein B (ApoB) protein levels at this locus.

**MATERIALS AND METHODS**

**Animals and diets**

Female DO mice (n = 292; J:DO, JAX stock number 009376) were obtained from the Jackson Laboratory (Bar Harbor, ME) as 146 full sibling pairs at 4 wk of age and at outbreeding generation 11 (G11) (received September 2012). The mice were group housed (n = 5 mice per cage) with nonirradiated pine bedding and provided with high-efficiency particulate air-filtered air and free access to sterile water in a climate-controlled facility under a 12-hr light/dark cycle. Mice were maintained on a defined synthetic diet, AIN-76A, until 6 wk of age to control for differences due to variable components of standard chow (D10001, Research Diets, New Brunswick, NJ); subsequently, 146 mice were transferred to a synthetic high-fat, cholic acid (HFCA) diet that contained 20% fat, 1.25% cholesterol, and 0.3% cholic acid, to induce atherosclerotic lesions, and 146 mice were maintained on a high-protein diet that contained 5% fat and 20.3% protein, which is not atherogenic (D12109C and D12083101, respectively; Research Diets, New Brunswick, NJ). One sibling from each of the 146 sibling pairs was randomly assigned to each one of the diets, Figure 1. The composition of all diets is listed in Supporting Information, Table S1.

The source of fat from the diets varied between the baseline diet (corn oil) fed to the mice from 4 to 6 wk of age and the dietary treatment groups (soybean oil plus cocoa butter) fed to the mice from 6 to 24 wk of age. All mice were maintained on their respective diets until 24 wk of age, for a total of 18 wk. All procedures were approved by the IACUC at University of North Carolina (UNC) Chapel Hill (IACUC Protocol Number 11-299).

**Plasma clinical chemistries**

To ensure that there were no spurious effects due to the potential variable composition of standard laboratory chow, these mice were maintained on a defined synthetic diet for 2 wk (from 4 to 6 wk of age). At 6 wk of age, the mice were anesthetized using isoflurane and blood was collected after 4 hr of fasting into ethylenediaminetetraacetic acid—containing microtubes from the retro-orbital sinus. This was repeated at 24 wk of age, after 18 wk of dietary treatment. Blood samples were centrifuged for 10 min at 9,000 rcf at 4°C and stored at −80°C. The plasma levels of total cholesterol, glucose, and triglycerides were quantitated using a Biolis 24i Analyzer (Carolina Liquid Chemistries, Winston-Salem, NC). Insulin was determined using the Alpco Mouse Ultrasensitive Insulin ELISA assay (Alpco Inc, Salem, NH); samples and controls were run in duplicate and optical densities were measured at 450 nm using a microplate reader and analyzed with Gen5 Data Analysis Software (Bio-Tek, Winooski, VT). Data are presented as mean ± SD and significance was determined using a Student’s t-test.

![Figure 1](image-url)
Atherosclerotic lesion size

Hearts, including the proximal aorta, were carefully dissected from 24-wk-old mice, perfused with 1X phosphate-buffered saline, and stored in 10% formalin at 4°C. A transverse cut, parallel to the atria, was made to remove the top portion of the hearts, which were then embedded in Optimal Cutting Temperature compound and stored at −80°C. Serial sections (10 μm thick) from the aortic sinus were mounted onto slides. Cross sections were arranged eight to a slide and approximately 10–14 total slides were obtained from each mouse heart, for a total of 80–112 cross sections through 400–560 μm of the aortic sinus per animal. Sections were stained with Oil Red O to measure lipid accumulation. To summarize, slides were fixed with formalin for 15 min, rinsed with water then 60% isopropanol, stained for 1 hr in freshly prepared Oil Red O solution, and rinsed with 60% isopropanol then water. Once stained, the slides were imaged using a Zeiss AxioCam MR3 (Zeiss, Munich, Germany), and atherosclerotic lesion area was quantified using Image J software (http://imagej.nih.gov/ij). Data are presented as the average lesion area in μm².

Apobec1 mRNA isoform expression

Quantitative polymerase chain reaction was performed in triplicate using a High-Capacity Reverse Transciptase Kit (Applied Biosystems, Foster City, CA). Following cDNA conversion, 1 μL of sample cDNA, 2 μL of KAPA Sybr Fast qPCR mastermix (KK4610), and 0.5 μM primers were added to each well of a 384-well plate. Apobec1 primers were custom-designed and ordered from Eurofins MWG Operon (Huntsville, AL). The long transcript was amplified using the following primer set 5’-cagcggtgtgactatccaga-3’ (left primer) and 5’-ttgccaatagctgtt-3’ (right primer). This primer set was designed to recognize the known Apobec1 transcript Apobec1-001 (ENSMUST00000112586.1). The short transcript was amplified using the following primer set 5’-cccatgagcgttg gatte-3’ (left primer) and 5’-icaacacgagccagcctt-3’ (right primer). This primer set was designed to recognize the known Apobec1 transcript Apobec1-004 (ENSMUST0000143356.1). A serial dilution of pooled samples was used to create a standard curve. Sterile water was used as a negative control.

