**Abstract**

Genetic factors have been estimated to account for at least 30% of a woman's risk to develop breast cancer. We have developed a rat model using Wistar Furth (WF) and Wistar Kyoto (WKy) strains to genetically identify mammary cancer susceptibility loci. The WKy allele of the mammary carcinogenesis susceptibility locus *Mcs5c* was previously shown to reduce carcinoma multiplicity after 7,12-dimethylbenz[a]anthracene (DMBA) exposure. In this study, *Mcs5c* was fine-mapped using WF:WKy congenic lines. *Mcs5c* was located to a region of approximately 176 kb on rat chromosome 5. One of the *Mcs5c* congenic lines containing a narrow *Mcs5c* WKy interval displayed a 40% decrease in average carcinoma number compared with WF-homozygous congenic controls after mammary carcinogenesis induction using two different models. As genetically mapped, the *Mcs5c* locus is located in a gene desert and thus is devoid of genes and annotated RNAs; thus, a genetic element in *Mcs5c* was hypothesized to regulate the expression of genes outside the locus. Tenascin C (*Tnc*) was identified as a candidate gene due to its reduced expression in thymus and ovarian tissues of *Mcs5c* WKy-homozygous congenic females compared with WF-homozygous congenic controls. This allele-specific differential expression is environmentally controlled.

**Introduction**

Breast cancer is the most common type of cancer and the second leading cause of cancer death in women in the United States (1). A woman’s risk of developing breast cancer is determined by genetic and environmental factors and interactions between these factors. Twin studies suggest that genetic factors account for at least 30% of a woman’s risk to develop breast cancer (2). High penetrance alleles, such as functional mutations in *BRCA1* and *BRCA2*, strongly increase breast cancer risk (3, 4). However, these rare mutations account for less than 5% of breast cancer risk (5). The majority of genetic risk is thought to be due to low penetrance alleles that are more common in a population (6). Estimates suggest that if all breast cancer susceptibility genes were identified, 88% of risk could be attributed to 50% of women (7).

Most breast cancer susceptibility alleles have yet to be identified. Candidate gene studies have focused on gene pathways thought to affect breast cancer (e.g., DNA repair and cell cycle control), and have not been as fruitful in identifying breast cancer genes as one might have hoped. Although such studies can be successful, as in the case of *AKAP9* (8), an analysis of more than 710 single nucleotide polymorphisms (SNP) in 120 candidate genes did not yield a significant association after adjusting for population stratification (9). One caveat of such candidate gene studies is a strong focus on coding exons of genes and pathways anticipated to affect breast cancer based on well-characterized biological function.

In the last few years, genome-wide association studies (GWAS), which are not biased to prior knowledge of gene function, have been used to identify SNPs that are associated with breast cancer risk in women. These studies have uncovered many novel independent loci associated with breast cancer (10–16). Although GWAS are useful in identifying breast cancer risk alleles, they are limited both in their discovery rate due to necessary statistical corrections that must be made for genome-wide multiple comparisons and in providing mechanistic insight.

We are using a comparative genomics approach to identify mammary cancer modifier genes in the rat and further evaluating their role as breast cancer modifier genes in women. Rats are a good model for mammary carcinogenesis, as most rat and human carcinomas have a ductal origin (17). A comparative genomics approach has been found to be successful for the rat locus *Mcs5a*. The human homologous region to *Mcs5a* contains 2 noncoding loci independently associated with breast cancer risk (18). As is the case for GWAS, such comparative genomics studies are not biased to genes or pathways expected to affect mammary cancer risk. More importantly, if a rat locus is found to...
be important for human breast cancer, congenic rats used to fine-map that locus provide an ideal experimental model in which to mechanistically characterize that locus.

Our laboratory characterized the Wistar Kyoto (WKy) strain for loci that modify mammary carcinogenesis susceptibility. Linkage mapping was done on a backcross between the [WKy × WF] F1 × WF strain. Four significant quantitative trait loci (QTL) were identified: mammary carcinoma susceptibility (Mcs) 5, Mcs6, Mcs7, and Mcs8. Mcs5, Mcs6, and Mcs8 are associated with resistance to mammary carcinogenesis, whereas Mcs7 is associated with increased susceptibility (19). Further characterization of the Mcs5 locus identified 3 loci, Mcs5a, Mcs5b, and Mcs5c. The Mcs5c candidate region was previously mapped to 4.5 Mb of rat chromosome 5, and rats homozygous for the Mcs5c WKy allele displayed a reduction in the development of mammary carcinomas (20).

