# Protective effects of prepubertal genistein exposure on mammary tumorigenesis are dependent on *BRCA1* expression

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## Abstract

This study investigated whether prepubertal dietary exposure to genistein reduces mammary tumorigenesis by up-regulating *Brca1* expression in mice. Heterozygous *Brca1+/−* mice and their wildtype (WT) littermates were fed control AIN93G diet or 500 ppm genistein supplemented AIN93G diet from postnatal day (PND) 15 to PND30, and then switched to AIN93G diet. Prepubertal dietary exposure to genistein reduced DMBA-induced mammary tumorigenesis and aggressivity of the tumors in the WT mice and up-regulated the expression of *Brca1* in their mammary glands. In contrast, prepubertal genistein diet neither significantly reduced mammary tumorigenesis or tumor aggressivity, nor increased *Brca1* mRNA expression in the *Brca1+/−* mice. These results may be related to the opposing effects of prepubertal genistein diet on the expression of *Rankl* and CK5/CK18 ratio (marker of luminal epithelial cell differentiation) in the mammary gland, and estrogen receptor (ER-α) and progesterone receptor (PgR) protein levels in the mammary tumor: these all were reduced in the WT mice or increased in *Brca1+/−* mice. Both the WT and *Brca1+/−* mice exhibited reduced levels of amphiregulin, CK5 and CK18, delayed ductal elongation and a reduction in terminal end bud number in the normal mammary gland, and reduced HER-2 protein levels in the mammary tumors; however, these effects were not sufficient to significantly reduce mammary tumorigenesis in *Brca1+/−* mice. Our results show that up-regulation of *Brca1* may be required for prepubertal dietary genistein exposure to reduce later mammary tumorigenesis, perhaps because in the absence of this up-regulation, mice do not exhibit genistein-induced down-regulation of ER-α, PgR and *Rankl*.

### Keywords

Genistein; Soy; BRCA1; Estrogen signaling; Breast cancer

## INTRODUCTION

Soy consumption during childhood and adolescence has been consistently linked to a marked reduction in breast cancer risk in Asians, American Asians and Caucasians (1–5). Genistein, a soy-derived isoflavone, is thought to play a key role in the cancer-preventive activity of pubertal soy intake. In agreement with this view, pubertal genistein exposure reduces mammary cancer risk in rodent models (6). The protective effect of soy/genistein

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may be limited to puberty, as an adult exposure does not alter the risk in mice or rats (6). Consumption of soy limited to adult life may not affect breast cancer risk in humans either (7).

The mechanisms mediating the protective effect of pubertal genistein exposure on mammary tumorigenesis remain to be established. Genistein is a weak estrogen receptor α (ER-α) agonist, but it binds relatively strongly to ER-β (8). However, with the exception of very low doses, genistein activates both estrogen receptors equally, similarly to estradiol (9). Previous studies indicate that genistein modifies the expression of ER-α *in vitro* and *in vivo* (10–13) and increases progesterone receptor (PgR) in human breast tumors (14), human breast cancer cells in a mouse model (15) and normal mammary tissue in animal models (16). Genistein also targets many other signaling pathways, including tyrosine kinases ErbB2/HER-2 (17). It has been shown that genistein down-regulates HER-2 in human breast cancer cells (18), and following pubertal genistein exposure, in rat mammary tumors (19). Prepubertal genistein exposure also affects mammary gland morphology. It reduces the number of targets for malignant transformation (terminal end buds, TEBs), and this is proposed to explain the cancer protective effects of genistein (6).

Because genistein targets several biological pathways, it is not clear which one of them may contribute to its breast cancer risk reducing effects. Our earlier study showed that prepubertal genistein exposure, similarly to prepubertal estradiol exposure, up-regulates the tumor suppressor gene BRCA1 (breast cancer susceptibility gene 1) (12). Others have reported that genistein up-regulates BRCA1 *in vitro* in human breast and prostate cancer cells (20), and *in vivo* in the mammary gland of ovariectomized rats (21). BRCA1 expression in the mammary gland is also elevated during pregnancy when estrogen and progesterone levels are high (22), and is reduced by ovariectomy (23). BRCA1 physically interacts with ER-α and PgR to inhibit their transcriptional activity (24). The link between BRCA1 and these steroid receptors as well as the role of BRCA1 in inducing differentiation of stem/progenitor cells to luminal cells (25) may explain why this tumor suppressor reduces breast cancer risk.

To further explore the role of *BRCA1* as a mechanism mediating the effects of prepubertal genistein exposure on the mammary gland, we used *Brca1*+/− mice. BRCA1+/− mutations are the main known cause for familial breast cancer (26), and women who have inherited a germline BRCA1 mutation have a 66–85% risk of developing breast cancer by age 70 (27). The BRCA1-induced changes were also investigated, focusing on ER-α and PgR and their target genes amphiregulin and receptor activator of nuclear factor-kappaB ligand (*Rankl*), respectively. Amphiregulin is an epithelial growth factor receptor (EGFR) ligand (28, 29). It is transcriptionally regulated by ER-α (30) and BRCA1 (31), and it mediates the proliferative actions of estradiol by activating the EGFR (30). Amphiregulin is required for mammary gland ductal morphogenesis at puberty (28), and its absence prevents duct-limited mammary progenitor cell proliferation, but has no effect on lobule-limited mammary progenitor cells (32). It is not known whether genistein alters amphiregulin expression. RANKL is a member of the tumor necrosis family (TNF), and mediates progesterone induced mammary cell proliferation downstream of PgR (33). Genistein down-regulates RANKL in the bone (34) and prostate cancer cells (35), but the effects on the mammary gland have not been explored.

We found that prepubertal dietary genistein exposure reduced mammary tumor incidence in the WT mice, but failed to do so in the *Brca1*+/− mice. In contrast to WT mice, prepubertal genistein diet did not increase Brca1 or reduce ER-α, PgR or *Rankl* expression in the mammary gland or tumor in these mice. However, prepubertal genistein exposure elevated CK5/CK18 ratio in the mammary gland of the *Brca1*+/− mice, indicative of reduced luminal

differentiation (36). Our results show that up-regulation of *Brca1* may be required for prepubertal dietary genistein exposure to reduce later mammary tumorigenesis.

# MATERIALS AND METHODS

## Animals

Brca1^tm1Cxd heterozygous (+/−) frozen mouse embryos at 129Sv/C57BL/6 background were obtained from the NCI mouse repository (Bethesda, MD). Frozen embryos were implanted in ICR female mice and a colony was established at the Georgetown University's Animal Facility. Mice were fed a semi-purified AIN93G (the American Institute of Nutrition) diet upon arrival. Animals were housed in a temperature- and humidity-controlled room under a 12-hour light-dark cycle. All animal procedures were approved by the Georgetown University Animal Care and Use Committee, and the experiments were performed following the National Institutes of Health guidelines for the proper and humane use of animals in biomedical research.