Quantification of ApoB in the DO mice

ApoB protein levels were measured using the mouse Apolipoprotein B Sandwich-ELISA method in 96-well format using the ApoB ELISA kit from Elabscience (Wuhan, China) in duplicate following the manufacturer’s instructions. Optical densities were measured at 450 nm using a microplate reader and analyzed with Gen5 Data Analysis Software (Bio-Tek, Winooski, VT).

Differential expression of genes in peak regions

Data were obtained from The Jackson Laboratory Gene Expression Strain Survey and used for the analysis of differential expression of candidate genes from liver tissue of female C57BL6/J, A/J, NOD/ShiLtJ, NZO/HiLtJ, WSB/Eij, CAST/Eij, PWK/Pbh, and 129S1/SvImJ mice (http://cgdjax.org/gem/strainsurvey26/v1). Mice were maintained for 11 wk on standard chow diet (4% fat content). Genes were identified as differentially expressed between the DO founder strains using analysis of variance and significant between strains differences were calculated using Tukey’s Post Hoc test. We used a Bonferroni correction to determine statistical significance and correct for multiple comparisons.

Genotyping

DNA was extracted and purified from tail biopsies taken from 6-wk-old mice using QIAGEN DNExtract kit according to the manufacturer’s instructions. Genotyping was performed using the Mega Mouse Universal Genotyping Array (MegaMUGA) by GeneSeek (Neogen, Lansing, MI) (Welsh et al. 2012). The MegaMUGA array is built on the Illumina Infinium platform and contains 77,800 SNP markers that are distributed throughout the genome at an average spacing of 33 kb. For the mapping, genomes were reconstructed based on the X and Y allele intensities from the array and founder haplotypes were reconstructed using a hidden Markov model. The reconstructed haplotypes were then used to perform linkage mapping.

QTL mapping

QTL mapping was performed using the R package DOQTL, version 0.99. To summarize, DOQTL reconstructs the genome in terms of founder haplotypes and performs QTL mapping by regressing the phenotypes on the founder haplotypes with an adjustment for kinship between the mice. Diet was included as an additive covariate for postdiet measurements, except lesion size, which was performed using only the subset of mice fed the HFCA diet (n = 146). Candidate genes were identified by position based on the Wellcome Trust Sanger mouse genomes database, www.sanger.ac.uk, release 1303, based on genome assembly GRCm38 (Yalcin et al. 2011). QTL support intervals were defined by the 95% Bayesian credible interval, calculated by normalizing the area under the QTL curve on a given chromosome (Sen and Churchill 2001).

The mapping statistic reported is log of the odds ratio (LOD). The significance thresholds were determined by performing 1000 permutations of genome-wide scans by shuffling phenotypic data in relation to individual genotypes. Significant QTL were determined at a genome-wide P-value of <0.05 and suggestive QTL were determined at a P-value of <0.63. The latter corresponds to one false positive per genome scan (Lander and Kruglyak 1995).

RESULTS

Effects of diet on clinical markers of cardiovascular disease in DO mice

Our studies using the DO mice were designed to examine nutrigenetic or gene × diet interactions and the overall design is outlined in Figure 1. Thus, we used several diets that we outline here. First, to standardize our measurements for this study and our planned future studies, all of the DO mice were placed on a synthetic, chemically-defined diet (AIN-76) upon arrival at the University of North Carolina, Chapel Hill. This diet is henceforth referred to as the baseline diet and was administered for 2 wk. After 2 wk of this defined diet, all 292 DO mice were phenotyped for a variety of clinical traits associated with cardiovascular disease risk. These data were used for QTL mapping. At the end of the 2-wk baseline diet, one mouse from each sibling pair was assigned to one of two diets for subsequent investigation of gene × diet interactions. These diets also were synthetic and chemically defined and included an HFCA diet designed to induce atherosclerosis and a low-fat, high-protein diet not expected to be atherogenic. The DO mice were fed these diets for 18 wk and then phenotyped at 24 wk of age.

