

## Selenoproteins reduce susceptibility to DMBA-induced mammary carcinogenesis

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**Selenium is an essential micronutrient in the diet of humans and other mammals. Based largely on animal studies and epidemiological evidence, selenium is purported to be a promising cancer chemopreventive agent. However, the biological mechanisms by which chemopreventive activity takes place are poorly understood. It remains unclear whether selenium acts in its elemental form, through incorporation into organic compounds, through selenoproteins or any combination of these. The purpose of this study was to determine whether selenoproteins mitigate the risk of developing chemically induced mammary cancer. Selenoprotein expression was ablated in mouse mammary epithelial cells through genetic deletion of the selenocysteine (Sec) tRNA gene (*Trsp*), whose product, designated selenocysteine tRNA, is required for selenoprotein translation. *Trsp* floxed and mouse mammary tumor virus (MMTV)-*cre* mice were crossed to achieve tissue-specific excision of *Trsp* in targeted mammary glands. Eight- to twelve-week-old second generation *Trsp*<sup>fl/+</sup>;wt, *Trsp*<sup>fl/+</sup>;MMTV-*cre*, *Trsp*<sup>fl/fl</sup>;wt and *Trsp*<sup>fl/fl</sup>;MMTV-*cre* female mice were administered standard doses of the carcinogen, 7,12-dimethylbenzylbenz[a]anthracene. Our results revealed that heterozygous, *Trsp*<sup>fl/+</sup>;MMTV-*cre* mice showed no difference in tumor incidence, tumor rate and survival compared with the *Trsp*<sup>fl/+</sup>;wt mice. However, 54.8% of homozygous *Trsp*<sup>fl/fl</sup>;MMTV-*cre* mice developed mammary tumors and exhibited significantly shorter survival than the corresponding *Trsp*<sup>fl/fl</sup>;wt mice, where only 36.4% developed tumors. Loss of the homozygous *Trsp* alleles was associated with the reduction of selenoprotein expression. The results suggest that mice with reduced selenoprotein expression have increased susceptibility to developing carcinogen-induced mammary tumors and that a major protective mechanism against carcinogen-induced mammary cancer requires the expression of these selenoproteins.**

### Introduction

Selenium is an essential micronutrient in the diet of many life forms including humans and other mammals. Numerous health benefits have

**Abbreviations:** DMBA, 7,12-dimethylbenzylbenz[a]anthracene; GPx1, glutathione peroxidase 1; GPx4, glutathione peroxidase 4; MMTV, mouse mammary tumor virus; Sec, selenocysteine; Sec tRNA<sup>[Ser]Sec</sup>, selenocysteine tRNA.

been attributed to this element including roles as a chemopreventive agent in cancer, heart disease and other cardiovascular and muscle disorders and roles in inhibiting viral expression, the onset of AIDS in HIV-positive patients, slowing the aging process, mammalian development and boosting the immune system (1). Among these beneficial effects, the one that has received the most attention is selenium's role in preventing cancer (see reviews in 2–14).

The anticarcinogenic properties of selenium, small molecular weight selenium-containing compounds (smw selenocompounds) and selenium-containing proteins (selenoproteins) have been reported in numerous *in vitro*, animal and epidemiological studies (1,11–14). However, despite these many studies, little is known about the underlying metabolic mechanisms of how selenium acts in preventing cancer; and there has been considerable debate in the selenium field whether small molecular weight selenium-containing compounds (4,5,6,7,9) or selenoproteins (11–13), or both (8,10), are the responsible agents. More recently, the emphasis appears to have shifted in favor of selenoproteins as the more likely selenium-containing components involved in cancer prevention (1,2,3,11–14). In addition, two recent studies have shown that selenoproteins are directly involved in preventing colon (15) and prostate cancers (16) and several other studies implicate specific selenoproteins in cancer prevention (1,11,12,14,17–19).

Selenocysteine (Sec) is the selenium-containing amino acid that is incorporated into protein in response to the codon, UGA, and is the 21st amino acid in the genetic code (20–22). Interestingly, Sec is biosynthesized, unlike any other known amino acid in eukaryotes, on its tRNA which is designated selenocysteine tRNA (Sec tRNA<sup>[Ser]Sec</sup>) (23). Sec tRNA<sup>[Ser]Sec</sup> is a single-copy gene in the mammalian genome, is designated as *Trsp* and its expression is essential for selenoprotein synthesis (24). As a consequence, any modulation of tRNA<sup>[Ser]Sec</sup> expression has a dramatic impact on selenoprotein expression. This feature of regulating selenoprotein expression has provided us with a means of elucidating the function of this protein class by generating various transgenic, standard knockout and conditional knockout mouse models involving wild type and mutant Sec tRNA<sup>[Ser]Sec</sup> transgenes and the loss, or targeted loss, of *Trsp* (reviewed in 24,25).

