Mammary Gland Morphogenesis Is Enhanced by Exposure to Flaxseed or Its Major Lignan During Suckling in Rats

KAH POH TAN, JIANMIN CHEN, WENDY E. WARD, AND LILIAN U. THOMPSON[1]

Department of Nutritional Sciences, Faculty of Medicine, University of Toronto 150 College Street, Toronto, Ontario M5S 3E2, Canada

The exposure of rats to 10% flaxseed (FS) or an equivalent level of its major lignan, secoisolariciresinol diglucoside (SDG), during suckling enhances mammary gland differentiation, which protects against mammary carcinogenesis at adulthood. We determined whether this diet-induced mammary gland differentiation is mediated through the estrogenic pathway via epidermal growth factor receptor (EGFR) and estrogen receptor (ER) signaling. Rats were fed the AIN-93G basal diet (BD) from day 7 of pregnancy until delivery and then randomized to consume BD, FS, or SDG during lactation. After weaning, female offspring were fed BD throughout the experiment. At postnatal day (PND) 21 and the proestrus phase on PND 49–51, mammary glands of offspring were analyzed for morphology, cell proliferation, and expression of EGFR, epidermal growth factor (EGF), transforming growth factor-α, ER-α, and ER-β. At PND 21, compared with the BD control, the number of terminal end buds (TEBs) and terminal ducts were increased by FS, whereas mammary epithelial cell proliferation was increased by both FS and SDG, suggesting that mammary morphogenesis was enhanced. Epithelial EGFR and stromal fibroblast EGF were increased by SDG, whereas epithelial ER-β was decreased by FS. Conversely, at PND 49–51, a lower number of TEBs but a higher ratio of lobules to TEBs with decreased expression of EGFR or EGF was observed in both treatment groups. EGFR expression was positively associated with EGF expression and cell proliferation in TEB epithelium at PND 21. Urinary lignans of lactating dams were related to their offspring's indices of mammary gland development. In conclusion, exposure to FS or SDG during suckling enhanced mammary gland morphogenesis by modulation of EGFR and ER signaling, which led to more differentiated mammary glands at PND 49–51. The physiological outcomes of FS and SDG were similar, which suggests that SDG is partly responsible for the mammary gland differentiation effect. Exp Biol Med 229:147–157, 2004

Key words: flaxseed; lignan; suckling; mammary gland differentiation; breast cancer

The relationship between mammary gland development, as influenced by hormonal and dietary exposures early in life, and lifetime breast cancer risk has gained much recent attention (1–3). Neonatal, prepubertal, or peripubertal exposure of female rodents to estrogen and/or progesterone (4, 5) or phytoestrogen genistein (6–8) has been shown to enhance early mammary gland differentiation, which then reduces mammary carcinogenesis during adulthood. Early mammary morphogenesis in rodents begins with the branching of mammary ducts, which elongate and end with the formation of terminal end buds (TEBs). TEBs, the most undifferentiated terminal ductal structures with a multilayer of highly proliferative epithelial cells, are highly vulnerable for chemical carcinogenesis (9). The incidence of carcinomas in rodents is directly associated with the density of TEBs in the mammary glands at the time of carcinogen administration (9, 10). In response to stimulation by the mammogenic hormones, particularly estrogens, TEBs differentiate to the more mature structures, namely, alveolar buds and lobules (LOBs), which are less susceptible to carcinogens (10, 11). Hence, early enhancement of mammary gland differentiation by hormonal induction may be a potential preventive strategy for breast cancer, with a protection basis similar to that produced by an early full-term pregnancy in humans (1, 2).

Flaxseed (FS) is the richest known source of the phytoestrogen lignan precursors, in particular secoisolariciresinol diglucoside (SDG; Ref. 12). On fermentation by the bacterial flora in the colon of mammals, SDG is converted to the mammalian lignans enterodiol (ED) and enterolactone (EL; Ref. 13). These biphenolic compounds have chemical structures that closely resemble that of endogenous 17-β estradiol and possess biphasic agonistic (estrogenic) and antagonistic (antiestrogenic) activities *in vitro* (14, 15) and *in vivo* (16–18). Serum level and urinary excretion of mamma-

This study was supported by the American Institute for Cancer Research Part of this paper was presented at Experimental Biology 2002, New Orleans. [Tan KP, Chen J, Thompson LU. Early mammary morphogenesis, reproductive indices, and splenocyte blastogenesis in rats exposed to flaxseed and secoisolariciresinol diglycoside (SDG) during suckling period. FASEB J 16:A742, 2002].

[1] To whom requests for reprints should be addressed at Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, 150 College Street, Toronto, Ontario M5S 3E2, Canada. Phone: 416-978-3523; Fax: 416-978-5882; E-mail: lilian.thompson@utoronto.ca

Received August 7, 2003.
Accepted October 2, 2003.

1535-3702/04/2292-0000$15.00

lian lignans, indicators of dietary lignan intake, have been associated with a lower breast cancer risk in retrospective epidemiological studies (19, 20). The high intake of foods containing vegetable oil and dietary fiber, which are often associated with lignans, during adolescence has been related as well to reducd risk of breast cancer (21). In agreement, we have shown, in a series of carcinogen-induced mammary cancer experiments in rats and human breast cancer xenografts in athymic mice, that FS, mainly because of its SDG, can inhibit mammary tumor development (20).

The results of *in vivo* studies have suggested that the dose, duration, and timing (stage of development) at which the exposure occurs are among the factors that influence the hormonal activities of lignans (16–18). In previous studies, we observed that the exposure of rats to 10% FS or its equivalent level of SDG during early life, especially suckling, enhanced mammary gland differentiation (17, 22). The transfer of mammalian lignans from rat dams consuming radiolabeled SDG to their suckling offspring via milk has been demonstrated elsewhere (16). In a recent rat tumorigenesis study, we further showed that suckling exposure to 10% FS or its SDG resulted in a reduced 7,12-dimethylbenz[a]anthracene (DMBA)–induced incidence of mammary cancer at adulthood (23).

The mechanism through which FS or its major lignan facilitates mammary gland differentiation remains unclear. The isoflavone genistein has been reported to upregulate epidermal growth factor receptor (EGFR) and its ligand, transforming growth factor (TGF)–α in TEBs of rat mammary glands at postnatal day (PND) 21 (24). This enhanced EGFR-signaling cascade was shown to be modulated by an estrogen-receptor (ER)–based mechanism (25). Because lignans and genistein share similarities in chemical structure and biological activities (19, 20), both phytoestrogens may enhance mammary gland differentiation through similar mechanisms.

Using a rat model, we sought to elucidate the mechanism(s) by which early mammary gland morphogenesis is facilitated by exposure to FS and SDG during suckling. We hypothesized that FS and its lignan participated in an estrogenic pathway via ER and EGFR signaling to mediate mammary gland morphogenesis. The development of mammary glands involves a complex mammary epithelial-stromal communication that is coordinated through autocrine and paracrine regulations of a myriad of hormones and growth factors (26). To understand how a diet-induced mammary gland differentiation was orchestrated, we determined differential expressions of growth factors and receptors in various tissue compartments of the mammary gland.

