Abstract

Rat strains differ dramatically in their susceptibility to mammary carcinogenesis. On the assumption that susceptibility genes are conserved across mammalian species and hence inform human carcinogenesis, numerous investigators have used genetic linkage studies in rats to identify genes responsible for differential susceptibility to carcinogenesis. Using a genetic backcross between the resistant Copenhagen (Cop) and susceptible Fischer 344 (F344) strains, we mapped a novel mammary carcinoma susceptibility (Mcs30) locus to the centromeric region on chromosome 12 (LOD score of ~8.6 at the D12Rat59 marker). The Mcs30 locus comprises approximately 12 Mbp on the long arm of rat RNO12 whose synteny is conserved on human chromosome 13q12 to 13q13. After analyzing numerous genes comprising this locus, we identified Fry, the rat ortholog of the furry gene of Drosophila melanogaster, as a candidate Mcs gene. We cloned and determined the complete nucleotide sequence of the 13 kbp Fry mRNA. Sequence analysis indicated that the Fry gene was highly conserved across evolution, with 90% similarity of the predicted amino acid sequence among eutherian mammals. Comparison of the Fry sequence in the Cop and F344 strains identified two non-synonymous single nucleotide polymorphisms (SNPs), one of which creates a putative, de novo phosphorylation site. Further analysis showed that the expression of the Fry gene is reduced in a majority of rat mammary tumors. Our results also suggested that FRY activity was reduced in human breast carcinoma cell lines as a result of reduced levels or mutation. This study is the first to identify the Fry gene as a candidate Mcs gene. Our data suggest that the SNPs within the Fry gene contribute to the genetic susceptibility of the F344 rat strain to mammary carcinogenesis. These results provide the foundation for analyzing the role of the human FRY gene in cancer susceptibility and progression.

Introduction

Breast cancer remains the most prevalent cancer among US women, with an estimated 226,870 new cases and 39,510 deaths occurring in 2012 (National Cancer Institute SEER Cancer Statistics). Although a multitude of genetic alterations and molecular pathways have been implicated in the pathogenesis of breast cancer [1,2], there remain significant gaps in knowledge regarding the biology and etiology, in particular, genetic susceptibility to this disease.
Rat strains vary widely in their genetic susceptibility to mammary carcinogenesis. On the assumption that tumor suppressors are conserved across mammalian species, investigators have carried out numerous genetic linkage studies in rats to identify genes responsible for this differential susceptibility [3,4,5,6,7,8,9,10]. Prior studies in rats identified 29 strain-specific mammary carcinoma susceptibility (Mcs) loci [11,12]. Several candidate Mcs genes, including Mc1b, Mcs5a1 and Mcs5c, were identified within several of these loci [6,7,8]. Identification and functional characterization of additional Mcs genes will enhance our understanding of the genetic basis for the differential susceptibility to mammary carcinogenesis.

Previous segregation analyses indicated that a genetic cross between the resistant Cop and the intermediately-sensitive F344 strain would involve a minimal number of genetic modifiers [13,14], and hence could facilitate genetic analyses. We therefore performed genetic linkage analysis and interval mapping in [F344 X Cop] F1 X F344] N2 backcross progeny to identify loci that conferred susceptibility to N-Methyl-N-Nitrosourea (NMU)-induced mammary carcinogenesis in the F344 rat. Using tumor number as the quantitative trait, we mapped a previously unreported susceptibility locus, Mcs30 (QTL30), to rat chromosome 12 (LOD score ~8.6). We identified and evaluated several candidate carcinoma susceptibility genes located within this locus. Comparative DNA sequence analysis, functional prediction, and analysis of FRY expression in rat mammary tumors and human breast cancer cell lines strongly indicate that Fry, the rat ortholog of Drosophila furry gene [15], encodes a Mcs gene within the susceptibility locus (QTL30) on rat chromosome 12 (RNO12).

Materials and Methods

Animals and Breeding Strategies

Ethics Statement: All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Fred Hutchinson Cancer Research Center (FHCRC). All experiments were performed in FHCRC vivarium, which is a fully accredited by AAALAC.

Copenhagen (Cop), Fischer 344 (F344) and Brown Norway (BN) rats were purchased from Harlan Sprague-Dawley, Inc. (Madison, WI). To generate hybrid F1 progeny, F344 females were mated with Cop males. Female (F344 X Cop) F1 progeny were then crossed with F344 males to generate the N2 backcross progeny. All animals were maintained on a standard diet of rat chow and acidified water ad libitum and housed in an approved facility with climate control and a 12-h light-dark cycle. A relational database developed in our laboratory was used to track all breeding, treatments, tumor sites, incidence, latencies, phenotypes and genotypes.

Carcinogen Treatment and Phenotyping

Female N2 and F344 rats were injected with a single intraperitoneal dose of NMU (50 mg/kg body weight) at 50–55 days of age. NMU (Ash-Stevens, Detroit, MI) was prepared as a stock solution of 10 mg/ml in a 0.9% NaCl solution acidified to pH 5.0 with acetic acid. Fresh solutions of NMU were prepared every 30 minutes. Following carcinogen exposure, N2 progeny were maintained ad libitum on acidified water and a high fat diet (rat chow supplemented with 30% fat) to promote tumor growth. Animals were palpated for tumor formation on a weekly basis, and animals were euthanized when moribund or when tumors reached a diameter of one centimeter. Mammary tumors and control normal mammary tissue from tumor-bearing animals were collected, frozen in liquid nitrogen and stored at −80°C. A section of each tumor was fixed in formalin and embedded in paraffin. Sections of each tumor specimen were stained with hematoxylin and eosin for histopathological analysis. The numbers and latency of mammary carcinomas arising were recorded for each animal (Figure S1 in File S1).