In an initial study, the targeted removal of *Trsp* in mammary tissues using *loxP-cre* technology was examined (26). Only a slight loss in most selenoproteins was found, since the *promoter-cre-recombinase* was specific to epithelial cells and epithelial cells represent only a small proportion of the cell population of mammary tissue in comparison with other cell types. Although mammary tissue consists of relatively few epithelial cells, the mammary gland remains an ideal tissue to examine the role of selenoproteins in cancer since both chemically induced and relevant genetic mouse mammary cancer models have been developed. Furthermore, this tissue is a major focus of cancer occurrence in women with known alterations in several genes that are involved in breast cancer development. For instance, BRCA1 and p53 tumor suppressor genes which are frequently altered in familial breast cancers play a central role in maintaining the genetic integrity of the cell (27–30). Most importantly, BRCA1 and p53 expression have been shown to be altered in the *Trsp* knockout model (26). Therefore, in the present study, we examined whether the reduction of selenoprotein expression in mammary epithelium would affect the incidence of mammary cancer induced through chemical carcinogenesis by targeting the removal of *Trsp* using *loxP-cre* technology (26). We found that homozygous deletion of *Trsp* in mammary epithelial cells resulted in a shortened time to tumor formation and shortened survival time compared with control animals. The reduction in Sec tRNA<sup>[Ser]Sec</sup> was accompanied by reduced expression of selenoproteins. Therefore, these results demonstrate that selenoproteins play a critical role in preventing

mammary cancer and that therapies to enhance the production of selenoproteins may be a useful strategy for chemoprevention.

## Materials and methods

### Chemicals

Paraformaldehyde, glutaraldehyde, NP-40, X-gal, K<sub>3</sub>Fe(CN)<sub>6</sub>, K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, MgCl<sub>2</sub> and Na-deoxycholate were purchased from the Sigma (St Louis, MO).

### Animals

Mice were handled in accordance with protocols approved by the NCI Animal Care and Use Committee and housed in microisolator cages on a 12 h light/dark cycle with food and water provided *ad libitum*. The mice were fed the Charles River Rat and Mouse 18% protein (Autoclavable) 5L79 diet (PMI Nutrition International, St Louis, MO). Mice exhibited normal weight gain, and no differences in weight were observed between the cohorts of mice. Generation of *Trsp* floxed (*Trsp*<sup>fl/fl</sup>) mice in a C57Bl/6 background (26), mouse mammary tumor virus (MMTV)-*cre* mice in an FVB/N background (31) and Rosa26R mice in a B6/129 background (32) have been described previously. Crosses of these mice to generate the animals used in the present study, their genotypes and strain backgrounds are summarized in the scheme shown in Table I. Briefly, F1 generation control mice were produced by crossing homozygous *Trsp*<sup>fl/fl</sup> mice with mice heterozygous for the MMTV-*cre* transgene resulting in *Trsp*<sup>fl/+</sup>;wt and *Trsp*<sup>fl/+</sup>;MMTV-*cre* offspring. The F2 generation was produced by crossing *Trsp*<sup>fl/+</sup>;MMTV-*cre* with *Trsp*<sup>fl/fl</sup>;wt mice to generate *Trsp*<sup>fl/+</sup>;MMTV-*cre*, *Trsp*<sup>fl/+</sup>;wt *Trsp*<sup>fl/fl</sup>;MMTV-*cre* and *Trsp*<sup>fl/fl</sup>;wt offspring. In addition, control animals were generated from MMTV-*cre* and Rosa26R crosses in order to determine whether recombination of an unrelated allele (Rosa26) by *cre* recombinase could influence carcinogen-induced tumor development. Rosa26R contain a flox-STOP-flox cassette upstream of the *lacZ* gene inserted into the Rosa26 locus. Upon *cre*-induced excision of the STOP cassette, *lacZ* is expressed. Wild-type FVB and C57Bl/6 mice were also treated with 7,12-dimethylbenz[a] anthracene (DMBA) to determine differences in tumor susceptibilities of these individual strains which have been reported to be different (31).

### Genotyping

DNA was extracted from mouse tissues using the Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions to determine the presence of floxed *Trsp* and the *cre* transgene by PCR as described (26). In addition, we also determined the presence of Rosa26R by PCR as described previously (32). Moreover, mammary tissue was randomly selected from Rosa26R mice to determine *cre* recombination through the expression of β-galactosidase. Tissues were fixed for 1–2 h in 2% paraformaldehyde, 0.25% glutaraldehyde, 0.01% NP-40 in PBS and stained for β-galactosidase activity (1 mg/ml X-gal, 30 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 30 mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, 2 mM MgCl<sub>2</sub>, 0.01% Na-deoxycholate, 0.02% NP-40, 1x PBS) overnight at 30°C. Stained and unstained tissues were viewed under an inverted microscope.

