

# Hormonal and Dietary Modulation of Mammary Carcinogenesis in Mouse Mammary Tumor Virus-*c-erbB-2* Transgenic Mice<sup>1</sup>

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## ABSTRACT

Exogenous and dietary estrogens have been associated with modification of breast cancer risk. Mammary cancer model systems can be used to explore interactions between specific transgenes, and hormonal and dietary factors. Transgenic mice bearing the rat wild-type *erbB-2* gene were used to study the effects of short-term hormonal exposure [17 $\beta$ -estradiol (E2) or tamoxifen] or a soy meal diet on mammary carcinogenesis. In mice fed a casein diet, mammary tumors developed at an earlier age after short-term E2 exposure during the early reproductive period. The median mammary tumor latency was shortest (29 weeks) for the high-dose estrogen as compared with the lowest dose of E2 treated or placebo control mice (33 and 37 weeks, respectively). The timing of short-term E2 exposure was also important, with the most significant changes observed in mice exposed to E2 between 8 and 18 weeks of age. E2 exposure was associated with the subsequent development of more aggressive tumors as determined by histologic grade, multifocal tumor development, stromal invasion, and pulmonary metastasis. In contrast, short-term tamoxifen-exposed mice generally failed to develop mammary tumors by 60 weeks of age. Mice fed a soy meal diet developed mammary tumors at a later age than casein-fed animals treated with E2 or placebo, whereas no differences were observed by diet for the tamoxifen-treated mice. Mammary tumor prevention was >80% in tamoxifen-treated mice on either diet. Novel histologic tumor types were identified, suggesting greater phenotypic diversity than described previously. Benign mammary gland morphogenesis was also significantly altered by short-term hormonal exposure or dietary factors, consistent with the modification of mammary tumor risk in specific treatment groups. Estrogenic modulation of the mammary tumor phenotype in wild-type *erbB-2* transgenic mice was observed. Histologic tumor types and clinical aggressivity not reported previously in this transgenic model were noted, suggesting greater biologic heterogeneity than reported previously. In addition, dietary phytoestrogens modified mammary development and tumor latency, suggesting a need for greater stringency in dietary assignment for transgenic mouse models of mammary neoplasia.

## INTRODUCTION

Factors associated with an increased risk of developing mammary gland (breast) cancer are both endogenous and exogenous, although genetic predisposition, patient age, and estrogen exposure are the most important. The association of estrogen and breast cancer risk is based on static analyses of human breast biopsies, population-based parity or hormonal exposure data, or randomized clinical trials using hormones or antihormonal treatments (1–4). Data suggest that exogenous estrogens may be particularly dangerous for women in high-risk subgroups (5). Modification of mammary cancer risk by the antiestrogen tamoxifen provides “proof of principle,” linking estrogenic promotion to

carcinogenesis (6). In the laboratory, breast-derived cell lines, carcinogen-induced murine cancer models, and more recently created transgenic mouse models provide the basis for *in vitro* analyses (7).

Several mechanisms have been proposed by which estrogen may exert a procarcinogenic effect, including: (a) increased cellular proliferation, enhancing the susceptibility for a genotoxic event to produce mutations, resulting in accumulated genetic errors (1, 8, 9); (b) tumor initiation, possibly via intermediate metabolites of estrogen (10); (c) tumor promotion via the induction of terminal end bud development (11); or (d) regulatory control of other genes. ER-mediated<sup>4</sup> activity includes the regulation of specific target genes in ER-positive mammary epithelial cells. However, ER  $\alpha$ -negative cells have also been shown to be indirectly estrogen responsive. Additional signaling mechanisms, in addition to the  $\beta$  form of the receptor, are likely, although the exact nature of these is still under investigation (12, 13).

*erbB-2* (HER-2/*neu*) is best known as a prognostic and predictive marker for invasive ductal carcinomas of the breast. Alteration (amplification and increased protein expression) of *erbB-2* is noted in one-third of invasive and up to two-thirds of *in situ* ductal breast cancers (14–17). *erbB-2* alterations of benign breast epithelium are rarely observed, although when present have been linked to an increased risk of breast cancer (18, 19). Cancer-associated genes affiliated with the receptor tyrosine kinase pathway and modulated either directly or indirectly by estrogen include: *erbB-2* (20, 21), *EGFR* (22–24), *EGF*, and *TGF- $\alpha$* , to name a few. *In vivo* interactions between the receptor tyrosine kinase and steroid hormone pathways are suggested by clinical trials that have shown a decrease in tamoxifen responsiveness for ER-positive patients with alterations of *erbB-2* or *EGFR* in their breast cancers (25).

Mammary gland morphogenesis and histology are similar between mice and women (26, 27). Proliferating subpopulations of epithelial cells within the terminal duct lobular unit (of women) or the lobuloalveolar unit (of mice) are believed to include the stem cells from which mammary cancers arise (7, 26, 28–31). Transgenic mice have been used recently to explore the effect of specific genes, combinations of genes, or the loss of genes on cancer development. Mice bearing the rat wt *erbB-2*, under control of the MMTV promoter, have been studied extensively during the past decade (31–45). The MMTV promoter/enhancer is active in the virgin mammary gland, and its activity is enhanced by pregnancy (responsive to prolactin, progesterone, and glucocorticoids but not estrogen). MMTV does not contain an estrogen response element and is not up-regulated by estrogens (34, 46–49).

In this transgenic mouse model, a single tumor histology and phenotype has been reported. Unifocal, well-circumscribed (nodular), intermediate cell type, ER-negative mammary cancers typically develop after a long latency (average 36 weeks; Refs. 41, 50). This

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<sup>4</sup> The abbreviations used are: ER, estrogen receptor; E2, 17 $\beta$ -estradiol; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; TGF, transforming growth factor; wt, wild-type; MMTV, mouse mammary tumor virus; MIN, mammary intraepithelial hyperplasia.

phenotype is generally considered strain-specific and indicative of the effect of the wt *erbB-2* rat transgene (50). The long latency associated with mammary tumors in the MMTV-wt *erbB-2* transgenic mouse is consistent with the acquisition of genetic defects in addition to *erbB-2*. Alterations of *p53* and *EGFR*, in addition to *erbB-2*, have been reported in some tumors (45). Mutations of *erbB-2* in the extracellular domain of the receptor have also been reported in some mammary tumors arising from this model system (33). Pregnancy has been shown to enhance tumorigenesis, presumably via promoter activation by prolactin, progesterone, and other factors (41, 42, 44).

A recent study using this transgenic strain has suggested that carcinogenic risk may be modified by the administration of tamoxifen (51). *i.p.* injection of tamoxifen beginning at 24 weeks of age accelerated tumor development (*i.e.*, a reduction in tumor latency) in contrast to administration of tamoxifen beginning at 12 weeks, which reduced tumor incidence by 50% (Ref. 51; see "Discussion"). In addition, others have recently reported prolongation of tumor latency in mice fed diets with the addition of genistein and diadzein from 7 weeks of age (52).

