Rat Mcs1b Is Concordant to the Genome-Wide Association-Identified Breast Cancer Risk Locus at Human 5q11.2 and MIER3 is a Candidate Cancer Susceptibility Gene

Aaron D. denDekker1, Xin Xu1, M. Derek Vaughn1, Aaron H. Puckett1, Louis L. Gardner, Courtney J. Lambring1, Lucas Deschenes1, and David J. Samuelson1,2

Abstract

Low-penetrance alleles associated with breast cancer risk have been identified in population-based studies. Most risk loci contain either no or multiple potential candidate genes. Rat mammary carcinoma susceptibility 1b (Mcs1b) is a quantitative trait locus on RN02 that confers decreased susceptibility when Copenhagen (COP)-resistant alleles are introgressed into a Wistar Furth (WF)-susceptible genome. Five WF.COP congenic lines containing COP RN02 segments were compared. One line developed an average of 3.4 ± 2.0 and 5.5 ± 3.6 mammary carcinomas per rat ± SD when females were Mcs1b-resistant homozygous and Mcs1b heterozygous, respectively. These phenotypes were significantly different from susceptible genotype littermates (7.8 ± 3.1 mean mammary carcinomas per rat ± SD, P = 0.0001 and P = 0.0413, respectively). All other congenic lines tested were susceptible. Thus, Mcs1b was narrowed to 1.8 Mb of RN02 between genetic markers ENSRNOG0000002740854 and g2UL2-27. Mammary gland-graft carcinoma susceptibility assays were used to determine that donor (P = 0.0019), but not recipient Mcs1b genotype (P = 0.9381), was associated with ectopic mammary carcinoma outcome. Rat Mcs1b contains sequence orthologous to human 5q11.2, a breast cancer susceptibility locus identified in multiple genome-wide association studies. Human/rat MAP3K1/Map3k1 and mesoderm induction early response (MIER; MIER3)/MIER3 are within these orthologous segments. We identified MIER3 as a candidate Mcs1b gene based on 4.5-fold higher mammary gland levels of MIER3 transcripts in susceptible compared with Mcs1b-resistant females. These data suggest that the human 5q11.2 breast cancer risk allele marked by rs889312 is mammary gland autonomous, and MIER3 is a candidate breast cancer susceptibility gene. Cancer Res; 72(22); 6002–12. ©2012 AACR.

Introduction

Low-penetrance breast cancer susceptibility alleles have been identified using human genome-targeted and genome-wide association study (GWAS) designs (1–13). Apart from identifying complex disease risk-associated genetic variation, human studies alone are limited in pinpointing candidate genes and functional characterization of susceptibility associated loci. Comparative genetics based on experimental organisms such as Rattus norvegicus (laboratory or Norway rat) recapitulate the complex genetics, and may complement and enhance human studies of breast cancer risk and prevention (14, 15).

The laboratory rat provides a good model of human breast cancer. Rat mammary carcinomas closely resemble human breast carcinomas in histopathology, hormonal responsiveness, and potential environmental etiologies (16–22). Rats develop spontaneous, carcinogen-, and oncogene-induced mammary carcinomas (23). Several rat strains representing different spectrums of genetic variation in susceptibility to mammary carcinogenesis exist (24–29). Genotypic differences in susceptibility are not necessarily because of carcinogen-induced differences in metabolism or DNA damage (26, 30).

Multiple rat mammary carcinoma susceptibility (Mcs) quantitative trait loci (QTL) have been identified (28, 29, 31). Rat Mcs QTLs that are concordant to human breast cancer risk alleles may be used to identify genes and mechanisms controlling breast cancer susceptibility in women. Comparative genetic approaches have been used to identify MCS5A1 and MCS5A2, which are common noncoding breast cancer risk alleles on human Chr 9 (1). Human MCS5A1 has been confirmed to associate with breast cancer risk in additional population-based studies (9). Another rat QTL, Mcs6, was mapped to an orthologous region of human Chr 12, which potentially associates with breast cancer risk (32). Rat genetics will continue to...
play an important role in elucidating breast cancer risk alleles, candidate genes, and molecular mechanisms.