## Genotyping

Wild-type (WT) and heterozygous *Brca1* (+/−) mice were genotyped using DNA extracted from the tail clips using the NaOH method. PCR amplification was performed using forward and reverse primers to obtain a 450 and 550 bp bands for wild type and KO alleles, respectively. Primer sequences were: forward primer (Brca1004) : 5′-CTGGGTAGTTTGTAAGCATGC3′ and reverse primer (Brca1005) : 5′-CAATAAACTGCTGGTCTCAGG-3′ and (Brca1007) : 5′-ATCGCCTTCTATCGCCTTCTTGACGAGTTC-3′. After PCR amplification, products were analyzed by agarose gel electrophoresis. The B004/B005 primer combinations generates a 450 bp product that corresponds to the wildtype Brca1 while the B004/B007 primer combination generates a 550 bp product that corresponds to the Neo cassette.

## Pre-pubertal dietary exposures

On postnatal day 15 (PND15), WT and *Brca1+/−* female mice were divided into two dietary groups (n= 30 mice per genotype/group): AIN93G diet (control) or genistein (500 ppm) supplemented AIN93G diet obtained from Harlan-Teklad (Madison, WI). The mice were kept on these diets until PND30, and all mice were then switched to the non-supplemented AIN93G diet.

## Effects on mammary tumorigenesis

*Brca1+/−* mice do not develop spontaneous mammary tumors, and therefore mammary tumors were induced by administration of a subcutaneous injection of 15 mg in 100 μl of Medroxyprogesterone Acetate (MPA, DepoProvera, Pfizer) to mice at 6 weeks of age, followed by administration of 1 mg 7,12-dimethylbenz(a)anthracene (DMBA) (Sigma, St. Louis, MO) weekly for four weeks (weeks 7, 8, 9, 10). The carcinogen was dissolved in corn oil and administered by oral gavage in a volume of 0.1 ml. This study included 22 WT mice fed the control diet, 21 WT mice fed prepubertally genistein diet, 25 *Brca1+/−* mice fed the control diet, and 26 *Brca1+/−* mice fed genistein diet. Animals were examined for mammary tumors by palpation once per week. The end-points for data analysis were (i) latency to tumor appearance, (ii) the number of animals with tumors (tumor incidence), and (iii) the number of tumors per animal (tumor multiplicity). During the follow-up, animals in which tumor burden approximated 10% of total body weight were euthanized, as required by the ethical guidelines of our institution. All surviving animals were sacrificed 20 weeks after the last does of DMBA administration.

**Hyperplastic alveolar nodules—**Mammary gland wholemounts were obtained from mice 20 weeks after DMBA exposure, processed as described above. Whole mounts were evaluated under the microscope to determine the number of hyperplastic alveolar nodules in each gland.

**Histopathological evaluation—**Mammary tumors were classified according to its histopathology by a veterinarian pathologist, through ARUP veterinarian services, Salt Lake City, Utah.

## Effects on mammary gland morphology and cytokeratin (CK) 5 and CK18 mRNA expression

To assess changes in mammary gland morphology, wholemounts of the 4th abdominal glands obtained on PND50 were processed as previously described (37). The total number of TEBs was counted. Identification of TEBs was based on the guidelines established by Russo and Russo (38). Ductal elongation (epithelial outgrowth) was measured as the growth, in centimeters, from the end of the lymph node to the end of the epithelial tree. All the analyses were done blindly using an Olympus dissecting microscope.

We also determined whether the mRNA expression of CK5 (committed progenitor cells) or CK18 (luminal cells) (36), or CK5/CK18 ratio were different between the WT and *Brca1+/−* mice, and whether prepubertal genistein exposure affected the expression of these cytokeratins.

## Mammary gland and tumor ER-α and HER-2 protein levels

Protein levels of ER-α and HER-2, were assessed by Western-blot in the mammary glands and tumors. Briefly, mammary glands and tumors were homogenized, centrifuged and the protein extract collected from the supernatant. 50 μg of protein extract was loaded onto a NuPAGE 12% Bis-Tris gel (Invitrogen Life Technologies, Carlsbad, CA), and gels were run at 150 V. Membranes were then washed with TBST and blocked in 5% milk in TBST for 30 min at room temperature. After blocking, membranes were incubated with antibodies against ER-α (1:100 dilution, Vector Laboratories, Burlingame, CA) and HER-2 (1:1000 dilution, Lab Vision Corp, Fremont, CA), overnight at 4°C. Next, membranes were incubated with secondary anti-rabbit IgG or mouse IgG horseradish peroxidase antibodies (1:5000 dilution, Amersham Pharmacia Biotech, Piscataway, NJ (anti-mouse) and developed using Super Signal (Pierce, Rockford, IL). Fold differences were calculated by normalization against beta-actin.

## Mammary gland and tumor PgR protein levels

Using immunohistochemistry, PgR expression was assessed in mammary glands and tumors collected at the end of the follow-up period. Briefly, tissues were fixed in 10% buffered formalin, embedded in paraffin and sectioned (5μm). Sections were deparaffinized in xylene, hydrated through graded alcohols and incubated with $H_2O_2$ to block endogenous peroxidases. Antigen retrieval was performed in a Target Retrieval solution (pH 9, Dako S2368) in a pressure cooker for 20 min + 2 h cool down. Tissue sections were incubated overnight with the primary antibody against PgR at a 1:400 dilution (polyclonal rabbit anti-human PgR, A0098, Dako CA). After several washes, sections were treated with secondary antibody (anti-rabbit) and developed using the Dako EnVision Dual Link System – HRP, DAB+ (K4065) as instructed by the manufacture. The PgR status of each sample was evaluated according to a modified version of the scoring system proposed by Allred *et al*. (39). The total score for each sample was calculted as the sum of the estimated proportion of positive-staining cells (0–7) plus the estimated intensity of positive-staining cells (0–4).

### Mammary gland amphiregulin, Rankl, CK5 and CK18 mRNA levels

Frozen tissue samples were weighed and homogenized using a PowerGen 35 hand held homogenizer (Fisher Scientific, Pittsburgh, PA) with RNAse free disposable OMNI-Tips (Fisher Scientific). RNA extraction was then performed according to the Qiagen's RNeasy Lipid Tissue kit (Qiagen Inc., Valencia, CA) instructions. The quantity and quality of RNA were measured by comparing the optical density ratios ($OD_{260}/OD_{280}$) obtained using a Nanodrop (ND-1000) Spectrophotomer (Thermo Scientific, Wilmington, DE). RNA samples were stored at −80°C until use.

