ONCOLOGY REPORTS 5: 609-616, 1998

Maternal genistein exposure mimics the effects of estrogen on mammary gland development in female mouse offspring

LEENA HILAKIVI-CLARKE[1,2], ELIZABETH CHO[1] and ROBERT CLARKE[1,3]

[1]Lombardi Cancer Center, Departments of [2]Psychiatry and [3]Physiology, Georgetown University, 3970 Reservoir Road, NW, Washington, DC 20007, USA

Received November 25, 1997; Accepted January 30, 1998

Abstract. Human and animal data indicate that a high maternal estrogen exposure during pregnancy increases breast cancer risk among daughters. This may reflect an increase in the epithelial structures that are the sites for malignant transformation, i.e., terminal end buds (TEBs), and a reduction in epithelial differentiation in the mammary gland. Some phytoestrogens, such as genistein which is a major component in soy-based foods, and zearalenone, a mycotoxin found in agricultural products, have estrogenic effects on the reproductive system, breast and brain. The present study examined whether *in utero* exposure to genistein or zearalenone influences mammary gland development. Pregnant mice were injected daily with i) 20 ng estradiol (E2); ii) 20 µg genistein; iii) 2 µg zearalenone; iv) 2 µg tamoxifen (TAM), a partial estrogen receptor agonist; or v) oil-vehicle between days 15 and 20 of gestation. E2, genistein, zearalenone, and tamoxifen all increased the density of TEBs in the mammary glands. Genistein reduced, and zearalenone increased, epithelial differentiation. Zearalenone also increased epithelial density, when compared with the vehicle-controls. None of the treatments had permanent effects on circulating E2 levels. Maternal exposure to E2 accelerated body weight gain, physical maturation (eyelid opening), and puberty onset (vaginal opening) in the female offspring. Genistein and tamoxifen had similar effects on puberty onset than E2. Zearalenone caused persistent cornification of the estrus smears. These findings indicate that maternal exposure to physiological doses of genistein mimics the effects of E2 on the mammary gland and reproductive systems in the offspring. Thus, our results suggest that genistein acts as an estrogen *in utero*, and may increase the incidence of mammary tumors if given through a pregnant mother. The estrogenic effects of zearalenone on the mammary gland, in contrast, are probably counteracted by the permanent changes in estrus cycling.

Correspondence to: Dr Leena Hilakivi-Clarke, Lombardi Cancer Center, Research Bldg., Room W405, Georgetown University, 3970 Reservoir Road, NW, Washington, DC 20007-2197, USA

Abbreviations: DES, diethylstilbestrol; EB, end bud; E2, estradiol; ER, estrogen receptor; LAU, lobulo-alveolar unit; TEB, terminal end bud; TGFα, transforming growth factor α

Key words: genistein, zearalenone, estradiol, tamoxifen, *in utero* exposure, mammary gland

Introduction

In animals studies, a high *in utero* estrogenic environment increases the risk to develop breast cancer (1-3), possibly by altering development of the mammary epithelial tree (4,5) and/or function of estrogen-regulated genes (6,7). Human studies also show that high birth weight, which reflects high maternal estrogen levels (8), increases breast cancer risk in women (9,10). Low pregnancy estrogen levels, which are associated with pre-eclampsia, reduce daughters' breast cancer risk (11,12). The primary exposure for estrogenic stimuli in pregnant women represents that originating from the placenta. However, other agents in our environment may intervene with pregnancy estrogenic activity. These include compounds from plants consumed in the diet (phytoestrogens) (13). Phytoestrogens are structurally and functionally similar to 17ß-estradiol (E2) or they produce estrogenic effects. Phyto-estrogens that have received most attention are genistein and zearalenone.

Genistein is an isoflavone present in soy-based food products (13). It interacts with both the 'classical' estrogen receptor (ERα) and the recently described ERß form (14). Similar to estrogens, isoflavones stimulate the growth of human breast cancer cells at physiological concentrations, and inhibit them when given in pharmacological doses (15). In the uterus and mammary gland in mice, genistein is also estrogenic (16). In addition, epidemiologic data suggest that genistein has estrogenic properties in female reproductive tissues (17-19) and the breast (20). There is a potential for high genistein exposure during early life. For example, plasma genistein levels are very high in infants fed a soy-based formula (21). Therefore, we studied whether *in utero* exposure to genistein affects the development of the mammary gland in a manner similar to estrogens.

Zearalenone is a resorcyclic acid lactone mycotoxin produced by several of the *Fusarium spp* molds, and is found in wheat, corn, rice, barley, and several other grains and cereals (22,23). Because of its estrogenic properties, zearalenone has been used as a contraceptive (24), an estrogen therapy in post-menopausal women (25), and an anabolic agent to enhance

growth in cattle and lambs (26,27). Zearalenone stimulates the growth of the MCF-7 human breast cancer cells (28), and increases the proportion of cells in S and G_2/M (our unpublished data). It also has been reported to enlarge the mammary gland and, at pharmacologic doses, induce spontaneous mammary tumors in mice (29) and rats (30).

The purpose of this study was to investigate the effect of a maternal exposure to genistein or zearalenone on mammary gland development in the female offspring. Altered mammary gland morphology is a potential marker of a prior exposure to high fetal estrogen levels, and may predict for an increased breast cancer risk (4,31). The mammary epithelial component consists of epithelial ducts that end in structures called terminal end buds (TEBs) (for reviews of mammary gland development see refs. 32,33). After puberty, TEBs begin to cleave into smaller buds, called alveolar buds (ABs), or they become atrophic and form terminal ducts (TDs). At the age of 4 months, most of the TEBs have differentiated into TDs or ABs. TEBs are the primary targets for neoplastic transformation in the rodent mammary gland, although some tumors may also arise in the TDs (34). Most mammary carcinomas in women originate from lobules type-1 (terminal ductal-lobular units; TDLU), which arise from TDs or TEBs (35). Differentiation of TEBs to TDs and ABs, which occurs especially during pregnancy, significantly reduces breast cancer risk both in humans and rodents (34).