**Rat Mcs1b** was initially mapped to a 16 cM region of rat Chr 2 (33). Positional mapping was completed in WF.COP congenics by introgression of a segment of resistant Copenhagen (COP) Chr 2 into a susceptible Wistar Furth (WF) genetic background. Mcs1b-resistant alleles decrease mammary carcinoma multiplicity compared with susceptible WF alleles. We have mapped rat Mcs1b to a shorter genomic interval, and found that it contains the rat ortholog to a 5q11.2 region that is marked by SNP rs889312 (2). This locus has been confirmed to associate with human breast cancer risk-associated allele at human Chr 5q11.2 that is marked by SNP rs889312 (2). We have further used rat Mcs1b congenic lines to investigate functional aspects of these alleles and identify mesoderm induction early response (MIER; MIER3) as a strong mammary cancer susceptibility gene.

**Materials and Methods**

**Animals and phenotyping**

Congenic rat lines were maintained in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)—approved facility on a 12-hour light/dark cycle and provided LabDiet 5001 Rodent Diet (PMI Nutrition International) and water *ad libitum*. All animal protocols were approved by the University of Louisville Animal Care and Use Committee (Louisville, KY). Congenics are defined as genetic lines that carry defined COP alleles introgressed into the inbred WF/NHsd (Harlan) genome. Information for genetic markers defining ends of COP alleles carried by each congenic line T, N3, F3, W2, U2, and 14 is available at the UCSC Genome Browser (www.genome.ucsc.edu). Rat Genome Database (http://rgd.mcw.edu/), NLM-National center for Biotechnology Information (NCBI), or Supplementary Table S1. At 50 to 55 days of age, 7,12-dimethylbenz(a)anthracene (DMBA, 20 mg/mL sesame oil) was given by a single oral gavage (65 mg DMBA/kg body mass). Mcs phenotypes were determined by counting (multiplicity) mammary carcinomas 3 × 3 mm² or more that developed 15 weeks after carcinogen (33).

**Mammary gland grafting**

Mammary gland grafting experiments used WF.COP line N3 females at N16F5, N16F6, and N16F7 generations to supply Mcs1b-resistant alleles. At the N15 generation, line N3 was backcrossed to the inbred WF/NHsd strain and reintrogressed into the inbred WF/NHsd (Harlan) genome. Congenics were fixed at the 10th generation. Genotypes were confirmed using standard protocols and assigned accession numbers at the National Center for Biotechnology Information (NCBI) or Supplementary Table S1. Congenics are defined as genetic lines that carry defined COP alleles introgressed into the inbred WF/NHsd (Harlan) genome. Information for genetic markers defining ends of COP alleles carried by each congenic line T, N3, F3, W2, U2, and 14 is available at the UCSC Genome Browser (www.genome.ucsc.edu).

**Quantitative PCR**

Total RNA was isolated with TRI-Reagent (Molecular Research Center) from flash-frozen and homogenized tissues. To reduce possible solvent and DNA contamination, RNA samples were further processed by a 1/10 v/v 3 mol/L sodium acetate and 2.5× v/v 100% ethanol wash on ice for 10 minutes followed by 80% ethanol wash followed by Turbo DNase (Life Technologies). Total RNA quantity and quality were measured with a Nanodrop 1000 (Fisher Scientific) and a Bioanalyzer with RNA 6000 NanoChips (Agilent). Reverse transcription reactions (20 μL f.v.) that contained 1 μg total RNA, 0.5× RNasearch, 5 μmol/L random hexamers, 25 ng/μL oligo(dT18), and 0.5 mmol/L dNTPs were incubated for 5 minutes at 65°C before adding 1× first-strand buffer, 100 mmol/L DTT, and 1 μL Superscript III (Life Technologies). Reactions were incubated for 5 minutes at 25°C, 1 hour at 50°C, and 15 minutes at 70°C. Quantitative PCR (qPCR) was conducted as previously published (1) using TaqMan MGB probes (Life Technologies) and primers in Supplementary Table S3. Except, 60 mmol/L each Rp1lp2 primer and 120 mmol/L VIC-labeled Rp1lp2 probe were used as an endogenous control. Fluorescence values were measured using SDS v2.3 software (Life Technologies).