There was considerable variation among the mice in the clinical traits measured while the mice were on the baseline diet (Table 1). After we administered the diets, we found there was a robust and statistically significant increase in plasma cholesterol in response to the HFCA diet (199.9 ± 68.6 mg/dL) compared with both the high-protein diet (91.7 ± 25.1 mg/dL) and baseline levels (97.6 ± 31.5 mg/dL) (Figure S1 and Table 1). Conversely, triglyceride levels decreased in response to the HFCA diet (59.3 ± 26.7 mg/dL, P < 0.05) and in mice on the high-protein diet (57.7 ± 30.8 mg/dL, P < 0.05) (Figure S1 and Table 1).
Identifying QTL for clinical markers of cardiovascular disease in DO mice at baseline

We performed QTL mapping for several clinical markers of cardiovascular disease in 6-wk-old mice fed the baseline (AIN-76) diet. Significant QTL were determined at a genome-wide P-value of <0.05 and the QTL support interval was defined using the 95% Bayesian credible interval (Table 2). We identified a highly significant QTL for triglycerides on Chromosome 9 with a peak SNP located at 51.4 Mb (LOD = 11.3; n = 262 mice) (Figure 2A). DO mice carrying the CAST/EiJ allele at the Chromosome 9 QTL have greater triglyceride levels (Figure 3A). There are 34 candidate genes within the QTL interval on Chromosome 9 (Figure 3B). We identified suggestive QTL for total cholesterol at baseline on Chromosome 13 with a peak SNP located at 30.4 Mb (LOD = 6.5; n = 277 mice) (Figure 2B) and for glucose at baseline on Chromosomes 5 and 7 with peak SNPs located at 92.6 Mb on Chromosome 5 (LOD = 7.0; n = 257 mice) and 27.2 Mb on Chromosome 7 (LOD = 6.5; n = 257 mice) (Figure 2C).

Identifying QTL for clinical markers of cardiovascular disease in the DO after dietary treatment

To assess the effects of diet in the DO, we determined the genetic architecture of several markers of cardiovascular disease by quantifying these phenotypes at 24 wk of age after dietary treatment. Because diet had significant effects on the phenotypes, it was included in the mapping model as an additive covariate. We observed a significant QTL for plasma cholesterol on Chromosome 9 with a peak LOD score of 7.54 at 48.3 Mb (47.84–70.04 Mb) (Figure 4A and Table 2). The CAST/EiJ founder haplotype was associated with high levels of cholesterol based on the founder allele contribution in the region (Figure 4B). This is a relatively large QTL interval of 22.2 Mb based on the Bayesian credible interval (P = 0.95) and the entire interval contains 391 known and predicted genes. The peak SNP is within 3 Mb of the peak SNP identified by mapping of baseline triglyceride levels in this population of mice, suggesting that this genes at this locus are critical in the overall regulation of lipid metabolism.

Differential expression of candidate genes in DO founder strain mice

To prioritize candidate genes within the QTL intervals we identified, we analyzed hepatic expression data from the founder strains for the 34 genes in the region from a publically available dataset (http://cgd.jax.org/gem/strainsurvey26). We first identified genes whose expression varies across the progenitor strains using 1-way analysis of variance analysis and using a Bonferroni correction based on the number of probes tested (0.05/68 probes representing the 34 genes in the locus). Based on this analysis, we identified 10 probes representing 9 genes that are differentially expressed: 1110032A03Rik, 1810046K07Rik, Alg9, Bco2, Cytb, Gm684, Pppl1b1, Pts, and Sik2 (Figure S2, A–I).

The DOQTL package identifies the contribution of the founder alleles to the QTL. For the Chromosome 9 triglyceride QTL, we observed that allelic contribution from the CAST/EiJ founder at this locus is associated with greater triglyceride levels, whereas comparatively low triglyceride levels are associated with allelic contribution from the other seven founder strains (Figure 3A). Thus, we next performed pair-wise comparisons using Tukey’s HSD for the nine differentially expressed

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**Table 1** Effects of high-protein, high-fat, cholic acid diets on cardiovascular risk factors in the DO mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Adj Mean Baseline (AIN-76A)</th>
<th>Adj Mean High Protein</th>
<th>Adj Mean High-Fat, Cholic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, mg/dL</td>
<td>277 91.7 ± 25.1</td>
<td>128 97.6 ± 31.5**</td>
<td>136 199.9 ± 68.6**</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>262 59.5 ± 26.5</td>
<td>128 57.7 ± 30.8**</td>
<td>136 32.3 ± 12.1**</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>257 155.2 ± 43.8</td>
<td>130 190.5 ± 49.9</td>
<td>137 177.9 ± 45.1</td>
</tr>
<tr>
<td>Insulin, ng/mL</td>
<td>235 0.8 ± 0.4</td>
<td>129 1.7 ± 1.1*</td>
<td>133 1.4 ± 0.7*</td>
</tr>
</tbody>
</table>