In the present study, we have fine-mapped the 4.5-Mb Mcs5c locus using additional congenic rats with smaller WKy intervals introgressed into a susceptible Wistar Furth (WF) background. The Mcs5c locus is now located in a 176-kb region of a rat gene desert that is devoid of known genes or small RNAs and was found to act after cancer initiation. It is hypothesized that Mcs5c, interacting with the host environment, affects mammary carcinogenesis through its regulation of the expression of the extracellular matrix protein gene Tnc.

Materials and Methods

Rat breeding

All animal experiments were conducted at our facility under protocols approved by the University of Wisconsin Medical School Animal Care and Use Committee. Wistar Furth (WF/NHsd) and Wistar Kyoto (WKy/NHsd) rats were purchased from Harlan. Rats were maintained in a 12-hour light/12-hour dark cycle and were provided with Teklad lab blox chow and acidified water ad libitum. Furth (WF/NHsd) and Wistar Kyoto (WKy/NHsd) rats were synthesized from 2 g of total RNA, diluted 1:8, and were treated with TRI reagent. TRI reagent and RNA extraction were performed using the Qiagen RNeasy kit (Qiagen, Valencia, CA). RNA was treated with TURBO-free DNase (Applied Biosystems). cDNA was synthesized from 2 µg of total RNA, diluted 1:8, and RNA-to-cDNA synthesis was performed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time quantitative PCR (QPCR) analyses were run using a 16-well plate with a 10-fold dilution series of the samples.

HER2/neu infusions

WKy-homozygous congenic rats from line 5C-11 (Fig. 1) and WF-homozygous congenic controls were bred at our facility. At 50–60 days of age, female rats were intraductally infused with the pJR-neu retroviral vector. Details on the construction and transfer of the pJR-neu retroviral vector into the mammary epithelium of the laboratory rat have been previously described (22). In brief, a suspension of replication-defective amphotropic retrovirus containing the activated HER2/neu oncogene was infused into the central duct of each of the 12 mammary glands. The rats were infused with a viral titer of 2 × 10^5 colony-forming units (CFU)/mL. At 12 weeks postinfusion, necropsies were conducted and the total number of carcinomas (≥3 × 3 mm) was recorded. Tumor multiplicity was analyzed with a generalized linear model using the family ‘quasi-Poisson’ in the program R.

Comparative genomic analysis

A rat, mouse, and human comparative genomics map of the Mcs5c region ± 500 kb was constructed using the UCSC genome browser November 2004, July 2007, and March 2006 assemblies, respectively. This analysis revealed a gap in the rat sequence. We identified 4 rat BAC clones that likely covered part of the gap. These clones were sequenced by the Genome Sequencing Center at Baylor College of Medicine to cover the gap in the rat sequence with coordinates chr5:80,748,116-80,798,115. Three of these clones BAC CH230-292A11, BAC CH230-433D12, and BAC CH230-431C16 were completely sequenced, whereas the fourth CH230-250D12 was partially sequenced. These 4 clones filled the gap in the rat sequence and are available in GenBank (AC229945, AC229947, and AC229946). The gap was found to be approximately 309 kb, which is consistent with the orthologous regions from the mouse and human genome sequences.

Real-time quantitative PCR

Real-time quantitative PCR (QPCR) analyses were run as previously described (18). Briefly, primers and probes for TaqMan QPCR (Applied Biosystems) were designed using Primer Express v 2.0 (Applied Biosystems). Tissues were collected from WKy-homozygous–resistant congenic rats and WF-homozygous congenic controls from DMBA-treated and nontreated rats 4 weeks post-DMBA exposure and from HER2/neu infused rats 12 weeks postinfusions. Tissues were homogenized in TRI reagent and RNA was extracted using MagMAX-96 for Microarrays kits (Applied Biosystems). RNA was treated with TURBO-free DNase (Applied Biosystems). cDNA was synthesized from 2 µg of total RNA, diluted 1:8, and 1 µL was used in a 16-µL QPCR reaction using the Applied Biosystems 7900 cycling conditions. The reaction components were 1× TaqMan Buffer A (Applied Biosystems); 5.5 mmol/L MgCl2; dATP, dCTP, dGTP, and dITP at 200 µmol/L each; 200 nmol/L TaqMan experimental probe (Applied Biosystems); 200 nmol/L rodent Gapdh probe; 0.4 units of Taq Gold DNA Pol (Applied Biosystems); and various concentrations of Gapdh and Mcs5c gene primers: 500 nmol/L Gapdh, 500 nmol/L Tnfsf15; 100 nmol/L Gapdh, 300 nmol/L Tnfsf8;
Figure 1. Fine-mapping of Mcs5c. Maps of the congenic lines used to fine-map the Mcs5c locus beginning with the previously defined 4.5-Mb region (20). Microsatellite markers in the Mcs5c region on rat chromosome 5 are shown along the Y-axis. Negative or susceptible congenic lines for which the WKy-homozygous rats develop the same average number of mammary carcinomas as WF-homozygous rats are shown in white. Resistant congenic lines, for which the WKy-homozygous rats average fewer mammary carcinomas compared with WF-homozygous rats, are shown in dark gray. Areas of recombination are shown in light gray. A, Mcs5c was originally localized within a 301-kb region that is contained in the resistant lines 5C-11 and 5C-18 but does not overlap with the susceptible line 5C-17. B, the Mcs5c interval is further reduced to a 176-kb region as defined by the overlapping congenic lines 5C-14 (resistant) and 5C-23 (susceptible).
100 nmol/L Gapdh, 100 nmol/L Tnc; 60 nmol/L Gapdh, 500 nmol/L Pappa in the ovary; and 100 nmol/L Gapdh, 300 nmol/L Pappa in the mammary gland. Tnfsf15 and Tnc expression was quantified in all 3 tissues. Pappa expression could not be quantified in the thymus, and Tnfsf8 expression was only quantified in the thymus.