**Plasmid construction and MIER3 expression**

*Homo sapiens* MIER3 ORF (NCBI/GenBank ref:NM_152622.3) from pooled human breast tumor total RNA (+AM6952, Life Technologies) was cloned into pEGFP-C1 vector at EcoRI and KpnI sites. Primer sequences for cDNA amplification were 5’-CGGAA-TTCCTATGGGGAGGCTTCTTTTGGAAGT and 3’-CGGGT-ACCCTCAGAGTGTAAGGCGAGGTCG. MDA-MB-231 (+HTB-26) and T47D (+HTB-133) cell lines were purchased from...
American Type Culture Collection (ATCC) in May 2011, cultured, respectively, in DMEM with 10% FBS and RPMI-1640 with 10% FBS and 0.2 U/mL bovine insulin, and cryopreserved after one passage for future use. Cell authentication was guaranteed by ATCC and morphology was confirmed under a phase-contrast light microscope. Cells were cultivated in 24-well plates using a phase-contrast or confocal (Olympus IX51 40× objective) microscope. Cells were grown on cover slips in chambers (Lab-Tek #177445), washed with PBS, fixed for 10 minutes with 10% paraformaldehyde, and washed again with PBS before 4′,6-diamidino-2-phenylindole (DAPI; 2 ng/mL PBS) staining for 5 minutes. Cover slips were mounted on microscope slides using fluorescent mounting solution (DAKO). Fluorescent images were captured using cellSens Dimension software (Olympus).

Genomics and statistical analysis

Genome assemblies used were Homo sapiens version GRCh37/hg19 and Rattus norvegicus version 3.4/rn4. Mammary carcinoma multiplicity phenotypes were compared by non-parametric Mann–Whitney tests. Results from mammary gland-grafting experiments were analyzed using logistic regression. Donor and recipient genotypes were incorporated as dependent variables. In independent models, graft site tumor outcome and grafting ability were used as independent variables. Quantitative qPCR data were analyzed using ANOVA and genotype and tissue source were independent variables for mammary carcinoma and non-diseased mammary tissue qPCRs. Fisher protected least significant difference (PLSD) tests were used to compare groups following a significant F test (α ≤ 0.05). Statview software (SAS Institute) was used.

Results

Fine-Mapping Mcs1b using WF.COP congenics

Subsequent comparative genetics work is reduced if QTLs are mapped to short syntenic intervals. Five congenic lines that contained different resistant COP rat Chr 2 segments of the Mcs1b candidate region from D2Wum17:D2Rat200 (Chr2:232051320–88762858) on a susceptible WF genetic background were tested to narrow Mcs1b (Fig. 1). Mcs phenotypes were determined using tumor multiplicity at 15 weeks following DMBA induction of mammary carcinogenesis. A shorter segment of COP Chr 2 that was contained in congenic line N3 conferred a decreased Mcs1b phenotype similar to line T (Table 1). Females of line N3 that were Mcs1b-resistant homozygous or heterozygous developed, respectively, 56% and 30% less mammary carcinomas per rat than those that were Mcs1b-susceptible homozygous (WF/WF) female littermates from line N3. Congenic lines F3, W2, U2, and H each contained different COP Chr 2 segments (Fig. 1). All these lines had Mcs phenotypes similar to littermates with susceptible WF genotypes (Table 1). Comparison of microsatellite DNA and published rat SNPs located in the 0.66 Mb of genomic sequence between the distal and proximal ends of lines N3 and H yielded no genetic variation between resistant COP and susceptible WF alleles (Supplementary Table S4). Therefore, we were unable to define a precise distal end to Mcs1b. When considered together, our congenic line data delimit Mcs1b to approximately 1.8 Mb of rat Chr 2 that spans from SNP ENSRNOSNP2740854 to microsatellite g2UL2-27, which corresponds to rat Chr2:42364155-44195382 (Fig. 1).

Mcs1b mammary gland-graft carcinoma susceptibility

To determine if the Mcs1b-resistant allele acted to reduce mammary cancer susceptibility in a mammary gland autonomic manner, we subjected animals with ectopically transplanted mammary gland tissue to DMBA-induced Mcs assays. Females used in mammary-grafting assays had a WF genetic background and either Mcs1b-resistant or -susceptible WF
alleles. We expected these animals to have compatible immune systems; and thus, not reject reciprocal mammary gland grafts. To test this expectation, it was determined empirically that recipients did not reject mammary tissue grafts from donors of different Mcs1b genotypes. The respective total number of recipients (r) and ectopic mammary graft–positive recipient (+r) females in susceptible (S) and resistant (R) reciprocal donor:recipient transplant groups are reported in Table 2. There were no statistically significant associations between mammary tissue grafting ability and donor or recipient genotype.