### Tumor induction by DMBA

7,12-dimethylbenz[a] anthracene was purchased from Sigma. Numbers of female mice with the following genotypes (8–12 week old) produced in the F2 generation received 0.1 ml of 10 mg/ml DMBA dissolved in sesame oil by gavage once a week for 6 weeks: 49 *Trsp*<sup>fl/+</sup>;MMTV-*cre*, 34 *Trsp*<sup>fl/+</sup>;wt, 31 *Trsp*<sup>fl/fl</sup>;MMTV-*cre*, and 33 *Trsp*<sup>fl/fl</sup>;wt. In addition, thirty 8- to 12-week-old female MMTV-*cre* and thirty wild-type mice and thirty-six 8- to 12-week-old female Rosa26R; MMTV-*cre* and 20 Rosa26R;wt mice received 0.1 ml of 10 mg/ml DMBA dissolved in sesame oil by gavage once a week for 6 weeks. Forty 8- to 12-week-old female mice from each FVB and C57Bl/6 group also received 0.1 ml of 10 mg/ml DMBA as described above. Mice were observed twice a week for the development of tumors that were measured by caliper. Mice were euthanized for humane reasons when a tumor reached 2 cm in diameter.

### Pathology

All mice were necropsied. The #4 mammary glands, mammary tumors, all tissues with tumors, lungs and liver were fixed in 4% paraformaldehyde (Fisher Scientific, Carlsbad, CA) overnight and then placed in 70% ethanol and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin (H&E) and subjected to a blind review by a single pathologist (M.H.).

### Western blot

Protein extracts were prepared from mammary glands by homogenizing the tissue in cold lysis buffer [50 mM Tris; pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Igepal and Complete Mini Protease Inhibitor (Roche)]. Protein was electrophoresed through 10% polyacrylamide gels, transferred onto PVDF

membranes and immunoblotted with antibodies against glutathione peroxidase 1 (GPx1) (Abcam, Burlingame, CA; 1:1000 dilution), glutathione peroxidase 4 (GPx4) (Epitomics, Cambridge, MA; 1:1000 dilution) or 15 kDa selenoprotein (Sep15) (Epitomics; 1:1000 dilution). Following incubation of the primary antibody, membranes were washed with tris-buffered saline (20 mM Tris/HCl, pH 7.5 and 150 mM NaCl) containing 0.1% Tween 20 and incubated in anti-rabbit horseradish peroxidase-conjugated secondary antibody (Thermo Scientific, Rockford, IL; 1:10 000). Following secondary antibody incubation, membranes were washed with tris-buffered saline, incubated in SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and exposed to X-ray film.

### Statistical analysis

Differences in tumor incidence between groups were tested using Fisher's Exact Test. Differences in median number of tumors were tested using the Wilcoxon or Kruskal–Wallis test as appropriate. Differences in time to tumor or survival time were tested using Kaplan–Meier methods and the Wilcoxon chi-square statistics.

## Results

### Generation of mammary-specific, *Trsp*-deficient mice

F1 and F2 generation mice were generated as shown in Table I to obtain animals with the desired genotypes for use in these studies. Table II indicates the number of mice used in each experimental group and the number of mammary tumors that developed in each group. Genotyping of mice was performed using PCR of DNA extracted from tail snips. The presence of the MMTV-*cre* transgene was confirmed by a 280 bp PCR fragment (Figure 1, upper panel). Confirmation of recombination of the floxed *Trsp* alleles in the mammary glands was performed by PCR on DNA extracted from mammary glands. The wild-type *Trsp* allele is detected as a 900 bp band, the *Trsp* floxed allele as a 1.1 kb band and the *Trsp* knockout allele as a 450 bp band (Figure 1, lower panel). Recombination of the floxed *Trsp* allele was detected only in mice carrying the MMTV-*cre*

**Table I.** Mouse genotypes, background strains, crosses, and resulting offspring used in the study

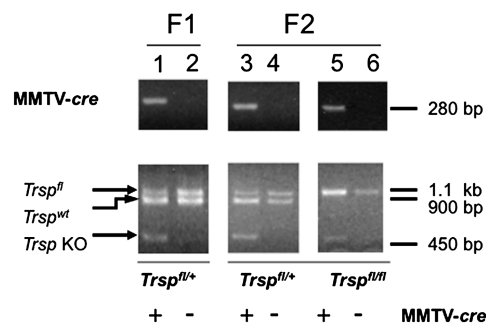
Mouse genotypes <sup>a</sup>	Crossed with	Mouse genotypes	Offspring <sup>b</sup>
F1 generation			
MMTV- <i>cre</i> (FVB/N)	X	Wt (C57Bl/6)	MMTV- <i>cre</i> wt
Rosa26R (B6/129)	X	MMTV- <i>cre</i> (FVB/N)	Rosa26R;MMTV- <i>cre</i> Rosa26R; wt
<i>Trsp</i> <sup>fl/fl</sup> (C57Bl/6)	X	MMTV- <i>cre</i> (FVB/N)	<i>Trsp</i> <sup>fl/+</sup> ;MMTV- <i>cre</i> <i>Trsp</i> <sup>fl/+</sup> ;wt
F2 generation			
<i>Trsp</i> <sup>fl/+</sup> ;MMTV- <i>cre</i> (C57Bl/6; FVB/N)	X	<i>Trsp</i> <sup>fl/fl</sup> ;wt (C57Bl/6)	<i>Trsp</i> <sup>fl/+</sup> ;MMTV- <i>cre</i> <i>Trsp</i> <sup>fl/+</sup> ;wt <i>Trsp</i> <sup>fl/fl</sup> ;MMTV- <i>cre</i> <i>Trsp</i> <sup>fl/fl</sup> ;wt

<sup>a</sup>Background strain in parentheses.

