Research Article

Cancer Prevention Research

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

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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. *Cancer Prev Res; 4(1); 97–106. ©2011 AACR.*

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 penetrant 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

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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*. Congenic rats with selected WKy alleles on a WF background were made from recombinants generated from congenic line Y (20).

DMBA phenotyping

At 50–55 days of age, WF.WKy congenic female rats (either WKy-homozygous or WF-homozygous) and WF controls were given DMBA (ACROS Organics, Fisher Scientific) in sesame oil as a single gastric intubation of 65 mg/kg of body weight as previously described (21). Mammary carcinomas $\geq 3 \times 3$ mm were counted 15 weeks after DMBA administration. Mammary carcinoma multiplicity was analyzed using the nonparametric Mann–Whitney test in Statview (SAS Institute) or using nonparametric Wilcoxon tests adjusted for multiple comparisons as applicable.

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 ($\geq 3 \times 3$ 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 chr*5*: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 (AC229948, 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 $MgCl_2$; dATP, dCTP, dGTP, and dTTP at 400 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: 60 nmol/L *Gapdh*, 500 nmol/L *Tnfsf15*; 100 nmol/L *Gapdh*, 300 nmol/L *Tnfsf8*;

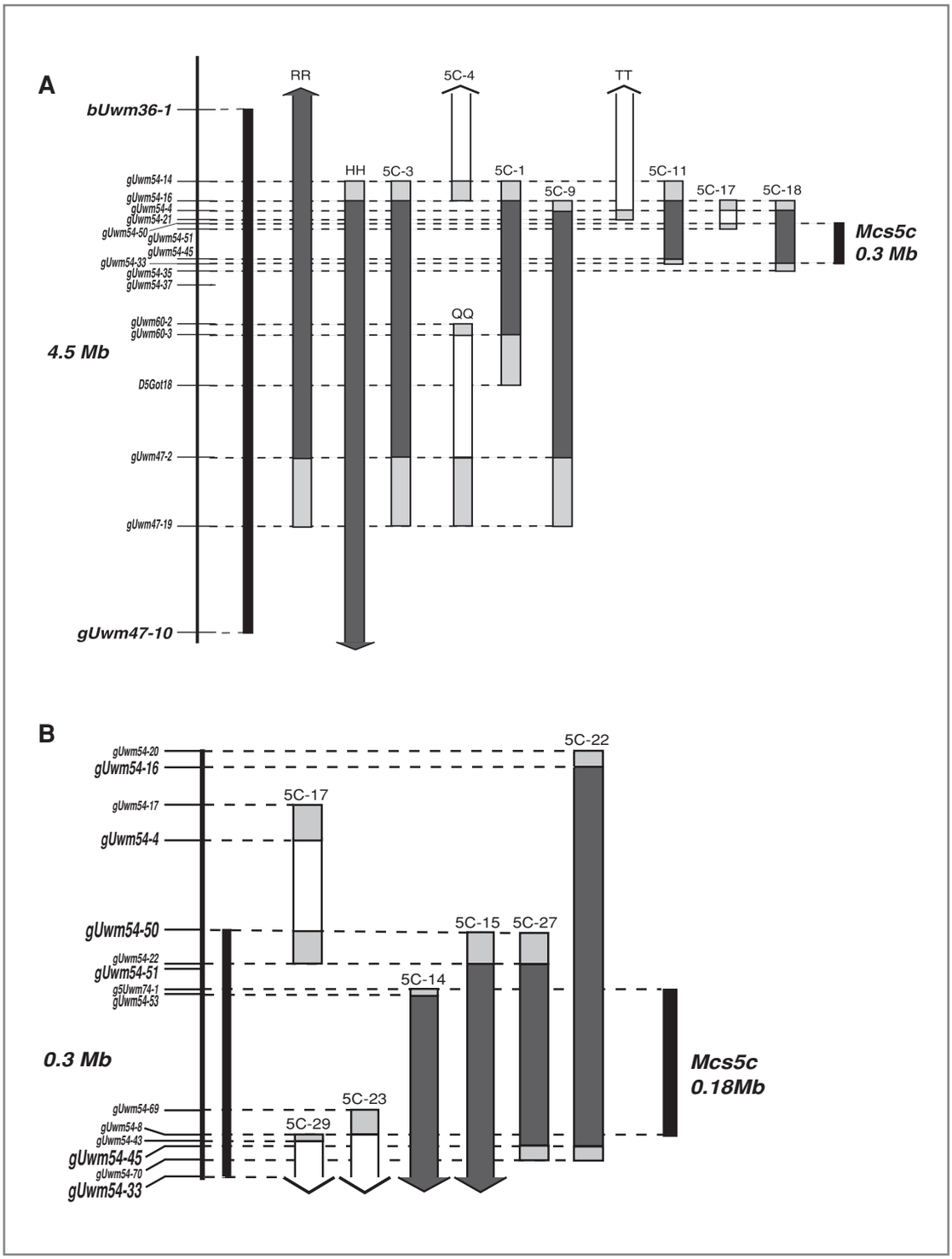

Figure 1. Fine-mapping of *Mcs5c.* Maps of the congenic lines used to fine-map the *Mcs5c* locus beginning with the previously defined 4.5-Mb region (20). Microsatellite markers in the *Mcs5c* region on rat chromosome *5* are shown along the *Y*-axis. Negative or susceptible congenic lines for which 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′-ATGTTTCCGACTGTGACAGA