The following primers and probes were used:

- **Tnfsf15** probe 5’-ATGGTTCCGACTTGAGACAGA
  - Tnfsf15 F 5’-GGACCTAGCCTCCCTCTGATGA
  - Tnfsf15 R 5’-GTGTGCTGAAAGGCCAGAC
- **Tnfsf8** probe 5’-AAGGAGAAATGCTC
  - Tnfsf8 F 5’-GACCTCCTACCTCATAACACTG
  - Tnfsf8 R 5’-AGGAGCCCATGACCTCITGGAT
- Tnc probe 5’-CGAGACTGTGACATGATAGA
  - Tnc F 5’-GGCTGTCAGAAGGCCAGA
  - Tnc R 5’-TGCCATGAAGGGATTTGAAGA
- **Pappa** probe 5’-ATGCCATAGGGTCAGAGTGA
  - Pappa F 5’-AGCCCCAAACCAACTCAACA
  - Pappa R 5’-GAATGTCACGCACCGTCAAG

The real-time QPCR cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A standard curve method was used to calculate transcript quantities, which were standardized by dividing the quantity of the gene of interest by the quantity of rodent Gapdh. Sample measurements are an average of 4 replicates. Data were analyzed using the Mann–Whitney test.

### Results

**Fine-mapping of Mcs5c**

WKy-homozygous rats from lines RR, HH, 5C-3, 5C-1, and 5C-9 (Fig. 1A) were found to exhibit the resistance attributed to the 4.5-Mb Mcs5c locus, displaying an average of approximately 50% decrease in carcinoma number, respectively ($P < 0.0001$), whereas WKy-homozygous rats from lines 5C-4, QQ, TT, and 5C-17 were found to be as susceptible to DMBA-induced mammary carcinogenesis as WF-homozygous congenic controls (Table 1). Therefore, the genetic element that leads to a decrease in carcinoma number is located in the region of overlap of lines RR, HH, 5C-3, 5C-1, and 5C-9 that does not overlap with susceptible lines.

WKy-homozygous rats from lines 5C-11 and 5C-18 averaged 4.8 ± 0.4 ($n = 58$) and 5.0 ± 0.5 ($n = 27$) carcinomas per rat compared with WF-homozygous congenic controls, which averaged 8.0 ± 0.5 ($n = 52$) and 8.1 ± 0.5 ($n = 47$) carcinomas per rat, respectively ($P < 0.0001$). Line 5C-17 was as susceptible to DMBA-induced mammary carcinogenesis as the WF-homozygous congenic controls. WKy-homozygous rats from line 5C-17, which overlaps with lines 5C-11 and 5C-18, averaged 8.8 ± 0.7 ($n = 29$) carcinomas compared with 7.7 ± 0.3 ($n = 34$) for the WF-homozygous congenic controls. These 3 lines localize Mcs5c to a 301-kb region of rat chromosome 5 from gUwm54-50 to gUwm54-33.