Our earlier data indicate that a perinatal exposure to 2-4 μg estradiol (E2) in mice, and *in utero* exposure to 20 ng E2 in rats, increases the number of TEBs in the mammary gland, and delays their differentiation to TDs and ABs (3,4). An increased number of TEBs, and their failure to differentiate, are also characteristic of transgenic mice that overexpress specific growth factors or oncogenes and consequently develop mammary tumors (36,37). The present study investigated whether *in utero* exposure to physiological doses of genistein or zearalenone, through a pregnant mother, affects the development of TEBs, TDs and ABs. The effects of early treatment with genistein and zearalenone on reproductive parameters were also assessed. *In utero* exposure to estogens, particularly diethylstilbestrol (DES), has several adverse effects on the reproductive system (38,39).

Materials and methods

Animals and treatments. Outbred female CD-1 mice (National Institutes of Health, Frederick, MD) arrived in our laboratory on day 7 of pregnancy. On gestation day 15, pregnant animals were assigned into the following treatment groups: i) 20 ng estradiol benzoate (E2) (Sigma, St. Louis, MO); ii) 20 μg genistein (Sigma, St. Louis, MO); iii) 2 μg zearalenone (Aldrich Chemicals, WI); iv) 2 μg tamoxifen (TAM) (Sigma, St. Louis, MO), a partial estrogen receptor agonist; or v) oil-vehicle. The phytoestrogen doses were chosen based on the levels of genistein exposure among Orientals, our previous study in rats in which an exposure to 20 ng E2 altered mammary gland development (3), and the potency of each compound, in relation to E2, to bind to ER. Americans are exposed to 1-3 mg of phytoestrogens per day, while in the Oriental countries the exposure varies between 25-100 mg per day (40). Approximately 50% of the daily isoflavone exposure in Orientals is composed of genistein (0.25-1 mg/kg) (40). It has been estimated that individuals living in the US are exposed daily to 1-5 mg/day of zearalenone (0.02-0.1 mg/kg) (41). Zearalenone and TAM are about 100 times weaker than E2 and genistein is 1,000 times weaker (28,42). Injections were given daily from day 15 until gestation day 20. Each group contained 4-6 litters.

Physical maturation. Within 24 h after birth, the number of offspring per litter and the offspring body weights were recorded. The offspring were then cross-fostered. The male pups were sacrificed and each nursing mother raised a litter consisting some of its biological offspring (2-3 pups) and some offspring (6-7 pups) of mothers exposed to a similar pregnancy treatment than the surrogate mother. Beginning on postnatal day 12, the litters were examined daily for eyelid opening. Body weights were recorded on days 25, 35 and 46.

Serum E2 levels. Female offspring (25- or 35-days-old), were anesthetized using methoxyflurane inhalant for the collection of blood by cardiac puncture. The mice were sacrificed immediately afterwards by cervical dislocation. In sexually mature animals (35-days-old), blood was collected when the animals were in estrus. Each group contained a total of 6-9 mice. Blood was placed in tubes, centrifuged, and stored at -70°C until total serum E2 concentrations were determined from the samples by using a specific double antibody kit from IN Biomedical, Inc. (Irvine, CA) according to the manufacturer's instructions.

Puberty onset (vaginal opening). We investigated whether *in utero* phytoestrogen manipulations influence the age when the female offspring become sexually mature. Sexual maturation in rodents can be determined by establishing the age when vaginal opening occurs, the first estrus occurring within a few days of this event (43). The animals were examined daily starting on postnatal day 25.

Estrus cycling. Two-month-old female mice were checked for reproductive cyclicity by examining vaginal smears taken between 8.00-10.00 a.m. each day for 2 weeks.

Mammary gland morphology. We have previously validated a visual scale to study the development of a mouse and rat mammary epithelial tree (3,4). Using this scale, we determined proliferation and differentiation of mammary epithelial structures in the whole mounts obtained from female offspring whose mothers were exposed to phytoestrogens during pregnancy. Whole mounts of the 4th abdominal gland of five 25-, 35- and 46-day-old female mice per treatment group were taken. These ages were chosen because they represent the times when critical events occur in the mammary gland. By day 25, rapid mammary epithelial proliferation begins and is characterized by a high number of TEBs. By day 35, TEBs begin to differentiate to TDs, and by age 46, the parenchymal structures have filled most of the fat pad and most TEBs have differentiated to TDs and ABs. The same animals were used for measuring serum E2 levels. The harvested glands were stained with carmine aluminum following a procedure developed by Dr Banerjee, referenced by Haslam (44).

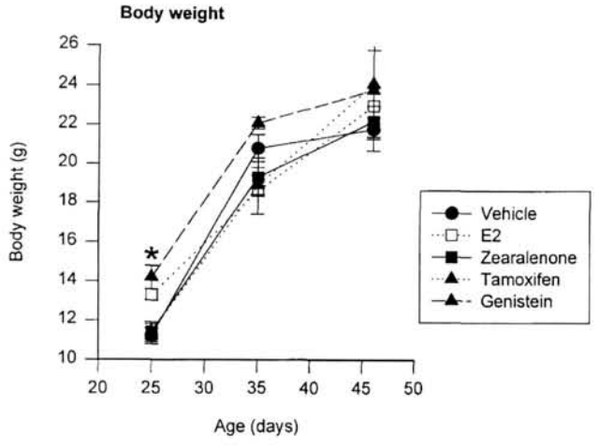

Figure 1. *In utero* treatment with E2 or genistein increases body weight on day 25, but not on days 35 or 46. Body weight gain in mice exposed to 20 ng E2, 2 µg zearalenone, 2 µg TAM or 20 µg genistein *in utero* (n=14-32 female mice per group). Significantly different from the controls: *p<0.05.

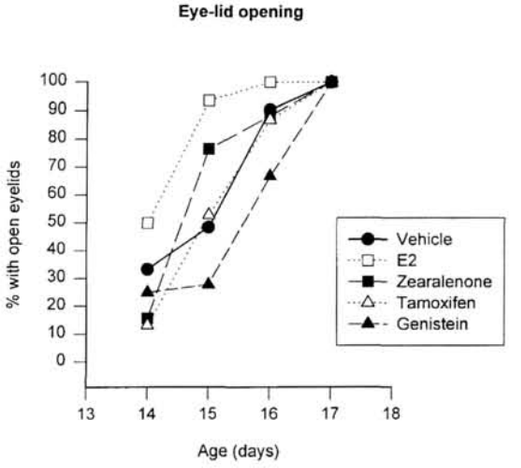

Figure 2. *In utero* treatment with E2 accelerates, while genistein delays, physical maturation (p<0.0001). Proportion of mice exposed to 20 ng E2, 2 µg zearalenone, 2 µg TAM or 20 µg genistein *in utero* with eyelid opening between postnatal days. Each group contained 15-30 mice.