Materials and Methods

Diets. All diets were prepared by Dyets Inc. (Bethlehem, PA). The basal diet (BD) was based on the phytoestrogen-free semipurified AIN-93G diet (27). The 10% FS diet was prepared by supplementing a modified AIN-93G formula with 10% (w/w) ground flaxseed (Linnott Variety; Omega Products, Melfort, Canada) after adjustment for total calories, macronutrients, and fiber contributed by the added flaxseed, as described elsewhere (18, 23). The SDG diet was prepared by adding to the BD the purified SDG at a level equivalent to that present in FS (200 mg SDG/kg BD), as determined by a high-performance liquid chromatography analysis (28). Fresh diet was stored at 4°C and provided to the rats every 2–3 days. Diet intake was monitored throughout the study.

Animals and Experimental Design. Eight-week-old, timed-pregnant Sprague-Dawley rats ($n = 36$) at day 7 of pregnancy (Charles River, Montreal, Canada) were caged individually at 22°–24°C with a daily 12-hr light-dark cycle. All rats received BD and water *ad libitum* throughout the rest of pregnancy. At parturition, lactating dams were randomized ($n = 12$/group) to consume one of the three experimental diets (BD, FS, or SDG) during lactation (21 days). All diets were given to the rat dams in tall jars to prevent access of the offspring to the diets. At PND 3, the pup number was reduced to eight, with five to six female and two to three male pups per litter. At PNDs 3 and 20, the anogenital distance (AGD) of suckling pups was measured. At the end of suckling (PND 21), the offspring were separated from their dams and fed BD throughout the remainder of the experiment. The rat dams, while still receiving their respective experimental diets, were placed in metabolic cages for a 3-day urine collection for urinary lignan analysis. From PND 21 to 49, female offspring were monitored for vaginal opening, an indicator of puberty onset, and the length of their estrous cycles was determined by daily vaginal smear examination using criteria described elsewhere (16, 18). At least one offspring per litter was sacrificed at the end of suckling (PND 21) and at the proestrus phase of the estrous cycle on PND 49–51. Their mammary glands were excised for later analysis, and their reproductive organs were weighed. To control for any circadian influences, rats were sacrificed at random between 0900 and 1230 hr. The remaining rats at proestrus phase (PND 49–51) were treated with the carcinogen DMBA and observed for tumor development. The results of the tumorigenesis study have been recently published (23). The experimental protocol of the study was approved by the University of Toronto Animal Care Committee. Animal care and procedures were in accordance with the *Guide to the Care and Use of Experimental Animals* (29).

Mammary Gland Histology and Morphometry. At necropsy, the right skin pelt of female offspring was removed, stretched and pinned on a corkboard, and then immediately fixed in 10% neutral phosphate-buffered formalin. After a 2–3-day fixation, the fourth right abdominal mammary gland explant was dissected from the skin pelt and dehydrated in ascending grades of ethanol. At PND 50, the most undifferentiated TEBs are mostly concentrated in the periphery of mammary gland, whereas the differentiated LOBs are present mostly in the central

region adjacent to the lymph nodes (11). To ensure that topological differences across the mammary gland were consistently assessed, a 15 × 20-mm section of mammary gland of rats at PND 21, which included the entire fourth right abdominal mammary gland and its neighboring lymph nodes, was excised. Rats at PND 49–51 had large mammary glands that could not be entirely included in the paraffin tissue preparation. To obtain the best representative sample of the mammary gland, a rectangular explant encompassing the fourth nipple and both the central (including two abdominal lymph nodes) and peripheral regions that measured 20 × 25 mm was consistently excised. The explants were firmly stretched and positioned horizontally during paraffin tissue preparations so that the whole cross-sectional portrait of mammary ductal tree could be obtained in subsequent tissue sectioning. Each tissue was sliced completely into sections of 5-μm thickness. Paraffin sections were secured on coated slides, with the slicing order of each section recorded. Sections were then stained with hematoxylin and eosin for mammary gland morphological assessment. All slides were coded and read blindly by a single observer under a light microscope at magnifications of ×100–200. Various terminal ductal structures (i.e., TEB, terminal duct [TD], and LOB) were identified and counted by means of simple point-counting morphometry based on established criteria (10).

Mammary Epithelial Cell Proliferation. To reduce interlitter variability, mammary gland samples (fourth right gland) at both PND 21 and PND 49–51 were taken among the littermates. The lot of serially cut paraffin sections of each sample that exhibited the maximum number of mammary ductal structures was examined. All sections were coded to ensure that the observer was unaware of the treatment group for each section. The cellular expression of proliferating cell nuclear antigen (PCNA) in the epithelium of terminal ductal structures was evaluated. In brief, after deparaffinization in toluene and rehydration in descending grades of ethanol, the endogenous peroxidase in the section was quenched in 3% H_2O_2 in methanol at room temperature for 10 mins. Antigens were retrieved by microwave heating the tissues in a pressure cooker filled with 10 m*M* citrate buffer (pH 6.0) at 92°–95°C for 20 mins. After cooling, sections were incubated with a blocking solution (2% normal goat serum) at room temperature for 45 mins, followed by incubation with 0.4 mg/L primary antibody solutions (mouse monoclonal antibody raised against a recombinant PCNA, Ab-1; Oncogene Research Products, MA) in a humidified chamber overnight (18 hrs) at 4°C. The subsequent immunoperoxidase method was done using a biotin-streptavidin system (DAKO, Carpinteria, CA). Sections were incubated with a biotinylated goat-origin secondary link followed by streptavidin–horseradish peroxidase conjugate, both for 1 hr, at room temperature. 3-amino 9-ethylcarbazole (AEC)-chromogen was used for antigen visualization, followed by brief counterstaining with hematoxylin. Sections were finally mounted with crystal-mount, dried at room temperature overnight, and coverslipped with Permount. At least two positive control tissues (normal rat colon) and two negative control tissues (mammary gland and positive control sections incubated with the antibody diluent precluding primary antibody) were included with the test tissues in each run of the staining. Stained sections were examined with a light microscope at magnifications of ×200–400. At least four randomly selected representatives of each terminal ductal structure (TEB, TD, and LOB) with >1200 epithelial cells were read. The cell cycle phases of PCNA-immunopositive cells were determined using the criteria of Foley *et al.* (30): (i) G_0, negative staining; (ii) G_1, light to moderate nuclear staining, patchy and uniform nucleus, no cytoplasmic staining; (iii) S, dark red nuclear staining, patchy and uniform nucleus, no cytoplasmic staining; and (iv) G_2/M, speckled nuclear staining, diffuse, cytoplasmic staining. Because the staining pattern for the G_1 and S phases was very similar and hardly distinguishable, cells undergoing these phases were combined and assessed as one measure (G_1/S), a method similarly adopted by others (31, 32). Results were expressed as %G_1/S and labeling index (LI), which are the percentage of total cells undergoing G_1/S and the percentage of total stained cells, respectively, to the total cells counted.