Tnfsf15 F 5′-GGACTTAGCACCCTCCTGATGA
Tnfsf15 R 5′-GTTGTGCTGAAGGGGCAGAC
Tnfsf8 probe 5′-AAAGGAGGAAATTGCTC
Tnfsf8 F 5′-GGACTCCACTCCAAAAACAACTG
Tnfsf8 R 5′-GTAGGCCCATGACTTCTTGGAT
Tnc probe 5′-CGAGAGCTGTGATTAGA
Tnc F 5′-GGCTGTCAGAAGGCCAGATG
Tnc R 5′-TGCCATGAAGGGATTTGAAGA
Pappa probe 5′-ATGCCATAGGGTCAGAGTG
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 ($P < 0.001$). 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-11and 5C-18, averaged 8.8 ± 0.7 ($n = 29$) carcinomas compared with 7.7 ± 0.5 ($n = 34$) 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

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

[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.

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

Primer name	Forward[a]	Reverse[a]
bUwm36-1	GATTCCTCTGTTCTTCTGGA	CCTGTGATTCCAACTCTAGG
gUwm54-14	ATCCTAATCTGCAAGTGAGC	CACCATTCCCTCTTAGTTCA
gUwm54-20	CAAGGCCAGAGAAATACAAG	ATAGCTCTCTCCCTGTCTCC
gUwm54-16	CACAAATGCATAGAAGCAGA	TATAGTCAAGGTGGGAGGTG
gUwm54-17	GGTCTCAATGGTAGAGGACA	AACAAATGATGCAGTTGACA
gUwm54-4	TCTGAAATAGAACCGGACAG	GGAATCCATCCCTAGTGTCT
gUwm54-21	AGACCTTCCATCTTGATCCT	GGTCCAGTTTATAGCACGAC
gUwm54-50	AGGAAAGCAGGGAAAGTAAG	CCAGGAAAGCAGATGAGTTA
gUwm54-22	TGGTGGTATTTTGTTGTTCA	GCTTGAAGGAAGAGGTAGGT
gUwm54-51	CTCTTCAAAAGCATGTGGAT	CAATGTCGAATTGAACACAC
g5Uwm74-1	ATAAATGACCAGGAAGCAAA	TGAGAGAAAACCAAACAACC
gUwm54-53	AGTTTCCTTGATGAAAACCA	CCATACCTGACACAGGAAAT
gUwm54-69	AAGTTTAACTGGGAGGAGGA	TACTATGGCCTCTGAAAGGA
gUwm54-8	TAAAGGCACCTAACCCTGTA	TTAACAGAGCACCATGAGGT
gUwm54-43	CAGGAGTCTATGGAATGAGG	TCTGGCCTCTGAACTAATGT
gUwm54-45	TCTATTGAAGATCCCCTTTG	CAGAGAGTAGCTGGCGTTAG
gUwm54-70	TCAAAGCAATGATCAGCATA	TGCAATTTGTCATGGTCTTA
gUwm54-33	AAGGTTGGACAGTGAAGAGA	AGTAGCCTCTGCATTCTCAG
gUwm54-35	TGAGAACAGCACAGAAATGA	GTCTCAGGCAAACTCAACTC
gUwm54-37	CTCAGGCATATGTCAAGAGC	CCATGGCATCTTATAAGTCA
gUwm60-2	GTTACACCATTAACCCTGGA	ACTGAATTGCTTGGTCTGTC
gUwm60-3	AGGATGCTTGAAAGATTGTG	GGGTTTGACAAGGATACAGA
gUwm47-2	TGGAAGTTGTGTAAGGATGA	CCTTCCAGATACGTGACATT
gUwm47-19	CCAGTTGTGAGCTCTGACTT	CAGTTGTTGTGTTTGTGTCC
gUwm47-10	GATAGGGACCATCCTAATCC	GCCAGAACAGAGTCTGAGTG