Data from ectopic mammary gland-graft–positive recipients were analyzed to test for associations of donor and recipient genotypes with carcinoma development at the ectopic mammary site. Donor mammary tissue from Mcs1b-resistant (R) females, when grafted into interscapular white fat pads of either R or susceptible (S) recipients resulted in fewer females developing ectopic mammary carcinomas compared with recipients of either genotype that received mammary tissue from S genotype donors (Fig. 2). Donor, but not recipient genotype, was significantly associated with graft-site mammary carcinoma outcome (Table 2). These results indicated that Mcs1b conferred resistance is mammary gland autonomous. This also suggested that work to characterize this locus should initially focus on mammary tissue.

Resequencing Mcs1b potential candidate open reading frames
As shown in Fig. 3, rat Mcs1b was found to contain 13 potential candidate gene transcripts as well as sequence

### Table 1. Mammary carcinoma multiplicity phenotypes (mean mammary carcinomas per rat ± SD) by genotype for WF.COP Chr 2 congenic lines used to map Mcs1b to 1.8 Mb

<table>
<thead>
<tr>
<th>WF.COP Chr2 region</th>
<th>Line</th>
<th>COP/COP (COP/WF) n</th>
<th>WF/WF n</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2Uwm17/g2UL2-30</td>
<td>T∂</td>
<td>3.5 ± 2.2 21</td>
<td>8.3 ± 3.3 19</td>
<td>0.0010</td>
</tr>
<tr>
<td>D2Uwm17/D2Utb4</td>
<td>F3</td>
<td>9.6 ± 4.1 32</td>
<td>8.8 ± 3.5 32</td>
<td>0.8433</td>
</tr>
<tr>
<td>D2Mgh2/g2UL1-5</td>
<td>N3</td>
<td>3.4 ± 2.0 25</td>
<td>7.8 ± 3.1 25</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>N3 (5.5 ± 3.6) 15</td>
<td></td>
<td>0.0413</td>
<td></td>
</tr>
<tr>
<td>D2Utb4/ENSRNOSNP2740854</td>
<td>W2</td>
<td>6.0 ± 1.8 18</td>
<td>5.9 ± 3.2 9</td>
<td>0.8498</td>
</tr>
<tr>
<td>D2Rat116/ENSRNOSNP2740854</td>
<td>U2</td>
<td>5.7 ± 3.9 6</td>
<td>6.3 ± 3.3 12</td>
<td>0.8866</td>
</tr>
<tr>
<td>g2UL2-27/D2Rat201</td>
<td>H4</td>
<td>9.3 ± 3.0 19</td>
<td>7.9 ± 3.7 13</td>
<td>0.2470</td>
</tr>
</tbody>
</table>

*P values from Mann–Whitney tests.
∂Line T phenotype published previously by Haag and colleagues (Cancer Research, 63:5808–5812, 2003).

### Table 2. Mammary gland graft-site and carcinoma outcome analyses

<table>
<thead>
<tr>
<th>Raw data from mammary gland grafting assays</th>
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</thead>
<tbody>
<tr>
<td><strong>Mcs1b donor:recipient genotype</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>S:S</strong></td>
</tr>
<tr>
<td>Total recipients (n)</td>
</tr>
<tr>
<td>MG-graft positive (n)</td>
</tr>
<tr>
<td>MG-graft AND tumor positive (n)</td>
</tr>
</tbody>
</table>

Logistic regression of graft site mammary gland outcome

<table>
<thead>
<tr>
<th>Effect</th>
<th>Coefficient</th>
<th>P</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>0.99</td>
<td>0.1869</td>
<td>2.69 (0.62–11.68)</td>
</tr>
<tr>
<td>Recipient</td>
<td>–1.31</td>
<td>0.1160</td>
<td>0.27 (0.06–1.38)</td>
</tr>
<tr>
<td>Intercept</td>
<td>2.70</td>
<td>0.0004</td>
<td></td>
</tr>
</tbody>
</table>

Logistic regression of mammary gland graft site tumor outcome

<table>
<thead>
<tr>
<th>Effect</th>
<th>Coefficient</th>
<th>P</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>1.48</td>
<td>0.0019</td>
<td>4.40 (1.73–11.18)</td>
</tr>
<tr>
<td>Recipient</td>
<td>–0.04</td>
<td>0.9381</td>
<td>0.96 (0.39–2.36)</td>
</tr>
<tr>
<td>Intercept</td>
<td>–1.22</td>
<td>0.0045</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: S, susceptible genotype; R, Mcs1b-resistant genotype; n, number of females; MG, mammary gland; CI, confidence interval.
orthologous to human 5q11.2, a GWAS-identified breast cancer risk-associated allele marked by SNP rs889312 (2). To prioritize potential candidates, we resequenced conserved protein-coding open reading frames (ORF) that were within the 1.8 Mb interval that delimited Mcs1b, and based on RT-PCR gel electrophoresis were expressed in mammary glands of susceptible WF and Mcs1b-resistant females (lines N3 and T).