Immunohistochemistry for EGFR, Its Ligands, and ER Proteins. From the same lot of tissues used for the PCNA immunostaining, the cellular expression of EGFR and its two main ligands, TGF-α and EGF, and ER-α and -β was evaluated in the epithelium of terminal ductal structures and periductal stromal fibroblasts, as well as adipose tissues surrounding the mammary ducts. The primary antibodies and their optimal concentrations used are as follows: EGFR, rabbit polyclonal antibody raised against a peptide at the carboxy terminus of human EGFR, 1005 (Santa Cruz Biotech, Santa Cruz, CA), 0.5 mg/L; TGF-α, mouse monoclonal antibody raised against a recombinant TGF-α, Ab-2 (Oncogene Research Products), 0.4 mg/L; EGF, rabbit antiserum raised against rat EGF (Biomedical Technologies, Stoughton, MA), 0.4 mg/L; ER-α, mouse monoclonal antibody raised against calf ER-α clones, ERA-11S (Alpha Diagnostic, San Antonio, TX), 3 mg/L, and ER-β, rabbit polyclonal antibody raised against a peptide of amino acids from rat and human ER-β (Upstate Biotech, Lake Placid, NY), 5 mg/L. The immunostaining procedures were similar to that for PCNA, except for the duration and extent of microwave heating during antigen retrieval and choice of blocking solutions (2% goat serum/2% dry milk/1% bovine serum albumin [BSA] or 2% goat serum/1% BSA), which were optimized for the individual antibody. The positive control tissues, which were included along with the negative control and test tissues in each run of staining, were rat skin for EGFR, rat kidney for EGF and TGF-α, and sexually mature rat uterus for ER-α and -β. The image of representative terminal ductal structures was examined at magnifications of ×100–400 in a microscopic imaging system (Leica Microsystems, Wetzlar, Germany) using

OpenLab image processing software (Improvision, Coventry, England). Because the immunostaining patterns across different mammary tissue compartments varied considerably, two immunohistochemical scoring (IHS) systems (one for ductal epithelium and the other for stromal fibroblasts and adipocytes) were used. The assessment for the ductal epithelium, which takes into consideration both the staining intensity and percentage of immunopositive cells, was based on a modified method of Harvey *et al.* (33) as follows: a score of 4.0, >50% immunopositive cells, mostly intense stains; 3.5, >50% immunopositve cells, mostly strong stains or 31%–50% immunopositive cells, mostly intense stains; 3.0, 31%–50% immunopositive cells, mostly strong stains; 2.5, 31%–50% immunopositive cells, mostly moderate stains or 15%–30% immunopositive cells, mostly strong stains; 2.0, 15%–30% immunopositive cells, mostly moderate stains; 1.5, 15%–30% immunopositive cells, mostly weak stains or <15% immunopositive cells, mostly moderate stains; 1.0, <15% immunopositive cells, mostly weak stains; and 0, negative stains.

Repeat analysis by the same observer using the above method showed a reproducibility of 86.2% and produced results that correlated significantly ($r = 0.89$; $P < 0.02$) with that of the immunoscoring method previously used in our laboratory (34). For stromal fibroblasts and adipocytes surrounding the mammary ducts, the overall staining was scored according to staining intensity as follows: 4, intense; 3, strong; 2, moderate; 1, weak; and 0, no staining (35). Eight to ten representative structures/compartments of each mammary gland section were assessed.

Urinary Lignan Analysis. Urinary lignans were analyzed as we previously described (23). In brief, the lignans ED, EL, and secoisolariresinol were extracted from the urine using a reverse-phase octadecylsilane bonded silica (C18) column (Scientific Products and Equipment Ltd., Concord, Canada). The methanol eluent containing the lignans were evaporated, and the residue was treated with β-glucuronidase (Helix pomatia; Sigma Chemical Co., St. Loius, MO) overnight at 37°C. The unconjugated lignans were then extracted from the hydrolysate by passing through another C18 column and then further purified and isolated on a DEAE-Sepadex ion exchange column (Pharmacia Biotech, Baie d'urfe, Canada). The lignans were derivatized and analyzed using a gas chromatograph (GC; Hewlett Packard 5890 Series II, Mississauga, Canada) mass spectrometer (Mass Selective Detector, Hewlett Packard). The GC was equipped with cross-linked methyl silicone gum phase with He as the carrier gas. The temperature program began at 100°C and increased to 250°C at a rate of 30°C/min.

Statistical Analyses. Statistical analyses were done using SigmaStat (Jandel Scientific, San Rafael, CA) or SPSS (SPSS Inc., Chicago, IL) software. Data are presented as means ± SEM. Differences in continuous variables such as the number of terminal ductal structure, PCNA LI and $\%G_1/S$, and IHS data of receptors and growth factors among groups were determined by one-way ANOVA for parametric data or Kruskal-Wallis one-way ANOVA on ranks for nonparametric data. When significant differences ($P < 0.05$) among groups were observed, differences between groups were further analyzed by post hoc, pairwise comparison tests—Tukey's test for parametric data and Dunn's test for nonparametric data. The relationship of paired variables with nonparametric distributions, such as the dams' urinary lignan levels and the indices of mammary gland differentiation of their offspring, was determined by Spearman's correlation coefficient. Pearson's correlation coefficient was used to determine the linear relationships among paired variables with normal distributions such as PCNA LI and IHS of growth factors and receptors.

Results

Food Intake, Weight Gain, Urinary Lignans, and Reproductive Indices. Food intake and weight gain of the lactating dams and their offspring throughout the experiment did not differ among groups (data not shown). Lactating dams that received FS and SDG diets excreted significantly higher ($P < 0.05$) amounts of total mammalian lignans than those fed BD. However, dams fed FS had much higher total urinary lignans levels than those fed its equivalent level of SDG (Table 1). This finding was consistent with the results of our previous studies (16, 17, 22, 23), and the factors that contribute to the disparity are explained in a recent article (23). Exposure to the treatment diets did not significantly alter the hormone-sensitive reproductive indices of offspring such as relative weights of reproductive organs, onset of puberty, and estrous cycle length (Table 1). These findings are similar to those of our previous study (36).

Mammary Gland Morphology and Cell Proliferation. At PND 21, the number of TEBs and TDs was higher in the FS and SDG groups than in the BD group, although a significant difference was noted only in the FS group (Table 2). Conversely, in rats at PND 49–51, the FS and SDG groups had significantly fewer TEBs compared with the BD group ($P < 0.05$). The number of LOBs did not differ significantly among groups. However, the number of LOB per unit of TEB, an index of mammary gland maturity, was significantly higher in both treatment groups than in the BD group (Table 2).

Positive control rat colon tissues showed an intense positive nuclear staining of PCNA (40%–60% LI) in mucosal epithelial lining (data not shown). In mammary gland test tissues, a higher percentage of proliferating cells was generally observed in the epithelium of the terminal ductal structures. Representative immunostaining for PCNA is shown in Figure 1. The PCNA LI and $\%G_1/S$ revealed a significantly higher cell proliferation of terminal ductal epithelium ($P < 0.05$) in rats exposed to the treatment diets than the BD at PND 21 but not at PND 49–51 (Table 2). No differences in the number of terminal ductal structures or cell proliferation were observed between FS and SDG groups at both ages.