Isolation of Genomic DNA and Genotyping

Genomic DNA was isolated from mammary tissues using the Qiagen DNeasy 96 Tissue Kit (QIAGEN Inc. Valencia, CA). Informative Simple Tandem Repeat (STR) markers that have different lengths in the Cop and F344 strains were selected from the Rat Genome Database [http://rgd.mcw.edu/]. Swept radii were calculated using the Haldane mapping functions, and used to estimate the optimum number of markers and N2 backcross progeny required for complete coverage of all 20 autosomes genome at the 95% confidence limits. No markers on the rat X chromosomes were included in the analysis. For low-resolution mapping, 77 polymorphic markers (Table S1 in File S1) across the genome were genotyped in 99 female N2 backcross progeny that were also phenotyped for susceptibility to NMU-induced mammary carcinogenesis. The longest distances in centiMorgan (cM) between markers and the farthest distance from the end of the map are listed in Table S2 in File S1. For high-resolution mapping (1–2 cM intervals whenever possible), we used informative markers from in and around the intervals that yielded significant or suggestive linkage scores in the low-resolution mapping.

PCR primers for STR markers were purchased from Research Genetics, Inc. (Madison, WI). The PCR-based genotyping assays were performed using one of two methods. If the lengths of the STRs differed by eight or more base pairs between the F344 and Cop alleles, the PCR reactions were performed with the unlabeled primers purchased from Research Genetics, and the genotypes were determined by amplicon length using polyacrylamide gel electrophoresis (PAGE). For STRs differing by six or less base pairs, PCR reactions were performed with the fluorescently labeled primers, and the genotypes were determined on an ABI PRISM® 3100 Genetic Analyzer System equipped with a G5 filter. The fluorescence data were analyzed with Genescan software, and the genotype calls were determined with Genotyper software (ABI). All the allelic calls, including those from regular PAGE analysis, were independently verified by at least one other person before importing into the database for further analysis.

Genetic Linkage Statistical Analyses

Linkage analyses were performed with MapManager QTX [16] using tumor number as the quantitative trait. Genetic interactions and associations were tested using two-way ANOVA, χ² tests and logistic analysis models using the SAS v.8.0 statistical software package (SAS Institute, Inc.).

Fluorescence In Situ Hybridization (FISH)

Fibroblasts from the BN/SsNhsdMcw, F344, Cop and F1 rat strains were cultured, blocked in mitosis with colcemid, and harvested to prepare metaphase spreads according to published procedures. Rat BAC clones that included the target STR marker sequences were purchased from the Children’s Hospital Oakland Research Institute (CHORI-230 BAC library) and verified by PCR amplification of the STR sequence. BAC DNA was isolated on an Autogen 740 system, biotinylated by nick-translation, hybridized to metaphase spreads in the presence of excess unlabeled rat Cot1 DNA, and detected with avidin-FITC as described elsewhere [17]. The chromosomes were QFH-banded by DAPI staining. DAPI and FITC images were collected.
separately, but in registration. Hybridization signals were analyzed in at least five, and more typically 10, metaphase cells per probe.

PCR Amplification and Sequencing of Candidate Ms Genes

PCR primers for amplification of selected candidate genes are shown in Table S3 in File S1 for Brea2 gene and Table S4 in File S1 for the rat Fry gene. For the longer exons, primers were designed to generate two or more overlapping amplicons. Following RNA extraction from normal mammary glands of the F344 and COP rats using a Qiagen RNeasy Maxi Kit (Qiagen Inc. Valencia, CA), CDNA was produced using reverse transcription reaction according to SuperScript® II Reverse Transcriptase instructions (Invitrogen, Carlsbad, CA). The amplified PCR products were separated by agarose gel electrophoresis (0.8–1.5%), and PCR products excised from the gel and purified with Qiagen gel extraction kits (Qiagen Inc. Valencia, CA).

DNA sequencing was performed using the Big Dye Cycle Sequencing Protocol (PerkinElmer Biosystems Inc. Boston, MA). Each PCR product was sequenced on both strands with the same primers used in the PCR amplification reaction. For the longer PCR products, additional sequencing primers were designed to generate overlapping amplicons. The labeled sequencing reaction products were separated on an automated, fluorescence-based sequencer (Model 377 from Applied Biosystems, Foster City, CA). Automated base calls were reviewed by visual inspection. Sequences were assembled using Sequencher 4.2 software (Gene Codes Corporation, Ann Arbor, MI).

Northern Blot Analysis

RNA was extracted from the normal mammary glands of F344 and COP rat strains using a Qiagen RNeasy Maxi kit (Qiagen Inc. Valencia, CA). Total RNA samples (30 μg) were separated by electrophoresis on a 0.8% (w/v) formaldehyde/agarose gel, transferred to Hybond-N nylon membranes (Amersham Pharmacia Biotech) and probed with a [32P]-dCTP labeled Brca2 peptide. The antiserum detected a 280 kDa protein in rat kidney (not shown), while the molecular weight was 120 kDa in human mammary tissue (Millipore, Billerica, MA). The membranes were incubated with this validated anti-FRY antisera (1:10,000 dilution) overnight at 4°C. The blots were then rinsed three times with TTBS for 10 min, 7 min, and 5 min, respectively, rinsed briefly with TBS and incubated with LI-COR anti-rabbit secondary antibody for 45 minutes under gentle agitation at 4°C. Blots were developed using the LI-COR Odyssey Infrared Imaging System. The FRY protein was verified through blocking the antibody (or the pre-serum) with the peptide. The antisera detected a 280 kDa protein in rat kidney (not shown), while the molecular weight was ~120 kDa in rat and human mammary cells, suggesting tissue-specific post-transcriptional regulation. FRY protein levels were normalized to β-actin.