#### Table 1. Tumor multiplicity of congenic lines

<table>
<thead>
<tr>
<th>Congenic line</th>
<th>WF-homozygous carcinoma #</th>
<th>n</th>
<th>WKy-homozygous carcinoma #</th>
<th>n</th>
<th>% reduction</th>
<th>P value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR</td>
<td>8.2 ± 0.4</td>
<td>63</td>
<td>3.2 ± 0.4</td>
<td>35</td>
<td>61</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HH</td>
<td>8.0 ± 0.7</td>
<td>29</td>
<td>4.2 ± 0.4</td>
<td>40</td>
<td>48</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5C-3</td>
<td>8.3 ± 0.6</td>
<td>33</td>
<td>3.8 ± 0.4</td>
<td>26</td>
<td>54</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5C-4</td>
<td>10.0 ± 0.8</td>
<td>26</td>
<td>8.6 ± 0.8</td>
<td>32</td>
<td>–</td>
<td>0.26</td>
</tr>
<tr>
<td>5C-1</td>
<td>8.9 ± 0.6</td>
<td>37</td>
<td>3.5 ± 0.4</td>
<td>27</td>
<td>61</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5C-9</td>
<td>7.6 ± 0.7</td>
<td>35</td>
<td>2.8 ± 0.3</td>
<td>32</td>
<td>63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>QQ</td>
<td>8.1 ± 0.6</td>
<td>39</td>
<td>7.4 ± 0.6</td>
<td>36</td>
<td>–</td>
<td>0.24</td>
</tr>
<tr>
<td>TT</td>
<td>6.8 ± 0.4</td>
<td>51</td>
<td>8.3 ± 0.7</td>
<td>26</td>
<td>–</td>
<td>0.11</td>
</tr>
<tr>
<td>5C-11</td>
<td>8.0 ± 0.5</td>
<td>52</td>
<td>4.8 ± 0.4</td>
<td>58</td>
<td>40</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5C-17</td>
<td>7.7 ± 0.5</td>
<td>34</td>
<td>8.8 ± 0.7</td>
<td>29</td>
<td>–</td>
<td>0.25</td>
</tr>
<tr>
<td>5C-18</td>
<td>8.1 ± 0.5</td>
<td>47</td>
<td>5.0 ± 0.5</td>
<td>27</td>
<td>38</td>
<td>0.0004</td>
</tr>
<tr>
<td>5C-17</td>
<td>n/d</td>
<td></td>
<td>8.1 ± 0.6</td>
<td>41</td>
<td>–</td>
<td>&gt;0.5$^c$</td>
</tr>
<tr>
<td>5C-29</td>
<td>n/d</td>
<td></td>
<td>6.9 ± 0.6</td>
<td>58</td>
<td>–</td>
<td>&gt;0.5$^c$</td>
</tr>
<tr>
<td>5C-23</td>
<td>n/d</td>
<td></td>
<td>6.3 ± 0.4</td>
<td>56</td>
<td>–</td>
<td>0.34$^c$</td>
</tr>
<tr>
<td>5C-14</td>
<td>n/d</td>
<td></td>
<td>3.9 ± 0.3</td>
<td>51</td>
<td>51</td>
<td>&lt;0.0001$^c$</td>
</tr>
<tr>
<td>5C-15</td>
<td>n/d</td>
<td></td>
<td>2.6 ± 0.6</td>
<td>18</td>
<td>67</td>
<td>&lt;0.0001$^c$</td>
</tr>
<tr>
<td>5C-27</td>
<td>n/d</td>
<td></td>
<td>3.4 ± 0.3</td>
<td>58</td>
<td>57</td>
<td>&lt;0.0001$^c$</td>
</tr>
<tr>
<td>5C-22</td>
<td>n/d</td>
<td></td>
<td>3.8 ± 0.8</td>
<td>12</td>
<td>52</td>
<td>0.0015$^c$</td>
</tr>
<tr>
<td>WF</td>
<td>7.9 ± 0.5</td>
<td>46</td>
<td>–</td>
<td>–</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$Tumor multiplicity is represented as the average number of carcinomas per rat ± standard error.

$^b$P values obtained using the nonparametric Mann–Whitney test.