**cDNA synthesis and quantitative real-time PCR (qRT-PCR) analysis—**Two hundred ng of total RNA per sample was used as a template for random primed cDNA synthesis with a recombinant Moloney murine leukemia virus reverse transcriptase (TaqMan MultiScribe Reverse Transcriptase and RT-PCR Reagents, AppliedBiosystems, Roche, New Jersey, USA), according to manufacturer's instructions. An RT enzyme minus control reaction was also included. The cDNA samples were then used as templates for quantitative real time PCR analysis with previously described specific primers for the target genes *Brca1* (40), amphiregulin (30), *Rankl* (41), and *CK5* and *CK18* (42) using QuantiTect SYBR green PCR kit (Qiagen Inc., Valencia, CA) and an ABI Prism 7900 Sequence Detection System. Each sample was run in triplicate, and qPCR run was repeated 1–2 times. Absolute gene expression levels were determined using Applied Biosystems' SDS2.3 software and the standard curve method. Concentration of each sample was normalized to the reference gene 18S rRNA (42).

### Statistical analyses

Data obtained on (i) mammary gland morphology (total number of TEBs and ductal elongation), (ii) mRNA levels, (iii) mammary gland and tumor HER-2 and ER-α protein levels, (iv) PgR staining and (v) number of hyperplastic alveolar nodules were analyzed by two-way ANOVA using genotype (WT or knockout) and treatment (control or genistein) as variables. Kaplan-Meier curves were used to compare differences in tumor incidence, followed by the log-rank test. Normalized quantitative RT-PCR results were analyzed with Kruskal-Wallis one-way ANOVA on Ranks and one-way ANOVA with Holm-Sidak method for pairwise multiple comparison procedures. All tests were performed using the SPSS SigmaStat software, and differences were considered significant if the p-value was less than 0.05. All probabilities are two-tailed.

## RESULTS

### Mammary tumorigenesis

Prepubertal genistein exposure significantly reduced mammary tumor incidence in the WT mice (p=0.029) (Figure 1A). Among the *Brca1+/−* mice, however, the difference in mammary tumor incidence failed to reach statistical significance (p=0.26) (Figure 1B). The number of HANs (Figure 1C), tumor multiplicity (the total number of tumors per animal; ranged between 1.2 and 1.6 in the WT and *Brca1+/−* mice) or tumor latency (time to tumor appearance; ranged between 10.1–11.9 weeks) were not affected by prepubertal genistein diet in either the WT or Brca1+/− mice (data not shown).

There was no evidence that the incidence of mammary tumors was higher in the *Brca1+/−* mice; in fact, a slightly lower number of these mice (14/25, 56%) developed mammary tumors, compared to WT mice (15/22, 68%). The number of pre-malignant lesions; i.e., hyperplastic alveolar nodules (HANs), was significantly higher in the *Brca1+/−* mice than in the WT mice (p=0.047) (Figure 1C). These findings suggest that the *Brca1+/−* mice have a

higher potential to develop mammary tumors than the WT mice do, but most of these HANs do not progress to malignancy in the *Brca1+/−* mice.

Histopathological analysis indicated that most mammary tumors were malignant carcinomas (Table 1) in all four groups. The incidence of benign adenomas was 10% and 8% in the WT and *Brca1+/−* mice fed the control diet, respectively, and 20% in both groups which were fed genistein containing diet before puberty. The malignant tumors consisted of adenocarcinomas and other carcinomas. These malignant carcinomas were divided into low grade and high grade tumors, and the ratio of low to high grade tumors was similar in the WT and *Brca1+/−* mice (Table 1). In the WT mice, the incidence of high grade malignant tumors dropped from 60% in the control diet group to 20% in the genistein group (p<0.001), but only from 67% to 50% in the *Brca1+/−* mice (non-significant). The tumor mitotic index, another indicator of tumor growth and aggressiveness, was reduced by prepubertal exposure to genistein in the WT mice (p<0.011), but genistein had no effect on the *Brca1*+/− mice (p=0.93) (Table 1).

### Mammary gland Brca1 mRNA levels

The expression levels of Brca1 mRNA in the mammary gland were determined on PND50 (Figure 1D). As expected, RT-qPCR showed lower levels of *Brca1* mRNA in *Brca1+/−* mice compared to WT mice (p<0.001), regardless of the diet. Higher levels of *Brca1* mRNA were observed in the mammary glands of WT mice fed genistein containing diet compared to those fed AIN93G control diet (p<0.042). No effects by genistein diet on *Brca1* levels were observed in *Brca1+/−* mice (Figure 2).

### Mammary gland morphology

Mammary gland morphology, and the expression of *CK5* and *CK18* were assessed at PND50. Progenitor cells, including committed myoepithelial and some ductal luminal progenitor cells, express CK5, and the expression is lost when the cells differentiate to mature myoepithelial and luminal cells (36). Differentiated luminal cells express *CK18*. Thus, mammary glands expressing high *CK5* and low *CK18* are more undifferentiated than glands which express low CK5/CK18 ratio.

*CK5* levels were significantly higher in the *Brca1+/−* mice fed the control diet than in the WT controls (p<0.016) (Figure 2A), but *CK18* levels were not altered (Figure 2B). Regardless of the genotype, the mice fed genistein during prepuberty exhibited significantly reduced levels of both cytokeratines, compared to the mice fed the control diet (*CK5*: p<0.002; *CK18*: p<0.003). The ratio between CK5 and CK18 was higher in the *Brca1+/−* mice than in the WT mice (p<0.022). The difference was particularly apparent in mice fed genistein during pre-puberty, because genistein reduced it in the WT mice, and significantly increased the ratio in the *Brca1+/−* mice (p<0.04) (p for interaction = 0.079) (Figure 2C).

The reductions in both CK5 and CK18 by genistein reflected changes in the mammary gland morphology. As seen in Figure 3A, mice fed genistein during prepuberty exhibited significantly smaller mammary epithelium than mice fed the control diet. No significant differences in the number of TEBs or ductal elongation were seen between the WT and *Brca1+/−* mice, when determined at PND50. However, prepubertal genistein exposure reduced the number of TEBs (p<0.001) and inhibited ductal elongation (p=0.007) in both the WT and *Brca1+/−* mice (Figure 3B).