Cell Culture

Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. MCF-10A cells were cultured in Mammary Epithelium Basal Medium (MEBM), supplemented with gentomicin sulfate, amphotericin-B, 20 ng/ml human epidermal growth factor, 10 μg/ml insulin, 5 μg/ml hydrocortisone and bovine pituitary extract using MEGM SingleQuots (Lonza, Allendale, NJ). HCC1954, T47D, MCF7 and MDA-MB-231 cells were cultured in Advanced DMEM with 10% heat inactivated FBS with 100 μg/ml penicillin-streptomycin and 20 ng/ml insulin, 5 μg/ml hydrocortisone supplemented with gentomicin sulfate, amphotericin-B, 20 ng/ml insulin, 5 μg/ml hydrocortisone and 0.32 μg/ml 40% bovine serum albumin. For cell culture, 2% CO2 was added to the atmosphere of 5% CO2 and 95% air. MCF-10A cells were maintained in a humidified atmosphere of 5% CO2 and 95% air. FRY protein was detected using the anti-FRY antibody. The antisera detected a 280 kDa protein in rat kidney (not shown), while the molecular weight was 120 kDa in human mammary tissue (Millipore, Billerica, MA). The membranes were incubated with this validated anti-FRY antisera (1:10,000 dilution) overnight at 4°C. The blots were then rinsed three times with TTBS for 10 min, 7 min, and 5 min, respectively, rinsed briefly with TBS and incubated with LI-COR anti-rabbit secondary antibody for 45 minutes under gentle agitation at 4°C. Blots were developed using the LI-COR Odyssey Infrared Imaging System. The FRY protein was verified through blocking the antibody (or the pre-serum) with the peptide. The antisera detected a 280 kDa protein in rat kidney (not shown), while the molecular weight was ~120 kDa in rat and human mammary cells, suggesting tissue-specific post-transcriptional regulation. FRY protein levels were normalized to β-actin.

Semi-quantitative Reverse Transcription (RT)-PCR

Tissues from normal Cop and F344 rats as well as mammary tumors and control mammary tissue from NMU-treated F344 rat were collected. Total RNA was isolated from these tissues and human breast cancer cell lines using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). Reverse transcription reactions were performed with the SuperScript® II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). The PCR conditions for DNA amplification in the linear range were established on a GeneAmp PCR System 7600 (PerkinElmer, Wellesley, MA). Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA) and primers for the Rat Fry gene (5- GCCCTGTTCATAGACTCT -3 ) and 5- TCAAAGACAAACGCCTCCA -3 ) and 5- GAGGTCCTTTAAGCAGTGTC -3 ). Human FRY gene (5- GTGTACAGAGACGCTTCT -3 and 5- CATGGGGCTGTGCATCTC -3 ) and human β-actin (5- CATGAGATCTCACCGA -3 and 5- AAGGGACTTCTTG -TACA -3 ) were used for DNA amplification. RT-PCR products were analyzed on 1% agarose gels, and expression was quantified using Image J software (NIH, Bethesda, MD) and normalized to β-actin.

Quantitative Real-time PCR Analysis

Total RNA was extracted from three independent cultures exponentially growing cells (70% confluence) using Qiagen RNeasy Mini protocol. Relative expression levels were measured using quantitative Real-time PCR chain reactions (QRT-PCR) and SYBR® Green intercalation to quantify amplification. Sequences of the human FRY specific forward and reverse primers were 5′- TCC CAT GTC TGG ATA TTT GC - 3′ and 5′ - ATA AGG CCA GGC ATA GCT GA – 3′, respectively. All reactions were performed in triplicate (technical replicates) and products verified by gel electrophoresis. CT values were normalized to those of β-actin. Expression levels are shown relative to MCF10A cells. Error bars indicate standard deviations.

Western Blot Analysis and Validation of the FRY Anti-peptide Antibody

To generate polyclonal antisera against the FRY protein, rabbits were immunized with a FRY-specific peptide conjugated to keyhole lymph hemocyanin. The immunogenic peptide used was chosen from a region of the FRY gene whose predicted amino acid sequence was conserved in mice, rats and humans.

Total cellular extracts (30 μg protein) were separated by electrophoresis in pre-cast 7.5% Tris-HCl gels (Bio-Rad, Hercules, CA) and transferred to Immobilon-FL PVDF membranes (Millipore, Billerica, MA). The membranes were incubated with this validated anti-FRY antisera (1:10,000 dilution) overnight at 4°C. The blots were then rinsed three times with TTBS for 10 min, 7 min, and 5 min, respectively, rinsed briefly with TBS and incubated with LI-COR anti-rabbit secondary antibody for 45 minutes under gentle agitation at 4°C. Blots were developed using the LI-COR Odyssey Infrared Imaging System. The FRY protein was verified through blocking the antibody (or the pre-serum) with the peptide. The antisera detected a 280 kDa protein in rat kidney (not shown), while the molecular weight was ~120 kDa in rat and human mammary cells, suggesting tissue-specific post-transcriptional regulation. FRY protein levels were normalized to β-actin.