$^c$P values obtained using nonparametric Wilcoxon tests adjusted for multiple comparisons.
In an independent series of DMBA-induced mammary carcinogenesis studies using additional congenic lines to fine-map the Mcs5c region, the 301-kb interval was further reduced to approximately 176 kb (Fig. 1B). Table 2 contains a list of primers used to amplify microsatellite markers for fine-mapping of the Mcs5c region. WKy-homozygous rats from overlapping Mcs5c congenic lines 5C-14, 5C-15, 5C-27, and 5C-22 were shown to be resistant to the development of mammary carcinomas ($P/C200.0015), as they averaged 3.9 ± 0.3 (n = 51), 2.6 ± 0.6 (n = 18), 3.4 ± 0.3 (n = 58), and 3.8 ± 0.8 (n = 12) carcinomas per rat, respectively, when compared with the WF control group 7.9 ± 0.5 (n = 46). Congenic lines 5C-29 and 5C-23 WKy-homozygous rats were as susceptible as WF rats to DMBA-induced mammary carcinogenesis, developing 6.9 ± 0.6 (n = 58) and 6.3 ± 0.4 (n = 56) carcinomas per rat on average (Table 1). Data from the resistant congenic line 5C-14 and the susceptible congenic line 5C-23 reduce the Mcs5c interval between the genetic markers g5Uwm74-1 and gUwm54-8 to a 176-kb region. Overall, the WKy-homozygous congenic rats having the Mcs5c region showed an approximately 50% reduction in the average number of DMBA-induced mammary carcinomas developing per rat.

### Mcs5c prevents HER2/neu-induced mammary cancer

The Mcs5c locus was identified because of its modifying effect on DMBA-induced mammary carcinogenesis. DMBA is a carcinogen that needs to be metabolized and activated in order to form DNA adducts and initiate carcinogenesis. To determine whether the effect of Mcs5c on carcinoma multiplicity is specific to carcinogenesis initiation by DMBA, we assayed the carcinoma response of WKy-homozygous congenic rats of line 5C-11 to mammary intraductal infusions of a retroviral vector expressing a HER2/neu oncogene. Congenic line 5C-11 WKy-homozygous rats were resistant to mammary cancer induced by HER2/neu infusions ($P = 0.03). These WKy-homozygous rats averaged 6.2 ± 1.7 carcinomas per rat compared with the WF-homozygous congenic controls that averaged 10.4 ± 1.5 carcinomas per rat. The degree of reduction in the number of mammary carcinomas after HER2/neu infusions is similar to that seen after DMBA-induced mammary carcinogenesis (Fig. 2).

### The Mcs5c locus is located in an intergenic region of rat chromosome 5

The Mcs5c locus is located in a 176-kb intergenic region of rat chromosome 5 that shows good conservation between rat, mouse, and human homologous regions. Currently, this interval does not contain any known gene or annotated microsatellite markers.

---

**Table 2. Primers to amplify microsatellite markers used to fine-map Mcs5c**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>bUwm36-1</td>
<td>GATTCCTCTGTTCTTCGGA</td>
<td>CCGTGATTTCCAACCTACTAGG</td>
</tr>
<tr>
<td>gUwm54-14</td>
<td>ATCTACTGCGGAACTGAGC</td>
<td>CACCATTCCCTCTAGTTC</td>
</tr>
<tr>
<td>gUwm54-20</td>
<td>CAAGGCCAGGAAATCAGA</td>
<td>ATAGCTCTCCCTGTCC</td>
</tr>
<tr>
<td>gUwm54-16</td>
<td>CACAAATGTCAGGAGGAGA</td>
<td>TATTGCAAGGGAGAGGAG</td>
</tr>
<tr>
<td>gUwm54-17</td>
<td>GGTGCTACTGTTAGAGGAG</td>
<td>AAAAATGATTGAGTATGA</td>
</tr>
<tr>
<td>gUwm54-4</td>
<td>TCGAAATAGACCCGAGGAG</td>
<td>GAAATCCATCCCTAGT</td>
</tr>
<tr>
<td>gUwm54-21</td>
<td>AGACCTCCCTATCGACCT</td>
<td>GGTCCAGTTTATAGCACG</td>
</tr>
<tr>
<td>gUwm54-50</td>
<td>AGGAAAGCAGGAAAGTATA</td>
<td>TTACTAAGCAGGACTAG</td>
</tr>
<tr>
<td>gUwm54-22</td>
<td>TGGTGCTATTTGTGCTGTC</td>
<td>CTGAGCATCTGAGGAG</td>
</tr>
<tr>
<td>gUwm54-51</td>
<td>CTCTCCGAAAAGACCTGA</td>
<td>GTCTGAGGACTAGGAG</td>
</tr>
<tr>
<td>gSUwm74-1</td>
<td>ATATATGGACCGAGCGAGAA</td>
<td>TGAAGAGGAAACCAAAAC</td>
</tr>
<tr>
<td>gUwm54-53</td>
<td>AGTTTCCTGTTAGAAGGACCA</td>
<td>CCACTAATCCAGAAGGAG</td>
</tr>
<tr>
<td>gUwm54-69</td>
<td>AAGTATTACGGAAGGGAGAGA</td>
<td>AGTACCCCTGAGTCTG</td>
</tr>
<tr>
<td>gUwm54-8</td>
<td>TAAAGGGCACTACATGAGTT</td>
<td>TTAAACAGGGACCATAGG</td>
</tr>
<tr>
<td>gUwm54-43</td>
<td>CAGAGGTCTATGGAATGAGG</td>
<td>TCTGACTCCTGAGAAG</td>
</tr>
<tr>
<td>gUwm54-45</td>
<td>TCTATTGGAATGCCCCTTGT</td>
<td>CAGAGGGATGCTGATGAG</td>
</tr>
<tr>
<td>gUwm54-70</td>
<td>TCAAGGAGATTAGACTGACATA</td>
<td>TCGAATTGTCTGAGTCTT</td>
</tr>
<tr>
<td>gUwm54-33</td>
<td>AAGTGTTGAGCATGAGCAGGAGA</td>
<td>AGTACCCCTGAGTCTG</td>
</tr>
<tr>
<td>gUwm54-35</td>
<td>TGAGAAGCGCRCAGAAGATAG</td>
<td>GTCTGAGGAAATCTCAAC</td>
</tr>
<tr>
<td>gUwm54-37</td>
<td>CTCAGGATATGTAAGGAGC</td>
<td>CCACTAATCCAGAAGGAG</td>
</tr>
<tr>
<td>gUwm60-2</td>
<td>GTACACATTTAACCTAGGGA</td>
<td>AGTACCCCTGAGTCTG</td>
</tr>
<tr>
<td>gUwm60-3</td>
<td>AGGTATCGTTGAAAGATTGTT</td>
<td>GGTGTTGACAGGATAAG</td>
</tr>
<tr>
<td>gUwm47-2</td>
<td>TGGAGTTGAATGTAAGGAGA</td>
<td>CTTCCGAGATGCTGACAT</td>
</tr>
<tr>
<td>gUwm47-19</td>
<td>CCAATCTGCTGAGCCTGACCT</td>
<td>CAGGTGTTGTGTTGTC</td>
</tr>
<tr>
<td>gUwm47-10</td>
<td>GATAGGACACACATCCTA</td>
<td>GCCGAGACAGGTCTGAG</td>
</tr>
</tbody>
</table>