The stained mammary whole mounts were examined under an Olympus dissecting microscope. The total mammary gland and epithelial cell area were measured using a caliper. In addition, the following characteristics of the mammary glands were evaluated double-blindly using a 4-point scale (0, absent; 1, low; 2, medium; 3, high): i) density of epithelial ducts; ii) TEBs; and iii) TDs and ABs.

Statistical analysis. Statistical tests were performed using the SOLO statistical system (BMDP Statistical Software, Los Angeles, CA, USA). Body weights and other general effects in the mother and offspring were analysed using one-way analysis of variance (ANOVA), followed by Fisher's least significance test to detect statistical differences between the vehicle-control and treatment groups. Data obtained on physical maturation and vaginal opening were analysed using Gehan-Wilcoxan test (proportion of animals with eyelid and vaginal openings on each day). Estrus cycling data were analysed using χ^2-test. Results for the size of mammary fat pad and parenchyma area, relative density of different parenchyma structures and number of TEBs, were determined using two-way ANOVA, using time and treatment as independent variables. All probabilities are two-tailed.

Results

General effects on pregnancy and offspring. No significant effects on pregnancy, the number of offspring born, or body weights on postnatal day 1 were noted among the female mice exposed to E2, genistein, zearalenone, or tamoxifen during pregnancy (data not shown). Body weights were significantly elevated on 25-day-old offspring of mothers exposed to E2 or genistein *in utero* [F(4,113)=8.4, p<0.0001] (Fig. 1). No significant alterations in body weights were noted after that age. Zearalenone or tamoxifen had no effect at the times studied.

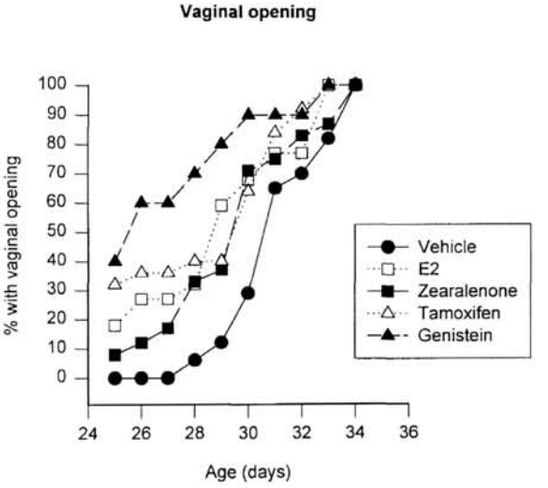

Figure 3. *In utero* treatment with genistein, TAM, and E2 induce precocious puberty (p<0.0001). Proportion of mice exposed to 20 ng E2, 2 µg zearalenone, 2 µg TAM or 20 µg genistein *in utero* with vaginal opening between days 25 and 34. Each group contained 10-25 mice.

Effects on physical maturation. Eyelid opening occurred significantly earlier in the E2-treated offspring (z-value = 3.44, p<0.0006) and later in the genistein-treated offspring (z-value = 2.11, p<0.035), when compared with respective controls. No effect was seen among the offspring exposed to zearalenone or TAM *in utero* (Fig. 2).

Effects on vaginal opening (puberty onset). Female mice exposed to either E2 (z-value = 2.42, p<0.016), genistein (z-value = 3.37, p<0.0007), or TAM (z-value = 2.97, p<0.003) *in utero* exhibited a significantly earlier vaginal opening than the controls (Fig. 3). Early zearalenone treatment did not affect the timing of vaginal opening.

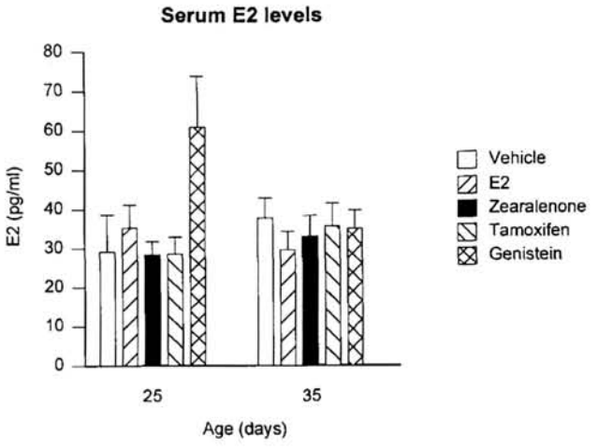

Figure 4. *In utero* treatment with phytoestrogens does not alter serum E2 levels. The mean E2 levels (± SEM) in 25- or 35-day-old female mice exposed to 20 ng E2, 2 μg zearalenone, 2 μg TAM or 20 μg genistein *in utero*. Each group contained 4-5 mice.

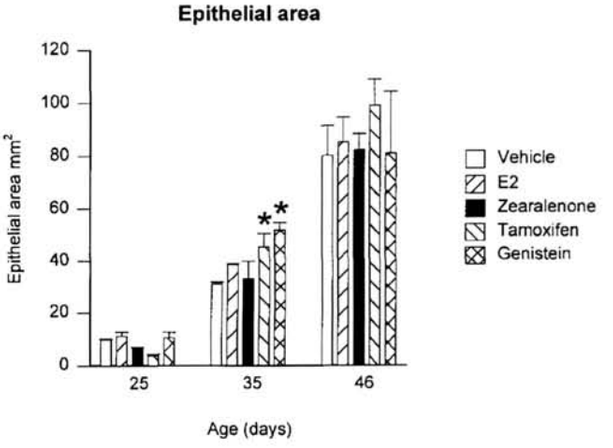

Figure 5. *In utero* treatment with genistein increases the epithelial cell area on day 35, but not on day 25 or 46. The total epithelial cell area (mm^2) in mice exposed to 20 ng E2, 2 μg zearalenone, 2 μg TAM or 20 μg genistein *in utero* (n=4-5 female mice per group). Significantly different from the controls: *p<0.05.