The values shown are means ± SD. Plasma clinical chemistries were measured at baseline (n = 6-wk-old mice), after 2 wk on the synthetic-defined diet AIN-76A and with 4 hr fasting. Mice were transferred to one of two diets, either a high-fat, cholic acid diet or a low-fat, high protein diet, and maintained on these diets for 18 wk. Plasma clinical chemistries were measured again (n = 24-wk-old mice) after diet treatment and with 4 hr fasting. Significant differences between the baseline diet measures and either diet treatment group (*, where P < 0.05) or significant differences between the two different diets after 18 weeks of diet treatment (**, where P < 0.05) are indicated.

**Table 2** QTL for cardiovascular risk factors in the DO mice at baseline and after dietary treatment

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Chr</th>
<th>Peak LOD</th>
<th>Position (Interval, Mb)</th>
<th>Significant (P &lt; 0.05)</th>
<th>Previously Known</th>
<th>KnownGene Candidates</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>9</td>
<td>11.3</td>
<td>51.4 (50.2–51.6)</td>
<td>Yes</td>
<td>Trig1q1 (40–70)</td>
<td>ApoA5</td>
<td>Suto and Sekikawa 2003</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>13</td>
<td>6.5</td>
<td>30.4 (28.7–43.8)</td>
<td>No</td>
<td>Hmgc1s1 (20–100)</td>
<td>Hmgc1</td>
<td>Welch 1996</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>7.0</td>
<td>92.6 (86.3–99.0)</td>
<td>No</td>
<td>Bglu13 (84–110)</td>
<td>Hnf1a, Pdx1</td>
<td>Zhang et al. 2012</td>
</tr>
<tr>
<td>Insulin</td>
<td>19</td>
<td>5.4</td>
<td>58.6 (22.6–60.8)</td>
<td>No</td>
<td>Tanidd1 (36–61)</td>
<td>Not Determined</td>
<td>Kim 2001</td>
</tr>
<tr>
<td>After diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>12</td>
<td>5.8</td>
<td>97.6 (49.6–102.3)</td>
<td>No</td>
<td>Tglq2 (55–89)</td>
<td>ApoB</td>
<td>Srivastava 2006</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>9</td>
<td>7.5</td>
<td>48.3 (47.8–70.0)</td>
<td>Yes</td>
<td>Cq4 (15-50 Mb),</td>
<td>ApoA4</td>
<td>Suto and Sekikawa 2003</td>
</tr>
<tr>
<td>Glucose</td>
<td>12</td>
<td>5.7</td>
<td>70.9 (26.7–75.1)</td>
<td>No</td>
<td>Bglu15 (10–42)</td>
<td>Adam17</td>
<td>Zhang et al. 2012</td>
</tr>
<tr>
<td>Insulin</td>
<td>13</td>
<td>7.0</td>
<td>8.7 (5.7–10.4)</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Clinical markers of cardiovascular disease were measured in study animals after 4 hr of fasting at both 6 and 24 wk of age. Baseline measures are from 6-wk-old fasted mice fed a synthetic diet. Measures at 24 wk of age, after diet, include mice on both the low-fat, high protein diet and the high-fat, cholic acid diet; diet was used as a covariate in the analysis. The statistic reported is log of the odds ratio (LOD), QTL, quantitative trait loci, DO, Diversity Outbred; NA, not available.
genes to identify genes that are differentially expressed in CAST/EiJ compared with the other founder strains and thus match the overall allelic effects at the QTL. Of the nine genes differentially expressed, Bco2 and Pp2r1b most closely match the allele effects of the QTL and are differentially expressed between CAST/EiJ and the other progenitor strains, Figure S2, A–B. We note that the locus we identified is adjacent to a region harboring genes known to influence plasma lipid levels, including Apoa1, Apoa4, Apoa5, and Nnmt. Both Apoa4 and Nnmt are differentially expressed among the strains, but only Nnmt is differentially expressed in CAST/EiJ mice (Figure S3).

Identification of Apobec1 as a candidate for regulating lesion size in DO mice
Atherosclerosis is a complex trait and studies using mice have identified numerous QTL for this trait (Wang et al. 2005); but this trait has not yet been evaluated in the DO mice. Based on previous studies, a HFCA diet can induce formation of atherosclerotic lesions (Paigen et al. 1987). Indeed, we found that none of the mice fed the high-protein diet exhibited any lesions, and our subsequent analyses focus only on the subset of DO mice that were fed the HFCA diet.