Table 1. Urinary Lignans of Dams and Selective Reproductive Indices in Female Offspring[a]

	BD	FS	SDG
Urinary lignans (μmol/d)[b]			
Enterodiol	0.01 ± 0.01*	12.44 ± 2.04***	2.60 ± 0.49**
Enterolactone	0.03 ± 0.00*	0.39 ± 0.06**	0.16 ± 0.03**
Secoisolariciresinol	—*	0.10 ± 0.01**	0.31 ± 0.03***
Total	0.04 ± 0.01*	12.94 ± 2.09***	3.07 ± 0.47**
Selective reproductive indices			
Relative Δ AGD (mm/100g body wt)[c]	15.7 ± 0.4	15.7 ± 0.3	16.2 ± 1.0
Ovary wt at PND 21 (mg/100 g body wt)	61 ± 7	59 ± 6	64 ± 8
Uterus wt at PND 21 (mg/100 g body wt)	66 ± 4	78 ± 4	72 ± 5
Ovary wt at PND 49–51 (mg/100 g body wt)	61 ± 3	59 ± 3	57 ± 3
Uterus wt at PND 49–51 (mg/100 g body wt)	238 ± 35	236 ± 32	319 ± 20
Onset of puberty (PND)	34 ± 2	33 ± 1	33 ± 1
Estrous cycle length (day)	4.1 ± 0.3	4.6 ± 0.5	4.7 ± 0.6

[a] Data are means ± SEM of six animals per group. Differences among diet groups were determined by one-way ANOVA and ANOVA on ranks for parametric and nonparametric data, respectively. When significant differences were achieved, Tukey's or Dunn's pair-wise comparison tests were performed to determine significant differences between groups. Asterisks within a row denote a significant difference at $P < 0.05$.

[b] Mean daily values of 3-day urinary lignan excretion of rat dams at the end of lactation; includes both conjugated and free lignans.

[c] Relative Δ AGD is [(AGD at PND 20) – (AGD at PND 3)]/[(body wt at PND 20) – (body wt at PND 3)].

Immunolocalization and Expression of EGFR and Its Ligands. EGFR was expressed intensely in the epidermis of the positive control rat skin, whereas EGF and TGF-α were mainly expressed in the tubular epithelium of the positive control rat kidney (data not shown). Representative immunostaining of mammary gland tissues for EGFR and EGF are depicted in Figure 1. Membrane and cytoplasmic staining of EGFR and cytoplasmic staining of EGF and TGF-α were found in the epithelium of terminal ductal structures and stromal fibroblasts adjacent to these ducts. EGFR and its two ligands were expressed uniformly across the terminal ductal epithelium and stromal fibroblast at PND 21. However, at PND 49–51, the expression of EGFR and its ligands in the epithelium of nonterminal mammary ducts was variable, with a higher intensity detected in the peripheral regions of the gland. As for mammary fat adipocytes, the immunoreactivity of EGFR and its ligands varied across the gland but was generally detected in the regions adjacent to the mammary ducts. At PND 21, EGFR expression in the epithelium of TEB and TD was higher in the treatment groups, with the SDG group results being significantly different from the BD group (Fig. 2). Although not statistically significant, a higher EGFR expression was observed in the periductal stromal fibroblast and adipocytes of the treatment groups (Table 3). EGF expression was upregulated by the FS and SDG exposures in the epithelium of TEBs and TDs (Fig. 2) as well as in stromal fibroblasts (Table 3), although only the latter showed a significant difference ($P < 0.05$) among groups. Conversely, in the epithelium of terminal ductal structures at PND 49–51, the IHS of EGFR in the SDG group and of EGF in the FS group was significantly lower ($P < 0.05$) than that in the BD group (Fig. 2). The treatment groups had also reduced EGFR and EGF expressions in the stromal fibroblast, as indicated by a marginally significant difference among groups (Table 3). The diet treatments did not affect TGF-α expression in all mammary compartments at both ages (Table 3 and Fig. 2). The FS and SDG groups did not differ from each other in the expression of these proteins.

Immunolocalization and Expression of ER-α and -β. Similar to earlier reports (37, 38), a strong immunoreactivity of both ERs was detected in the luminal and granulose epithelium of the positive control rat uterus. The expression of ER-α was also found in the myoepithelium of rat uterus (data not shown). Figure 1 shows a representative immunostaining of ER-α and -β in the mammary gland tissues. ER-α immunostaining was predominantly localized in the epithelial cell nuclei of terminal ductal structures. High ER-α expression was also found in the nuclei of stromal fibroblast adjacent to terminal ducts but was generally more intense in mammary glands at PND 21 than those at PND 49–51. ER-β demonstrated both cytoplasmic and nuclear staining in the epithelium of terminal ducts, as well as in stromal fibroblasts adjacent to these ducts. Moderate nuclear staining of both estrogen receptors was found in the fat adipose nuclei adjacent to the mammary ducts at PND 21, whereas, at PND 49–51, the immunoreactivity of both receptors was sparingly detected in some adipocytes, particularly those surrounding the peripheral regions of the mammary ductal tree. The results indicate reduced ER-β expression in the epithelium of TEBs and TDs at PND 21 in the treatment groups, with that of the FS group being significantly lower ($P < 0.05$) than that of the BD group (Fig. 3). A lower expression of ER-α was also observed in the terminal ductal epithelium (Fig. 3) and stromal fibroblasts (Table 3) in the treatment groups, but the difference was only marginally significant ($P = 0.059$)

Table 2. Number of Terminal Ductal Structures and Ductal Epithelial Cell Proliferation[a]

	BD	FS	SDG
PND 21			
TEBs			
No.[b]	43 ± 5[a]	74 ± 9[b]	54 ± 8[ab]
%G_1/S	18.6 ± 0.9[a]	23.9 ± 2.2[ab]	25.7 ± 0.8[b]
LI	19.8 ± 1.0[a]	26.4 ± 2.3[b]	27.3 ± 0.9[b]
TDs			
No.[b]	45 ± 6[a]	84 ± 15[b]	61 ± 10[ab]
%G_1/S	15.5 ± 1.0[a]	19.7 ± 0.8[b]	23.8 ± 1.3[b]
LI	16.6 ± 1.1[a]	22.0 ± 1.0[b]	26.1 ± 1.3[b]
PND 49-51			
TEBs			
No.[b]	25 ± 4[b]	12 ± 3[a]	14 ± 2[a]
%G_1/S	18.7 ± 2.8	16.4 ± 2.3	17.7 ± 2.4
LI	19.6 ± 2.8	17.3 ± 2.3	18.4 ± 2.5
LOBs			
No.[b]	51 ± 8	60 ± 7	57 ± 9
%G_1/S	17.3 ± 2.2	11.7 ± 1.9	11.9 ± 2.3
LI	17.9 ± 2.2	12.0 ± 2.0	12.1 ± 2.3
LOBs/TEBs	2.1 ± 0.3[a]	6.7 ± 1.4[b]	7.2 ± 1.0[b]

[a] Data are means ± SEM of six animals per group. Differences among diet groups were determined by one-way ANOVA and ANOVA on ranks for parametric and nonparametric data, respectively. When significant differences were achieved, Tukey's or Dunn's pair-wise comparison tests were performed to determine significant differences between groups. Different superscript letters within a row denote a significant difference at $P < 0.05$. Cell proliferation was determined by the PCNA immunostaining of ≥4 representative terminal ductal structures.