Table I. Estrus cycling in 2-month-old female mice exposed to 20 ng E2, 2 μg zearalenone (ZER), 2 μg tamoxifen (TAM), or 20 μg genistein *in utero*.[a]

% Animals exhibiting	Control	E2	ZER[b]	TAM	Genistein
4-5 day cycle	100	67	7	66	34
Persistent estrus	0	0	66	17	33
Not cycling	0	23	17	17	33

[a]Six females per group; [b]Significantly different from the controls.

Effects on serum E2 levels. Serum E2 levels were not significantly different in 25- or 35-day-old female mice exposed to genistein, zearalenone, E2, TAM, or vehicle *in utero* (Fig. 4).

Effects on estrus cycling. When compared with the controls, the females in the zearalenone group significantly more often exhibit persistent cornification of the vaginal smears (χ^2=8.6, df=2, p<0.05) (Table I). No other differences were noted.

Effects on mammary gland morphology. Mammary gland wholemounts, obtained from 25-, 35- and 46-day-old female mice exposed to *in utero* phytoestrogen, E2 or tamoxifen manipulations, were analysed for the size of mammary fat pad and epithelial area, and the density of mammary parenchymal pattern, TEBs, and TDs and ABs, and pattern of epithelial ductal crossing. The results indicate that the groups differed from each other in terms of the density of all the parenchymal measures, but not of fat pad areas (Figs. 5-7).

Fat pad and epithelial cell areas. There were no differences among the groups in the size of the total mammary fat pad area (data not shown). The epithelial cell area was significantly larger in the offspring of mothers exposed to genistein or tamoxifen *in utero* than in the vehicle-treated controls by day 35 [F(4,19)=3.49, p<0.027] (Fig. 6). The areas were similar in the 25 and 46-day-old offspring of different treatment groups.

Epithelial density. The epithelial density was significantly higher in the 46-day-old female mice exposed to 2 μg zearalenone *in utero* than in the vehicle controls [F(4,52)=4.66, p<0.0027] (Fig. 7). No differences in epithelial density were seen among the genistein-, E2- or vehicle-exposed offspring.

Terminal end buds. The density of TEBs, the structures that are the targets of neoplastic transformation in the carcinogen-treated rodents (34,35), was influenced by the maternal phyto-estrogen exposure. The density of TEBs was significantly increased in the mammary glands of female offspring of mothers treated with genistein during pregnancy, when compared with the vehicle treated offspring, on days 35 and 46 (p<0.05) [F(4,52)=4.28, p<0.0045] (Fig. 7). Density of the TEBs was higher than in the vehicle-controls by days 35 and 46 in the tamoxifen-exposed offspring, by day 35 in the zearalenone-exposed offspring, and by day 46 the E2-exposed offspring.

Differentiation. Differentiation of the mammary parenchymal TEBs to TDs and ABs will reduce the likelihood that malignant growth will occur in the mammary gland (34). In the present study, there was a significant interaction between age and treatment on the density of TDs and ABs [F(6,37)=5.73, p<0.0011] (Fig. 7). Post-hoc analysis revealed that at the age of 35 days, the density of TDs and ABs was lower in the female rats exposed to genistein *in utero* than in the vehicle-controls, while at the age of 46 days, their density was similar

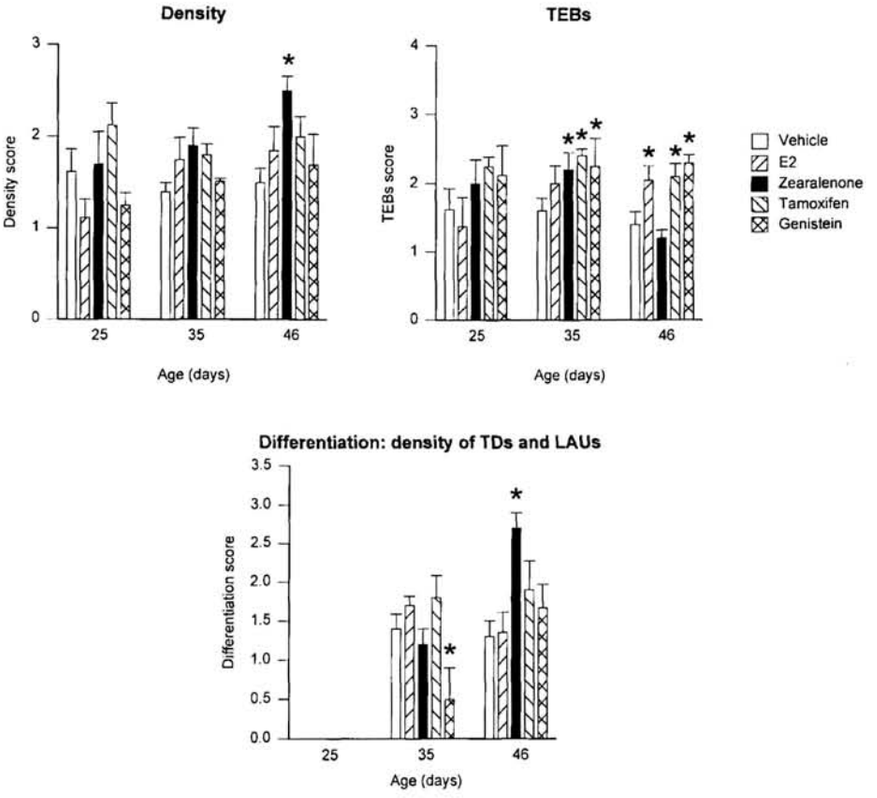

Figure 6. *In utero* treatment with phytoestrogens alters mammary parenchymal patterns. The density of mammary epithelial tree, TEBs, and TDs and LAUs, and the pattern of epithelial ductal growth in mice exposed to 20 ng E2, 2 μg zearalenone, 2 μg TAM or 20 μg genistein *in utero* (n=4-5 female mice per group). Significantly different from the controls: *p<0.05.