<sup>b</sup>Represent the offspring that were dosed with DMBA.

**Table II.** Listing of experimental groups, number of mice and number of mice developing mammary tumors in each group

Experimental groups	Number/group	Number with tumors (%)
1) wt FVB/N + DMBA	40	17 (42.5)
2) wt C57/Bl6 + DMBA	40	9 (22.5)
3) Rosa26R; wt + DMBA	20	6 (30.0)
4) Rosa26R;MMTV- <i>cre</i> + DMBA	36	11 (30.5)
5) <i>Trsp</i> <sup>fl/fl</sup> ;wt + DMBA	33	12 (36.4)
6) <i>Trsp</i> <sup>fl/fl</sup> ;MMTV- <i>cre</i> + DMBA	31	17 (54.8)
7) <i>Trsp</i> <sup>fl/+</sup> ;wt + DMBA	34	21 (61.8)
8) <i>Trsp</i> <sup>fl/+</sup> ;MMTV- <i>cre</i> + DMBA	49	26 (53.1)



**Fig. 1.** Excision of floxed-*Trsp* alleles in the mammary glands of *Trsp<sup>fl/fl</sup>;MMTV-cre* mice. Upper panel shows a 280 bp band for the MMTV-*cre* transgene in lanes 1, 3 and 5 as demonstrated by PCR. Lower panels show heterozygous and homozygous floxed *Trsp* mice wherein *Trsp<sup>fl/+</sup>* is represented by a 1.1 kb band, *Trsp<sup>wt</sup>* by a 900 bp band and *Trsp<sup>fl/fl</sup>* by a 450 bp band. F1 and F2 generations are shown in lanes 1 and 2 and in lanes 3, 4, 5 and 6, respectively.

transgene. The 450 bp knockout allele appears relatively weak compared with the other allele bands that is due to the fact that only a relatively small percentage of cells in the mouse mammary gland are epithelial cells that undergo *cre*-recombination in this model system (26). Body weights of the groups of mice in the F2 generation (*Trsp<sup>fl/fl</sup>;MMTV-cre*; 25.7 gm  $\pm$  3.47, *Trsp<sup>fl/fl</sup>;wt*; 25.5 gm  $\pm$  3.41, *Trsp<sup>fl/+</sup>;MMTV-cre*; 24.8 gm  $\pm$  1.67,  $p = 0.705$ ) were not significantly different from one another suggesting that the loss of *Trsp* in the mammary gland did not affect the overall health of the mice. Our previous study of selenoprotein loss in the normal mammary gland using *Trsp<sup>fl/fl</sup>;MMTV-cre* mice demonstrated strong *cre* expression in the mammary epithelial cells with evidence of moderate expression in the skin and spleen and virtually undetectable expression in the kidney (26).

#### DMBA-induced tumor susceptibility in FVB, C57Bl/6, MMTV-*cre* and Rosa26R mice

Since mice used in this study were hybrids between different strain backgrounds, we compared the response of FVB and C57Bl/6 wild-type mice with the induction of tumors by DMBA (Figure 2). There was no statistically significant difference in the mammary tumor incidence [FVB, 42.5% (17/40) versus C57Bl/6; 22.5% (9/40),  $p = 0.09$ ] (Table II) or median tumor number (1.0 versus 1.0,  $p = 0.99$ ) comparing FVB and C57Bl/6 mice. However, following exposure to DMBA, FVB mice developed mammary tumors in a significantly shorter period of time compared with C57Bl/6 mice (166 versus 273 median days, respectively;  $p = 0.0001$ ) (Figure 2A and Table III) and exhibited a significantly reduced survival rate (about 132 versus 180 median days, respectively;  $p = 0.004$ ) (Figure 2B and Table III). These data are consistent with previous reports demonstrating that DMBA-induced mammary tumors arise earlier and are more aggressive in FVB than in C57Bl/6 mice (31). Since the floxed model system that we utilized in this study introduced a recombination event in the mouse genome, we examined the possibility that such an event could modify the risk of DMBA-induced carcinogenesis unrelated to the *Trsp* allele. Therefore, to control for *cre* recombination, we determined whether tumor incidence following exposure to DMBA differed between Rosa26R; MMTV-*cre* mice and Rosa26R; wt mice in the same genetic background. No significant differences in mammary tumor incidence (30.5% versus 31.6%; respectively;  $p = 0.739$ ) (Table II), median tumor number (1.0 versus 1.0; respectively;  $p = 0.363$ ) or survival (about 238 versus 249 median days; respectively;  $p = 0.845$ ) were observed between these groups (Table III). The results indicate that *cre*-mediated recombination of an allele unrelated to tumorigenesis does not increase tumor susceptibility (see Supplementary Figure 2A and B is available at *Carcinogenesis* Online).