[b] The no. of each terminal ductal structure was averaged from three (PND 21) and five (PND 49–51) random representative sections of each mammary gland sample.

among groups. It is noteworthy that, unlike ER-α, ER-β expression in the stromal fibroblasts was unaffected by the treatments (Table 3). At PND 49–51, no differences in the expression of either receptor were found among groups for all mammary compartments (Table 3 and Fig. 3). Similarly, the ratio of ER-α:ER-β of all mammary compartments did not differ significantly among groups (data not shown). ER-α and -β expression in stromal fat cells was not altered by the dietary exposure (Table 3). There was no difference in the IHS of both ERs between FS and SDG groups.

Correlation Analyses. A summary of significant correlations is depicted in Table 4. At the end of suckling (PND 21), the total urinary excretion of lignans of lactating dams was positively correlated with the number of TEBs and the number of TEBs plus TDs of their female offspring. However, at PND 49–51, the urinary lignans of dams had an inverse association with the number of TEBs but a positive correlation with the maturity index LOBs/TEBs of their female offspring. The epithelial EGFR expression was positively correlated with the PCNA LI at both PND 21 and PND 49–51. Significant positive correlations were also observed between epithelial expressions of TGF-α and EGF and of ER-α and ER-β. In the TEB epithelium, EGF expression was positively associated with PCNA LI at PND 21, as well as with EGFR at both PND 21 and PND 49–51. The EGF expression in stromal fibroblasts was also positively correlated with the TEB epithelial PCNA LI at PND 21. No correlations between ER-α or ER-β and PCNA LI, TGF-α, or EGFR were found in or between different tissue compartments.

Discussion

The present study provides the first evidence on the mechanism relating the reduction in mammary tumor development observed in our recent study (23) to the lignan-induced mammary gland differentiation determined in this and our previous studies (17, 22). The enhancement of mammary gland morphogenesis upon exposure to FS and SDG during suckling, as evidenced by increased TEBs and TDs and epithelial cell proliferation at PND 21, led to more differentiated mammary glands, with a reduced number of TEBs but a higher ratio of LOBs to TEBs by early adulthood (PND 49–51). This finding was in agreement with the results of our earlier studies, which used the whole mount analysis of mammary glands (22). A higher rate of cell proliferation in normal mammary gland development corresponds to an increased cellular differentiation (10). Moreover, the urinary lignans of dams significantly correlated with the mammary gland development parameters of their offspring at both ages.

Immunostaining data have suggested that FS and SDG may target, specifically or preferentially, different tissue compartments, via the paracrine and autocrine regulation of EGFR and ER signaling, to exert mitogenic and differentiation effects on the mammary gland. Compared with the control group, exposure to FS resulted in significantly reduced epithelial ER-β at PND 21 and less EGF at PND 49–51. On the other hand, SDG exposure resulted in significantly higher epithelial EGFR and fibroblast EGF at PND 21 but lower EGFR at PND 49–51. However, the difference between the FS and SDG groups in the expression of these proteins was very small and not statistically significant. Both treatment groups did not consistently differ significantly versus the BD group, perhaps because of the small sample size ($n = 6$/group). The presence of components in flaxseed such as α-linolenic acid–rich oil, proteins, dietary fiber, and other phytochemicals may also have modified the effect of SDG on estrogen metabolism and, hence, cellular signaling (3, 20, 28).

Overall, the offspring exposed to FS or SDG expressed higher epithelial EGFR and fibroblast EGF but lower ERs, particularly ER-β, at PND 21. An upregulation of EGFR in TEBs (24) and increased phosphorylated EGFR but reduced epithelial ER-α at PND 21 (25) were similarly reported after the pharmacological administration of genistein (500 mg/kg body wt) to prepubescent rats. Genistein was found to