Estrogenic modulation of this well-defined transgenic model allows interactions between these two important signaling pathways to be explored *in vivo*. This report summarizes several years of transgenic mouse studies evaluating hormonal and dietary modulation of *erbB-2*-mediated mammary carcinogenesis. Unusual features of our studies are the number of mice analyzed, the degree of morphological and histological examination of mammary glands, tumors, and other tissues via necropsy in a model system that has been characterized previously. We believe that modifications of disease patterns associated with these epigenetic factors and the natural heterogeneity we observed in tumor histology are unique. These data suggest that transgenic model systems may be more dynamic than appreciated, with histological and disease pattern heterogeneity worthy of more rigorous histological analyses. These findings may have potential implications for human breast development and individuals with an inherited breast cancer risk.

## MATERIALS AND METHODS

**Estrogen Exposure Studies and Dietary Groupings.** Animal care was provided in accordance with institutional guidelines, and was reviewed and approved by the Institutional Animal Care and Use Committee. Three-hundred thirty-eight 4–5-week-old female FVB/N-TgN (MMTV-*neu*) mice were obtained from Jackson Labs (Bar Harbor, ME) and had been raised on dietary soy chow (Purina 5K52; Ralston Purina, St. Louis, MO) at that facility since weaning. Mice were divided into two separate groups and fed either our institutional standard animal chow (based on soy protein, Purina 5001; Ralston Purina) or a similar casein-based rodent chow (0 estrogenic activity, Purina 5K96). Both diets were initiated on arrival of the mice to our facility at 4–5 weeks. Mice were maintained on either of these diets for the duration of their life. Mice were implanted *s.c.* with a 0.5-mg (resulting in serum levels approximately 225–300 pg/ml), 0.36-mg (approximately 150–200 pg/ml), or a 0.18-mg (approximately 75–100 pg/ml) 60-day "constant" release estrogen pellet, or a 5.0 mg/pellet (~1.5 ng/ml serum) constant release tamoxifen pellet or a placebo pellet (Innovative Research of America, Sarasota, FL) in the lateral neck *s.c.* tissue at 4, 8, 12, or 18 weeks of age (see "Results" for detail of each experiment). Not all of the mice originally implanted with pellets were used in the final analyses.

Animals were monitored daily for tumor development and euthanized at 15, 20, 25, 32, 38, 45, and 60 weeks of age, or when a palpable tumor reached 1.2 cm in diameter. Tumor development was recorded as the animal age when a tumor was detected by palpation. Nonpalpable, grossly identifiable, and microscopically confirmed tumors were recorded as identified at the age of euthanasia. A full necropsy was performed on all of the mice. Mammary glands were removed and processed for whole mounts, fixed in formalin, and embedded for histology or frozen. Grossly palpable tumors were subdivided

and frozen, formalin fixed for histology, or minced to provide cells for primary tissue culture. Over 50 novel mouse mammary carcinoma lines have been generated.<sup>5</sup> Lung tissue was also fixed and processed for examination of metastatic lesions. For these studies, a human breast pathologist and a veterinary pathologist with a background in chemical mammary carcinogenesis blindly analyzed the derived tumors and tissues.

**Dietary Considerations.** The Purina 5001 chow contains 23.4% crude protein (derived entirely from soy), 5.5% crude fat, and 5.3% crude fiber. This diet has been reported to contain 277  $\mu\text{g/g}$  daidzein and 214  $\mu\text{g/g}$  genistein (total of 491  $\mu\text{g/g}$ ), or an equivalent of 4.3 ppb of calculated diethylstilbestrol activity (53). The casein-based Purina 5K96 contains 19% crude protein, 4% crude fat, and 5% crude fiber. The later is not known to have endogenous estrogenic activity. We recognize that these diets are not rigorously matched; hence, verification studies using matched, open casein *versus* soy formulas have been initiated.

**E2 Serum Level Measurements.** Preliminary mouse experiments used a 0.5-mg E2 pellet, implanted at 8 weeks of age in parental FVB mice (strain controls;  $n = 20$ ), to obtain estimates of serum E2 levels and variance over time after implant. Animals were euthanized at regular intervals (day 1, day 3, and every 7 days for 8 weeks) and serum E2 analyses were performed using a RIA (54) in duplicate for each sample (performed in the core laboratory of R. T. C.).

**Mammary Tissue Processing.** Formalin-fixed, paraffin-embedded tissue sections were prepared using standard methods. H&E-stained sections were microscopically evaluated for epithelial pathology by a human breast pathologist (A. T.) and a veterinary pathologist with experience in mammary gland lesions (S. K.). Tumors were evaluated for histological subtype (using standardized murine terminology when possible; Ref. 55), at other times using terminology reflecting similarities with human breast cancers), invasion, vascular invasion, borders, and other features. Each tumor was subtyped and graded using a low, intermediate, and high grade system that considered three factors: proliferation (mitoses), nuclear features (size, nucleoli, and regularity), and growth pattern. In addition, benign findings such as MIN, duct branching and complexity, and calcifications were also documented but will be reported in detail elsewhere.

Whole mount preparations stained with H & E were also made of the fourth right and left mammary glands on all of the mice for an evaluation of the mammary microanatomy and development. For the whole mounts pertinent microscopic features included the branching pattern of ducts and buds (end bud, side bud, and lobular development), and the growth of the ducts into the mammary fat pad. Whole mount images were generated using a Polaroid slide scanner. Other microscopic analyses were performed using standard light microscopy.

**Statistical Analysis.** Comparisons of mice with tumors between groups (soy *versus* casein, E2 *versus* placebo, E2 *versus* tamoxifen, doses of estrogen, or timing of E2 implantation) were calculated using the two-sided Student's *t* test. All of the analyses were carried out at the completion of each experiment after euthanasia and histopathologic review of mammary glands. The tumor-free interval for mice in different treatment groups on the same diet, or on the same treatment group on different diets (*i.e.*, the survival curves) were performed using the Cox proportional hazards model and the log rank statistic. The first palpable tumor was used to calculate the tumor latency for animals that had multiple tumors. Animals euthanized without tumors were censored at the date of euthanasia for tumor free survival and incidence analysis. Censoring was necessary because animals euthanized early in the study (used to compare morphological and histological differences between groups or diets) had not developed tumors by the date of euthanasia. In addition, mice that died suddenly ( $n = 21$ ) without necropsy were also censored for incidence or survival analyses. Comparisons between the number of involved mammary glands was performed using unpaired means analyses and *t* tests. Statistical calculations were carried out using Stat View 5.01 software (SAS Institute Inc., Cary, NC).