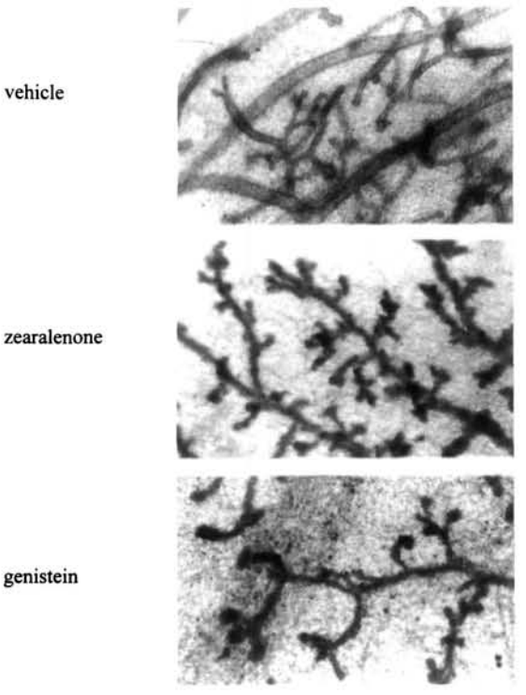

Figure 7. *In utero* treatment with phytoestrogens alters mammary parenchymal patterns. Representative wholemounts of 46-day-old female mice exposed to vehicle, 2 μg zearalenone or 20 μg genistein *in utero*. Photographs of the wholemounts were taken through a dissecting microscope under 6.3x magnification. By day 46, the zearalenone glands contained more differentiated TDs and LAUs, and the genistein glands more TEBs than the vehicle-control glands.

in both groups. Among the zearalenone-exposed offspring, by day 35 the density of TDs and ABs was the same, or marginally lower than in the vehicle-group, while by day 46 the density of these structures in the zearalenone mice was significantly increased. The offspring of tamoxifen- or E2-treated mothers did not differ from the vehicle-treated offspring in terms of the density of ABs.

Discussion

Maternal exposure to genistein during pregnancy, at a dose comparable to that consumed by Oriental women (40), has profound effects on mammary gland of female mouse offspring. The mammary epithelial structures in the offspring exposed to 20 μg genistein daily during days 15 and 20 of gestation (last third of pregnancy) through their pregnant mother, closely resemble those of female mice exposed to 2 μg E2 during postnatal days 1, 2 and 3 (4). These treatment groups exhibit more TEBs, and fewer differentiated epithelial structures, when compared with appropriate controls. Thus, *in utero* exposure to either genistein or E2 increases the number of epithelial targets for malignant transformation. In the present study, *in utero* exposure to 20 ng of E2 has a less dramatic effect on mammary parenchymal patterns than postnatal exposure to 2 μg E2 in our earlier study (4). These differences may reflect higher level of E2 exposure during the postnatal period than *in utero*. However, consistently in both studies, the density of TEBs is elevated, when compared with the vehicle group. Our data, thus, suggest that genistein

acts as an estrogen in the fetal mammary gland. This is in line with findings from studies of either animals (16) or humans (20) exposed to genistein as adults. Dietary genistein at 750 μg/g feed stimulates ductal and lobulo-alveolar development in the mammary gland of ovariectomized mice in a manner similar to that of 1 μg/g feed estradiol (16). In humans, consumption of soy protein causes changes generally linked to high estrogens, i.e., it increases the volume of nipple aspirate fluid and appearance of hyperplastic epithelial cells (20).

Emerging epidemiological evidence suggests that high maternal estrogenic environment during pregnancy increases breast cancer risk among daughters. Women who at birth were heavy, i.e., had high birth weight, have a higher incidence of breast cancer than women who had a low birth weight (9,10). High birth weight reflects high maternal estrogenic activity (8), possibly caused by a high pregnancy weight gain and/or a high-fat diet (3,45,46). Daughters of women who had low pregnancy estrogen levels due to pre-eclampsia, are at a significantly reduced risk to develop breast cancer (11,12). Animal studies strongly support the human data and indicate that *in utero* or early postnatal exposure (postnatal days 1-3) to estrogenic stimuli, including DES, E2, or a high-fat exposure, increases the incidence of spontaneous or carcinogen-induced mammary tumors (1-3). If genistein acts as an estrogen *in utero*, it also may increase breast cancer risk. Our data support this assumption and show that a maternal exposure to 20-300 μg genistein during pregnancy causes a dose-dependent increase in mammary tumorigenesis among the offspring (Hilakivi-Clarke L, *et al*, Am Assoc Cancer Res 38: abs. 111, 1997).

Studies comparing the low incidence of breast cancer in the East with the higher incidence in Western countries have been used to argue for the possible link between soy intake and breast cancer risk (47). The evidence in animals generally support this assumption (48,49). However, the major weakness of the animal studies is the use of pharmacological instead of physiological doses of genistein. These doses are likely to have multiple other mechanisms of action than binding to estrogen receptor; e.g., high genistein doses inhibit tyrosine kinases (50), DNA topoisomerases II (51), and angiogenesis (52). In addition, case-control studies investigating soy intake and breast cancer risk have produced contrasting results (53-59). Therefore, the role of genistein in preventing breast cancer remains to be established.

The partial ER agonist tamoxifen, when administered during fetal life through a pregnant mother, only affects the number of TEBs in the mammary gland. This effect is similar to that of genistein and E2. The ER activating properties of fetal tamoxifen exposure on the mammary gland are in accordance with the data in human and mouse reproductive system. Tamoxifen acts as an ER activator in the human and mouse fetal vagina and uterus, and in the uterus of post-menopausal women and mice (60,61; Sinchak K, *et al*, Society for Neuroscience Abstracts 16, 1990). Our findings indicate that tamoxifen is an ER activator also in the fetal mouse mammary gland.

In several models, zearalenone acts like an estrogen agonist, increasing the growth of uterine cells (28,42,62) and inducing expression of estrogen-regulated genes (63). The estrogenicity of zearalenone is currently being used to promote growth among cattle in the USA (26,27). *In utero* exposure to zearalenone in the present study has some effects on the developing mammary gland that mimic those of E2, and some effects that appear opposite to E2. Similar to E2, zearalenone increases the number of TEBs. In contrast to E2, zearalenone increases epithelial differentiation. This latter effect could be caused by altered reproductive functions in the zearalenone offspring. Previous studies have reported that an administration of zearalenone during postnatal days 1-10, at the doses of 3-30 μg per animal, delays vaginal opening, and induces persistent estrus and sterility (64). The early zearalenone treatment also causes ovarian dysfunction and preneoplastic and/or neoplastic changes in the cervico-vaginal epithelium (65). We found that *in utero* zearalenone exposure only induces persistent estrus, but does not affect puberty onset. Serum E2 levels during estrus also are normal. However, since the zearalenone offspring exhibit persistent cornification of vaginal smears, their serum estrogen levels probably do not exhibit estrus cycle-dependent changes, and this may have an impact on mammary gland development.