Transcripts from Gpbp1, Map3k1, MIER3, Ankrd55, Il6st, Il31ra, Ddx4, Slc38a9, and Ppap2a genes were detected in mammary gland total RNA pools from each genotype by RT-PCR. No genetic variants were identified between susceptible WF and Mcs1b-resistant genotype ORFs or 3’ UTRs for these transcripts. Nucleotide sequences were submitted to NLM-NCBI.

Four of the Mcs1b potential candidate genes were predicted transcripts (gray bars in Fig. 3). Rat Actbl2 (Fig. 3A) is a pseudogene located outside rat genomic sequence orthologous to the human 5q11.2 haplotype block that associates with breast cancer risk. Predicted transcript ENSRNOG00000013098 (Fig. 3B) was listed on the Ensembl genome browser (40). We found no evidence by RT-PCR of a transcript from Actbl2 or ENSRNOG00000013098 in total RNA samples from multiple susceptible and Mcs1b-resistant mammary glands or in rat-mixed tissue total RNA samples that included embryo, brain, testes, ovary, thymus, spleen, and liver. Because cDNA was not attainable and Actbl2 was predicted to be a single exon, we sequenced genomic DNA spanning this predicted pseudogene and found no sequence differences between WF and COP alleles.

A rat orthologous transcript (Fig. 3C) to predicted C5ORF35 was not present in any total RNA samples tested from various rat tissues. We successfully amplified C5ORF35 from human thymus, spleen, and ovary, but not human breast tissue cDNA (Supplementary Fig. S1). However, in an Oncomine (41) search we found that other groups have reported detection of C5ORF35 in human breast carcinoma and nondiseased breast tissue. We noted that the annotated 5’- and 3’-UTRs of human C5ORF35 are poorly conserved between humans and rodents (Supplementary Fig. S1); therefore, we concluded that C5ORF35 is a human, but not a rat transcript.

A predicted small nuclear RNA at rat Chr 2:43765811-43765918:1 named U6 or ENSRNOG00000034909 (Fig. 3D) is estimated to be 108 bp on the forward strand. We noted that ENSRNOG00000034909 sequence aligned to multiple regions of...
the rat genome using both NCBI/BLAST and UCSC/BLAT (42, 43). Thus, because of the highly repetitive nature of the sequence, we were unable to design specific probes to determine if this predicted single exon gene was transcribed from rat *Mcs1b*.

**Mcs1b potential candidate gene transcript levels**

Rat *Mcs1b* did not contain any protein coding genetic variation between *Mcs1b*-susceptible and-resistant alleles; therefore, rat *Mcs1b* may contain variation in one or more nonprotein-coding regulatory elements that differentially control gene expression between mammary cancer susceptible and resistant genotypes. To test this hypothesis, we measured mammary gland transcript levels of genes located at *Mcs1b* in 12-week-old virgin female rats that were exposed to DMBA at 50 to 55 days to induce mammary carcinogenesis and age-matched controls without DMBA. We focused on mammary gland transcript levels due to the mammary gland autonomous nature of *Mcs1b*. Twelve-week-old animals were used because this age is after acute DMBA toxicity and before frank mammary carcinomas are detectable.

Potential candidate gene transcript levels between *Mcs1b* genotype and DMBA exposure were analyzed by 2-way ANOVA (Table 3). Effect of *Mcs1b* genotype was statistically significant for *Gppp1*, *MIER3*, *Map3k1*, and *Il6st*. There was a significant effect of DMBA exposure on *Map3k1* transcript levels. The interaction between *Mcs1b* genotype and DMBA exposure approached statistical significance for *Map3k1*. When mammary cancer-susceptible and *Mcs1b*-resistant genotypes were compared by exposure (with DMBA or without), mammary gland transcript levels were significantly different between susceptible and *Mcs1b*-resistant females that were not carcinogen-induced for *Gppp1*, *MIER3*, and *Map3k1*. Transcript levels of *Gppp1* and *Map3k1* were not different between genotypes when DMBA-exposed females were evaluated. Significant expression differences between susceptible and *Mcs1b*-resistant genotypes were sustained only for *MIER3* when females given DMBA were compared between genotypes. We did not observe statistically significant differences in transcript levels of *Ankrd55*, *Il31ra*, *Ddx4*, *Sic38a9*, or *Ppap2a* between susceptible and *Mcs1b*-resistant genotype mammary glands with DMBA or without.