We found that 76% of the DO mice were susceptible to atherosclerosis with lesions induced by the HFCA diet, with a range in lesion size from 38 to 33,200 μm². Based on this highly variable phenotype, we were able to identify a highly significant QTL on Chromosome 6 (LOD = 10.7; 122.6–122.7 Mb) (Figure 5A). Although our Chromosome 6 peak SNP, UNC11996440, is within the 95% confidence interval of a previously reported QTL, Ath37, the interval is refined to sub-Mb resolution using the DO. The 95% confidence interval reported for this previously defined QTL encompasses an 11.8-Mb interval, whereas our Chromosome 6 atherosclerosis peak maps to a refined 100,000-kb region containing six genes (Figure 5C). One candidate gene within the Chromosome 6 peak region is Apobec1, the apolipoprotein B mRNA-editing enzyme, which plays a role in the regulation of ApoB, a critical component of LDL, by editing ApoB mRNA to produce the short ApoB48 isoform. Misregulation of Apobec1 results in altered ApoB isoform editing. For example, Apobec1−/− mice have greater levels of ApoB100 compared with the edited isoform, ApoB48 (Nakamuta et al. 1996) and transgenic rescue of Apobec1 in Apobec1−/− animals has been shown to directly alter chylomicron production (Blanc et al. 2012). Apobec1 is an attractive candidate gene for influencing diet-induced lesion size based on the known function of Apobec1 in lipid homeostasis and a causal allele for Apobec1 has not been previously identified as associated with atherosclerosis. Additionally, we found that total cholesterol levels after dietary treatment were significantly correlated with both the short and long isoforms of Apobec1 (r = 0.6 and r = 0.57, respectively), but not ApoB levels in these mice (r = 0.06).
whereas A/J and C57BL/6J express similar levels of the short isoform of Apobec1 increased in both A/J and C57BL/6J mice compared with long isoform of diet and found that on the synthetic diet A/J expresses more of the animals expressing the A/J-specific demonstrate genetic regulation of mRNA editing by More importantly, using AXB and BXA recombinant inbred strains they animals in response to the HFCA diet. The long isoform of Apobec1 was recently identified between A/J and C57BL/6 mice, where C57BL/6 mice express a truncated Apobec1 protein (Hassan et al. 2013). More importantly, using AXB and BXA recombinant inbred strains they demonstrate genetic regulation of mRNA editing by Apobec1, such that animals expressing the A/J-specific isoform of Apobec1 exhibit greater editing efficiency of Apobec1 targets compared with C57BL/6J animals.

We quantitated the expression of Apobec1 in liver tissue from A/J and C57BL/6J mice fed either the HFCA diet or the control synthetic diet and found that on the synthetic diet A/J expresses more of the long isoform of Apobec1 compared with C57BL/6J (Figure 6B), whereas A/J and C57BL/6J express similar levels of the short isoform of Apobec1 while on a synthetic diet (Figure 6A). In response to the HFCA diet, expression of both isoforms of Apobec1 is significantly increased in both A/J and C57BL/6J mice compared with Apobec1 levels in the mice on the synthetic diet (P > 0.05). The short isoform of Apobec1 is induced 2.5-fold in C57BL/6J animals and 3.5-fold in A/J animals in response to the HFCA diet. The long Apobec1 isoform is induced 2.5-fold in C57BL/6J animals and fourfold in A/J animals.

cis-eQTL for Apobec1 exhibit isoform-specific allele effects patterns in the DO mice

Based on the differences in expression of Apobec1 between A/J and C57BL/6J mice, we hypothesized that the expression of Apobec1 may be genetically regulated. Thus, we surveyed two publically available databases for expression QTL data to determine whether a natural variant near the Apobec1 gene affects its expression. Specifically, we queried a panel of inbred strains of mice called the hybrid mouse diversity panel and The Jackson Laboratory’s Diversity Outbred eQTL viewer, data located at http://cgd.jax.org/apps/eqtlviewer-beta/, respectively (Ghazalpour et al. 2012). Apobec1 expression varied significantly among the hybrid mouse diversity panel strains and the major locus regulating expression mapped directly over the Apobec1 gene, at 122 Mb on Chromosome 6 (data not shown). Similarly, the Jackson Laboratory’s DO eQTL viewer identifies a cis-eQTL associated with Apobec1 expression (Figure S4A). Similar to our results, these data demonstrate that the A/J allele is associated with greater expression of Apobec1 (Figure S4B).