[a]Primer sequences are shown 5′ to 3′.

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 ($P \leq 0.0015$), 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

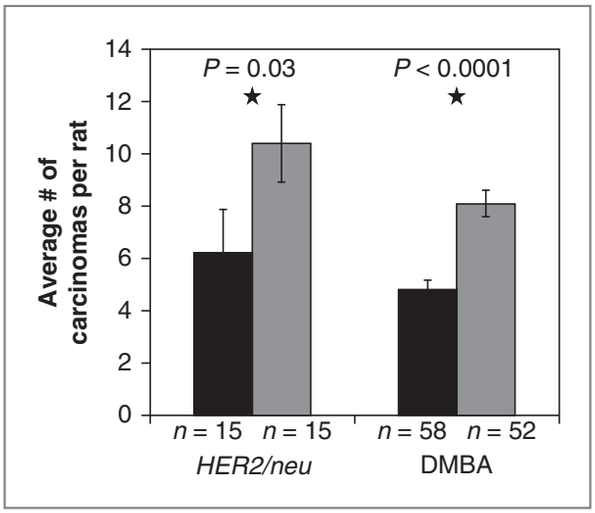

Figure 2. *Mcs5c* equivalently decreases mammary carcinoma multiplicity following DMBA administration and *HER2/neu* infusions. Average number of carcinomas per rat ± standard error is shown for congenic line 5C-11 WKy-homozygous animals (black) and WF-homozygous congenic controls (gray) for each of the 2 treatment groups. *P* values were obtained using the nonparametric Mann–Whitney test for the DMBA study and using a generalized linear model for the *HER2/neu* experiment.

transcripts (UCSC http://genome.ucsc.edu/ and Ensembl http://uswest.ensembl.org/index.html genome browsers). 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 *Msc5c* 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