Mammary gland transcript levels were lower in *Mcs1b*-resistant genotype females for all genes with a significant difference between genotypes. *MIER3* mean transcript levels were approximately 4.5-fold lower in *Mcs1b*-resistant compared with susceptible genotype mammary glands whether animals were exposed to DMBA or not (Table 3). Thus, exposure to mammary carcinogen had no appreciable effect on *MIER3* differences between susceptible and *Mcs1b*-resistant genotype females. No significant differences in *MIER3* transcript levels were detected between *Mcs1b*-resistant and susceptible genotypes in spleen, thymus, ovary, or brain tissues (Supplementary Fig. S2). This suggests that *MIER3* transcript level differences between *Mcs1b* alleles may be specific to mammary gland tissue.

A loss in statistical significance between DMBA-exposed susceptible and *Mcs1b*-resistant females for *Map3k1* was due to

<table>
<thead>
<tr>
<th>Table 3. Analysis and statistics of <em>Mcs1b</em> potential candidate gene mammary gland transcript levels in <em>Mcs1b</em>-resistant and susceptible genotypes at 12 weeks of age</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td><em>Gppp1</em></td>
</tr>
<tr>
<td><em>MIER3</em></td>
</tr>
<tr>
<td><em>Map3k1</em></td>
</tr>
<tr>
<td><em>Ankrd55</em></td>
</tr>
<tr>
<td><em>Il6st</em></td>
</tr>
<tr>
<td><em>Il31ra</em></td>
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<td><em>Ddx4</em></td>
</tr>
<tr>
<td><em>Sic38a9</em></td>
</tr>
<tr>
<td><em>Ppap2a</em></td>
</tr>
</tbody>
</table>

Abbreviations: n, number of females; G, genotype; E, exposure.

*Fisher PLSD test P-values from comparing susceptible and *Mcs1b*-resistant genotypes by exposure.
a statistically significant ($P = 0.0003$) increase in the mean level of Map3k1 in the Mcs1b-resistant genotype females with DMBA compared with age-matched controls of the same genotype without DMBA (Table 3). Map3k1 levels were not different ($P = 0.2038$) between susceptible WF mammary glands with DMBA or without.

**Effect of Mcs1b genotype on body weight**

Travis and colleagues detected a significant association between human breast cancer risk-associated SNP rs889312 and stature in women (44). To determine whether rat Mcs1b might also exhibit pleiotropy, we analyzed rat body weight, which is the information that we routinely collect and is relevant because body weight is genetically correlated to stature in humans (45). Significant effects of Mcs1b genotype ($P < 0.0001$) and DMBA exposure ($P = 0.0014$) on body weight at 12 weeks of age were detected (Fig. 4A). The interaction between Mcs1b genotype and DMBA exposure was also significant ($P = 0.0004$). Females with the Mcs1b-resistant genotype had mean ± SD body weights of $200 \pm 11$ g with DMBA ($n = 47$) and $201 \pm 7.7$ g without DMBA ($n = 33$), which were not significantly different ($P = 0.7880$). Comparatively, mammary cancer–susceptible females had higher ($P < 0.0001$) mean ± SD body weight at $192 \pm 11$ g with DMBA ($n = 45$) than unexposed susceptible females ($n = 34$) who had a mean ± SD body weight of $180 \pm 12$ g.

**Higher MIER3 transcript levels in mammary carcinomas**

Transcript levels of MIER3, Il6st, Gpdp1, and Map3k1 in DMBA-induced mammary carcinomas that developed in susceptible ($n = 28$) and Mcs1b-resistant genotype ($n = 25$) mammary glands were measured by qPCR to determine if there was an effect of Mcs1b genotype on levels of any of these transcripts in mammary carcinoma tissue. These genes were evaluated because significant effects of Mcs1b genotype on mammary gland transcript levels of these genes were detected (Table 3). Furthermore, Il6st was included because it had been reported to be higher in rat mammary carcinoma compared with mammary gland tissues (46). We collected total RNA from DMBA-induced mammary carcinomas ($n = 1$ or 2 per rat) and adjacent non-diseased mammary gland tissue from 21- to 23-week-old females ($n = 6$ per genotype). There were no statistically significant differences in mammary carcinoma transcript levels between Mcs1b genotypes for any of the 4 genes tested. However, as shown in Fig. 4B, MIER3 transcript levels were significantly higher (1.8-fold) in mammary carcinomas compared with non-diseased mammary tissue. We also observed that Il6st was potentially different between mammary carcinomas and non-diseased mammary glands; however, this comparison did not meet our statistical significance criterion (Fig. 4B).