Considering our observations of the effect of genetic background on Apobec1 expression in liver tissue and recent observations that mouse genetic background regulates isoform usage in macrophages, we next asked whether there was a difference in the genetic regulation of each of these isoforms among the DO founder strains. We performed QTL mapping of mRNA expression levels of either the short or long isoform of Apobec1 from RNA isolated from liver tissue from the present DO mouse study population. We identified highly significant cis-eQTL for both isoforms on Chromosome 6 with the peak SNP located at 121.8 Mb with a maximum LOD score of 9.9 associated with expression of the short isoform (P < 0.05, n = 252 mice) and at 123.4 Mb with a maximum LOD score of 15.7 associated with expression of the long isoform (P < 0.05, n = 251 mice) (Figure 7, A and B). When we estimate the effect of each founder at each marker along Chromosome 6, we see that CAST/EiJ alleles are associated with greater expression of the short isoform (Figure 7C), whereas A/J alleles are associated with greater expression of the long isoform (Figure 7D). We also mapped the ratio of Apobec1 isoforms and observe a highly significant cis-eQTL with a max LOD score of 41.0 at 122.6 Mb (P < 0.05, n = 251 mice) (Figure 7, E and F).

Apobec1 and ApoB levels are dependent on the genotype of UNC11996440, the Chromosome 6 peak SNP associated with atherosclerosis

Based on the association of the A/J founder with atherosclerotic lesion size, we hypothesized that a causal allele located in the Apobec1 sequence would be private to A/J. However, there are no documented SNPs that are private to A/J within the refined peak region of 122.6–122.7 Mb on Chromosome 6. The peak SNP for this locus, UNC11996440, is located

![Figure 4](image_url)
distal to the *Apobec1* gene on Chromosome 6 at 122.67 Mb, whereas *Apobec1* lies between 122.5 and 122.6 Mb. In addition to the cis-eQTL data for *Apobec1* expression, this finding suggests that a distant SNP affecting *Apobec1* expression may be responsible for the association between the A/J haplotype and atherosclerotic lesion size. However, if the association of the A/J haplotype with increased lesion size in the DO mice is mechanistically related to *Apobec1* expression, then we would expect *Apobec1* expression levels to differ based on the genotype of UNC11996440. We identified T as the major allele (frequency = 0.69) and C as the minor allele (frequency = 0.31) at the tagging SNP UNC11996440. The genotype at this SNP significantly affects *Apobec1* mRNA expression levels ($P > 0.001$) (Figure 8A). We next hypothesized that the association at this locus may be mechanistically related to the role of *Apobec1* in editing its primary target, *ApoB*, as elevated plasma *ApoB* is a known risk factor for atherosclerosis. Therefore, we quantified plasma *ApoB* levels in duplicate using two mouse-specific *ApoB* Sandwich-ELISA plates (N = 80 mice). Plasma samples from only the HFCA diet-fed mice were used and samples were chosen to represent a range of atherosclerotic lesion sizes. As shown in Figure 8B, *ApoB* levels are significantly different between the genotype groups at the tagging SNP UNC11996440 ($P > 0.001$). Therefore, in addition to identifying the A/J-specific isoformal of *Apobec1* as associated with atherosclerotic lesion size in the DO mice, these data suggest that this association may be attributable to the role of *Apobec1* in editing *ApoB*.

**DISCUSSION**

The DO was designed to be a high-resolution genetic mapping panel. The use of DO mice for mapping offers a number of advantages over classical approaches to mouse genetics, including high mapping resolution, increased heterozygosity, and uniformly distributed genetic background. This study demonstrates the utility of DO mice for identifying genetic loci associated with atherosclerosis and provides insights into the molecular mechanisms underlying this disease.
variation across the genome (Svenson et al. 2012). Using the newly
developed DO mouse resource, we were able to refine the positions
and identify new candidate genes for previously mapped QTL for
atherosclerosis, a highly complex phenotype associated with human
disease. We further interrogate this QTL by using publically available
data, by quantitating mRNA levels for Apobec1, and by investigating
the protein target of Apobec1 to identify a potential mechanism for
this QTL. We discuss each of these in detail.