<sup>5</sup> J.S. Jeruss, N.X. Liu, Y. Cheung, G. Magrane, F. Waldman, S. Edgerton, X. Yang, and A.D. Thor. Characterization and chromosomal instability of novel derived cell lines from a wt-*erb-2* transgenic mouse model, manuscript in press.

## RESULTS

**E2 Pellet Implantation and Serum E2 Levels in Parental Controls.** An initial experiment was performed to evaluate the amount and variance of serum E2 after implantation of the constant release pellet. These preliminary studies used a 0.5-mg E2 pellet, implanted at 8 weeks of age in parental FVB (control) mice, fed a soybean meal diet, to quantitate serum E2 levels and variance over time. E2 levels peaked at ~600 pg/mg (day 1), fell rapidly to an average of 200 pg/ml by 25 days, and then declined slowly to barely detectable levels by day 60 (~8 weeks; see Fig. 1). Mammary tumors were not identified in any of the parental controls, either E2 or placebo treated.

**E2-associated Procarcinogenesis and Tamoxifen Prevention.** In the first study using wt *erbB-2* transgenic mice, we studied the *in vivo* effects of hormonal modulation of mammary tumorigenesis by comparing groups of mice treated with an estrogen or tamoxifen pellet implanted at 8 weeks of age. The controls were transgenic mice with implantation of a placebo pellet at 8 weeks. Mice were monitored for tumor formation, latency, multiplicity, and gland histology. wt-*erbB-2* transgenic mice implanted with a 0.5-mg E2 pellet (Fig. 2, circles;  $n = 48$ ) developed mammary tumors with the shortest latency (mean 29 weeks). All of these mice demonstrated mammary tumor formation by 45 weeks of age. Transgenic mice treated with a placebo pellet (Fig. 2, squares;  $n = 40$ ) had a significantly longer latency (mean 37 weeks) with each developing mammary neoplasia by 58 weeks ( $P < 0.0001$ ). Mice implanted with a tamoxifen pellet (Fig. 2, trian-

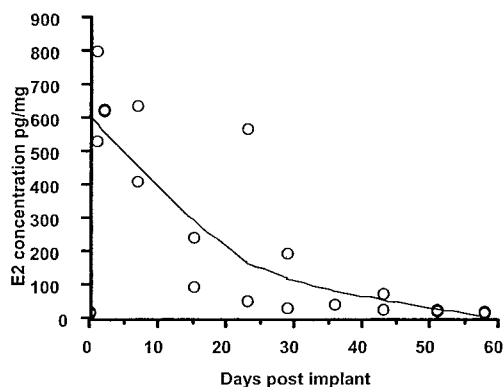


Fig. 1. Serum study of FVB mice implanted with E2 pellet: E2 levels with time. This graph shows the results of serum E2, determined by RIA methods after implantation of a 0.5-mg estrogen pellet at 8 weeks of age in parental (FVB) mice ( $n = 20$ ).

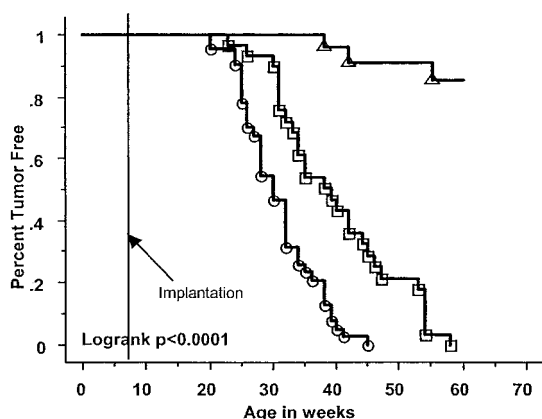


Fig. 2. Mammary gland tumor development by age and treatment group (E2, placebo, or tamoxifen). Kaplan-Meier curves showing the age of palpable tumor formation in MMTV-wt-*erbB-2* transgenic mice on a casein diet implanted at 8 weeks of age (hormonal release until ~16 weeks) with a 0.5-mg estrogen pellet ( $\circ$ ,  $n = 48$ ), 5.0-mg tamoxifen pellet ( $\Delta$ ,  $n = 23$ ), or placebo pellet ( $\square$ ,  $n = 40$ ).

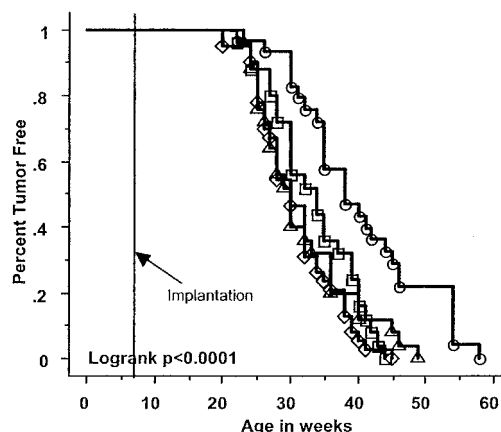


Fig. 3. Dosage study. Mice on a casein diet were implanted with a 0.5-mg E2 ( $\diamond$ ,  $n = 48$ ), a 0.36-mg estrogen ( $\Delta$ ,  $n = 30$ ), a 0.18-mg estrogen ( $\square$ ,  $n = 30$ ), or a placebo pellet ( $\circ$ ,  $n = 40$ ) at 8 weeks of age. Significant differences in the tumor-free interval were observed between placebo and each E2 dosage:  $P < 0.0001$  for E2 0.5 mg,  $P = 0.0007$  for E2 0.36 mg, and  $P = 0.0015$  for E2 0.18 mg, respectively.

gles;  $n = 34$ ) generally failed to develop mammary tumors by 60 weeks. Median latency could not be estimated for tamoxifen-treated mice, because so few developed tumors [3 of 18 (17%) of tamoxifen-treated, casein-fed mice followed for 60 weeks]. Tamoxifen-treated mice had a significantly longer tumor latency than placebo-treated mice ( $P < 0.0001$ ). In a three-way comparison, the tumor-free interval was significantly different ( $P < 0.0001$ ). Stromal invasion was also analyzed in each treatment group as an *in vivo* measure of tumor aggression. For this comparison, invasive tumors (single or clusters of tumor cells beyond the main focus of tumor and separated by stromal elements) were separated from well-circumscribed, nodular tumors with a pseudocapsule. In the E2-treated mice 23 of 74 (31%) mouse tumors showed evidence of stromal invasion, whereas among the placebo treated mice, only 1 of 39 (2.6%) showed similar stromal invasion ( $P = 0.0003$ ). Comparisons of stromal invasion in the tamoxifen-treated mice could not be performed, because too few mice developed tumors. In this first experiment, each of the mice were fed Purina 5K96 chow (casein-based, 0 estrogenic activity) from receipt (4–5 weeks of age) until their death. In the parental FVB mice ( $n = 152$ ) treated similarly, none developed mammary tumors with observation until 50 weeks of age.