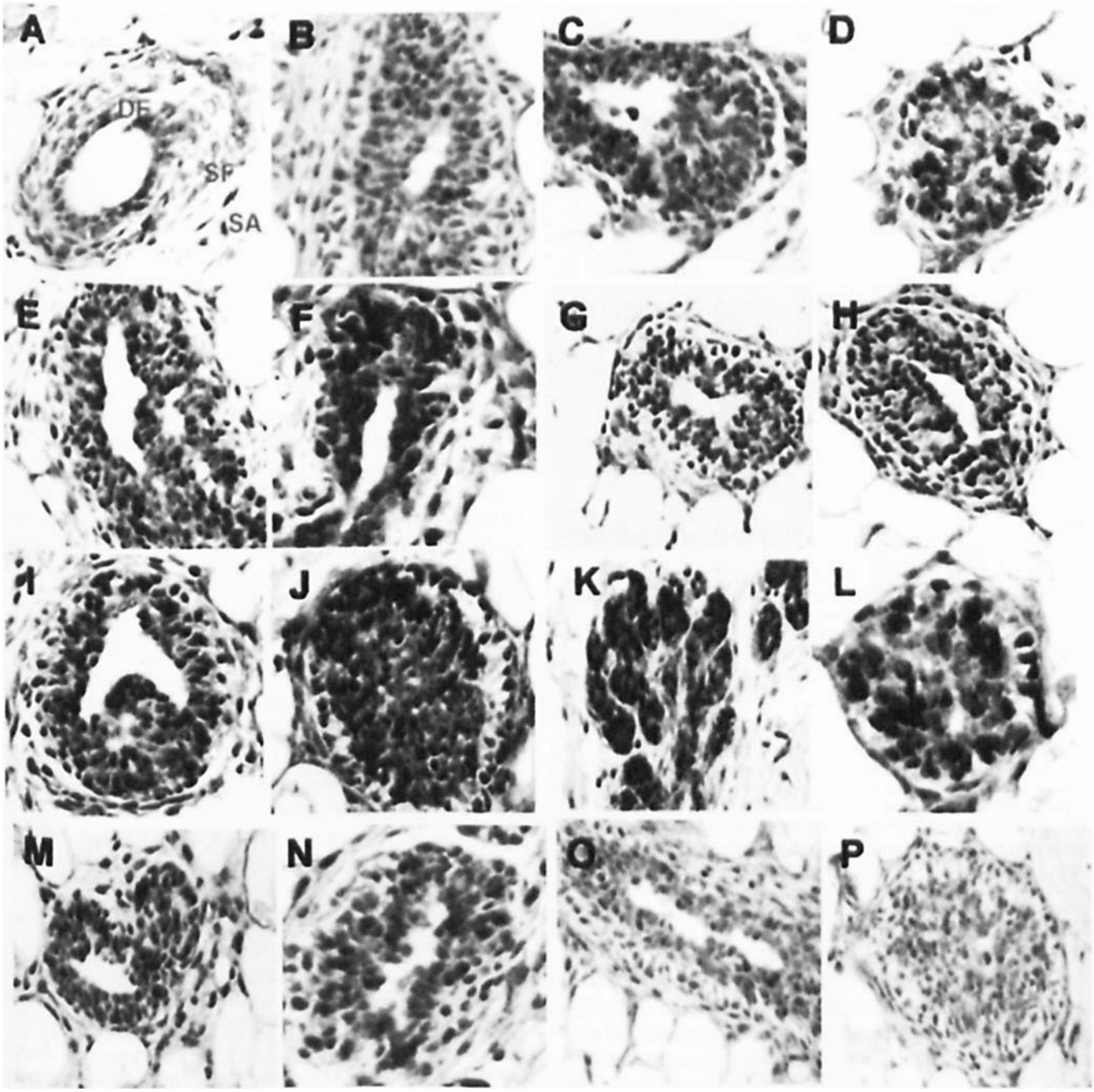

Figure 1. Representative slides immunostained for expressions of PCNA, EGFR, EGF, ER-α, and ER-β in the terminal ductal structures of mammary glands. Immunopositive cells were stained brownish red by AEC. All sections shown were magnified at ×400, unless otherwise indicated. (A) TEB (negative control) with no positive staining across the ductal epithelium (DE), stromal fibroblasts (SF), and adipocytes (SA). (B) TEB of the BD group at PND 21, with lower percentage of proliferating cells (PCNA immunostaining) than that in the FS group (C). (D) Terminal ductal structure of the BD group at PND 21 (×450), with lower expression of EGFR than that of the FS group (E). (G) TEB of the FS group at PND 49–51, with lower expression of EGFR than in the TEB of the BD controls (F). (H) TEB of the BD group at PND 21, with lower EGF expression than in the FS group (I) (×450). TEB (J) (×450) and LOBs (K) of the BD group at PND 49–51, showing higher EGF expression than the TEB of the FS group (L) (×500). (M) TEB of the BD group at PND 21, with higher expression of ER-α than the FS group (N) (×450). TEB of the FS group at PND 21 (O), with lower expression of ER-β than the BD group (P).

simulate the action of a synthetic estrogen (estrogen benzoate) in prepubertal mammary glands that uses an ER-mediated pathway (25).

Unlike genistein, which has been shown to activate ER-mediated gene products by direct binding to both ER subtypes, but preferentially to ER-β (39), there are currently no data on a direct binding of lignans to ERs. However, several lines of evidence have suggested that lignans may mediate estrogenically through direct or indirect interactions with the ERs. EL at concentrations of 0.5–10 μ*M* increased the growth and DNA synthesis of ER-dependent human breast cancer MCF-7 cells cultured in low (0.01 n*M*) or the

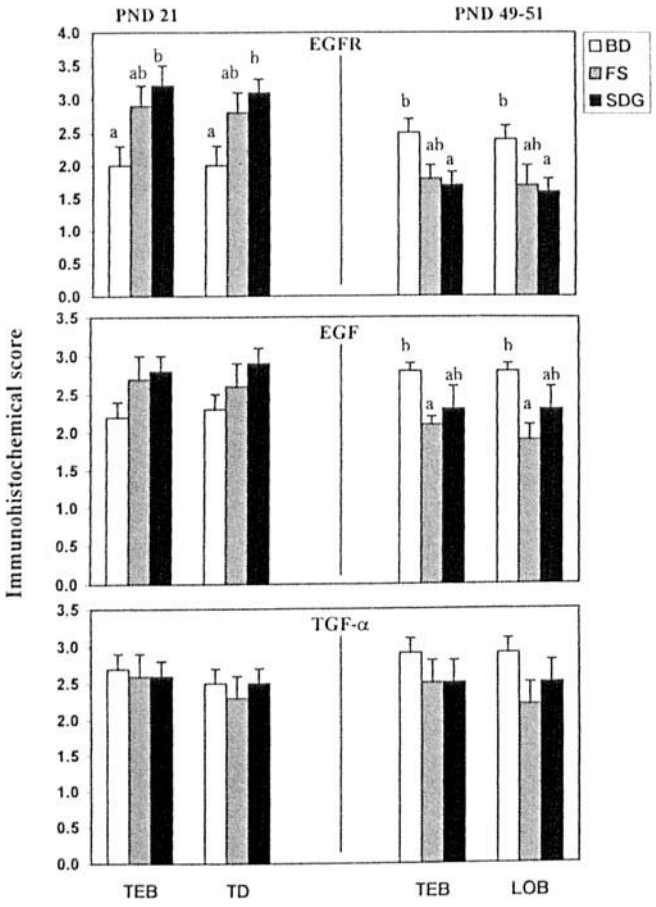

Figure 2. IHS for EGFR and its ligands EGF and TGF-α in the epithelium of terminal ductal structures. Bars and errors bars are means ± SEM of six animals per group. Differences among groups were tested by one-way ANOVA for parametric data or ANOVA on ranks for nonparametric data. When significant differences were achieved, the post hoc analyses for parametric and nonparametric data were Tukey's and Dunn's pairwise comparison tests, respectively. Different letters within bars of diet groups represent a significant difference at $P < 0.05$.

absence of exogenous estradiol (14, 15). In a stable, transfected ER reporter cell system, EL dose-dependently induced the transcriptional responses of both ER-α and ER-β at concentrations >1 μ*M* (40). *In vivo* studies have also shown that the continuous exposure of rats to FS or SDG from gestation through suckling results in reproductive alterations indicative of an estrogenic effect (16–18). This timing of exposure has been implicated in an increased serum estradiol level in female offspring at PND 21 (16, 18), but such an effect was not observed on exposure to the same diets during suckling (Ward and Thompson, unpublished data). Therefore, increasing the systemic synthesis of estrogens may not be the primary mechanism for the enhancement of mammary gland differentiation by lignan. It is possible that lignans elicit estrogenic activities when the physiological estrogen milieu is low, particularly during prepubertal or suckling periods (20, 22).

The developmental role of ER-α in mammary gland morphogenesis is well documented, whereas ER-β is thought to play a role in terminal ductal differentiation, functional silencing, and/or the dominant repressing of ER-α toward estrogen activation (41–43). A significant down-regulation of epithelial ER-β in the TEBs of the FS group may denote a higher sensitivity of ER-α–positive cells in these structures toward estrogen- or lignan-inducing proliferation. Nevertheless, no correlations were observed between either ER subtype and PCNA LI, EGFR, or its ligands, which suggests that the mitogenic regulation of ERs does not correspond linearly to the regulation of respective growth factors and receptor or that changes in epithelial ERs are independent of EGFR-signaling and cell proliferation.

We have shown that EGF expression in the stromal fibroblast at PND 21 was increased by suckling exposure to treatment diets, particularly in the SDG group. Because this was not evident with genistein exposure (24), the mechanism by which FS enhances the mammary gland development is not entirely similar to that of genistein. EGF stimulates a mitogenic effect on ductal growth (44), and its regulation is reciprocally influenced by multiple growth factors and hormones such as estrogen (45). In the present study, EGF expressions in both TEB epithelium and stromal fibroblast correlated positively with PCNA LI in the TEB epithelium at PND 21, which indicates that the upregulation of epithelial and stromal EGF may be involved in TEB epithelial cell proliferation via autocrine and paracrine pathways. FS or SDG may act through this mechanism of action to stimulate epithelial cell proliferation. No study to date has reported an association of EGF regulation with exposure to phytoestrogens in mammary gland morphogenesis. However, exposure to methoxychlor, a xenoestrogen with estrogenic and antiestrogenic activities, has been shown to activate a GC-rich element located on the promoter site of the lactoferrin gene, which exerts and regulates EGF responses in mouse uterus (46).