5′ and 3′ RACE Reaction

Mammary tissues from normal Cop and F344 rats were collected. Total RNA was isolated from these tissues using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). The 3′ and 5′ RACE were used to determine the open reading frame (ORF) of the rat Fry mRNA sequence. Amplifications were carried out with the Marathon cDNA Amplification Kit (BD-Clontech, Palo Alto, CA). Two gene-specific primers, 5′ - GTGGGTCACACGCTCTGCCTGCGTAC - 3′ (3′-RACE) and 5′ - TCAAATAGTCCTGCTGCTGCTGCTAC - 3′ (5′- RACE), were designed from the nucleotide sequences within the sequenced DNA fragments. Double stranded cDNA prepared using the Marathon cDNA Amplification Kit was amplified and cloned using the TOPO TA kit for DNA sequencing.
SNAP Analysis

The SNAP (Screening for Non-Acceptable Polymorphisms) computational tool predicts the functional consequences of single amino acid substitutions in both binary (neutral/non-neutral, with respect to wild type function) and scored from −100 to +100, where negative predictions are neutral, positive are non-neutral, and higher absolute values of scores indicate higher reliability of the binary prediction [18]. Using SNAP, we determined whether the non-synonymous SNPs in the Fisher 344 rat strain are indicative of functional effects when present in the rat as well as the human FRY genes. The SNAP output scores were evaluated for each amino acid substitution at the codons in question. Additionally, we assessed the likely functional (as opposed to structural) importance of each amino acid in the protein sequence by computing the SNAP-SubMat score — for each wild-type residue, the average of all SNAP scores of substitutions allowed by the BLOSUM62-matrix at cutoff >0 [19]. SNAP prediction of non-neutral functional effects for substitutions expected to be functionally neutral (as indicated by common, BLOSUM62-defined exchangeability of the involved residues in evolution) is indicative of the functional importance of the protein sequence position in question. As with regular SNAP scores, SNAP-SubMat scores <0 indicate that a specific sequence position is not likely functionally significant, and a score >0 indicates that this location is probably functionally significant.

Results

Genome-wide Screen for Mcs (QTL) Loci

We performed linkage analysis using a genetic backcross between two rat strains with differential susceptibility to NMU-induced mammary carcinomas (the resistant Cop and the susceptible F344 strains). We selected a panel of 77 informative STR markers (Table S1 in File S1) that provided an estimated 95% coverage of the twenty rat autosomes (Table S2 in File S1). Low-resolution linkage of 50 N2 progeny with and 49 without mammary tumors provided suggestive evidence for linkage of tumor susceptibility to the short arm of rat chromosome 12 (RNO12) (normal font in Table 1). About 58% of the N2 progeny developed mammary tumors over the course of one year after NMU exposure (Figure S1 in File S1). We used the number of mammary tumors induced within the first 210 days after NMU exposure as the quantitative trait (Figure S1 in File S1). We initiated high-resolution QTL mapping using an additional 225 female N2 progeny. All 324 of the NMU-treated N2 progeny were genotyped at all informative markers (bold font in Table 1) from the candidate locus on RNO12. Markers bounded by and including D12Rat57 and D12Arb2 yielded highly significant linkage, with a maximum LOD score of 8.6 at the D12Rat59 marker (Table 1), demonstrated the presence of a new Mcs locus. Interval mapping [20] also indicated highly significant linkage to the same intervals on RNO12 (Figure 1 A and B) confirming the presence of a new Mcs locus. The direction of the additive effect confirmed that the presence of the Cop FRY allele of the Mcs was negatively correlated with susceptibility. This new susceptibility locus on RNO12 was designated as Mcs30 (QTL30) and was assigned the RGD ID: 7243062.

FISH Mapping of the Putative Mcs Locus on RNO12

At the time we performed these studies, both genetic linkage [21,22] and the rat radiation hybrid mapping [23] placed all the STRs that defined the Mcs30 locus on the short arm of RNO12 [24]. The D12Rat59 marker was also depicted within the short arm of RNO12 on the UCSC Genome browser (http://genome.ucsc.edu/). However, rat chromosome 12p is a satellite, comprised of tandem repeat sequences that show strain-specific variations in length [25,26]. Using several Bacterial Artificial Chromosome (BAC) clones harboring the D12Rat59 marker (LOD ~8.6), we physically mapped the Mcs30 locus by fluorescence in situ hybridization (FISH). We used the sequence of the highly linked D12Rat59 marker (LOD ~8.6) for a BLAST search of the available rat genome sequences, and assembled a contig comprising three overlapping BAGs: CH230-85G15; CH230-381M14; and CH230-275K13 (Figure S2 in File S1).

Table 1. Calculated LOD scores for markers on rat chromosome 12.

| Marker Name | Number of Rats Genotyped | LOD |
|-------------|--------------------------|-----|
| D12Rat57    | 324                      | 3.3 |
| D12Rat1     | 324                      | 6.0 |
| D12Rat59    | 324                      | 8.6 |
| D12Arb2     | 324                      | 2.1 |
| D12Rat3     | 99                       | 2.7 |
| D12Rat35    | 99                       | 0.8 |
| D12Rat36    | 99                       | 0.5 |

Note: LOD, logarithm of the odds ratio. doi:10.1371/journal.pone.0070930.t001

Each of these BAC clones was physically mapped by fluorescence in situ hybridization (FISH) to metaphase chromosomes prepared from embryo fibroblasts of Cop, F344, and (F344 X Cop)F1 hybrid strains, as well as the BN/SsNHsdMcw strain used for rat genomic sequencing (Figure S3 in File S1) [24]. BAGs CH230-123F8 and CH230-151L24 were used as controls, which include the EPO and PAI1A1 gene sequences, respectively to RNO12q12 and RNO12q11 (Table 2) [27,28]. All of the BAGs tested were FISH-mapped to the long arm of rat chromosome 12. BAC CH230-85G15, which includes the highly linked D12Rat59 marker, mapped to the 12q11-12 region of RNO12, close to the centromere (Figure 2). FISH analyses performed using embryo fibroblasts from the F344 and Cop strains, as well as F1 hybrids yielded identical results. Together these results indicated that the Mcs30 locus mapped to the centromeric region on the long arm of rat chromosome 12 (RNO12q). FISH analysis did not reveal any gross differences between the Cop and F344 in the region defined by the BAGs, although there is slight heteromorphism in the pericentromeric region proximal to the FISH signals.