Atherosclerosis in the DO
Atherosclerotic lesion development is the most common cause of
cardiovascular disease. Here, we characterize for the first time the
development of diet-induced atherosclerotic lesions in the newly developed
DO mouse population. In the present study, 292 mice on either a HFCA
diet designed to induce atherosclerotic lesions or a nonatherogenic, low-
fat, high-protein diet were phenotyped for atherosclerosis. We found that
none of the mice fed the high-protein diet exhibited any lesions. We
found that 76% of the DO mice were susceptible to lesions induced by
the HFCA diet, with a range in lesion size from 38 to 33,200 μm². We
detected one locus on Chromosome 6 for atherosclerosis that maps
within the 95% confidence interval of a previously reported QTL,
Ath37 (Ghazalpour et al. 2006), which was identified in studies using
sub-congenic mice between C57BL/6J and CAST/EiJ. The locus in the
current study contains Apobec1, which was originally described as the
enzyme responsible for deamination of a cytosine in mature ApoB
mRNA, resulting in a premature stop codon and production of the
truncated protein ApoB48. Lipoproteins containing ApoB48 are more
efficiently cleared from the circulation as demonstrated by adenoviral
overexpression studies that reduce plasma lipid levels (Teng et al. 1997);
in contrast, Apobec1−/− mice synthesize only ApoB100 and have in-
creased atherosclerosis when crossed to Ldlr−/− mice (Powell-Braxton
et al. 1998).

Figure 6 A/J preferentially expresses the long isoform of Apobec1 in response to a high-fat, cholic acid diet. Apobec1 expression levels of the short (A) and long (B) isoforms from RNA from liver tissue from A/J and C57BL/6J founder strain mice. A/J mice on a high-fat, cholic acid diet exhibit increased expression of both the long and short transcripts of Apobec1 in a diet-dependent manner, *P > 0.05. Apobec1 levels for each sample were normalized relative to RPS20. Fold changes are reported as the relative expression in A/J vs. C57BL/6J samples. Data are presented as mean ± SD, and significance was determined using a Student’s t-test.

Figure 7 Cis-eQTL for hepatic Apobec1 short and long isoforms in the DO mice. Genome-wide QTL scan for eQTL regulating expression of the short (A) and long (B) Apobec1 isoforms in the DO mice. Chromosomes 1 through X are represented numerically on the x-axis, and the y-axis represents the LOD score. The relative width of the space allotted for each chromosome reflects the relative length of each chromosome. Colored lines show permutation-derived significance thresholds (N = 1000) at P = 0.05 (LOD = 7.57, shown in red), P = 0.10 (LOD = 7.17, shown in orange), and P = 0.63 (LOD = 5.79, shown in yellow). The eight coefficients of the QTL model show the effects on the phenotype contributed by each founder haplotype on Chromosome 6 for mapping of the short (C) and long (D) isoforms of Apobec1. Shading identifies the 95% Bayesian credible interval around the peak. eQTL, expression quantitative trait loci; DO, Diversity Outbred; LOD, log of the odds ratio.
Atherosclerosis. Genotyping was performed using the Mega Mouse Universal Genotyping Array (MegaMUGA). Apobec1 mRNA levels were measured by quantitative polymerase chain reaction from liver tissue from the DO mice. Expression levels of the Apobec1 long isoform differed significantly between genotype classes (P > 0.001) (A). ApoB protein levels were measured in a subset of the mice phenotyped for atherosclerosis (N= 80 mice) using a mouse Apolipoprotein B Sandwich-ELISA method. ApoB expression levels differed significantly between the genotype classes (P > 0.001) (B).

Closer examination of the locus we identified here indicates that A/J mice contain a susceptible allele, which is unexpected as A/J has classically been defined as an atherosclerosis-resistant mouse strain (Paigen et al. 1985). However, it is possible that if Apobec1 editing is affected by an allele carried by the A/J strain, this effect can be masked by transgressive variation or epistasis in inbred A/J animals. When we queried the Sanger SNP database for alleles private to A/J within 10 kb of the Apobec1 gene sequence (122,567,890–122,612,426 Mb), we found that there are no documented SNPs that are private to A/J. However, the association of distant SNPs influencing gene expression of genes as far as 300 kb away is not uncommon (Guenther et al. 2014). Although our data suggest that a distant SNP affecting Apobec1 expression may be responsible for the association between the A/J haplotype and atherosclerotic lesion size, we have not yet ruled out an association between lesion size and the other five genes in the locus, nor have we ruled out nearby genes with A/J-specific alleles. Additionally, in our study we did not find lesion size to be correlated with Apobec1 expression (r = −0.02) although total cholesterol was correlated with this phenotype (r = 0.60).

Recently, Hassan et al. (2013) have reported that genetic differences between C57BL/6J and A/J at the Apobec1 gene affect global RNA editing patterns between these strains. Indeed, this study identified an A/J-specific isoform of Apobec1 in murine macrophages that increased the editing efficiency of this enzyme for ApoB as well as other Apobec1 targets (Rosenberg et al. 2011; Hassan et al. 2013). Thus, differences in Apobec1 structure or expression could affect editing or perhaps overall levels of ApoB or another of Apobec1 targets. Our initial studies, in a subset of the DO mice used in this study, indicate that total ApoB levels may be influenced by this locus.