The fact that a significant positive association was observed between ductal epithelial EGFR and PCNA LI, particularly at PND 21, implies that EGFR (or perhaps its signaling) has a role in mammary epithelial cell proliferation. That EGFR signaling is essential, especially the role of EGFR and ErbB2 in postnatal mammary ductal morphogenesis, has been established in a gene-knockout mice model of tissue recombinant experiments (47, 48). Although EGFR in stroma, rather than in ductal epithelium, has been suggested to be indispensable for ductal morphogenesis, the functional importance of epithelial EGFR cannot be trivialized. Studies that have used truncated dominant negative forms of EGFR under control of mouse mammary tumor virus promoter showed a strong mitogenic activity of mammary epithelial EGFR (49) and that truncated EGFR expressed exclusively in the mammary epithelium could ablate proliferation and branching of mammary ducts (50). In support of this, a more differentiated mammary gland phenotype observed in rats exposed to FS and SDG may possibly derive from the corresponding increased epithelial EGFR and cell proliferation.

Table 3. IHS for EGFR, Its Ligands, and ER-α and -β in Stromal Fibroblast and Adipose Tissues[a]

	BD	FS	SDG
PND 21			
EGFR			
Fibroblasts	1.4 ± 0.3	2.0 ± 0.2	2.2 ± 0.2
Adipocytes	1.7 ± 0.5	2.0 ± 0.4	2.2 ± 0.5
EGF			
Fibroblasts	2.0 ± 0.2[a]	2.8 ± 0.3[ab]	2.8 ± 0.2[b]
Adipocytes	1.5 ± 0.6	2.0 ± 0.5	2.0 ± 0.4
TGF-α			
Fibroblasts	2.5 ± 0.2	2.4 ± 0.3	2.1 ± 0.3
Adipocytes	2.2 ± 0.3	1.8 ± 0.5	2.2 ± 0.3
ER-α			
Fibroblasts**	2.6 ± 0.1*	1.9 ± 0.3*	1.7 ± 0.4*
Adipocytes	1.7 ± 0.5	1.7 ± 0.6	1.8 ± 0.6
ER-β			
Fibroblasts	2.4 ± 0.2	2.4 ± 0.2	2.4 ± 0.3
Adipocytes	2.0 ± 0.5	2.0 ± 0.4	1.7 ± 0.6
PND 49-51			
EGFR			
Fibroblasts***	2.0 ± 0.2*	1.3 ± 0.2*	1.3 ± 0.2*
Adipocytes	1.5 ± 0.4	1.5 ± 0.6	1.3 ± 0.6
EGF			
Fibroblasts****	3.0 ± 0.2*	2.3 ± 0.2*	2.6 ± 0.2*
Adipocytes	2.0 ± 0.4	1.5 ± 0.4	1.5 ± 0.4
TGF-α			
Fibroblasts	2.4 ± 0.3	1.9 ± 0.3	2.2 ± 0.3
Adipocytes	1.5 ± 0.6	1.7 ± 0.5	1.7 ± 0.4
ER-α			
Fibroblasts	1.3 ± 0.3	1.8 ± 0.3	1.2 ± 0.3
Adipocytes	1.0 ± 0.4	1.2 ± 0.5	0.8 ± 0.3
ER-β			
Fibroblasts	2.4 ± 0.2	2.2 ± 0.2	1.8 ± 0.4
Adipocytes	1.2 ± 0.3	1.2 ± 0.3	1.0 ± 0.3

[a] Data are means ± SEM of six animals per group. Differences among diet groups were determined by one-way ANOVA and ANOVA on ranks for parametric and nonparametric data, respectively. When significant differences were achieved, Tukey's or Dunn's pair-wise comparison tests were performed to determine significant differences between groups. Different superscripts within a row denote a significant difference at $P < 0.05$. Overall staining intensity of stromal adipose tissues and 8–10 representative stromal fibroblasts adjacent to mammary ducts were examined for each mammary gland sample. See Materials and Methods section for details of analysis.

* Marginally significant difference among groups; ** $P = 0.054$; *** $P = 0.052$; **** $P = 0.051$.

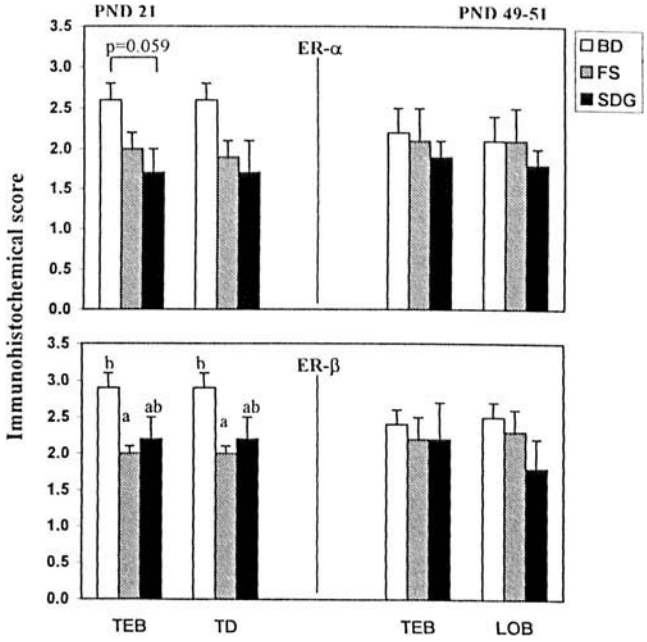

Figure 3. IHS for ER-α and ER-β in the epithelium of terminal ductal structures. Bars and errors bars are means ± SEM of six animals per group. Differences among groups were tested by one-way ANOVA for parametric data or ANOVA on rank for nonparametric data. When significant differences were achieved, the post hoc analyses for parametric and nonparametric data were Tukey's and Dunn's pairwise comparison tests, respectively. Different letters within bars of diet groups represent a significant difference at $P < 0.05$.

In addition to glandular differentiation, exposure to mammogenic hormones or compounds with hormone-like activity may cause persistent alterations in the systemic hormonal milieu, which subsequently leads to permanent biochemical changes in the mammary gland (2). Parous rats were found to have lower levels of mammary ER and EGFR than age-matched virgin rats (51). These cellular changes may modulate the subsequent mitogenic potential of the mammary epithelial cells against carcinogen challenge (2, 51). Significant reductions in the expression of EGFR by SDG and of EGF by FS were observed in the epithelium of TEBs and TDs of rats at PND 49–51. A similar down-regulation of EGFR in TEB was observed on prepubertal exposure to genistein (24). A reduced expression of these mitogenic-related proteins in mammary glands may be another chemopreventive mechanism of FS and SDG against chemically-induced mammary tumorigenesis.

The mammary gland differentiation effect due to lignan exposure during the prepubertal period may, in part, explain the recent epidemiological findings in which a high intake of foods containing vegetable oil and dietary fiber, which are often rich sources of lignans, during adolescence was associated with a lower risk of breast cancer (21). The intake of 10% FS diet by rats is estimated to be equivalent to a daily intake of 25–50 g of FS by human adults, depending on their total food intake (20, 52). The metabolism of lignans in rats and humans appears to be similar, as has been suggested by the similarity in the production of mammalian lignan metabolites and the time-course changes in plasma and urinary lignans in response to dietary lignan loads in both species (52). However, whether lactating vegetarian or FS-fed mothers provide their nursing daughters with a protection against future breast cancer development remains to be investigated.