The Mcs Gene on Rat Chromosome 12 is Distinct from Brca2

The Mcs30 locus comprises approximately 12 Mbp on the long arm of RNO12 that probably incudes the centromere (Figure 3). A comparison of the draft human and rat genome sequences indicated that the Mcs30 locus includes a 5.6 cM region on RNO12q whose syteny is conserved on human chromosome 13q12 to 13q13. Mcs30 harbors at least 186 genes (RGD Rat Genome V3.4 Assembly, down loaded on 5/19/13). We used available functional data (e.g., data from KEGG, GeneCards, etc.) to select several candidate genes whose functions could be related to cancer susceptibility. These candidate included the rat orthologs of the human BRCA2 gene, the St aud1 gene, also known as DLC2 (Deleted in Liver Cancer 2), and the insulin receptor (Insr).
Brca2 gene is ~0.6 Mbp distal to the highly linked D12rat59 marker, raising the possibility that Brca2 gene was a Mcs gene in QTL30. A previous study demonstrated that the known haplotypes within exon 11 of the rat Brca2 gene did not segregate with the susceptibility phenotype [29]. However, these observations could not rule out the possibility that the rat Brca2 gene includes haplotype blocks that segregate with susceptibility.

To rule out strain-specific epigenetic silencing, we compared levels of Brca2 expression in mammary tissue from F344 and Cop rats. Northern blot analysis showed that Brca2 was expressed at comparable levels in mammary glands of both F344 and Cop rats (Figure 4A). We next asked if the Brca2 gene from the two strains differed at the nucleotide sequence level (See Table S5 in File S1 for all Accession Numbers). The genomic sequencing results showed that the coding sequence of the Brca2 genes from the Cop and F344 strains (Genbank accession numbers for Brca2: AH014113.1 (Cop) and AH014114.1 (F344)) differed by only two synonymous SNPs at nucleotides 5380 and 9239 (Figure 4B), one of which (nucleotide 5380) was reported in previous study, which also showed that this SNP was not linked to genetic susceptibility [29]. We found no strain-specific sequence differences within the Brca2 splice junctions or adjacent sequences. Together, these results suggested that differences in the sequence, transcription or splicing rat Brca2 did not contribute to the susceptibility of the F344 rat strain to NMU-induced mammary carcinogenesis.

We also found no strain-specific alterations in DNA sequence for several other candidate genes within the Mcs locus, including the domain containing STARD 13 (Stard13) and the insulin receptor (Insr): the sequences of the Stard13 and Insr genes in both the Cop and F344 strains were identical to those previously reported (NM_001109060 and NM_017071.2, respectively) (See Table S5 in File S1 for all Accession Numbers). Moreover, the mRNA levels of both genes were comparable between Cop and F344 rat, and NMU-treatment did not affect the expression levels of Insr and Stard13 (Figure S4 in File S1).

**Table 2.** BACs used for FISH. BACS containing sequences from the D12Rat1, D12Rat59, Epo and PAI1A1were used from physical mapping of loci to Rat chromosome 12.

| BAC Identification | Sequences within BAC | Chromosomal Localization |
|--------------------|----------------------|--------------------------|
| CH230-152N10       | D12Rat1              | RNO12q11-12              |
| CH230-85G15        | D12 Rat59            | RNO12q11-12              |
| CH230-381M14       | D12Rat59             | RNO12q11-12              |
| CH230-275K13       | D12 Rat59            | RNO12q11-12              |
| CH230-123F8        | PAI1A1               | RNO12q12                 |
| CH230-151L24       | EPO                  | RNO12q12                 |

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![Figure 1. Interval mapping of putative quantitative trait loci (QTL) on rat chromosomes 12 that confer susceptibility to NMU-induced mammary carcinogenesis.](http://www.plosone.org/doi/abs/10.1371/journal.pone.0070930.g001)
Analysis of mRNA Sequence and Tissue Specific Expression of the Rat Fry Gene

The Mcs30 locus also included the Fry gene, previously designated as a hypothetical gene similar to the human CG003 (predicted) gene. Since the rat Fry ortholog was only 30 Kbp distal to the D12Rat59 marker, we decided to evaluate its potential role in genetic susceptibility. We designed a series of PCR primers to amplify and sequence overlapping fragments comprising the predicted open reading frame of the rat ortholog (Table S4 in File S1). Sequencing failed to reveal a transcription start site within the predicted open reading frame. We therefore performed 5' and 3' RACE (rapid amplification of cDNA ends) using mRNA extracted from mammary tissue. The results demonstrated that the transcript in rat mammary tissue includes 10792 nucleotides encoding Fry, the rat ortholog of the Drosophila furry gene. Rat Fry mRNA encodes a predicted protein comprising 3011 amino acids.

RT-PCR analyses demonstrated that the rat Fry gene is highly expressed in the heart and brain, with relatively lower expression levels in several other tissues including the mammary gland, kidney, pancreas, colon, uterus, and liver (Figure 5A). We found no-strain specific differences in the expression of the Fry gene in any of the organs tested from Cop and F344 rat, including mammary glands.