#### Loss of Sec-tRNA<sup>[Ser]<sup>Sec</sup> increases susceptibility to mammary cancer</sup>

DMBA is known to cause multiple pathologies in mice leading to death including a significant incidence of lymphomas (33). We

determined overall survival curves for each group of mice (Figure 3). Mammary tumor incidence was calculated as the number of mice developing a mammary tumor (confirmed histologically)/total number of mice treated in each group (Table III). The median time of tumor onset was determined for the mice in each group that developed mammary tumors (Table III). We compared the time with mammary tumor onset and survival between *Trsp<sup>fl/fl</sup>;wt*, *Trsp<sup>fl/+</sup>;MMTV-cre* and *Trsp<sup>fl/fl</sup>;MMTV-cre* mice from the F2 generation in response to DMBA exposure as described in the Materials and methods (Figure 3A and B). There was no significant difference between *Trsp<sup>fl/fl</sup>;MMTV-cre* and *Trsp<sup>fl/fl</sup>;wt* mice in overall mammary tumor incidence [54.8% (17/31) versus 36.4% (12/33), respectively;  $p = 0.21$ ] (Table II) and median tumor number (1.0 versus 1.0;  $p = 0.30$ ). However, *Trsp<sup>fl/fl</sup>;MMTV-cre* mice developed mammary tumors more rapidly than *Trsp<sup>fl/fl</sup>;wt* mice (154 versus 356 median days, respectively;  $p = 0.03$ ) and had a shorter survival period compared with *Trsp<sup>fl/fl</sup>;wt* mice (146 versus 214 median days;  $p = 0.018$ ) (Figure 3A and Table III). Compared with *Trsp<sup>fl/+</sup>;wt* mice, however, *Trsp<sup>fl/+</sup>;MMTV-cre* mice showed no differences in mammary tumor incidence [61.8% (21/34) versus 53.1% (26/49), respectively;  $p = 0.50$ ] (Table III), median tumor number (1.0 versus 1.0;  $p = 0.94$ ), time to tumor development (294 versus 300 median days, respectively;  $p = 0.18$ ) and survival (242 versus 264 median days, respectively;  $p = 0.21$ ) (Table III). This is consistent with our previous study showing that *Trsp<sup>fl/+</sup>;MMTV-cre* mice produce sufficient levels of selenoproteins to provide adequate selenoprotein function (26). The histology of the mammary tumors generated by DMBA were mostly of the adenocarcinoma type, although other phenotypes included tubuloacinar adenocarcinoma, solid carcinoma, carcinosarcomas, spindle cell sarcoma and keratoacanthoma (see Supplementary Table I and Figure 1 is available at *Carcinogenesis* Online).

#### Loss of Sec-tRNA is associated with reduced GPx1, GPx4 and Sep15

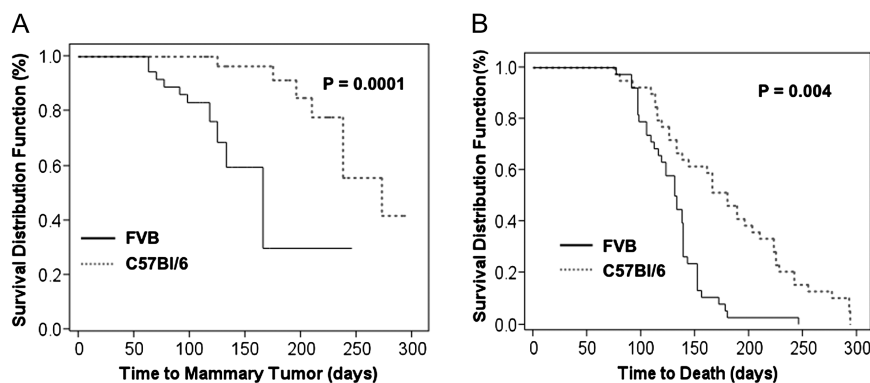
Since loss of Sec-tRNA results in the loss of selenoprotein expression, we evaluated the expression levels of representative selenoproteins, Sep15, GPx4 and GPx1 from DMBA-induced mammary tumors generated in *Trsp<sup>fl/fl</sup>;MMTV-cre* and *Trsp<sup>fl/fl</sup>;wt* mice (Figure 4). Western blot analyses demonstrated that mammary tumors arising in *Trsp<sup>fl/fl</sup>;MMTV-cre* mice expressed reduced levels of Sep15, GPx4 and GPx1 selenoproteins as compared with tumors from *Trsp<sup>fl/fl</sup>;wt* mice. Since tumors are composed of a mixture of epithelial cells in which the *Trsp* knockout has occurred as well as stromal cells containing wt *Trsp*, we did not observe a complete loss of these proteins by western blotting. Although Sec tRNA<sup>[Ser]<sup>Sec</sup> expression was reduced in *Trsp<sup>fl/fl</sup>;MMTV-cre* mice in our earlier study (26), it was apparent from the level of expression that there was substantially more cell types in addition to epithelial cells in mammary tissue used for tRNA analysis. Unfortunately, no reagents are available to perform immunohistochemistry on the tumor samples to demonstrate loss of the proteins within the epithelial cells.</sup>