Besides zearalenone, other estrogenic compounds often alter reproductive functions (38,39). In the present study, maternal exposure to E2 during pregnancy increases body weight, and advances the day when eyelid opening and vaginal opening occurs in the offspring. However, no changes in estrus cycling or serum E2 levels are noted, which is in agreement with our previous study in rats (3). Thus, E2 acts as a growth promoter, possibly through effects on estrogen-regulated growth factors (EGF and transforming growth factor α), which accelerate physical maturation (66). Similarly to E2, *in utero* exposure to genistein increases early body weight, and causes advanced puberty onset. It does not influence estrus cycling or serum estrogens. Several other investigators have found that early phytoestrogen exposure, including genistein, affects the timing of puberty (38,67,68). Tamoxifen, when given through a pregnant mother, also induces early puberty onset. Thus, an exposure to some extraplacental estrogens *in utero* appears to advance the timing of puberty, and possibly affect prepubertal weight gain.

The exact mechanisms by which genistein and zearalenone affect mammary gland development remains to be defined. These phytoestrogens activate estrogen receptors, which appear in the mouse mammary gland on day 14 of gestation (69). However, genistein interacts with both the 'classical' ERα and the recently described ERß form. The relative binding affinities (relative to E2=100) for binding genistein have been reported as ERα=5, ERß=36 (14). Thus, genistein is likely to be more biologically active (perhaps up to 5-fold) through its interactions with ERß. Zearalenone, on the other hand, is a more potent estrogen than genistein in all assays that have used MCF-7 cells (preferentially express ERα) (70). However, zearalenone does not exhibit a differential selectivity for ERß and has a lower affinity for ERß than genistein, since the relative binding affinities for its major metabolite zearalenol, are reported as ERα=16, ERß=14 (14). The biological responses of genistein and zearalenone are likely to depend upon the relative levels of expression of each ER form, any differences in transcriptional regulation between

these forms, and the concurrent presence and concentration of other estrogenic compounds.

Genistein also may influence ER function through alterations in circulating estrogens. However, the reported effects are not consistent: both an increase in the level of serum estradiol during follicular phase (17) or throughout the cycle (20), or a reduction (71) by a high soy intake in premenopausal women have been found. Soy does not appear to affect serum estrogens in postmenopausal women (19). In the present study, serum E2 levels were not altered in the offspring.

In summary, maternal exposure to genistein at the level comparable to that seen in Oriental women, alters the development of a mouse mammary gland. These alterations are similar to those in the rodents exposed to estrogens perinatally (4), and who exhibit an increased incidence of mammary tumors (1-3). Since Oriental women have a significantly lower risk to develop breast cancer than Caucasian women who consume low levels of genistein (47), it is possible that either the life-time genistein exposure, or exposure before the onset of puberty, opposes the fetal effects of genistein. It is to be noted that daughters of Oriental women born in the USA, who presumably have been exposed to high levels of genistein *in utero* and during early life, but may have acquired more westernized dietary habits during adult life (55), have the same or higher breast cancer risk as American women (72). Our study also indicates that maternal exposure to tamoxifen is estrogenic to the fetus. *In utero* exposure to zearalenone increases mammary gland differentiation, and may therefore protect against mammary tumorigenesis. However, fetal zearalenone exposure alters development of the reproductive system, as also reported by other investigators (64). More research is required to determine whether a maternal intake of phytoestrogens is safe during pregnancy.

Acknowledgments

This work was supported by grants from the American Cancer Society (CN-80420) and the Lombardi Cancer Center Animal Shared Resource Facility, U.S. Public Health Service Grant 2P30-CA51008.