We then studied the effect of E2 by dose on mammary tumor development in these high-risk transgenic mice fed a casein-based diet. Three groups of virgin, FVB/N-TgN (MMTV-*neu*) mice were implanted at 8 weeks of age with a single constant release estradiol (E2) pellet, at 0.5 mg (approximately 225–300 pg/ml serum level;  $n = 48$ ), 0.36 mg (approximately 150–200 pg/ml;  $n = 30$ ), or a 0.18 mg total dose (approximately 75–100 pg/ml;  $n = 30$ ). Forty control mice were similarly implanted with a placebo pellet. The median tumor-free survival was 29, 29, and 33 weeks (not significantly different;  $P > 0.3$  in a three-way comparison) for the 0.5, 0.36, and 0.18 mg E2 treatment groups, respectively, as compared with 37 weeks for placebo controls (significantly different from each E2 dose;  $P < 0.0001$ ,  $P = 0.0007$ , and  $P = 0.0015$ , respectively; Fig. 3). The frequency of mice with single tumors, as compared with multiple tumors (multifocality), increased with the age of the mouse in both the E2 and placebo-treated (casein-fed) mice. Of all of the mice with tumors analyzed in this study, 171 of 245 (70%) demonstrated multiple mammary glands with tumors. E2 treatment was more often associated with multifocal tumor development (150 of 206; 73%) as compared with placebo (20 of 32; 63% of mice with multifocal tumors),  $P =$  not significant or placebo + tamoxifen groups (21 of 39;



54%;  $P < 0.018$ , compared with E2). Tamoxifen-treated mice generally showed no multifocal tumor development (1 of 7; 14%; observed for 60 weeks).

**Age and Risk Associated with E2 Exposure.** We hypothesized that the timing of estrogen exposure might affect the phenotype or biological features of mammary tumorigenesis, consistent with a risk window (concept based on chemical carcinogenesis rat models and human epidemiological studies). Transgenic *wt-erbB-2* mice ( $n = 48$ ) treated with a 0.5-mg estrogen pellet implanted at 8 weeks of age were compared with 28 mice similarly implanted at 4 weeks of age, 27 mice similarly implanted at 12 weeks of age, and 27 evaluable mice implanted at 18 weeks of age (each fed a casein diet). Mammary tumors developed with approximately the same tumor-free interval, and incidence in the 8- and 12-week implantation groups. Mice implanted at 4 or 18 weeks had significantly longer tumor-free intervals, as compared with mice implanted at 8 or 12 weeks, with survival curves super-imposable on placebo-treated mice ( $P = 0.0004$  in the five-way comparison; see Fig. 4).

Histological examination of the tumors from these animals showed no major difference in pattern or grade by tumor implantation date. Significant differences in the numbers of mammary glands with tumors per mouse (multifocality) between mice treated with 0.5-mg estrogen pellets at 8, 12, or 18 weeks in the timing study were also not observed.

**Differences in Tumor Phenotype by Treatment.** A detailed microscopic examination of all of the mammary and pulmonary tissues from each mouse, as well as necropsy examination, was performed. The majority of tumors (~80%) were of the nodular, low grade, intermediate cell type histology (illustrated Fig. 5A) and as reported by others (41). Some variance in tumor histology was observed in ~20% of cases. Two histological subtypes not reported previously for this *wt-erbB-2* model are demonstrated in Fig. 5, B and C (a gland-forming invasive cancer and a poorly circumscribed, invasive comedo carcinoma). A more complete report illustrating the wide range of histology is in preparation.

In general, E2 exposure was associated with a more aggressive phenotype as compared with tumors that arose in tamoxifen or placebo-treated mice. For example, the highest dose (0.5 mg pellet) of E2 was more often associated with a higher (more aggressive) histological grade of tumor as compared with the lower dose E2 or placebo pellet-treated mice ( $P < 0.0001$  and  $P = 0.0009$ , respectively, using the unpaired means comparison; see Table 1). Significant differences in tumor grade between the moderate and low E2 dose, and between

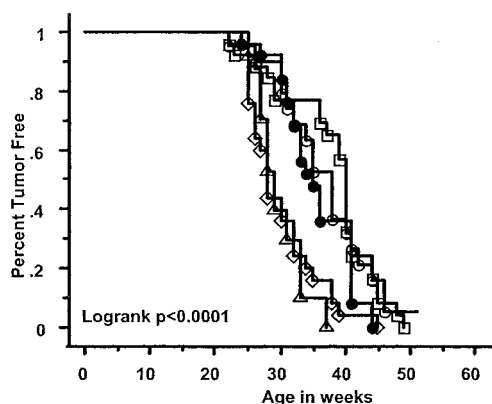


Fig. 4. Timing study. Kaplan-Meier disease-free survival curves for the transgenic *wt-erbB-2* mice on the casein diet, using a single 0.5-mg estrogen pellet implanted at 4 weeks of age ( $\square$ ,  $n = 28$ ), 8 weeks of age ( $\diamond$ ,  $n = 48$ ), 12 weeks of age ( $\triangle$ ,  $n = 27$ ), or 18 weeks of age ( $\bullet$ ,  $n = 27$ ). The control pellet ( $\circ$ ,  $n = 40$ ) was implanted at 8 weeks of age.

the low E2 dose and placebo arms were also observed ( $P = 0.0057$  and  $P = 0.0238$ , respectively).

In a similar fashion, we investigated tumor aggression by determining the percentage of mice with pulmonary metastases. Pulmonary metastases were never observed in the absence of mammary tumors. Metastases were more often identified in E2-treated mice (31 of 190 in the E2 group as compared with 4 of 24 in the placebo group). However, these data were not statistically different. Metastatic tumors were confined to the lungs, as compared with visceral organs, lymph nodes, or skeletal structures. Metastatic foci were vasocentric (as reported by others, image not shown).

**Dietary Phytoestrogens and Mammary Tumor Incidence.** To assess the impact of dietary phytoestrogens in this model system, we studied tumor incidence, latency, and other factors in E2 or tamoxifen-treated, soy-fed mice. In brief, virgin FVB/N-TgN (MMTV-*neu*) mice were fed Purina 5001 (Ralston Purina), a closed chow with a relatively high isoflavone content (491  $\mu\text{g/g}$ ). These mice were implanted with a single “constant” release estradiol E2 ( $n = 19$ ), tamoxifen (estimated circulating serum level 1.5 ng/ml;  $n = 45$ ), or a placebo pellet ( $n = 15$ ) similar to the casein-fed mice described above. Each of the mice in the soy group had been fed the soy meal-based diet, Purina 5K52, at the Jackson Laboratory (until 4–5 weeks of age). Each was placed on Purina 5001 on arrival at our animal facility and maintained on that chow for life.