*Primer sequences are shown 5’ to 3’.*

The mouse and human homologous regions also lack protein coding genes and small RNAs. Three potential genes or spliced ESTs were identified in the human homologous region to Mcs5c: EU250754, CN315134, and CD250067. None of these transcripts had homologues in the rat or mouse genomes and were thus eliminated as potential candidate genes responsible for the rat Mcs5c phenotype. No additional spliced ESTs were identified in the mouse region homologous to Mcs5c.

As there were no expressed sequences found in the Mcs5c region, we looked at the intervals with the highest species conservation. We found 2 highly conserved noncoding sequences (CNS) greater than 100 bp in length and showing approximately 70% identity between the rat and chicken genome sequences. These 2 elements, CNS1 and CNS2, were sequenced in both the WF and Mcs5c congenic rats. Two SNPs were identified within CNS1. Neither of these SNPs fell in any of the predicted transcription factor binding sites identified in the UCSC human genome browser. No SNPs were identified in the second conserved element CNS2.

We hypothesized that a genetic element within Mcs5c may regulate the expression of a gene outside the locus. The Mcs5c transcript map was expanded approximately 500 kb upstream and downstream of Mcs5c (Fig. 3). Four known genes are present in all 3 species investigated: tumor necrosis factor ligand superfamily 15 (Tnfsf15), tumor necrosis factor ligand superfamily (Tnfsf8), tenascin c (Tnc), and pregnancy-associated plasma protein A (Pappa). Three additional genes were present in the human genome database, EST_YD1, DEC1, and CTS9, none of which shared homology with the rat or mouse genomes. The 4
known genes that were found in all 3 species were evaluated further for expression differences between WKy-homozygous congenic rats having the Mcs5c interval and WF-homozygous congenic controls.

**Reduced Tnc expression is associated with the Mcs5c cancer phenotype**

We have tested for differences in expression levels of Tnfsf15, Tnfsf8, Tnc, and Pappa in the mammary gland and in 2 tissues that could potentially influence mammary carcinogenesis (i.e., thymus--immune system and ovary--endocrine system) of Mcs5c WKy-homozygous congenic animals compared with WF-homozygous congenic controls using TaqMan QPCR. These genes are not expressed ubiquitously and could not all be amplified in all tissues. Tnfsf8 expression could only be quantified in the thymus, Pappa expression was quantified in the mammary gland and the ovary, and Tnfsf15 and Tnc expression was quantified in all 3 tissues (Fig. 4).