## Discussion

Although epidemiological data have associated a protective effect of selenium in the prevention of prostate and lung cancer in humans, the relationship between selenium and breast cancer risk is less clear (1,11,12). Since selenium may exist in various forms within an organism, recent studies have attempted to elucidate what role each form of selenium may play in cancer chemoprevention. This element may exist as elemental selenium, small molecular weight selenium-containing compounds and selenium incorporated into selenoproteins as the amino acid, Sec. All three forms of selenium appear to have positive effects on preventing cancer (2–14) (1,3,15). The current study has demonstrated, by using a genetic approach, that selenoproteins are protective against chemical carcinogen-induced mammary cancer in mice.

Our previous study in which *Trsp* was knocked out in wild-type mouse mammary glands revealed a reduction in Sec tRNA<sup>[Ser]<sup>Sec</sup> and</sup>





**Fig. 2.** Mammary tumor development and survival rates in FVB and C57Bl/6 control mice. (A) Time to mammary tumor formation following DMBA exposure in control FVB (solid line) and C57Bl/6 mice (dotted line). Represents the percent of mice that develop mammary tumors over time. (B) Time to death of control FVB (solid line) and C57Bl/6 mice (dotted line) from all causes.

**Table III.** Median time to death and time to mammary tumor onset

Group	Median	Confidence interval		P-value
		Lower 95%	Upper 95%	
<b>Time to death</b>				
FVB	132	119	146	0.004
C57Bl/6	180	138	210	
<i>Trsp<sup>fl/fl</sup></i> ;MMTV- <i>cre</i>	146	126	216	0.018*
<i>Trsp<sup>fl/+</sup></i> ;MMTV- <i>cre</i>	264	234	322	0.21*
<i>Trsp<sup>fl/fl</sup></i> ;wt	214	168	266	0.068#
<i>Trsp<sup>fl/+</sup></i> ;wt	241	196	286	
Rosa26R; MMTV- <i>cre</i>	238	168	287	0.845
Roas26R; wt	249	133	295	
<b>Time to mammary tumor onset</b>				
FVB	166	133	197	0.0001+
C57Bl/6	273	238	306	
<i>Trsp<sup>fl/fl</sup></i> ;MMTV- <i>cre</i>	236	154	261	0.03*
<i>Trsp<sup>fl/+</sup></i> ;MMTV- <i>cre</i>	300	265	412	0.18*
<i>Trsp<sup>fl/fl</sup></i> ;wt	356	294	377	
<i>Trsp<sup>fl/+</sup></i> ;wt	294	188	384	

\*p-value when compared with *Trsp<sup>fl/fl</sup>*;wt. #p-value comparing *Trsp<sup>fl/fl</sup>*;wt versus *Trsp<sup>fl/+</sup>*;wt. \*p-value when compared with *Trsp<sup>fl/fl</sup>*;wt. +p-value comparing FVB versus C57Bl/6.

selenoprotein levels but the reduction was only partially observed due to the low percentage of epithelial cells among other cells in mammary tissue (26). Herein, we expanded the earlier study and explored whether the loss of *Trsp* and, therefore, the loss of selenoprotein expression increased the risk of development of mammary tumors induced by DMBA using *Trsp<sup>fl/fl</sup>*;MMTV-*cre*, *Trsp<sup>fl/+</sup>*;MMTV-*cre* and *Trsp<sup>fl/+</sup>*;wt mice. Previous studies have shown that different strains of mice respond differently to DMBA. Hennings *et al.* (31) showed that FVB mice were more susceptible to developing DMBA tumors than C57Bl/6 mice. Therefore, we carefully controlled for genetic background of the mice in these experiments to exclude genetic background as a confounding variable in influencing the outcome of our results.