For placebo and E2-treated groups, mice fed the soy meal-based diet developed mammary tumors at a significantly older age than the casein-fed controls ( $P = 0.0324$ , mean 40 weeks for casein versus 49 weeks for soy and  $P = 0.0083$ , mean 31 weeks for casein versus 37 weeks for soy, respectively; see Fig. 6). Differences in tumor incidence or latency were not observed in the tamoxifen-treated mice by dietary group. Very few of the soy chow-fed, tamoxifen-treated mice developed mammary gland tumors by 60 weeks of age (4 of 21; 19%).

**Alterations in Mammary Gland Development by Treatment and Diet.** Using whole mount preparations, morphogenic variance in breast development could be assessed by treatment group and age. As shown in Fig. 7, mice maintained on a soy meal-based diet for life demonstrated less infiltration of the mammary fat pad by ducts and lobuloalveolar structures (as compared with casein-fed mice at 25 weeks of age; Fig. 8). In E2-treated mice, the most significant differences in morphogenesis by diet were observed. E2-treated, casein-fed mice demonstrated the greatest end and side bud development, and diffuse gland complexity (Fig. 8B). In comparison, E2-treated, soy meal-fed mice showed relatively fewer side buds and greater differentiation of the distal end buds (Fig. 7B). Tamoxifen-treated mice on either diet showed immature duct structures with simple end and side buds (Fig. 7C; Fig. 8C). Morphogenic variability by dose of E2 was less pronounced than morphogenic variability by treatment group (data not shown). The morphogenesis of placebo-treated mouse mammary glands are demonstrated in Fig. 7A (soy) and Fig. 8A (casein). With age, E2-treated mice demonstrated earlier development of MIN (data not shown).

## DISCUSSION

The MMTV-*wt-erbB-2* transgenic mouse model is an established transgenic model first reported in 1992 (35). In this report, we describe a comprehensive study of the effects of epigenetic hormonal factors (E2 and tamoxifen) and diet (soy versus casein) on *wt-erbB-2*-mediated mammary cancer development. A key period of vulnerability (or so called “risk window,” Ref. 28) appears to be from approximately 8–18 weeks. During this period, the lobuloalveolar units of mice undergo a complex process of growth and differentiation. The mammary epithelium becomes fully responsive to estrogen,

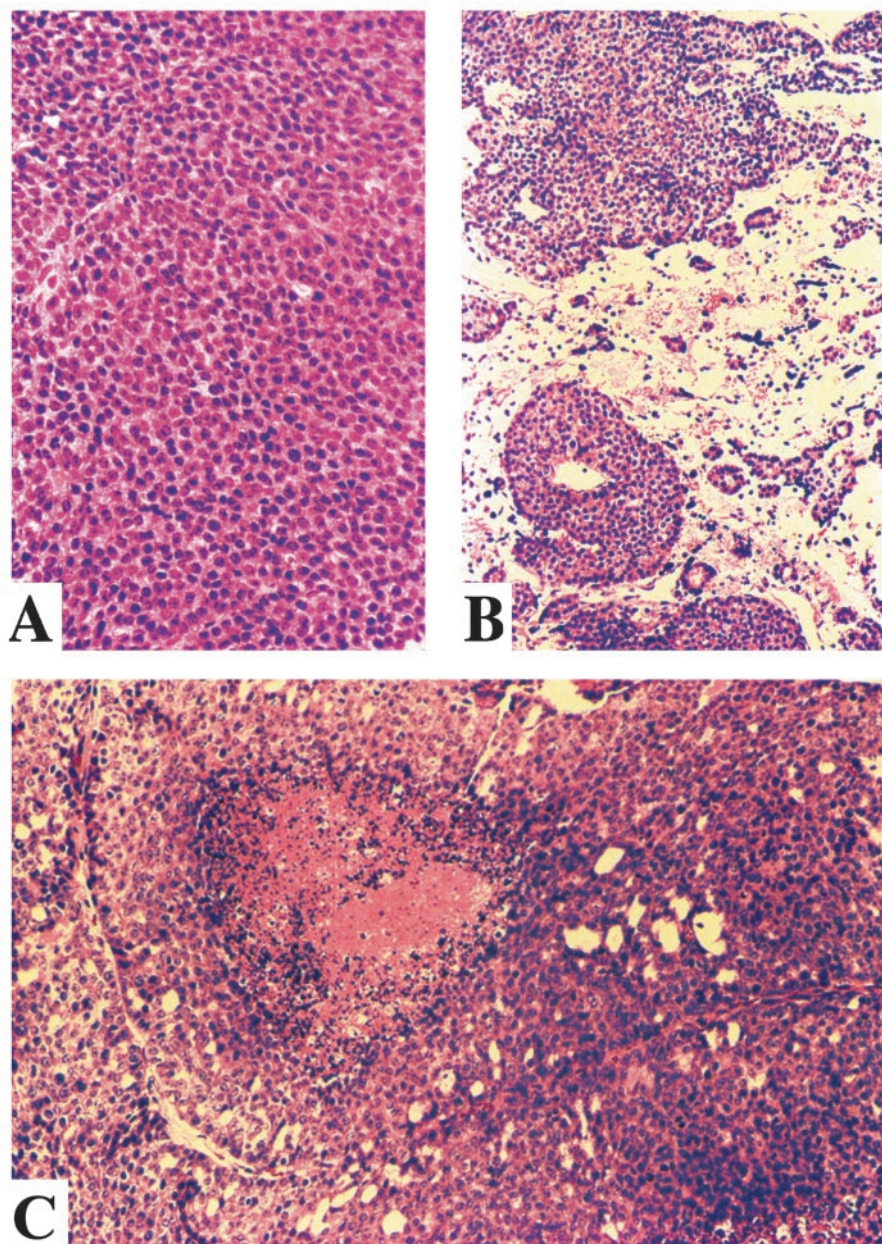


Fig. 5. Histological subtypes. Photomicrographs of H&E-stained, 4  $\mu$ m sections of fixed embedded mammary tumors. **A**, low-grade, intermediate cell type, nodular mammary tumor, classically associated with this *wt-erbB-2* transgenic model ( $\times 40$ ). Note uniformity of nuclear size, low mitotic count, lack of tubule formation, abundant eosinophilic cytoplasm, and scant vascularity. **B**, intermediate grade, invasive acinar mammary adenocarcinoma with small and large nests of cancer cells infiltrating the mammary stroma ( $\times 20$ ). Some of the cell groups demonstrate tubule formation. Large nests demonstrate focal gland lumina. Nuclei are somewhat more atypical than **A**, but less atypical than **C**. Mitotic figures are rarely noted. The highly invasive pattern and tubule formation have not been described for this model. **C**, a high-grade, solid, invasive comedo carcinoma ( $\times 40$ ). Note the solid nests of invasive cancer, remarkably high mitotic figure count ( $>10$  in the image shown), central comedo necrosis, high-grade nuclei (large, open, and vesicular with prominent irregular nucleoli). This histological subtype has not been reported previously for this model system.

progesterone, and other hormones as ducts elongate, divide, and differentiate (28, 30). Subsequent division of terminal buds into alveolar units, type 1 and 2 lobules, occurs. Type 1 lobules (early and undifferentiated), in particular, are believed to be particularly susceptible to procarcinogenic agents, estrogen/proliferation-associated influences, or mutagens (28). Similar periods of risk during human adolescence are suggested based on epidemiological studies of radiation exposure (56) and calorie restriction (57).