Tnc was the only gene that showed differential expression between Mcs5c WKy-homozygous congenic rats compared with WF-homozygous congenic controls. No differential expression of Tnfsf15, Tnfsf8, and Pappa was observed in any of tissues examined, with or without DMBA exposure (Fig. 4 for DMBA treatment; data not shown for no DMBA treatment). Tnc expression was reduced by 40% in thymus and 30% in ovaries from WKy-homozygous congenic animals (P < 0.01; Fig. 5A and B). This difference in gene expression was detected only post-DMBA administration (4 weeks after exposure, before palpable carcinomas arise) but not in aged-matched rats that were not treated with DMBA. There was no difference in Tnc expression in the mammary gland of WKy-homozygous congenic rats compared with WF-homozygous congenic animals (Fig. 5C). Tnc expression was also significantly decreased in the thymus and ovary of rats post-HER2/neu infusions (12 weeks, P < 0.001, Fig. 5D). Based on these results, Tnc is a strong candidate gene for eliciting the mammary carcinogenesis phenotype of Mcs5c.

**Discussion**

Rats homozygous for the WKy allele of the 176-kb Mcs5c locus have a 50% decrease in mammary carcinoma development per rat after DMBA administration. As DMBA is a synthetic polycyclic aromatic hydrocarbon that requires metabolic activation to lead to mammary carcinomas, we asked whether these congenic rats were also resistant to mammary carcinomas induced by the HER2/neu oncogene. In this model, a replication-deficient retrovirus expressing a HER2/neu oncogene is directly infused into the mammary ducts of female rats, giving rise to mammary carcinomas. Congenic rats that were WKy-homozygous for the Mcs5 locus were also resistant to mammary cancer induced by a HER2/neu oncogene. They exhibited a similar reduction in carcinoma numbers per rat regardless of whether mammary cancer was DMBA- or HER2/neu-induced, suggesting the effect of Mcs5c is not carcinogen specific and that Mcs5c acts past the initiation stage of carcinogenesis.

The Mcs5c locus is located in a noncoding intergenic desert region of rat chromosome 5. Thus far, all Mcs loci that have been fine-mapped to regions <500 kb are located in noncoding regions of the genome (ref. 18; Gould and colleagues unpublished data). Mcs5c is located between Tnc and Pappa, which are approximately 420 and 530 kb away, respectively. The Mcs5c locus is located in a 1.1-Mb gene desert. We asked whether the noncoding Mcs5c locus may be regulating the expression of genes located directly outside the region. Previous studies of intergenic regions have
expression (D). Phosphorylation expression was also decreased in the thymus and ovary of WKy-homozygous expression was reduced in the thymus (A) and ovary (B) but not in the tissues of the age-matched rats that did not receive DMBA (A – DMBA treatment. Differential expression was not seen in any of these compared with WF-homozygous congenic rats after DMBA treatment and values were obtained using the Mann–Whitney test. Mean relative expression ± standard errors are shown, with 10 or more rats per group. Tnc expression is reduced in the ovary and thymus of WKy-homozygous–resistant congenic rats compared with WF-homozygous congenic rats 4 weeks post-DMBA administration. No differential expression of Tnc was detected in the mammary gland between the 2 rat strains. It is possible that the lack of difference in the mammary gland could be due to the glands heterogenous nature. For example, if a difference existed in one cell type of the mammary gland, it could be diluted by the lack of difference in a more plentiful cell type.

Both the immune system and ovarian hormones are known to be involved in rat mammary carcinogenesis. Ovariectomy of rats prior to or shortly after DMBA administration suppresses the growth of mammary carcinomas (27). Thymectomy was also shown to affect the growth of DMBA-induced mammary carcinomas (27). Furthermore, the degree of immune system depression and individual carcinoma growth is correlated in DMBA-treated rats (27, 28). The significance of ovarian hormones and the immune system on human breast cancer has also been described (29, 30). Because of the importance of ovarian hormones and immune system in breast cancer, the reduced expression of Tnc in the ovary and thymus of Mcs5c congenic rats may be relevant to the mammary phenotype of Mcs5c animals. Reduced expression of Tnc observed in tissues outside the mammary gland of Wky-homozygous Mcs5c animals is hypothesized to lead at least in part to mammary cancer resistance in these rats. It is interesting to note that the Mcs5a locus is believed to act to reduce mammary cancer through activity in immune cells (18). Furthermore, there is an epistatic interaction between Mcs5a and Mcs5c (20), providing support of a possible immune cell role for Mcs5c activity.