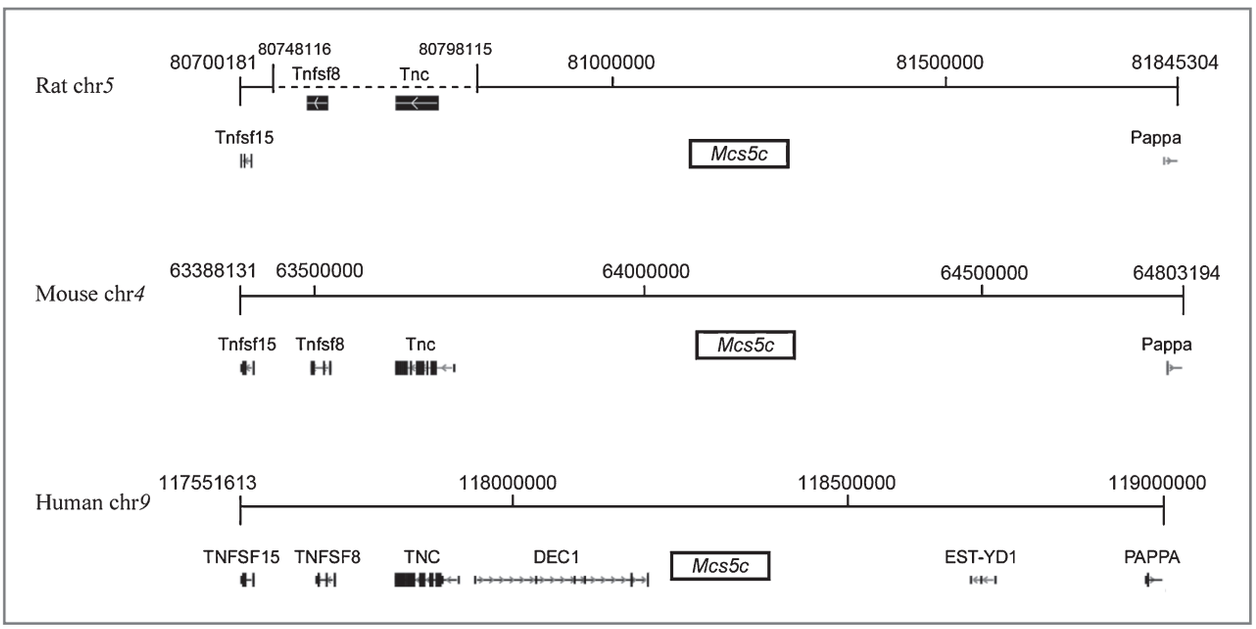

Figure 3. Comparative genomic map of the *Mcs5c* locus. The gene-poor 176-kb *Mcs5c* locus ± approximately 500 kb and its orthologous regions in the mouse and human are shown. The potential candidate genes *Tnfsf15*, *Tnfsf8*, *Tnc* and *Pappa* are indicated. The dashed line indicates the sequence gap in the rat genome database, which was covered by sequencing the BAC clones. The map coordinates were obtained using the November 2004, July 2007, and February 2009 UCSC genome browser assemblies for the rat, mouse, and human, respectively.

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.*

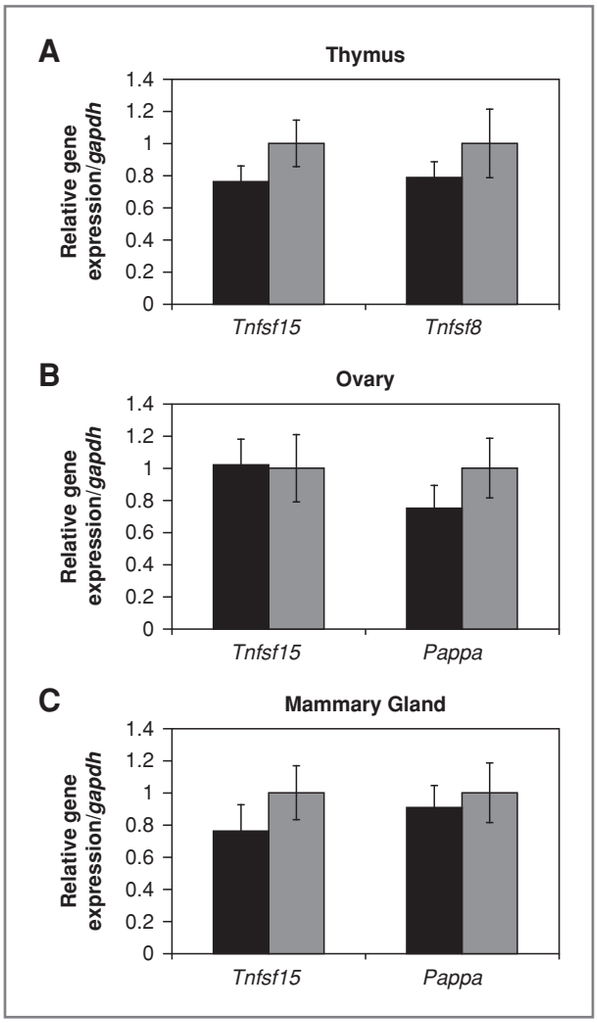

Figure 4. Relative expression of *Tnfsf15*, *Tnfsf8*, and *Pappa* in the thymus, ovary, and mammary glands of *Mcs5c* WKy-homozygous rats 4 weeks post-DMBA. No differential expression of *Tnfsf15*, *Tnfsf8*, and *Pappa* was observed in (A) the thymus, (B) the ovary, and (C) the mammary gland of WKy-homozygous congenic rats having the *Mcs5c* region (black) versus the WF-homozygous congenic controls (gray) at 4 weeks post-DMBA administration. *Tnfsf8* expression was only quantified in the thymus; *Pappa* expression could not be quantified in the thymus. Mean relative expression ± standard errors are shown, with 10 or more rats in each group.