## Mammary gland and mammary tumor protein expression levels of ER-α, ER-β, PgR and HER-2, and their down-stream targets amphiregulin and RANKL

Because genistein acts by binding to ER-α and ER-β (8, 9), and up-regulation of PgR is indicative of ER-α activation, we determined the expression of these receptors. We also tested pathways downstream of ER-α and PgR; i.e., the expression of amphiregulin and RANKL. In addition, genistein is a tyrosine kinase inhibitor (17), and therefore HER-2 levels were assessed. ER-α and HER-2 protein levels were detected by Western blot, *ER-β*, amphiregulin and *Rankl* mRNA levels by RT-qPCR, and PgR by RT-qPCR and immunohistochemistry.

**Changes in mammary glands—**The assays revealed that mammary glands and tumors were positive for ER-α, ER-β, PgR and HER-2 expression. Protein levels of ER-α (Figure 4A, mammary gland) and HER-2 (Figure 4B) in mammary gland tissues were not different between the genotypes or dietary groups. PgR protein levels were higher in the mammary glands of *Brca1+/−* mice than in the WT mice (p<0.033) (Figure 4C). Genistein decreased the PgR protein levels in the WT mice (p<0.030). ER-β protein levels were not different among the groups (data not shown).

**Changes in mammary tumors—**Assessment of expression of the different receptors in the mammary tumors revealed that ER-α protein levels were not different in the mammary tumors between WT and *Brca1+/−* mice fed the control diet. The expression of ER-α was reduced in the tumors by prepubertal genistein exposure in the WT but not in the *Brca1+/−* mice (p for interaction <0.04) (Figure 4A, **mammary tumor**).

In contrast to normal mammary gland tissue, the levels of PgR were significantly *lower* in the mammary tumors of the *Brca1+/−* mice than in the WT mice (p=0.007) (Figure 4C). Further, although the WT mice exposed to genistein at pre-puberty exhibited a significant reduction in PgR expression, no reduction was seen in the in mammary tumors of the *Brca1+/−* mice (p for interaction <0.034).

Mammary tumors of *Brca1+/−*⁻ mice expressed higher levels of HER-2 than the tumors in the WT mice, regardless of diet (p<0.03) (Figure 4B). Prepubertal exposure to genistein diet decreased the expression levels of HER-2 in mammary tumors of WT and *Brca1+/−* mice, compared to their respective controls (p<0.002).

## Amphiregulin and Rankl expression in the mammary gland

Although mammary gland ER-α levels were similar in the WT and *Brca1+/−* mice, amphiregulin mRNA levels were lower in the mammary glands of *Brca1+/−* mice than WT mice (p<0.011) (Figure 5A). Prepubertal genistein exposure significantly reduced the levels in both groups (p<0.001), and consequently no differences in amphiregulin levels between the two genotypes were seen.

PgR's down-stream target *Rankl* was significantly higher in the control diet fed WT mice than in the *Brca1+/−* mice on PND50 (p<0.018), but the difference was lost in mice fed genistein during prepuberty (Figure 5B). This is because among the WT mice genistein reduced *Rankl*, but among the *Brca1+/−* mice genistein exposure increased *Rankl* mRNA (p<0.026 for interaction).

## DISCUSSION

### Effect of prepubertal genistein diet on mammary tumorigenesis

We found that in the WT mice prepubertal dietary exposure to 500 ppm genistein significantly reduced carcinogen-induced mammary tumor incidence, in accordance with data previously reported in (6). Since the dose we used generates blood genistein levels comparable to those seen in the Asians (43), our study adds to the convincing evidence obtained in human studies that childhood soy intake protects against the development of breast cancer later in life (1–5).

However, no significant reduction in mammary tumorigenesis was seen in the *Brca1+/−* mice fed the genistein diet before puberty onset. Further, in contrast to the WT mice, these mice did not exhibit an increase in *Brca1* expression, possibly because the remaining Brca1 allele was already maximally expressed in the heterozygous *Brca1+/−* mice. The increase in *Brca1* expression in the WT mice is in agreement with the earlier findings obtained in both *in vitro* and *in vivo* studies indicating that genistein up-regulates BRCA1 (12, 20, 21). Our results thus suggest that the genistein-induced up-regulation of *Brca1* may be required for prepubertal genistein exposure to reduce breast cancer risk.

### Mammary tumorigenesis in the Brca1+/− mice: role of PgR signaling

Palpable mammary tumor incidence was not different between the genotypes, although *Brca1+/−* mice developed more premalignant HAN lesions than WT mice did. Since *Brca1+/−* mice expressed higher levels of PgR than those of the WT mice; a finding which is consistent with the data by Poole et al (44) in conditional *Brca1−/− /p53−/−* mice, these genetically modified mice should be at least as sensitive as the wildtype mice to the MPA exposure, which is required in both wildtype and various genetically modified mouse models for DMBA to induce mammary tumors (45). This was confirmed by determining that MPA similarly increased *Rankl* expression and TEB number in the WT and *Brca1+/−* mice (data not shown).

### Differences in the response to prepubertal genistein diet between the genotypes

Prepubertal genistein diet affected some end-points in the mammary glands and tumors differentially, and some similarly in the WT and *Brca1+/−* mice. This diet had an opposite effect on the following end-points in the two genotypes: *Rankl* expression and CK5/CK18 ratio in the mammary gland, PgR expression in the mammary gland and tumor, ER-α expression in the tumor, and histopathology of the tumor. In the WT mice, prepubertal genistein exposure reduced the expression of *Rankl* in the mammary gland and PgR both in the mammary gland and tumor. The effect on the PgR is in agreement with the study by Pei et al (46) showing that prepubertal genistein exposure reduced PgR expression in the (rat) mammary gland. Thus, consumption of genistein during prepuberty may induce persistent down-regulation of PgR. Down-regulation of *Rankl* by genistein is consistent with previous data in bone and prostate (34, 35). Since reduced expression of PgR and *Rankl* may be associated with a reduction in the mammary progenitor cell proliferation (47), one of the mechanisms by which prepubertal genistein exposure reduces breast cancer risk may be through increased mammary luminal cell differentiation. Changes in these three end-points could have occurred through up-regulation of BRCA1, which is known to suppress progesterone signaling (24) and induce luminal specific differentiation of mammary progenitor cells (25).

Prepubertal genistein diet affected *Rankl* and CK5/CK18 ratio differently in the *Brca1+/−* mice. Genistein treated *Brca1+/−* mice expressed more *Rankl*, and higher CK5/CK18 ratio than the control diet fed mice. Since high CK5/CK18 ratio is indicative of increased

presence of undifferentiated luminal cells (36) as is high *Rankl* expression (47), prepubertal genistein exposure may have led to reduced luminal cell differentiation in the *Brca1+/−* mice.