Oncomine (41) was used to query The Cancer Genome Atlas (cancergenome.nih.gov) gene expression database to find that levels of human MIER3 were, respectively, 1.33- and 1.20-fold higher in invasive ductal ($n = 392$) and invasive lobular ($n = 36$) breast carcinoma samples compared with pathologically normal breast tissues ($n = 61$; $P = 2.8 \times 10^{-13}$, ductal; $P = 6.3 \times 10^{-7}$, lobular; $t$ tests, Fig. 4C). Thus, both human/rat MIER3/MIER3 transcript levels are higher in breast/mammary carcinoma compared with non-diseased breast/mammary tissues.

**Localization of human MIER3 protein to nuclei**

MIER3 encodes an uncharacterized member of the MIER family of proteins. The gene product of family member MIER1 is a transcription factor targeted to the nucleus (47). Therefore, we determined whether MIER3 may also encode a transcription factor targeted to the nucleus. We cloned a human MIER3...
ORF into an enhanced eGFP expression vector and transiently transfected this expression vector into MDA-MB-231 and T47D breast cancer cell lines. In vitro expression of MIER3 linked to eGFP resulted in green fluorescence in distinct foci compared with eGFP alone (Supplementary Fig. S3). We confirmed that foci were nuclei by determining that MIER-3-eGFP colocalized with DAPI-staining in both cell lines (Fig. 5).

**Discussion**

Rat Mcs, like human breast cancer risk, is complex as both are controlled by multiple susceptibility alleles and environmental factors. We have mapped rat Mcs1b to a 1.8 Mb region of rat chromosome 2 using multiple congenic lines. We found that rat Mcs1b is highly relevant to human breast cancer susceptibility as it contains genomic sequence orthologous to a low-penetrance breast cancer risk allele at 5q11.2. This human susceptibility allele was first reported by Easton and colleagues (34) and was later confirmed by Gould and colleagues (48) on the use of rat models. In a rodent complex disease susceptibility QTL with a GWAS-identified concordant human ortholog that had a probability of association below a stringent significance level of \( P < 10^{-7} \), which is widely deemed to be required for genomewide studies.

An experimental organism with a segregating concordant susceptibility allele implies that functional genetic studies may translate directly to human biology and disease. For example, Gould and colleagues (48) reported that rat Mcs5a, a Wistar Kyoto (WKY) strain resistance QTL that is concordant to human MCSSX4, acted in a nonmammary cell-autonomous manner that involves immune cells (48). Here, we used rat genetic lines to show that Mcs1b controls mammary cancer susceptibility by an undetermined mechanism that is autonomous to mammary gland tissue. While our result is in agreement with previous work that concluded a majority, but not all, of the COP rat strain resistance to mammary cancer is mammary gland autonomous (49), it further highlights that the WKY and COP strains may achieve mammary carcinoma resistance through different genetically determined cellular and molecular mechanisms; mechanisms that are likely genetically determined in humans.

Most common genetic variation associated with human complex disease susceptibility seems to be located in nonprotein-coding DNA. Because we found no genetic variation between susceptible and resistant allele Mcs1b ORFs, we conclude that Mcs1b is likely a noncoding gene regulatory element(s), such as a transcription factor binding site or noncoding RNA. This would be similar to the hypothesized identity of the human 5q11.2 breast cancer risk-associated element. Human polymorphisms that are contained in public databases and are highly correlated with human 5q11.2 breast cancer risk-associated SNP rs889312 are in nonprotein-coding DNA. There are no known noncoding RNAs in either the human or rat ortholog; therefore, another type of gene regulatory element is likely responsible for or associated to susceptibility differences.

Our studies suggest that MIER3 is a strong candidate breast cancer susceptibility gene at human 5q11.2. We have identified MIER3 as a strong Mcs1b candidate gene in this study based on different MIER3 mammary gland transcript levels between susceptible and Mcs1b-resistant genotypes. Lower levels of MIER3 in Mcs1b-resistant genotype females were genetically determined and not dependent on the induction of mammary carcinogenesis by DMBA. We also found MIER3 levels to be significantly lower in nondiseased rat mammary tissue compared with mammary carcinoma. Furthermore, we queried The Cancer Genome Atlas gene expression database and noted that human MIER3 levels were higher in both ductal and lobular breast carcinomas compared with breast tissue.