Our data confirmed that tumor development in the *wt-erbB-2* transgenic mice was specifically associated with the transgene, as parental FVB mice failed to develop mammary tumors even with E2 exposure. In the MMTV-*wt-erbB-2* transgenic mice, all of the E2 and nearly all of the placebo-treated groups developed mammary tumors by 60 weeks of age. Blockade of mammary tumor development in  $>80\%$  by short-term tamoxifen (at 8–16 weeks of age) was also demonstrated. These data provide proof of the principle that estrogens contribute to mammary gland tumor formation in this transgenic model system (even if retained in the virgin state). These data also

suggest that brief tamoxifen exposure can render mammary epithelial cells resistant to tumorigenesis for the lifetime of the mouse and that this model system may be useful to study this phenomenon.

Table 1 Comparison of tumor grade by estrogen dose or placebo by Student's *t* test

Using the unpaired means comparison, the value of differences between the means of two groups could also be calculated. Comparison between the high-dose E2 (mean grade 1.9) and placebo (mean grade 1.5) showed significant differences by tumor grade ( $P = 0.0009$ ; 95% CI, 0.17–0.64). Comparison between the high and moderate dose (mean grades 1.9 and 1.5, respectively) were also significant ( $P < 0.0001$ ; 95% CI, 0.21–0.61). Comparisons between the means for the high versus low dose and placebo versus moderate dose failed to show statistical significance. Comparisons of the means for the low (1.8) and moderate E2 dose, and low E2 and placebo also showed statistical significance ( $P = 0.0057$ ; 95% CI, 0.09–0.52), and  $P = 0.02$ ; 95% CI, 0.04–0.56, respectively).

Dose	<i>n</i>	Grade 1	Grade 2	Grade 3	<i>P</i>
0.00 mg	39	20 (51%)	18 (46%)	1 (2.6%)	0.0007
0.18 mg	70	25 (36%)	33 (47%)	12 (17%)	
0.36 mg	77	43 (16%)	29 (38%)	5 (6.5%)	
0.50 mg	73	17 (23%)	45 (62%)	11 (15.1)	



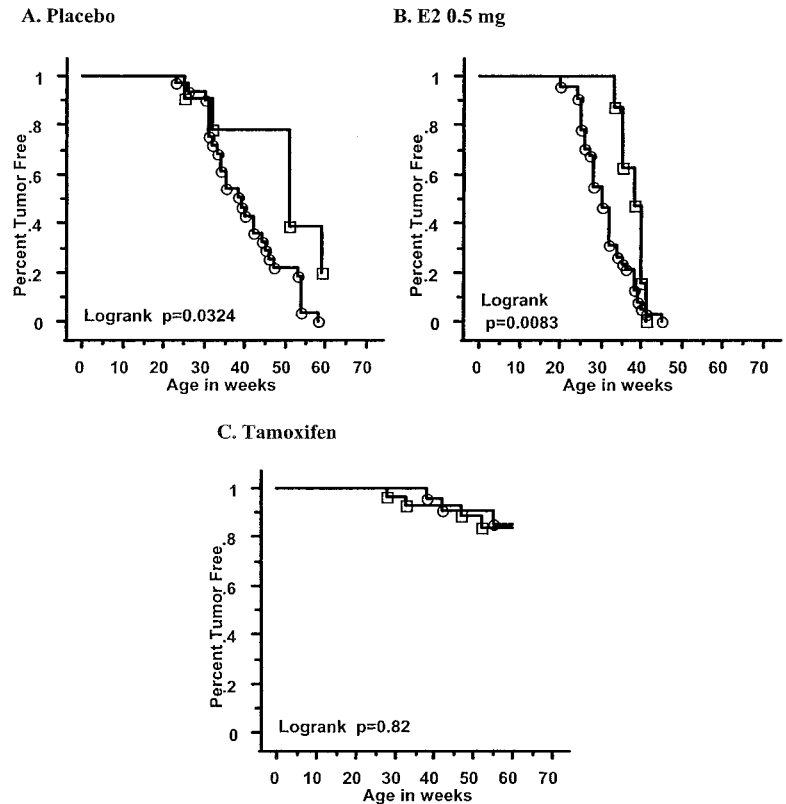


Fig. 6. Dietary effects. Kaplan-Meier survival curves for MMTV-wt-*erbB-2* mice fed casein or soy diets (refer to Ref 53 and “Materials and Methods” for additional detail on dietary components). Pellets were implanted at 8 weeks of age for all groups. *A*, placebo group. Mice treated with placebo pellet at 8 weeks. Casein ( $\circ$ ,  $n = 40$ ), soy meal ( $\square$ ,  $n = 15$ ). *B*, E2 group. Mice treated with a 0.5-mg (approximately 225–300 pg/ml) E2 pellet at 8 weeks and fed on either casein ( $\circ$ ,  $n = 48$ ) or soy meal diet ( $\square$ ,  $n = 17$ ). *C*, tamoxifen group. Mice treated with 5-mg (1.5 ng/ml) tamoxifen pellet at 8 weeks, fed either a casein ( $\circ$ ,  $n = 34$ ) or soy meal-based diet ( $\square$ ,  $n = 44$ ).