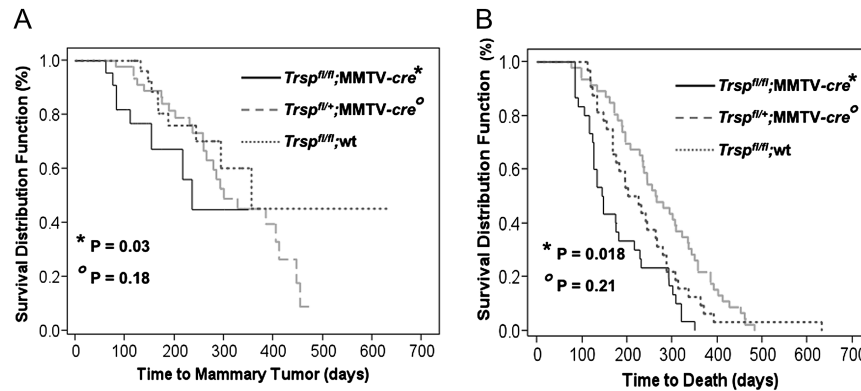
Furthermore, we observed that genomic recombination alone did not increase the incidence of DMBA-induced mammary carcinogenesis. Genomic recombination by *cre* has been shown to cause DNA damage in mammalian cells (34), and thus, we evaluated whether *cre* recombination in the mammary gland in an unrelated allele contributes to a higher risk of developing DMBA-induced mammary tumors. Rosa26R; MMTV-*cre* mice undergo homologous recombination at the Rosa26 locus in the mammary epithelium resulting in the

loss of Rosa26 protein expression which is replaced by expression of *lacZ*. No differences in tumorigenesis were observed in Rosa26R; MMTV-*cre* mice exposed to DMBA compared with Rosa26R; wt mice. Therefore, we conclude that the differences in mammary tumor development observed in *Trsp* knockout mice compared with wild type is not the result of nonspecific *cre* recombination.

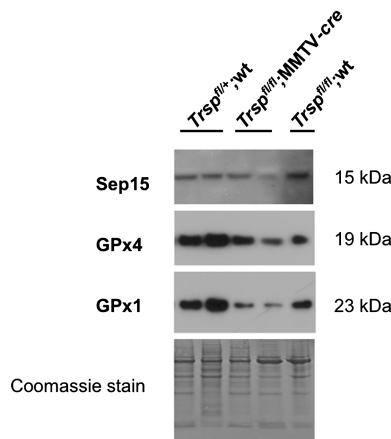
Hu *et al.* showed that loss of heterozygosity at the GPx1 locus is a common event in cancer of head and neck, breast, lung and colon (18,35–37). GPx1 was shown to protect against hypoxia in human breast cancer cells (38). In addition, a gene variant of GPx1 was shown to be associated with breast cancer (18). Furthermore, loss of heterozygosity of Sep15 was shown to be associated with breast cancer (3). Our results are consistent with these studies wherein we found that reduction in the expression of selenoproteins (see Figure 4) is associated with accelerated mammary tumor development. Unfortunately, we were not able to directly compare activities of these selenoproteins in normal mammary tissues (due to the low numbers of mammary epithelial cells in the gland) or tumor tissue since the other cell types present have not lost the *Trsp* alleles. However, our earlier study of *Trsp* loss in normal mammary epithelium has demonstrated that this model system does knock-out selenoprotein expression in the mammary gland (26). We demonstrated a reduction in levels of the selenoproteins GPx1, GPx4 and Sep15 in the present study.

Clearly, selenoproteins are not expressed in mammary epithelial cells due to the dependence of their synthesis on the presence of Sec tRNA<sup>[Ser]<sub>Sec</sub></sup> which is absent by the targeted removal of *Trsp* (26). Interestingly, loss of both *Trsp* alleles and lack of selenoprotein expression in mammary epithelium resulted in a significantly shortened time to tumor onset and reduced survival time but did not reduce overall tumor incidence or significantly impact tumor phenotype. The shortened time to tumor onset and reduced survival time might be explained by the ability of selenoproteins to delay tumor promoting events induced by the carcinogen, but the inhibition is eventually overcome. As cancer cells suffer from oxidative stress and the major role of most characterized selenoproteins is to serve as antioxidants (39), such a proposal would seem to be a logical possibility. Our results appear to be significant since they suggest that chemoprevention may be improved by identifying mechanisms that increase selenoprotein expression, but it should also be noted that other prevention targets may also be necessary in order to ultimately prevent the development of mammary cancer. If translatable to preventing human breast cancer, then combinations of chemopreventive agents along with enhanced selenoprotein production may be critical in reducing the development of breast cancer.