Prepubertal genistein diet reduced mammary tumor grade and tumor mitotic index, both of which are indicators of aggressiveness of a tumor, in the wildtype but not *Brca1+/−* mice. In addition, tumors in the prepubertally genistein fed wildtype mice expressed significantly lower levels of the ER-α, PgR and HER-2 than tumors in the control diet fed wildtype mice. Again, mammary tumors in *Brca1+/−* mice failed to show similar changes, with the exception of down-regulation of HER-2 in the genistein fed mice. These findings indicate that genistein failed to reduce aggressiveness of the mammary tumors in the mice lacking one *Brca1* allele.

## Similarities in the response to prepubertal genistein diet in the two genotypes

Genistein had some effects on the *Brca1+/−* mice which were similar to those seen in the WT mice; i.e., it reduced the number of targets for malignant transformation (TEBs), inhibited ductal elongation, down-regulated amphiregulin expression in the mammary gland and reduced HER-2 expression in the tumors. Some of these changes have been previously reported to occur by genistein (6, 17, 48), but we are not aware of any studies showing that genistein delays ductal elongation or inhibits amphiregulin expression.

Activation of ER-α increases amphiregulin expression which then binds to the epidermal growth factor receptor (EGFR/ErbB1/HER-1) and induces cell proliferation (30). ErbB2/HER-2 does not have any known ligands, but its activity and possibly expression can be modified through hetero-dimerization with amphiregulin bound EGFR (49, 50). Amphiregulin expression was lower in the control diet fed *Brca1+/−* mice than in the WT controls suggesting that ER-α signaling may be reduced in the *Brca1+/−* mice, although no differences in the ER-α expression in the normal mammary gland were seen between the genotypes. Our results also indicate that both in the *Brca1+/−* and WT mice, prepubertal genistein diet induced a persistent reduction in mammary amphiregulin levels. Lamber et al. have reported that BRCA1 suppresses amphiregulin (31), and therefore the reduction in the WT mice may be due to genistein-induced up-regulation of Brca1. However, since amphiregulin was reduced also in the genistein exposed *Brca1+/−* mice, but genistein failed to up-regulate their Brca1 expression, other mechanisms than genistein-induced up-regulation of Brca1 suppressed their amphiregulin expression.

Amphiregulin was recently found to mediate self-renewal of stem/progenitor cells in the mammary duct (32); its inhibition by siRNA reduced mammosphere formation by preventing the expansion of ductal progenitor cells. Reduction in amphiregulin expression by prepubertal genistein diet could thus explain the significant delay in ductal elongation observed here in the postpubertal mammary gland in both WT and *Brca1+/−* mice. Since amphiregulin also is expressed in the TEBs (51), genistein-induced reduction in TEB number could perhaps be explained by a reduction in amphiregulin expression. Mammary gland morphology on PND50 in the control diet fed WT and *Brca1+/−* mice was similar; i.e., loss of one Brca1 allele did not affect gross mammary gland morphology. In humans, mammographic density also is similar in germline BRCA1 mutation carriers and controls (52, 53). Animal studies have generated conflicting data and reported that conditional or mammary specific BRCA1 mutations impair mammary ductal development (16, 54–56) or induce ductal elongation (57).

## Conclusions

In this study we showed that a pre-pubertal dietary exposure to genistein in mice reduces the risk of developing mammary tumors later in life and up-regulates *Brca1* mRNA expression in an adult mammary gland. We also showed that the protective effect was not seen in mice with a germline *Brca1* mutation, and neither was the remaining *Brca1* allele up-regulated by genistein in these mice. WT mice fed genistein before puberty onset exhibited reduced expression of PgR and *Rankl*; changes in the opposite direction were seen in the *Brca1+/−* mice, and they also exhibited increased CK5/CK18 ratio. These findings are indicative of reduced progenitor cell proliferation and increased luminal differentiation in the genistein exposed WT mice, but reduced differentiation in the genistein exposed *Brca1+/−* mice (36, 47).

Genistein down-regulated amphiregulin expression and inhibited ductal elongation and reduced TEBs in the mammary glands of both the WT and *Brca1+/−* mice, and also reduced the expression of Her-2 in the mammary tumors, suggesting that prepubertal dietary genistein exposure induces some breast cancer risk protective effects in the mammary gland, regardless of the level of *Brca1* expression. However, these effects do not offer full protection to *Brca1+/−* mice, indicating that up-regulation of Brca1 is required for prepubertal genistein to prevent cancer.