**Mapping clinical markers of cardiovascular disease in the DO**

Measures of cholesterol, triglycerides, and glucose are commonly used markers of cardiovascular disease. Therefore, we were interested in investigating the genetic architecture of these traits in the DO mice. We identified highly significant QTL for triglycerides in mice at baseline. The significant QTL on Chromosome 9 for baseline triglycerides is 1.4 Mb in size and is coincident with a ~30 Mb QTL previously identified as associated with triglyceride levels in C57BL/6J × KK-Ay/a F2 mice, Trigq1. Trigq1 maps to 61 Mb on Chromosome 9 with a peak LOD score of 4.2 at the marker D9Mit163 (Suto and Sekikawa 2003). The large Trigq1 locus includes multiple gene candidates known to regulate lipid levels such as the ApoA5-ApoA4-ApoA3-ApoA1 gene cluster and Lipc. Although the ApoA5-ApoA4-ApoA3-ApoA1 gene cluster is located at 46.2 Mb, just proximal to the QTL interval we identified here and Lipc is located 20 Mb distal to our peak region, these genes would seem to be excluded based on our results. However, at least one of these genes, ApoA5, has been well characterized as associated with triglyceride levels, (Charlton-Menys and Durrington 2005). In humans, a mutation in ApoA5, also called the Delhi gene, is linked to extremely elevated triglyceride levels and increased risk of cardiovascular disease (Sinha et al. 2010; Ramakrishnan et al. 2011). Hepatic lipase, encoded by the Lipc gene, hydrolyzes triglycerides and phospholipids in lipoprotein particles and is therefore also likely to be functionally associated with heart disease. We used publicly available gene expression data to prioritize genes whose expression may be genetically regulated. These analyses are not able to identify candidate genes that have a functional variant that affects protein function, protein stability, or binding. Further study to confirm that one of the genes in the triglyceride QTL identified in this study is a causal gene remains to be performed.

This region of Chromosome 9 clearly contains multiple genes that are important for the regulation of cardiovascular disease risk factors. We also identified the same region as associated with total cholesterol after dietary treatment in our DO mouse population. Additionally, the human syntenic region of the Chromosome 9 QTL we identified as associated with both baseline triglycerides and total cholesterol after dietary treatment has been identified in several human GWAS studies as associated with multiple atherosclerosis risk factors across multiple human populations (Hu et al. 2013; Weissglas-Volkov et al. 2013; Zhou et al. 2013). When one QTL contains more than one causal variant, the Bayesian interval calculation may fail to differentiate genetic signals from the closely linked genes. Several studies have suggested the use of 2-LOD support intervals for generating conservative estimates of genes for candidate testing. Indeed, in our study the 95% Bayesian credible intervals represent approximately 1-LOD support intervals for baseline triglycerides and for total cholesterol after diet...
treatment and if we broaden these intervals to 2-LOD support intervals, the apoA5-ApoA4-ApoA3-ApoAl gene cluster and Lipt gene falls within the more conservative interval estimate.

We identified a suggestive QTL for total cholesterol on Chromosome 13 with a peak SNP located at 30.4 Mb (LOD = 6.5; n = 277 mice) and for glucose on Chromosome 5 with a peak SNP located at 92.6 Mb (LOD = 7.0; n = 257 mice). These suggestive QTL co-localize with previously reported QTL. The Chromosome 13 locus associated with baseline cholesterol levels is just proximal to Lipp2, which was identified in a backcross between MOLF/EiJ and C57BL/6J mice on with an Ldrr−/− mutation (Welch et al. 2001). The suggestive QTL associated with blood glucose on Chromosome 5 is less than 2 Mb from the peak SNP reported for the QTL, Bglu13, which was identified in an F2 intercross cross of mutant C3H/HeJ and C57BL/6J carrying the ApoE−/− mutation (Zhang et al. 2012).

In summary, we demonstrate here the use of the DO mice for high-resolution mapping of traits related to atherosclerosis. We identify several candidate genes for triglycerides in mice on a synthetically defined diet. Perhaps the most interesting result is the identification of an atherosclerosis QTL on Chromosome 6 that is 100 kb in size. This locus contains the candidate gene Apobec1 which is known to alter circulating lipoprotein composition, but also has widespread RNA editing capabilities, perhaps indicating an additional mechanism by which susceptibility to atherosclerosis is regulated.

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