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

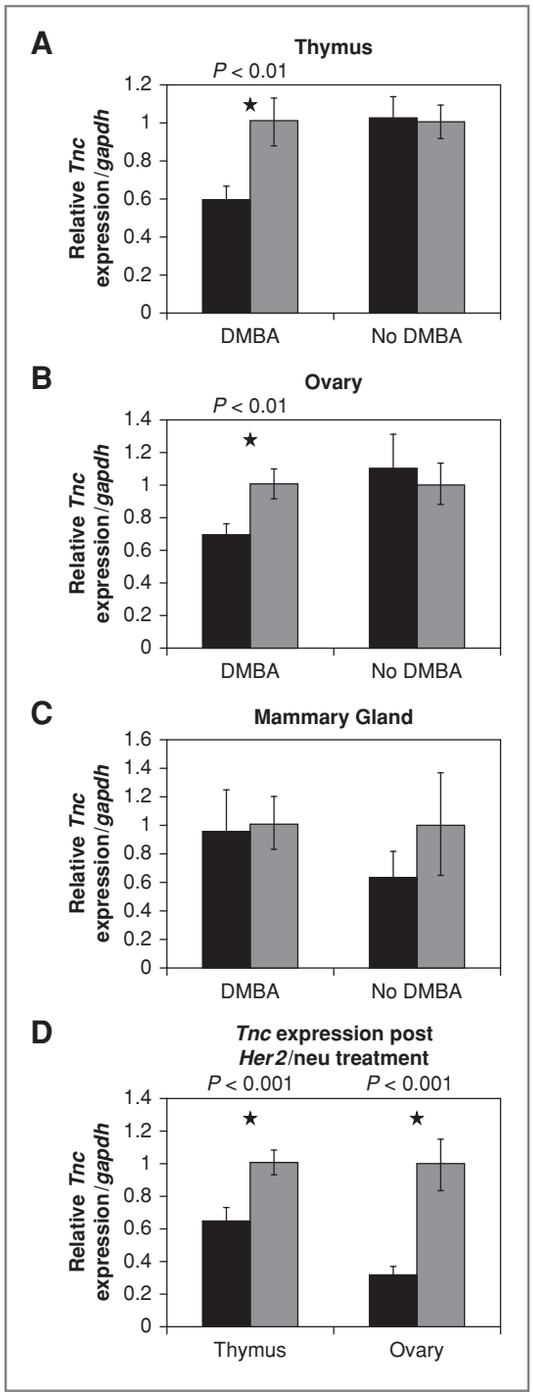

Figure 5. *Tnc* expression is reduced in the ovary and thymus of *Mcs5c* congenic rats after DMBA treatment and *HER2/neu* treatment. *Tnc* expression is reduced in the thymus (A) and ovary (B) but not in the mammary gland (C) of WKy-homozygous–resistant congenic rats (black) compared with WF-homozygous 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.

suggested that they may contain regulatory elements that can function over large distances. For example, analysis of the human *DACH* gene desert led to the discovery of 7 conserved elements with enhancer activity located up to 780 kb away from the *DACH* gene (23). Further evidence of distal intrachromosomal or interchromosomal regulatory activity has also been reported (24–26).

We investigated whether any of the 4 genes located within approximately 500 kb of the *Mcs*5c locus were differentially expressed between WKy-homozygous–resistant congenic rats and WF-homozygous congenic controls. *Tnc* was found to be differentially expressed only following the initiation of the carcinogenesis process (by DMBA administration or *HER2/neu* infusions). *Tnc* expression was reduced by 40% in the thymus and 30% in the ovary 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

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 *Mcs5a* 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 *Mcs5c* 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 ductally 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 *Mcs5c* 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 *Mcs5c* locus to a 176-kb region of rat chromosome *5*. *Mcs5c* 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.

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