## Acknowledgments

## Reference List

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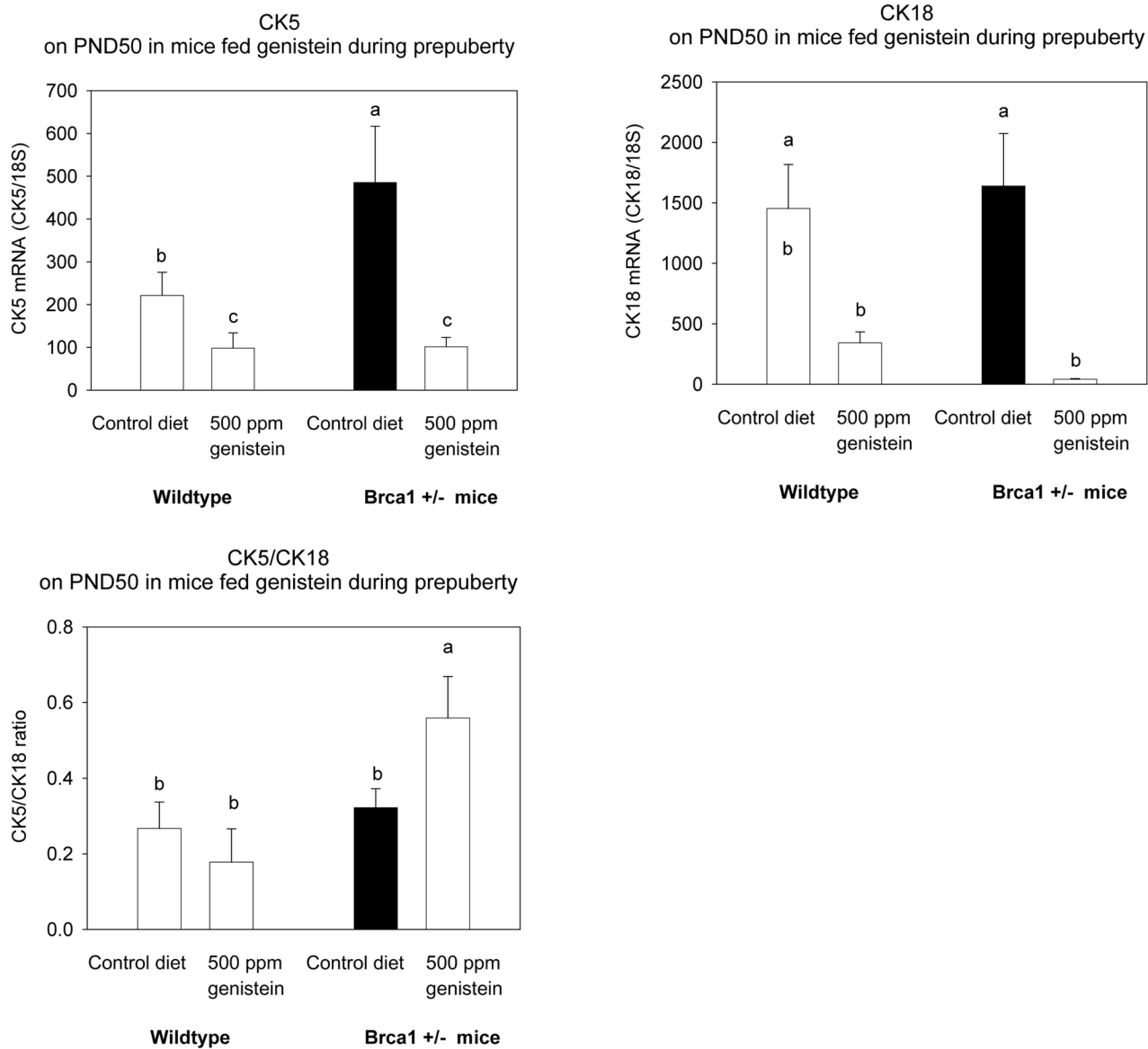
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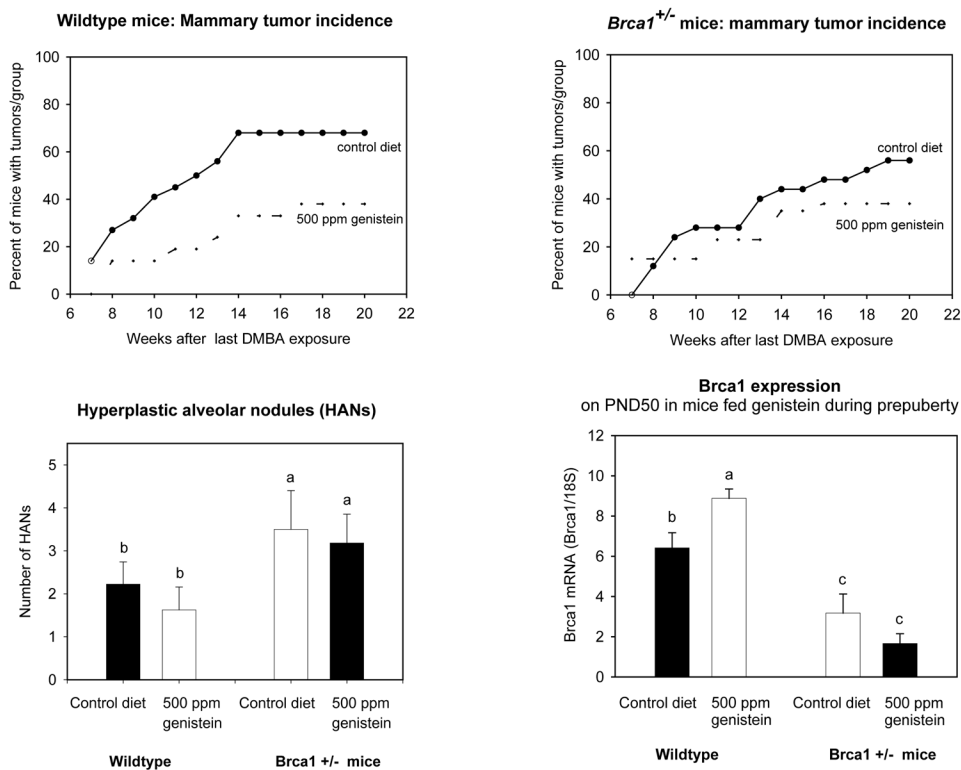
**Figure 1. Mammary tumorigenesis and Brca1 expression**
Effect of prepubertal dietary exposure to 500 ppm genistein on (A) tumor incidence in wildtype mice (n=21–22 mice/group) and (B) in *Brca1+/−* mice (n=25–26 mice/group), (C) hyperplastic alveolar nodules (HANs) in these mice, and (D) *Brca1* mRNA levels (n=4–6 mice per group) on postnatal day 50. Means ± SEM of *Brca1* mRNA are shown; bars with different letters are significantly different from each other, p<0.05. Prepubertal genistein exposure significantly reduced mammary tumorigenesis in WT mice (p<0.029), but not in *Brca1+/−* mice (p<0.26).