We next compared Fry expression levels in normal rat mammary tissue with those in mammary carcinomas collected from same rat. The results showed that the rat Fry gene was expressed at low levels in a majority of mammary tumors relative to mammary tissue of age-matched control rats (Figure 5B and 5C).

The Genome of the Inbred F344 Rat is Homozygous for the Mutant Fry Allele

Since there were no strain-specific differences in the expression of Fry in mammary cells, we asked if the F344 Fry gene harbors any loss of function mutations. We sequenced the Fry gene both in the Cop (NCBI Accession EU563851) and F344 (NCBI Accession EU563850) rat strains. DNA sequence comparison of the Fry gene identified 22 SNPs, two of which were non-synonymous. The first of these (codon 661) substitutes an Aspartic acid (D) residue in the Cop protein with a Glutamic acid (E) residue in the F344 protein. The SNP in codon 2170 replaces an Alanine (A) in the Cop protein with a Serine (S) residue in the F344 protein.

To investigate if these amino acid substitutions occurred in conserved sequences, we (multiple sequence) aligned the sequences of all vertebrate orthologs of the fry gene (OrthoDB, group id EOG6PCBZN) [30] and the COP and F344 strain sequences using ClustalOmega [31]. The results showed that these two amino acid residues were identical to those of the resistant Cop strains in most species tested (Figure 6), which harbors conservative amino acid substitutions, and a few orthologs are missing this region altogether. These observations are consistent with the possibility that the variant allele present in F344 rat alters the function of the Fry protein. Nonetheless, if the alignment is performed with protein families rather than the FRY sequence, this region of the FRY protein was not explicitly conserved.
A.

**Figure 4. Comparison of Brca2 mRNA levels in mammary tissue and DNA sequences in Cop and F344 rats.** (A) Steady-state levels of the 13 kb Brca2 mRNA were compared by Northern blot analysis. Brca2 mRNA was expressed at comparable levels in both strains. (B) Comparison of Brca2 cDNA sequences in Cop and F344 rat strains. DNA sequence analysis detected two synonymous single nucleotide polymorphisms (SNP); a T to C transition in Exon11 and C to T transition in Exon25.

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We therefore used SNAP [18] to further investigate whether these non-synonymous SNPs in the Fisher 344 rat strain are indicative of functional effects, or if they were to occur in the human protein. The human FRY protein sequence was taken from the UniProt [32] (Q5TBA9; FRY_HUMAN). The SNAP scores for an amino acid change at rat Fry codon 661 (where the F344 rat had an Aspartic acid to Glutamic acid mutation) in the rat and human protein were −51 and −44, respectively, indicating that this mutation is not likely to cause functional consequences. SNAP-SubMat scores of 2 and −11 for position 661 in the rat and human proteins, respectively, further suggested that this amino acid residue may not be functionally significant. We also analyzed the SNAP and SNAP-SubMat scores for the human protein residue 2171, which coincided with the F344 rat Alanine to Serine mutation in codon 2170. The SNAP scores for an amino acid substitutions at rat Fry codon 2170 and human FRY codon 2171 were score −74 and −67 respectively, indicating that an amino acid change at this location is also not likely to have functional consequences. The SNAP-SubMat scores of −16 and −37 for amino acid substitutions in this residue in the rat and human proteins, respectively, also indicated that this location is may not be functionally significant in the FRY protein.

SNAP-SubMat scores are a good way to measure functional significance of protein residues within a sequence. Our results indicate that neither of the mutations affects a clearly functional site in the protein. However, SNAP does not assess the functional effects of newly introduced amino acids, such as acquiring de novo phosphorylation sites. Since *Drosophila melanogaster* and *Saccharomyces cerevisiae* Fyr orthologs regulate the function of serine/threonine protein kinases [15,33], we next asked if the introduction of Serine residue created a de novo phosphorylation site in the mutant Fry protein in F344 rats. Using the NetPhos2.0 [34] phosphorylation site prediction software, we confirmed that the serine residue in the F344 rat Fry sequence was within a peptide sequence with a 90.6% probability of being a substrate for several protein kinases (Table 3). Moreover, replacing the alanine in the human protein with a serine, also produced a predicted phosphorylation site.

**Altered Expression and Mutation of FRY in Human Breast Cancer Cell Lines**

We next measured human FRY gene mRNA levels in several human breast cancer cell lines. As shown in Figure 7A, the level of FRY mRNA expression in all breast cancer cell lines evaluated was reduced by at least 40% relative to the non-tumorigenic MCF10A human mammary epithelial cell line. This result was further confirmed by qRT-PCR analysis. FRY protein levels were decreased in 3 of 4 breast cancer lines evaluated (Figure 7B). Although we observed a higher level of FRY protein in the MCF7 breast cancer cell line, our studies showed that the FRY gene is mutated and probably dysfunctional in this cell line. DNA sequence analysis showed that the coding sequence of the FRY gene in the MCF7 cell line harbors 10 SNPs relative to the sequence published in the NCBI database (NM_023037.2), four of which have been previously reported in the NCBI database. Among these 10 SNPs are three non-synonymous changes (Figure 7C). The Leucine (L) to Proline (P) substitution at amino acid 1177 of the MCF7 FRY protein has a SNAP score +18, indicating that the substitution is highly likely associated with a functionally disruption. The SNP in codon 1968 changes the amino acid from Glycine (G) to Serine (S); the SNP in codon 2544 substitutes a serine (S) with a Proline (P) in MCF7 FRY protein (Figure 7C). However, SNAP analysis suggested that these two substitutions may not lead to functional changes. Taken together, our observations in human and rat mammary tumor cells are consistent with the possibility that decreased FRY activity and/or function is associated with an increased risk of carcinogenesis.