TNC is an extracellular matrix protein involved in tissue interactions during fetal development (31), in tissue remodeling, and disease in adults (32). Tnc exerts immunomodulatory functions, such as inhibiting T-cell activation, and influencing T cells in anti-inflammatory processes (33–36). In the ovary, the remodeling of the extracellular matrix is crucial for folliculogenesis, ovulation, and corpus luteum formation (37). As Tnc may affect both the immune system and ovarian functions, it is currently unclear through which of these mechanisms Tnc may exert its effect on mammary carcinogenesis. It is possible that altered Tnc expression in

Figure 5. Tnc expression is reduced in the ovary and thymus of Mcs5c congenic rats after DMBA treatment and HER2/neu treatment. Tnc expression is reduced in the thymus (A) and ovary (B) but not in the mammary gland (C) of Wky-homozygous–resistant congenic rats (black) compared with Wf-homozygous congenic controls (gray) at 4 weeks post-DMBA treatment. Differential expression was not seen in any of these tissues of the age-matched rats that did not receive DMBA (A – C). Tnc expression was also decreased in the thymus and ovary of Wky-homozygous–resistant congenic rats 12 weeks post HER2/neu infusions (D). P values were obtained using the Mann–Whitney test. Mean relative expression ± standard errors are shown, with 10 or more rats per group.
thus far uncharacterized tissues may also play a role in cancer resistance. Interestingly, when Tnc is overexpressed in breast cancer cell lines, an enhancement in growth and invasion is observed (38).

In contrast to what was observed for the previously characterized Mc5 locus, where differential expression of the candidate genes was seen irrespective of DMBA treatment (18), the differential expression of Tnc in rats WKy-homozygous for Mc5 versus WF-homozygous congenic controls is seen after carcinogen exposure (4 weeks post-DMBA) but not in untreated age-matched rats. At 4 weeks post-DMBA, frank carcinomas have not yet arisen although early lesions may be present. Differential expression of Tnc does not seem to be specific to DMBA-induced mammary cancer as Tnc was also differentially expressed in the thymus and ovary of rats dually infused with a HER2/neu oncogene. This HER2/neu-induced expression difference was observed 12 weeks postinfusions, at which time the rats averaged 6 to 10 mammary carcinomas per rat. Rats in both treated groups have been stressed because of exposure to a carcinogen or an oncogene. The mammary glands of these animals have also started to undergo mammary transformation and may have preneoplastic lesions, carcinomas, or both. Early disregulation of gene expression has been observed in the mammary gland as early as 3 weeks post-DMBA administration (e.g., NF-κb, ref. 39), suggesting that there are major changes in the mammary glands of carcinogen-treated animals whether or not frank carcinomas are present. Further characterization of resistant WKy-homozygous versus susceptible WF-homozygous congenic rats will be needed to determine whether a response to stress or to preneoplastic changes in the mammary gland may be responsible for the observed differential expression of Tnc. Thus, the Mc5c WKy allele is an example of a locus polymorphism that interacts with the host environment and xenobiotic exposure to modulate its gene regulatory effects.

In conclusion, we have fine-mapped the 4.5-Mb Mc5c locus to a 176-kb region of rat chromosome 5. Mc5c is a noncoding locus hypothesized to affect mammary carcinogenesis by long-range regulation of the extracellular matrix protein Tnc transcript. Further validation of Tnc as a candidate gene and elucidation of the mechanism by which it may affect rat mammary carcinogenesis will shed light on how this gene could modulate breast cancer susceptibility.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Kim Worley and the Baylor Genomics Center for sequencing clones covering a rat genome sequence gap in our region of interest, Dr. Stephan Wodinischa for assistance with HER2/neu mammary gland infusions and Dr. Bob Mau for his assistance with statistical analyses.

Grant Support

NIH grants CA077994 and CA123272 to M.N.G., the DOD postdoctoral fellowship DAMD17-03-1-0280 to D.J.S, and the DOD-W81XWH-05-1-0611 predoctoral fellowship to A.L.V.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 5, 2010; revised October 4, 2010; accepted October 20, 2010; published online January 4, 2011.

References


Mcs5c: A Mammary Carcinoma Susceptibility Locus Located in a Gene Desert that Associates with Tenascin C Expression


Updated version  Access the most recent version of this article at: http://cancerpreventionresearch.aacrjournals.org/content/4/1/97

Cited articles  This article cites 36 articles, 12 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/4/1/97.full#ref-list-1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.