## CK5
### on PND50 in mice fed genistein during prepuberty



## CK18
### on PND50 in mice fed genistein during prepuberty



## CK5/CK18
### on PND50 in mice fed genistein during prepuberty

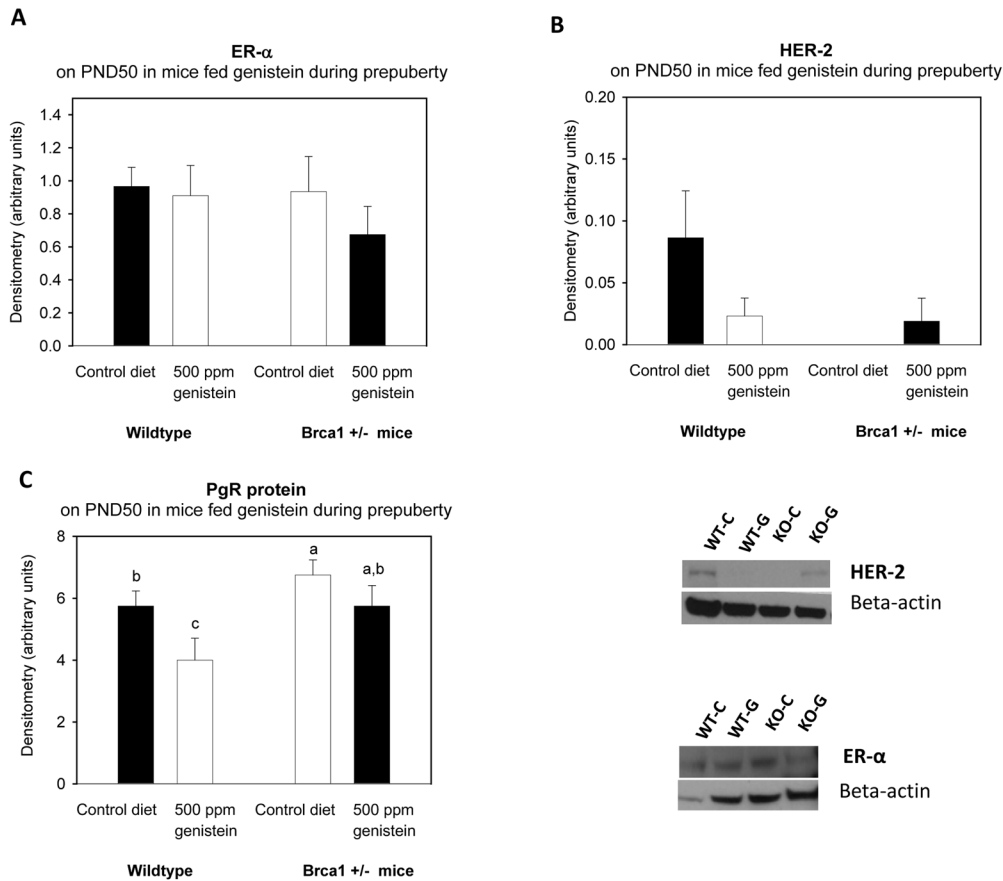

**Figure 2. CK5 and CK18 expression, and CK5/CK18 ratio**
Effect of prepubertal dietary exposure to 500 ppm genistein on CK5, CK18 expression and CK5/CK18 ratio in 50-day-old wildtype and *Brca1+/−* mice. Mean ± SEM of 4–6 mice/ group are shown; bars marked with different letters are significantly different from each other: p<0.05. Prepubertal genistein exposure tended to have a different effect on CK5/ CK18 ratio in the wildtype and *Brca1+/−* mice (p<0.079 for interaction).
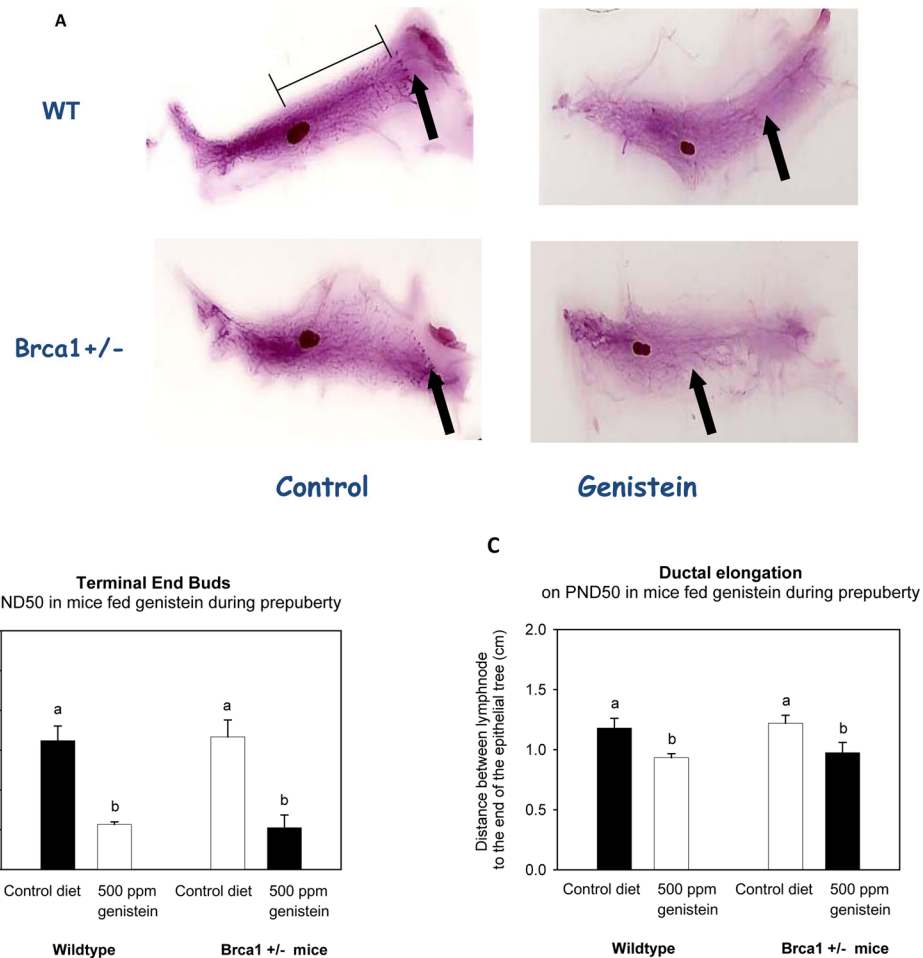
**Figure 3. Mammary gland morphology**