**Discussion**

Over the past two decades investigators have made significant progress in elucidating the genetic basis for the differential susceptibility of inbred rat strains to mammary carcinogenesis, identifying 29 susceptibility (*Mcs*) loci [6,7,8,11,12]. In the present study, we used N2 backcross progeny generated by mating female (F344 X Cop) F1 progeny with F344 males to identify additional *Mcs* loci by linkage analysis. All informative markers on chromosome 12 within the region bounded by and including the D12Rat59 and D12Arh2 markers yielded highly significant LOD scores, with a maximum (LOD=8.6) at the D12Rat59 marker. This interval, deemed *Mcs30* (QTL30), encodes almost 200 genes and includes a −5.6 Mbp region on the rat chromosome 12 whose synteny is conserved on human chromosome 13g12 to 13g13. We therefore used available data to identify candidate *Mcs* genes within this region, including the rat orthologs of the human *BRCA2* gene, the *Stard13* gene (also known as *DLC2*) that encodes a Rho GTPase activating protein with growth suppressor function [35,36], and the Insulin Receptor (*Insr*). However, comparison of DNA sequences and mRNA levels indicated that these genes did not differ between strains and hence were unlikely to encode the *Mcs* gene within the *Mcs30* locus RNO12q9. We therefore focused our attention on *Fry*, a highly conserved gene, located less than 30 Kbp from distal to the highly linked D12Rat59 STR marker.

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We first sequenced and established the entire open reading frame and splice sites of the *Fry* gene in Cop and F344 rat strains. The rat *Fry* gene shared significant sequence similarity with the *Drosophila* *furry* gene, and was 80% similar to human (*FRY*) and mouse (*Fry*) orthologs. Our analysis further revealed a total of 22 single nucleotide polymorphisms between the *Fry* alleles of Cop and F344 strains, two of which resulted in amino acid substitutions in the susceptible F344 relative to the resistant Cop strain. One approach to the identification of functional SNPs is to use comparative genomics [37], which predicts that amino acids important to the normal function of a protein will be highly conserved through evolution and show very little species variation. By contrast, amino acids that have little effect on function will be less conserved and more likely to harbor non-synonymous SNPS in distantly related species. Our analyses indicated that while the F344 SNPs are conserved in FRY orthologs, they were not

![Figure 5. Expression of rat Fry mRNA in rat tissues and mammary tumors.](image)

Semi-quantitative RT-PCR analysis of steady-state *Fry* mRNA levels in somatic tissues of Cop and F344 rats. β-actin expression was used for normalization (lower panel). (B) Semi-quantitative RT-PCR analysis of steady-state *Fry* mRNA levels in normal mammary tissue, mammary tumors and normal mammary tissue collected from the matched tumor bearing F344 rats. β-actin mRNA of rat was used for normalization (lower panel). (C) Image densitometry in (B) was quantified using Image J software.

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explicitly conserved in protein families with related sequences. Since Drosophila melanogaster and Saccharomyces cerevisiae Fry orthologs regulate the function of serine/threonine protein kinases [15,33], it is plausible that the introduction of Serine residue could lead to altered Fry phosphorylation and regulation. Consistent with this possibility, the NetPhos2.0 phosphorylation site prediction software indicated that Ser at residue 2170 creates a de novo phosphorylation site in the mutant Fry protein in F344 rats. Semi-quantitative PCR analysis further indicated Fry gene was expressed at reduced levels in mammary tumors when compared to normal mammary of non-tumor bearing rats. Although the findings were not confirmed by the sensitive quantitative PCR

Table 3. Prediction of a novel phosphorylation site in the F344 Fry allele using NetPhos2.0 phosphorylation prediction software.

| Name                          | Position | Context     | Score   | Predicted          |
|-------------------------------|----------|-------------|---------|--------------------|
| **Sequence**                  | 2170     | AERISQVCL   | 0.986   | *S*                |
| **Site**                      |          |             |         |                    |
| S-2170 protein kinase, DNA activated, catalytic polypeptide (predicted) [Rattus norvegicus] | 0.51     |             |         |
| S-2170 ataxia telangiectasia mutated homolog (human) (mapped) [Rattus norvegicus] | 0.55     |             |         |
| S-2170 protein kinase, cAMP-dependent, catalytic, [Rattus norvegicus] | 0.60     |             |         |

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techniques such as 5’ endonuclease based QPCR [38,39], we did use qRT-PCR to confirm that levels of FRY mRNA expression are reduced in all human breast cancer cell lines examined (Figure 7a), indicating that loss of FRY expression is also associated with human breast cancer. More importantly, database mining revealed that FRY mRNA and protein expression are reduced in a human cancers arising from a wide variety of different tissue types, and that that reduced FRY expression is associated with tumor progression and clinical outcomes (Jessica Graham, Xuefeng Ren and Helmut Zarbl, submitted for publication).

Together, these findings indicate that the rat Fry gene encodes a Mcs gene within the Mcs30 (QTL30) locus. However, these findings do not preclude the possibility that the Mcs30 locus harbors additional susceptibility genes.