Our data shows greater tamoxifen associated prevention (>80%) than the study by Menard *et al.* (Ref. 51; 50%) using i.p. tamoxifen injections (5 days/week for life) starting a 12 weeks ( $n = 16$ ). In other mice, similar tamoxifen injections beginning at 24 weeks failed to

prevent tumorigenesis and in fact accelerated tumor formation ( $n = 16$ ; Ref. 51). In aggregate, these data suggest that tamoxifen-mediated chemoprevention is particularly robust at the early stage of mammary gland development. Our data extends the data of Menard *et*

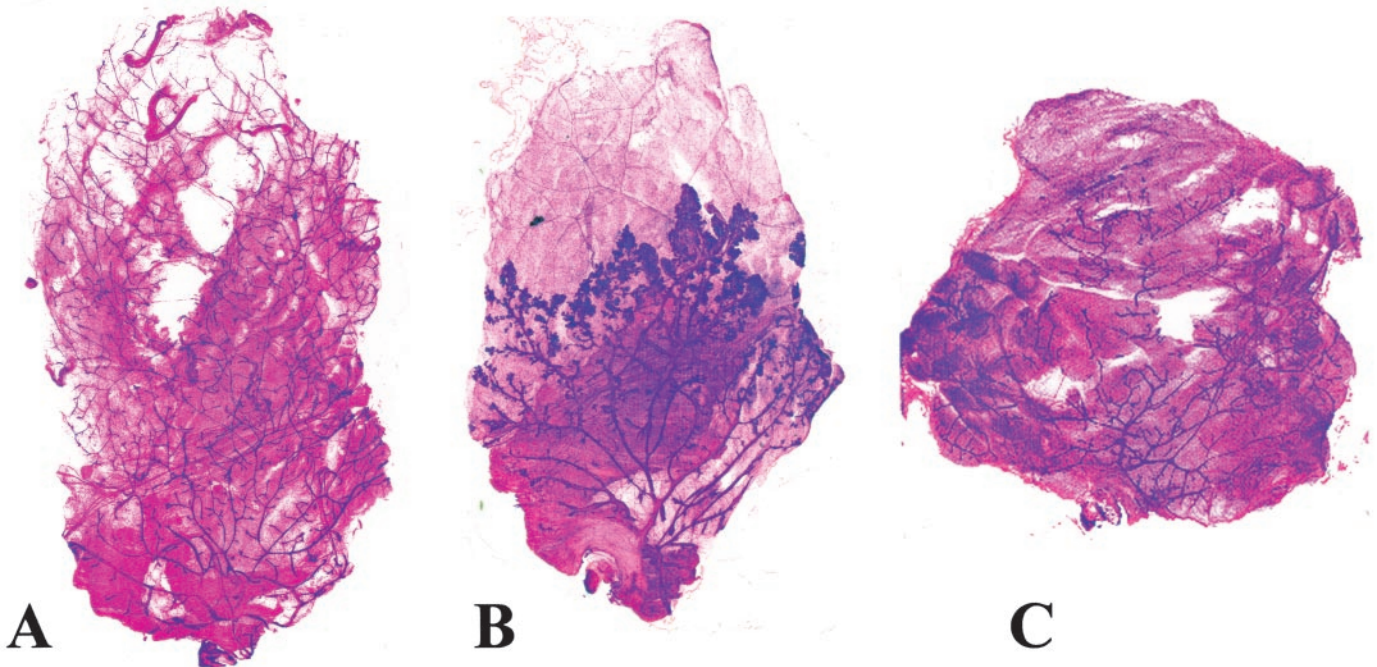


Fig. 7. Morphogenesis by treatment group in soy-fed mice. Whole mounts of entire mammary gland, oriented with the nipple in the lower portion of each image. Stroma is lightly stained by eosin, ducts stain blue from hematoxylin. *A*, placebo pellet-treated mouse, casein diet. Note complex duct branching and side bud development. End buds and terminal lobuloalveolar units (predominantly type 1) are more developed than the tamoxifen mice (*C*) and less hyperplastic and complex than the E2 mice (*B*) on a casein diet. *B*, mammary tissue from a 0.5-mg E2-treated mouse. The large, proximal duct structure is similar to the placebo mouse (*A*), but end and side bud complexity and lobuloalveolar development are more hyperplastic (predominantly type 2, some type 3) with focal atypical hyperplasia (MIN). *C*, relatively undeveloped ducts and duct branches with few side and end buds in a tamoxifen-treated mouse at the same age, 25 weeks as *A* and *B*. These findings are roughly similar to a parental mouse at ~6 weeks.

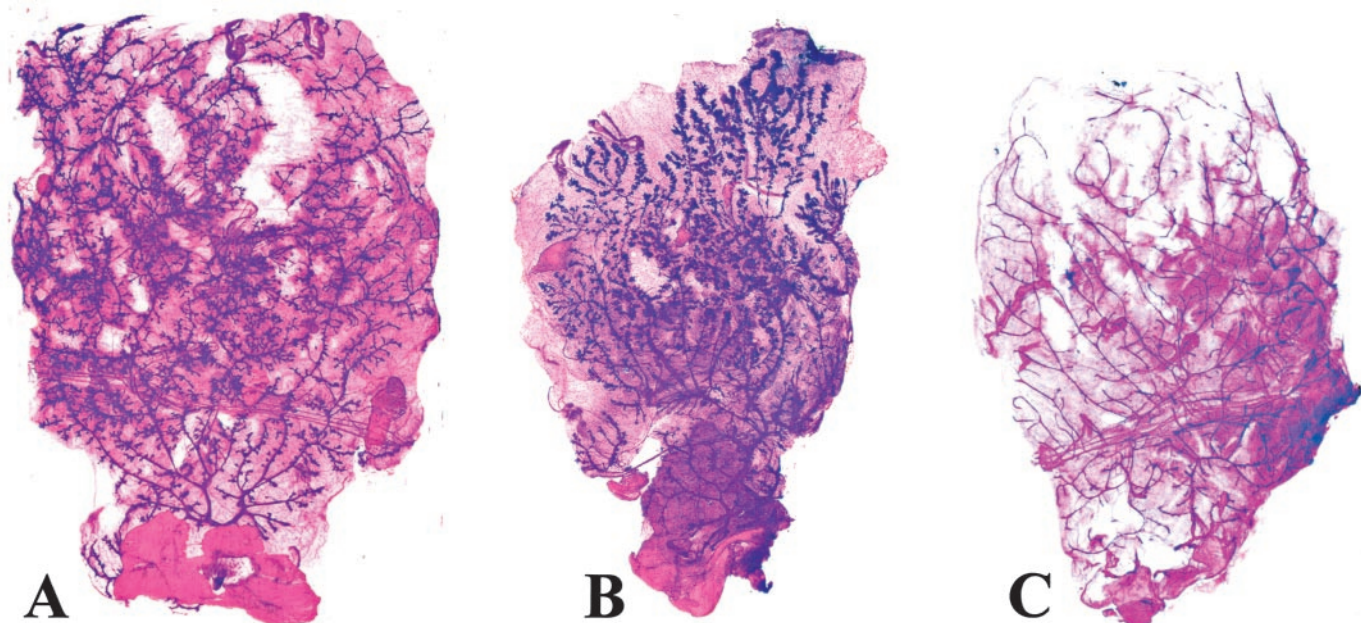


Fig. 8. Morphogenesis by treatment group in casein-fed mice. *A*, placebo: note more complex branching, end and side buds when compared with Fig. 7*A* (soy diet, same treatment group). *B*, E2: mammary tissue from a 0.5-mg E2-treated mouse. Note increases in side and end bud development, a generalized hyperplasia, and atypical hyperplasia. These changes are more pronounced than observed in the mammary glands of mice fed a soy diet (Fig. 7*B*). *C*, tamoxifen. Mammary glands from mice treated with a single 5-mg tamoxifen pellet demonstrates a lack of terminal differentiation and less branching than E2 (*B*) or placebo (*A*) mice fed the same casein diet. Mammary glands from tamoxifen-treated mice have greater similarities between dietary subgroups (Fig. 7*C* soy, Fig. 8*C* casein) than E2 or placebo-treated mice.