References

1. Walker BE: Tumors in female offspring of control and diethylstilbestrol-exposed mice fed high-fat diets. J Natl Cancer Inst 82: 50-54, 1990.
2. Lopez J, Ogren L, Verjan R and Talamantes F: Effects of perinatal exposure to a synthetic estrogen and progestin on mammary tumorigenesis in mice. Teratology 38: 129-134, 1988.
3. Hilakivi-Clarke L, Clarke R, Onojafe I, Raygada M, Cho E and Lippman ME: A maternal diet high in n-6 polyunsaturated fats alters mammary gland development, puberty onset, and breast cancer risk among female rat offspring. Proc Natl Acad Sci USA 94: 9372-9377, 1997.
4. Hilakivi-Clarke L, Cho E, Raygada M and Kenney N: Alterations in mammary gland development following neonatal exposure to estradiol, transforming growth factor alpha, and estrogen receptor antagonist ICI 182,780. J Cell Physiol 170: 279-289, 1997.
5. Jones LA and Bern HA: Cervicovaginal and mammary gland abnormalities in BALB/cCrgl mice treated neonatally with progesterone and estrogen, alone or in combination. Cancer Res 39: 2560-2567, 1979.
6. Hilakivi-Clarke LA, Raygada M, Stoica A and Martin M-B: Consumption of a high-fat diet during pregnancy alters estrogen receptor content, protein kinase C activity and morphology of mammary gland in the mother and her female offspring. Cancer Res (In press).
7. Nelson KG, Sakai Y, Eitzman B, Steed T and McLachlan J: Exposure to diethylstilbestrol during a critical developmental period of the mouse reproductive tract leads to persistent induction of two estrogen-regulated genes. Cell Growth Differ 5: 595-606, 1994.
8. Gerhard I, Vollmar B, Runnebaum B, Klinga K, Haller U and Kubli F: Weight percentile at birth: II prediction by endocrinological and sonographic measurements. Eur J Obstet Gyn Reprod Biol 26: 313-328, 1987.
9. Michels KB, Trichopoulos D, Robins JM, Rosner BA, Manson JE, Hunter D, Colditz GA, Hankinson SE, Speizer FE and Willett WC: Birthweight as a risk factor for breast cancer. Lancet 348: 1542-1546, 1996.
10. Sanderson M, Williams M, Malone KE, Stanford JL, Emanuel I, White E and Daling JR: Perinatal factors and risk of breast cancer. Epidemiology 7: 34-37, 1996.
11. Ekbom A, Trichopoulos D, Adami HO, Hsieh CC and Lan SJ: Evidence of prenatal influences on breast cancer risk. Lancet 340: 1015-1018, 1992.
12. Ekbom A, Hsieh CC, Lipworth L, Adami HO and Trichopoulos D: Intrauterine environment and breast cancer risk in women: a population-based study. J Natl Cancer Inst 89: 71-76, 1997.
13. Adlercreutz H, Mousavi Y, Clark J, Hockerstedt K, Hamalainen E, Wahala K, Makela T and Hase T: Dietary phytoestrogens and cancer: *in vitro* and *in vivo* studies. J Steroid Biochem Mol Biol 41: 331-337, 1992.
14. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S and Gustaffson JA: Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138: 863-870, 1997.
15. Wang TT, Sathyamoorthy N and Phang JM: Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis 17: 271-275, 1996.
16. Santell RC, Chang YC, Nair MG and Helferich WG: Dietary genistein exerts estrogeneic effects upon the uterus, mammary gland and the hypothalamic/pityuitary axis in rats. J Nutr 127: 263-269, 1997.
17. Cassidy A, Bingham S and Setchell KDR: Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. Am J Clin Nutr 60: 333-340, 1994.
18. Wilcox G, Wahlqvist ML, Burger HG and Medley G: Oestrogenic effects of plant foods in postmenopausal women. BMd J 301: 905-906, 1990.
19. Baird DD, Umbach DM, Lansdell L, Hughes CL, Setchell KDR, Weinberg CR, Haney AF, Wilcox AJ and McLachlan JA: Dietary intervention study to assess estrogenity of dietary soy among postmenopausal women. J Clin Endocrinol Metab 80: 1685-1690, 1995.
20. Petrakis NL, Barnes S, King EB, Lowenstein J, Wiencke J, Lee MM, Miike R, Kirk M and Coward L: Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women. Cancer Epidem Biomarker Prev 5: 785-794, 1996.
21. Setchell KDR, Zimmer-Nechemias L, Call J and Heubi JE: Exposure of infants to phytoestrogens from soy-based infant formula. Lancet 350: 23-27, 1997.
22. Hagler WM, Tyczkowska K and Hamilton PB: Simultaneous occurrence of deoxynivalenol, zearalenone, and aflatoxin in 1982 scabby wheat from the Midwestern United States. Appl Environ Microbiol 47: 151-154, 1984.
23. Luo Y, Yoshizawa T and Katayama T: Comparative study on the natural occurrence of fusarium mycotoxins (trichothecenes and zearalenone) in corn and wheat from high- and low-risk areas for human esophageal cancer in China. Appl Environ Microbiol 56: 3723-3726, 1990.
24. Hidy PH and Baldwin RS: Method of preventing pregnancy with lactone derivates. US Pat June 22: 3,966,274, 1976.
25. Utian WH: Comparative trial of P-1496, a new non-steroidal oestrogen analogue. BMJ 1: 579-581, 1973.
26. Ralston AT: Effect of zearalanol on weaning weight of male calves. J Anim Sci 47: 1203-1206, 1978.
27. Wiggins JP, Rothenbacher H, Wilson LL, Martin RJ, Wangness PJ and Ziegler JH: Growth and endocrine responses of lambs to zearanol implants: effects of preimplant growth rate and breed of sire. J Anim Sci 49: 291-297, 1979.