(A) Histological depiction of the 4th abdominal mammary gland whole-mounts (arrow indicates where the epithelial tree ends); (B) number of terminal end buds (TEBs); and (C) ductal elongation (distance, in cm, from the end of the lymph node to the end of the epithelial tree) on PND50 in wildtype and Brca1+/− mice fed 500 ppm genistein during pre-puberty. Mean ± SEM of 4–6 mice/group are shown; bars marked with different letters are significantly different from each other: $p < 0.05$.
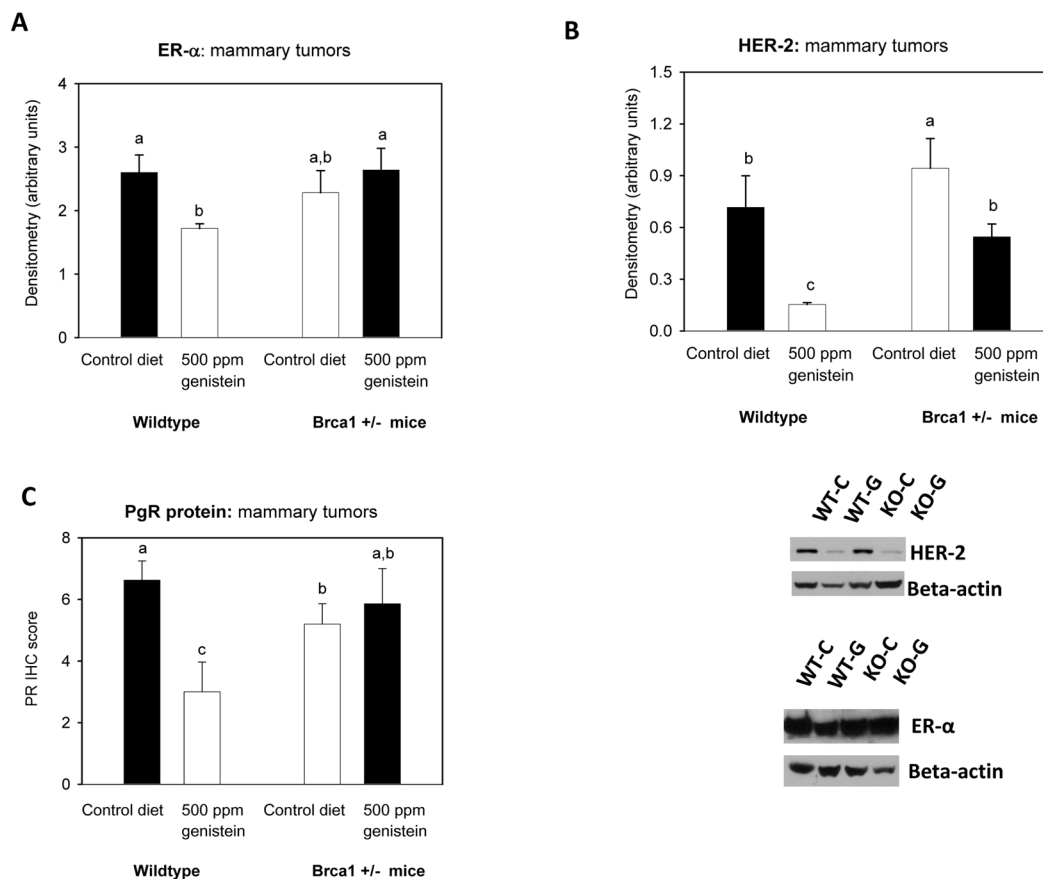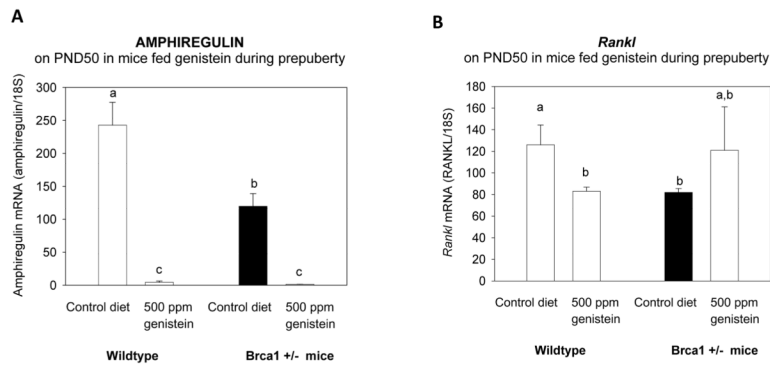
**MAMMARY GLAND**

**A**

**ER-α**
on PND50 in mice fed genistein during prepuberty



**B**

**HER-2**
on PND50 in mice fed genistein during prepuberty



**C**

**PgR protein**
on PND50 in mice fed genistein during prepuberty

**MAMMARY TUMOR**

**A**



ER-α: mammary tumors

**B**



HER-2: mammary tumors

**C**



PgR protein: mammary tumors



**Figure 4.**

Figure 4a. Mammary gland and tumor protein levels of ER-α, PgR and HER-2.

Protein levels of (A) ER-α, (B) HER-2 and (C) PgR determined on PND50. All values are expressed as the mean ± SEM of 4–6 mice/group. ER-α and HER-2 levels were determined by western blot, and PR levels were determined by IHC. Bars marked with different letters are significantly different from each other: $p<0.05$.

Figure 4b. Mammary tumor protein levels of ER-α, PR and HER-2.

Protein levels of (A) ERα, (B) HER-2 and (C) PgR were determined in the DMBA-induced adenocarcinomas. All values are expressed as the mean ± SEM of 5–7 mice/group. Bars marked with different letters are significantly different from each other: $p<0.05$. Genistein had opposing effect on ER-α ($p<0.04$ for interaction) and PgR expression ($p< 0.034$ for interaction) in wildtype and *Brca1+/−* mice

**Figure 5. Expression of amphiregulin and Rankl in the mammary gland**
mRNA levels of (A) amphiregulin, and (B) *Rankl* in the mammary gland on PND50,
determined using qRT-PCR. All values are expressed as the mean ± SEM of 4–6 mice/
group. Bars marked with different letters are significantly different from each other: p<0.05.
Genistein reduced amphiregulin expression in both the wildtype and *Brca1+/−* mice, but had
opposing effect on *Rankl* expression in the two genotypes (p<0.026 for interaction).

**Table 1a**

Mammary tumor multiplicity and latency in DMBA-exposed wildtype (WT) and *Brca1 +/−* mice fed a control or genistein supplemented AIN93G diet during prepuberty. Mean±SEM are shown.

| Group | Number of tumors | Tumor multiplicity (number of tumors/tumor bearing mice) | Tumor latency (weeks) |
|---|---|---|---|
| **WT control** | 10 | 1.2 ± 0.4 | 10.1 ± 0.7 |
| **WT genistein** | 5 | 1.6 ± 0.3 | 11.6 ± 1.2 |
| ***Brca1*+/− control** | 12 | 1.2 ± 0.3 | 11.9 ± 1.0 |
| ***Brca1*+/− genistein** | 10 | 1.2 ± 0.2 | 10.8 ± 1.1 |

**Table 1b**

Mammary tumor histopathology in DMBA-exposed wildtype (WT) and *Brca1+/−* mice fed a control or genistein supplemented AIN93G diet during prepuberty

| Group | Benign Adenoma | Malignant Adeno-carcinoma | Carcinoma (Other) | Tumor grade Low | High | Mitotic Index (mean±SEM) |
|---|---|---|---|---|---|---|
| **WT control** | 1 (10%) | 6 (60%) | 3(30%) | 3(33%) | 6(67%)[a] | 5.0±0.8[a] |
| **WT genistein** | 1 (20%) | 1 (20%) | 3 (60%) | 3(75%) | 1(25%)[b] | 1.4±1.1[b] |
| ***Brca1*+/− control** | 1 (8%) | 9 (75%) | 2 (17%) | 3(27%) | 8(63%)[a] | 2.1±0.7[b] |
| ***Brca1*+/− genistein** | 2 (20%) | 4 (40%) | 4 (40%) | 3(37%) | 5(63%)[a] | 2.0±0.8[b] |

Values within a column which are marked with a different letter are significantly different from each other, p<0.05.