*al.* (51) in that it is not necessary to treat these mice with a lifetime of tamoxifen to prevent tumor formation. Our study also suggests that earlier initiation of tamoxifen treatment and or a shorter duration of treatment is beneficial.

Significant differences in tumor biology and phenotype by hormonal treatment were also observed. E2-treated mice developed more aggressive mammary tumors (as demonstrated by tumor grade, stromal invasion, and metastasis). These data suggest that estrogens are requisite for mammary gland tumorigenesis, and at higher levels may modulate cell processes enough to result in unique phenotypic and biological properties. Because transcription of the *erbB-2* transgene is driven by the MMTV promoter in this model, we have questioned whether E2 or dietary treatments may modulate *erbB-2* expression directly. It is known that the MMTV promoter contains a hormonal regulatory element, which can be induced by progesterone, glucocorticoids, androgens, or prolactin (58–60). MMTV does not contain an estrogen response element and is not known to be up-regulated by estrogens (34, 46–49). To explore this issue *in vivo*, we have performed studies on additional *wt-erbB-2* mice. We implanted a 0.5-mg E2 or placebo pellet short term (24 h or 7 days) at 9 weeks of age. Four mice were used in each treatment group, (E2/casein and placebo/casein). Another 4 mice were exposed at the same age to 24 h of a soy diet. Benign mammary glands were harvested at 24 h after hormone or soy treatment, or 7 days after E2 treatment for RNA extraction and *erbB-2* RNA expression studies analyzed by reverse transcription-PCR. We observed no significant differences in *erbB-2* RNA levels between E2 *versus* placebo groups on either diet (data not shown), consistent with no direct effect of E2 or soy on the MMTV promoter.

In this model system, others have reported *erbB-2* gene mutation (leading to activated receptor) as well as up-regulation of EGFR resulting in signal pathway activation (32, 33, 36, 61–64). EGFR up-regulation is one mechanism by which mammary cancer cells increase their sensitivity to autocrine or paracrine growth stimulation (65–68). Of interest, estrogen induces components of this pathway including EGF, EGFR, and TGF- $\alpha$  via direct binding to an estrogen

response element in their promoter regions (22, 23, 69). We have detected up-regulation of EGFR and down-regulation of ER in benign mammary glands of E2-treated mice (immunohistochemical data not shown; studies in progress). Of interest, blockage of EGFR has been linked with tumor suppression in MMTV/*neu* + MMTV/TGF- $\alpha$  bigenic mice (70). In addition, genistein in the diet reduces carcinogenesis in a dose-dependent manner by down-regulating both the EGFR and ER $\alpha$  (71).

In assessing the relevance of this model system, we are struck by consistencies with data relating to human breast cancer. Alterations of EGFR and *erbB-2* have been particularly associated with breast cancers that arise in premenopausal women with higher endogenous estrogen levels (14–16). We have demonstrated recently inverse age-associated correlations with measures of tumor growth and genetic instability (proliferation, nuclear grade, and p53 positivity), as well as growth factor receptor (*erbB-2* and EGFR) overexpression in ~3800 human primary breast cancers (72). Male breast cancers that arise in the absence of significant estrogenic stimulation have not been associated with EGFR or *erbB-2* alterations (73–75), in keeping with this age/hormonal milieu hypothesis. It is likely that altered mammary gland structure is a modulator of mammary tumor risk as well. Increasing or decreasing the epithelial cell number or altering cellular differentiation may affect molecular responses to hormonal or dietary factors.

In addition, dietary isoflavones (particularly in premenopausal women) has been inversely associated with breast cancer risk (76) similar to what we observed in this model system. In one study, self-reported soy intake has been inversely associated with mammographic parenchymal density of the breast (77), a surrogate marker for breast cancer risk. In certain countries like Japan, adults typically consume 1–2 servings of traditional soy foods per day (35–40 mg of isoflavones; Ref. 78). This level of intake has been associated with a wide variety of benefits, including reductions in cardiovascular risk,<sup>6</sup>

<sup>6</sup> Internet address: <http://www.cfsan.fda.gov/~dms/fdsoypr.html>.



osteoporosis, and breast cancer (79). In the United States, the intake of soy-rich foods or soy-derived products like meat analogues (which contain soy protein isolate and/or soy protein concentrate) has increased significantly in the last decade. In particular, breast cancer survivors and individuals who perceive they are at an increased risk for the breast or prostate cancer have demonstrated significant interest in soy as a disease or risk-modulating factor.

Many articles have now demonstrated that dietary factors (lipids, calorie restriction, fiber, and retinoids) may influence the development of mammary gland neoplasia in transgenic model systems (80–82). The potential effects of dietary factors on model systems have been reviewed recently in detail (53, 83). In particular, prepubertal exposure to genistein has been shown to modify breast carcinogenesis via enhanced gland differentiation (84) and in conjunction with modulation of TGF- $\alpha$ , EGF, and EGFR in rat carcinogen models (85). The experiments and derived data summarized above brought to our attention the importance of diet on rodent models of cancer. Institutional choices of diet or switches in dietary formulas can cause major changes in the derived data (as we have experienced first hand). Such changes are often made without investigator disclosure. Furthermore, much of the rodent model literature does not cite dietary formulas. The importance of dietary components and their impact on *in vivo* model systems are anything but trivial and have been discussed in detail by others (53, 83). However, until such changes were noted in our model system we were unaware of the impact dietary formulas might have.

In summary, we have used a well-described transgenic model and modified the pattern of mammary growth, tumor development, and phenotypic aggression via estrogens, antiestrogens, and/or a diet rich in isoflavones (plant-based phytoestrogens). We have also recognized at least two new histological variants, and recognized that epigenetic factors may effect histological features and phenotype in this transgenic model. These data suggests that the “five biological rules of mammary transgenes” (86) may need modification. As published, the five rules include: (a) mammary development is related to the type and amount of transgene expressed; (b) dysplasias and tumors develop from secondary mutations; (c) transgenes determine tumor phenotype; (d) transgenes may activate dominant oncogenic pathways; and (e) the oncogenic pathway determines prognosis (86). We propose an additional sixth rule: that genetic predisposition may be modified by epigenetic factors. These data may have implications for model system development and investigation at large, as well as in genetically at-risk women, because exposure to dietary or exogenous forms of estrogens or antiestrogens are common, and may modify breast development and subsequent patterns of tumorigenesis.

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