Two well-characterized selenoproteins that serve as antioxidants are TR1 and GPx1 and the loss of their antioxidant properties most certainly has an impact on the role that this selenium-containing protein class has in maintaining reduced oxidative, cellular stress in



**Fig. 3.** Tumor development and survival rates in *Trsp* knockout and control mice. (A) Time to tumor formation in *Trsp*<sup>fl/fl</sup>;MMTV-*cre*<sup>\*</sup> (solid line), *Trsp*<sup>fl/+</sup>;MMTV-*cre* (dashed line) and *Trsp*<sup>fl/fl</sup>;wt mice (blue dashed line). Represents the percent of mice that develop mammary tumors over time. (B) Time to death of *Trsp*<sup>fl/fl</sup>;MMTV-*cre* (solid line), *Trsp*<sup>fl/+</sup>;MMTV-*cre* (dashed line) and *Trsp*<sup>fl/fl</sup>;wt mice (blue dashed line) from all causes. *P* values are compared with *Trsp*<sup>fl/fl</sup>;wt and *Trsp*<sup>fl/+</sup>;wt mice. There was no significant differences between *Trsp*<sup>fl/fl</sup>;wt and *Trsp*<sup>fl/+</sup>;wt mice. Only the *Trsp*<sup>fl/fl</sup>;wt mice are graphed.



**Fig. 4.** Selenoprotein expression in DMBA-induced mammary tumors. Western blot analyses of protein extracts from carcinogen-induced mouse mammary tumors showing Sep15, GPx4 and GPx1 protein levels are reduced in *Trsp*<sup>fl/fl</sup>;MMTV-*cre* tumors compared with *Trsp*<sup>fl/+</sup>; wt and *Trsp*<sup>fl/fl</sup>;wt tumors. Genotypes of tumors are indicated at the top of the figure.

normal cells (40). TR1 is known to have a role in protecting normal cells from cancer as it is one of the major redox regulators in mammals having roles in cell proliferation, transcription and angiogenesis, contributing to the antioxidant defense and acting as a redox regulator of cell signaling (reviewed in 41). One of the principal functions of TR1 in normal cells is to maintain thioredoxin in the reduced state (42) and thus its loss in mammary epithelium ablates the thioredoxin system. As noted above, cancer cells suffer from oxidative stress and must depend on a strong antioxidant system to maintain their malignant properties. The interrelationship between the glutathione and thioredoxin systems and the upregulation of the glutathione system in malignant cells following the downregulation of the thioredoxin system has been thoroughly reviewed recently (43–45).

It should also be noted that GPx1 has a role in serving as an antioxidant in the glutathione system (reviewed in 46,47), but, the targeted loss of the GPx1 gene in mice resulted in no observable phenotype unless the mouse was subjected to stress (46,47). It would appear, therefore, that the loss of GPx1 expression contributes to the shortened time to tumor onset and reduced survival time observed in the present study. It should also be noted that although selenoproteins play an important role as antioxidants, they have other functions which may also be critical in chemoprevention. Further studies will be required to address this latter point.

Our results may have important translational implications in light of the failure of the Selenium and Vitamin E Cancer Prevention Trial (SELECT) (48). The SELECT Trial was based upon observations made during the Nutritional Prevention of Cancer Trial wherein an association was made between increased prostate cancer risk and low baseline levels of serum selenium (49). In the prospectively designed SELECT Trial, L-selenomethionine, vitamin E (all rac- $\alpha$ -tocopheryl acetate) or a combination of both were administered to men to determine whether these compounds could reduce the risk of prostate cancer. The study concluded that there was no reduction in prostate cancer for these compounds at the doses administered. Thus, the chemopreventive properties of selenium may be related to the levels of selenoprotein expression and not simply the ingestion of selenium-containing compounds. Our results suggest that an important strategy for cancer chemoprevention may be to identify mechanisms that increase selenoprotein expression. Combinations of chemopreventive agents along with enhanced selenoprotein production may be critical in reducing the development of breast cancer.

There are at least two other studies that have shown a direct connection between selenoprotein deficiency and enhanced carcinogenesis. Irons *et al.* (15) and Diwadkar-Navsariwala *et al.* (16) demonstrated that selenoprotein deficiency generated by using a specific transgenic mouse model that causes a reduction in certain selenoproteins resulted in enhanced colon cancer and prostate cancer, respectively. The current study provides another example of a direct role of selenoproteins, and likely their role as antioxidants, in affecting cancer development, and more specifically, in the onset and degree of intensity in breast cancer.

While this study supports the role of selenoproteins in delaying mammary tumor progression in this model system, our results indicate that mammary tumors ultimately develop. This indicates that while selenoproteins are protective against chemically induced carcinogenesis in the mammary glands, they may be insufficient as a completely chemopreventive agent. Other strategies may need to be combined with enhanced selenoprotein expression to provide full chemoprevention. Alternatively, it may be possible that further mutations occur in lesions that are initially suppressed by selenoproteins leading to the eventual development of mammary tumors. Further work will be required to dissect more detailed mechanisms of how selenoproteins retard mammary tumor formation and whether the protective effect is more pronounced in certain subtypes of human breast cancer.

In summary, the current study demonstrates that loss of *Trsp* expression in mammary epithelial cells accelerates the development of carcinogen-induced tumorigenesis associated with reduced expression of selenoproteins and supports a chemopreventive mechanism through the enhanced expression of selenoproteins.

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## Supplementary material

Supplementary Table I and Figures 1–2 can be found at <http://carcin.oxfordjournals.org/>

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