MIER3 or MIER1, family member 3 (GenBank ref|NM_152622) is an uncharacterized gene. We determined that MIER3 localized to the nucleus. Human and rat MIER3/MIER3 (GenBank ref|NP_689835.3 and NP_001161472.1) gene products share 93% amino acid sequence identity, and human MIER3 and MIER1 (GenBank ref|NP_001071172.1) have 54% identical amino acids based on BLAST (42). MIER1 physically interacts with estrogen receptor alpha, Sp1, and Creb-binding protein (50–52). MIER1 contains 1, whereas MIER3 has 2 conserved LXXLL sequences, which is a motif that facilitates nuclear hormone receptor interactions (53). A potential functional difference between MIER1 and MIER3 may be that a difference in the number of LXXLL motifs between them results in physical interactions with different nuclear hormone receptors (54).

In addition to MIER3, MAP3K1 and CSFBP35 reside within the human 5q11.2 haplotype block that associates with breast cancer risk. Even though there are no published studies in support, MAP3K1 is often considered the candidate breast cancer susceptibility gene at 5q11.2 due to its location within the breast cancer risk-associated haplotype.
block and known function as a serine/threonine kinase. In our rat studies, Map3k1 was differentially expressed between susceptible and Mcs1b-resistant congenic rats that had not been induced to undergo mammary carcinogenesis; however, mammary glands that had been induced to undergo mammary carcinogen did not show a difference in Map3k1 levels between Mcs1b alleles. An interesting result in our study with respect to Map3k1, which may have important implications for human studies of potential genotype–environment interactions, is that the exposure to mammary carcinogen resulted in increased mammary gland Map3k1 levels for the Mcs1b resistant, but not the susceptible genotype. We found no evidence of a rat orthologous transcript to human C5ORF35 in multiple rat tissues. Furthermore, exonic elements of C5ORF35 have not been conserved in the rat. Therefore, we conclude that MAP3K1 and C5ORF35 are not as likely as MIER3 to be breast cancer susceptibility genes.

We noted that both rat Mcs1b and human 5q11.2 exhibit pleiotropy. Travis and colleagues reported that carriers of the increased risk allele at human 5q11.2 were significantly shorter in height than noncarriers (44). In our study, high-risk female rats had lower body weight than Mcs1b-resistant females. We noted on the Rat Genome Database that there is a predicted rat body weight QTL named Bw1 that overlaps Mcs1b and is associated with mesenteric body fat amount (55). Both human and rat study results are counter intuitive as one might expect taller women and heavier rats to be at greater cancer risk. Thus, it is important to note that, as expected with low-penetrance alleles, the quantitative difference between the means for each human genotype were subtle with overlapping distributions. Mean height difference was 7 mm between noncarriers and carriers of the increased risk allele. In our study, we analyzed only body weight, and not specific components of body weight, such as bone density or fat amounts. Thus, better descriptive traits would likely be more informative. It is notable that the pleiotropic effects of these alleles opens the possibility that other experimental organisms, approaches, and study designs without focus on breast or mammary cancer may be useful to functionally characterize breast cancer risk-associated genetic variation at 5q11.2.

In conclusion, rat Mcs1b is mammary gland autonomous allele and a nonprotein-coding genetic element that is orthologous to the GWAS-identified human 5q11.2 breast cancer susceptibility locus. We propose that MIER3 is a strong candidate breast cancer susceptibility gene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: D.J. Samuelson

Development of methodology: X. Xu, M.D. Vaughn, D.J. Samuelson

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.D. denDekker, X. Xu, M.D. Vaughn, L. Deschenes, D. J. Samuelson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.D. denDekker, X. Xu, M.D. Vaughn, C.J. Lambring, L. Deschenes, D.J. Samuelson

Writing, review, and/or revision of the manuscript: A.D. denDekker, D.J. Samuelson

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.D. denDekker, X. Xu, M.D. Vaughn, A. H. Puckett, L.L. Gardner, L. Deschenes, D.J. Samuelson

Study supervision: D.J. Samuelson

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Aaron D. denDekker, Xin Xu, M. Derek Vaughn, et al.


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