28. Martin PM, Horwitz KB, Ryan DS and McGuire W: Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology 103: 1860-1867, 1978.
29. Schoental R: Role of podophyllotoxin in the bedding and dietary zearalenone on incidence of 'spontaneous' tumors in laboratory animals. Cancer Res 34: 2419, 1974.
30. Schoental R: Trichothecenes, zearalenone, and other carcinogenic metabolites of fusarium and related microfungi. Adv Cancer Res 45: 217-290, 1985.
31. Anbazhagan R, Bartek J, Monaghan P and Gusterson BA: Growth and development of the human infant breast. Am J Anat 192: 407-417, 1991.
32. Imagawa W, Bandyopadhyay GK and Nandi S: Regulation of mammary epithelial cell growth in mice and rats. Endocrine Rev 11: 494-523, 1990.
33. Daniel CW and Silberstein GB (eds): Postnatal development of the rodent mammary gland. In: The Mammary Gland: Development, Regulation, and Function. Plenum Press, New York, 1987.
34. Russo J and Russo IH: Biological and molecular bases of mammary carcinogenesis. Lab Invest 57: 112-137, 1987.
35. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR and van Zwieten MJ: Comparative study of human and rat mammary tumorigenesis. Lab Invest 62: 244-278, 1990.
36. Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH and Merlino GT: TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61: 1137-1146, 1990.
37. Krane IM and Leder P: NDF/heregulin induces persistence of terminal end buds and adenocarcinomas in the mammay glands of transgenic mice. Oncogene 12: 1781-1788, 1996.
38. Burroughs CD, Mills KT and Bern HA: Reproductive abnomalities in female mice exposed neonatally to various doses of coumestrol. J Toxicol Environ Health 30: 105-122, 1990.
39. Talamantes F and Browing HC: Effect of neonatal administration of norethynodrelmestranol on the reproductive system of female mice. Tex Rep Biol Med 30: 361-369, 1972.
40. Messina M, Persky V, Setchell KDR and Barnes S: Soy intake and breast cancer: a review of the *in vitro* and *in vivo* data. Nutr Cancer 21: 113-131, 1994.
41. Kuiper-Goodman T: Uncertainties in the risk assessment of three mycotoxins: aflatoxin, ochratoxin, and zearalenone. Can J Physiol Pharmacol 68: 1017-1024, 1990.
42. Mayr U, Butsch A and Schneider S: Validation of two *in vitro* test systems for estrogenic activities with zearalenone, phytoestrogens and cereal extracts. Toxicology 74: 135-149, 1992.
43. Eckstein B, Golan R and Shani J: Onset of puberty in the immature female rat induced by 5alpha-androstane-3beta, 17beta-diol. Endocrinology 92: 941-945, 1973.
44. Haslam SZ: Progesterone effects on deoxyribonucleic acid synthesis in normal mouse mammary glands. Endocrinology 122: 464-470, 1988.
45. Villar J, Cogswell M, Kestler E, Castillo P, Menendez R and Repke JT: Effect of fat and fat-free mass deposition during pregnancy on birth weight. Am J Obstet Gynecol 167: 1344-1352, 1992.
46. Susser M: Maternal weight gain, infant birth weigh, and diet: causal consequences. Am J Clin Nutr 53: 1384, 1990.
47. Adlercreutz CH, Goldin BR, Gorbach SL, Hockerstedt KAV, Watanabe S, Hamalainen E, Markkanen MH, Makela TH, Wahala KT, Hase TA and Fotsis T: Soybean phytoestrogen intake and cancer risk. J Nutr 125: 757S-770S, 1996.
48. Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S and Lamartiniere CA: Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. Carcinogenesis 17: 1451-1457, 1996.
49. Barnes S: Effect of genistein on *in vitro* and *in vivo* models of cancer. J Nutr 125: 777S-783S, 1995.
50. Akiyama T, Ishida J, Nakagawa S, Ogawa H, Watanabe S, Itou N, Shibata M and Fukami Y: Genistein, a specific inhibitor of tyrosine-specific protein kinease. J Biol Chem 262: 5592-5595, 1987.
51. Okura A, Arakawa H, Oka H, Yoshinari T and Monden Y: Effect of genistein on topoisomerase activity and on the growth of (val 12) Ha-*ras*-transformed NIH 3T3 cells. Biochem Biophys Res Commun 157: 183-189, 1988.
52. Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R and Schweigerer L: Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis. Proc Natl Acad Sci USA 90: 2690-2694, 1993.
53. Lee HP, Gourley L, Duffy SW, Esteve J, Lee J and Day NE: Dietary effects on breast cancer risk in Singapore. Lancet 337: 1197-1200, 1991.
54. Nomura A, Henderson BE and Lee J: Breast cancer and diet among the Japanese in Hawaii. Am J Clin Nutr 31: 2020-2025, 1978.
55. Wu AH, Ziegler RG, Horn-Ross PL, Nomura AMY, West DW, Kolonel LN, Rosenthal JF, Hoover RN and Pike MC: Tofu and risk of breast cancer in Asian-Americans. Cancer Epidem Biomarker Prev 5: 901-906, 1996.
56. Hirohata T, Shigematsu T, Nomura AMY, Nomura Y, Horie A and Hirohata I: Occurrence of breast cancer in relation to diet and reproductive history: a case-control study in Fukuoka, Japan. Natl Cancer Inst Monogr 69: 187-190, 1985.
57. Witte JS, Ursin G, Siemiatycki J, Thompson WD, Paganini-Hill A and Haile RW: Diet and premenopausal bilateral breast cancer: a case-control study. Breast Cancer Res Treat 42: 243-251, 1997.
58. Yuan JM, Wang QS, Ross RK, Henderson BE and Yu MC: Diet and breast cancer in Shanghai and Tianjin, China. Br J Cancer 71: 1353-1358, 1995.
59. Ingram D, Sanders K, Kolybaba M and Lopez D: Case-control study of phytoestrogens and breast cancer. Lancet 350: 990-994, 1997.
60. Wakshlak A and Weinstock M: Neonatal handling reverses behavioral abnormalities induced by prenatal stress. Physiol Behav 48: 289-292, 1990.
61. Sato T, Ohta Y, Okamura H, Hayashi S and Iguchi T: Estrogen receptor (ER) and its messenger ribonucleic acid expression in the genital tract of female mice exposed neonatally to tamoxifen and diethylstilbestrol. Anat Rec 244: 374-385, 1966.
62. Kiang DT, Kennedy BJ, Pathre SV and Mirocha CJ: Binding characteristics of zearalenone analogs to estrogen receptors. Cancer Res 38: 3611-3615, 1978.
63. Mayr UE: Estrogen-controlled gene expression in tissue culture cells by zearalenone. FEBS Lett 239: 223-226, 1988.
64. Ito Y and Ohtsubo K: Effects of neonatal administration of zearalenone on the reproductive physiology of female mice. J Vet Med Sci 56: 1155-1159, 1994.
65. Williams BA, Mills KT, Burroughs CD and Bern HA: Reproductive alterations in female C57BL/Crgl mice exposed neonatally to zearalenone, an estrogenic mycotoxin. Cancer Lett 46: 225-230, 1989.
66. Smith JM, Sporn MB, Roberts AB, Derynck R, Winkler ME and Gregory H: Human transforming growth factor α causes precocious eyelid opening in newborn mice. Nature 315: 515-516, 1985.
67. Levy JR, Faber KA, Ayyash L and Hughers CLJ: The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. Proc Soc Exp Biol Med 208: 60-66, 1995.
68. Burroughs CD, Bern HA and Stokstad ELR: Prolonged vaginal cornification and other changes in mice treated neonatally with coumestrol, a plant estrogen. J Toxicol Environ Health 15: 51-61, 1985.
69. Holderegger C and Keefer D: The ontogeny of the mouse estrogen receptor: the pelvic region. Am J Anat 177: 285-297, 1986.
70. Dotzlaw H, Leygue E, Watson PH and Murphy LC: Expression of estrogen receptor-beta in human breast tumors. J Clin Endocrinol Metab 82: 2371-2374, 1996.
71. Lu L-JW, Anderson KE, Grady JJ and Nagamani M: Effects of soya consumption for one month on steroid hormones in premenopausal women: implications for breast cancer risk reduction. Cancer Epidem Biomarker Prev 5: 63-70, 1996.
72. Ziegler RG, Hoover RN, Pike MC, Hildesheim A, Nomura AMY, West DW, Wu-Williams AH, Kolonel LN, Horn-Ross PL, Rosenthal JF and Hyer MB: Migration patterns and breast cancer risk in Asian-American women. J Natl Cancer Inst 85: 1819-1827, 1993.