Although the mammalian FRY genes have not been functionally characterized, orthologs in lower eukaryotes regulate cell differentiation. The D. melanogaster (furry) and S. cerevisiae (tau3) orthologs regulate cell morphogenesis, polarity, cell wall biosynthesis, and proliferation and cytokinesis. Fry proteins mediate these processes by interacting with and maintaining the functions of a class of Serine/threonine protein kinase, such as Tricornered and Warts kinases in the D. melanogaster and the Cbk1 kinase in S. cerevisiae [15,33]. Orthologs to these kinases in humans are members of the nuclear Dbf2-related protein-kinase (NDR) family of kinases. NDR kinases are essential components of pathways that control important cellular processes, such as epithelial cell morphology and polarity, mitotic exit, cytokinesis, cell proliferation and apoptosis from yeast to humans [40]. Recent studies suggest that NDR kinases may also have tumor suppressor activity [41,42], indicating that FRY regulates susceptibility to carcinogenesis by regulating the activity of NDR kinases. Significantly, computational approaches indicate that the Fry protein is likely to include an ATP binding domain, suggesting that it may be regulated by ATP binding or have intrinsic kinase activity. Moreover, the
prediction that the F344 specific serine residue at position 2170 generates a de novo consensus target sequence for multiple protein kinases suggests that the activity of the mutant allele may be altered by aberrant phosphorylation, a common carcinogenic event [43].

Interestingly, at least three previous studies reported that deletions on the long arm of human chromosome 13q12 to 13q22 were very common genetic aberrations in lymph node-negative breast cancer [44,45,46]. A recent study of Scandinavian families also suggested the presence of a third human breast cancer susceptibility locus that is distinct from BRCA2, on the long arm of human chromosome 13 [47]. These authors identified recurrent somatic chromosomal deletions on HSA13q21, with a minimal region of loss between 13q21-q22, although specific loss of the wild type locus was not demonstrated in these tumors. Genotyping indicated the segregation of a shared germline HSA13q21 haplotype by all affected individuals in this high-risk family. Targeted linkage analysis in 77 breast cancer families with markers from this chromosomal region provided further evidence for a putative predisposition locus (BRCA3). However, a subsequent study using 129 high-risk breast cancer families of Western European ancestry failed to corroborate these findings, and indicated that if a BRCA3 locus did exist on HSA13q, it only accounted for a small fraction of heritable, early onset breast cancers [48].

One possible explanation for the discrepancy between these two studies is the effects of gene-environment interactions. Rat mammary tumors induced by exposure to estradiol showed frequent loss of RNO12, while those induced by DMBA did not show this chromosomal loss [49,50], indicating that gene-environment interactions can select for the loss of susceptibility loci on rat chromosome 12. If the human populations used in these studies had different environmental exposures, such gene environment interaction effects might also explain the differences observed between human mapping and somatic deletion studies. Thus, the possibility remains that the human FRI1 gene encodes a putative susceptibility gene on human Chromosome 13q, and studies are in progress to further define its role in human breast cancer.

Supporting Information

File S1 Contains: Table S1. Complete list of Rat Simple Tandem Repeat (STR) Markers used for low-density linkage analysis. Table S2. Coverage of the genome (autosomes) by STR markers used in low-density linkage analysis. Table S3. Primers used to generate amplicons and sequence the Brca2 gene from F344 and Cop rat strains. Table S4. Primers used to generate amplicons and sequence the Rat Fry gene from F344 and Cop rat strains. Table S5. Genbank Accession numbers for all genes sequenced in this study. Figure S1. Histogram showing the incidence (frequency on left axis) of mammary tumors detected in N2 backcross progeny on each of the indicated days post exposure to NMU. The incidence mammary tumors (left axis) and percent of animals with tumors (right axis) as a function of time after exposure is indicated by the green dotted line. Figure S2. Schematic representation of the contig comprising overlapping Bacterial Artificial Chromosome (BACs) containing rat genomic DNA assembled using the DNA sequence of the D12Rat59 STR marker as the seed (not drawn to scale). Rat BAC clones encompassing particular STR markers were obtained from the Children’s Hospital Oakland Research Institute (CHORI-230 BAC library) and verified by PCR amplification to contain the STR sequence. R and F refer to the forward and reverse orientation. Vertical lines indicate sequence overlap. Figure S3. Metaphase chromosome spreads prepared from mouse embryo fibroblast fibroblasts isolated from (F344 X Cop)/F1 progeny were hybridized with Bacterial Artificial Chromosomes containing STR markers that comprise the Mes30 locus or genes on RNO12 used as hybridization controls (see Table 2). Hybridization signals are pseudo-colored red for clarity, and DAPI banding is displayed as grey values. BAC 1: CH230-152N10 containing D12Rat1 hybridized to 12q11-12. BAC 2: CH230-301M14 containing D12Rat59 hybridized to 12q11-12. BAC 3: CH230-275K13 containing D12Rat59 hybridized to 12q11-12. BAC 4: CH230-85G15O containing D12Rat59 hybridized to 12q11-12. BAC 5: CH230-151L24 containing the Epo gene hybridized to 12q11-12. BAC 6: CH230-123F6 containing the Prf1/ Iai gene hybridized to 12q11-12.

Figure S4. Relative mRNA expression levels of candidate genes from the Mes30 locus on Chromosome 12 in normal rat mammary tissue before and after NMU exposure. [A] RNA was isolated from normal mammary tissue of 55 day-old female Fischer 344 and Copenhagen rats, before and 6 h, one day and 30 days after exposure exposing female F344 and Copenhagen rats at a carcinogenic dose of NMU. Gene expression levels of Insr and Stad13 on chromosome 12 were compared by semi-quantification PCR analysis. Expression levels were normalized to expression of beta-actin mRNA; [B] Image densitometry in [B] was quantified using Image J software. (DOC)

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Author Contributions

Conceived and designed the experiments: XR, LG, LJ, HZ. Performed the experiments: XR, JG, LJ, AM, YG, JL, HX, ASK, XS, CF, GV, MF. Analyzed the data: XR, JG, LJ, VB, HZ. Wrote the paper: XR, JG, HZ.

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