Table 4. Significant Correlations Between Urinary Lignans of Lactating Dams and Indices of Mammary Gland Development of Their Offspring and Among PCNA LI and IHS of EGFR, Its Ligands, ER-α and -β[a]

Paired variables	r	P
PND 21		
Urinary lignans vs. TEB no.[c]	0.52[b]	0.01
Urinary lignans vs. TEB and TD no.[c]	0.55[b]	0.01
EGFR vs. LI	0.63	<0.0001
TGF-α vs. EGF	0.47	0.004
ER-α vs. ER-β	0.51	0.03
EGF vs. EGFR[d]	0.52	0.03
EGF vs. LI[d]	0.52	0.001
EGF vs. LI[e]	0.55	0.01
PND 49–51		
Urinary lignans vs. TEB no.[c]	−0.59[b]	0.009
Urinary lignans vs. LOB/TEB[c]	0.65[b]	0.004
EGFR vs. LI	0.44[b]	0.008
TGF-α vs. EGF	0.80	<0.0001
ER-α vs. ER-β	0.84	0.0002
EGF vs. EGFR[d]	0.84	<0.0001

[a] Because of the large number of analyses, only paired variables that showed significant correlations are presented. Analyses were conducted by Pearson's correlation coefficient test on IHS of the epithelium of terminal ductal structures, unless otherwise indicated.
[b] Spearman's correlation coefficient.
[c] Urinary lignan values of the lactating dams were paired with the indices of mammary gland development of their female offspring.
[d] IHS of paired variables in the TEB epithelium.
[e] IHS of EGF in stromal fibroblast versus LI in TEB epithelium.

Exposure to FS or its SDG during suckling altered the development of mammary glands without causing significant changes to other hormone-sensitive organs and reproductive indices in female offspring, as was seen in our previous studies (23, 36) or those of others in which dietary genistein was administered during early life (6). The reason for this variable sensitivity of mammary glands versus other reproductive organs to hormonal effects of lignan remains to be elucidated. It is possible that lignan exerts hormonal effects in a tissue-specific manner, depending on the expression and distribution of ER subtypes and the local tissue estrogen metabolism. As opposed to other sex organs, the prepubertal mammary gland is mainly composed of estrogen-synthesizing fat tissues (11) and contains high levels of both ER-α and -β, with the latter being the predominant subtype (41). The mammalian lignans, which are known to exert ER-mediated responses (40) and interfere with aromatase activity (53), may preferentially target the mammary gland. Another possibility is that, because most mammary gland development occurs postnatally (11), exposure to high levels of phytoestrogens during postnatal and/or prepubertal stage of the life cycle may have a greater effect on the development of this organ.

In conclusion, the exposure of rats to FS or its SDG during suckling could enhance mammary gland morphogenesis by increasing mammary cell proliferation, possibly via the modulation of EGFR- and ER-signaling. These changes led to more differentiated mammary glands, with a downregulation of EGFR or EGF at PND 49–51. Consistent with our previous observations (17, 22), the physiological outcomes of FS and SDG in inducing mammary gland morphogenesis were similar, which suggests that SDG in FS is, at least in part, responsible for the mammary gland differentiation effect.

We thank Zhen Liu for technical assistance in urinary lignan analysis, Matthew Ma for assistance in animal handling, Omega Products for supplying the flaxseed, and the American Institute for Cancer Research for financial support.

1. Russo J, Russo IH. Hormonally induced differentiation: a novel approach to breast cancer prevention. J Cell Biochem 22:58–64, 1995.
2. Sivaraman L, Medina D. Hormone-induced protection against breast cancer. J Mamm Gland Biol Neoplasia 7:77–92, 2002.
3. Hilakivi-Clarke L, Cho E, deAssis S, Olivo S, Ealley E, Bouker KB, Welch JN, Khan G, Clarke R, Cabanes A. Maternal and prepubertal diet, mammary development and breast cancer risk. J Nutr 131:154S–157S, 2001.
4. Guzman RC, Yang J, Rajkumar L, Thordason G, Chen X, Nandi S. Hormonal prevention of breast cancer: mimicking the protective effect of pregnancy. Proc Natl Acad Sci U S A 96:2520–2525, 1999.
5. Sivaraman L, Stephen LC, Markaverich BM, Clark JA, Krnacik S, Conneely OM, O'Malley BW, Medina D. Hormone-induced refractoriness to mammary carcinogenesis in Wistar-Furth rats. Carcinogenesis 19:1573–1581, 1999.
6. Fritz WA, Coward L, Wang J, Lamartiniere CA. Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in rats. Carcinogenesis 19:2151–2158, 1998.
7. Hilakivi-Clarke L, Onojafe I, Raygada M, Cho E, Skaar T, Russo I, Clarke R. Prepubertal exposure to zearalenone or genistein reduces mammary tumorigenesis. Br J Cancer 80:1682–1688, 1999.
8. Lamartiniere CA, Cotroneo MS, Fritz WA, Wang J, Mentor-Marcel R, Elgavish A. Genistein chemoprevention: timing and mechanisms of action in murine mammary and prostate. J Nutr 132:552S–558S, 2002.
9. Russo J, Russo IH. DNA labeling index and structure of the rat mammary gland as determinants of susceptibility to carcinogenesis. J Natl Cancer Inst 61:1451–1495, 1978.
10. Russo J, Wilgus G, Russo IH. Susceptibility of the mammary gland to carcinogenesis: I. Differentiation of the mammary gland as determinant of tumor incidence and type of lesion. Am J Pathol 96:721–736, 1979.
11. Russo J, Russo IH. Biology of Disease: biological and molecular bases of mammary carcinogenesis. Lab Invest 57:112–137, 1987.
12. Thompson LU, Robb P, Serraino M, Cheung F. Mammalian lignan production from various foods. Nutr Cancer 16:43–52, 1991.
13. Axelson M, Sjovall J, Gustafsson BE, Setchell KD. Origin of lignans in mammals and identification of a precursor from plants. Nature 298:659–660, 1982.
14. Sathyamoorthy N, Wang TTY, Phang TM. Stimulation of pS2 expression by diet-derived compounds. Cancer Res 54:957–961, 1994.
15. Wang C, Kurzer MS. Effects of phytoestrogens on DNA synthesis in MCF-7 cells in the presence of estradiol or growth factors. Nutr Cancer 31:90–100, 1998.
16. Tou JC, Chen J, Thompson LU. Flaxseed and its lignan precursor, secoisolariciresinol diglycoside, affect pregnancy outcome and reproductive development in rats. J Nutr 128:1861–1868, 1998.

17. Tou JC, Thompson LU. Exposure to flaxseed or its lignans component during different developmental stages influences rat mammary gland structures. Carcinogenesis 20:1831–1835, 1999.
18. Tou JC, Chen J, Thompson LU. Dose, timing, and duration of flaxseed exposure affect reproductive indices and sex hormone levels in rats. J Toxicol Environ Health A 56:555–570, 1999.
19. Adlercreutz H. Phyto-oestrogens and cancer. Lancet Oncol 3:364–373, 2002.
20. Thompson LU. Flaxseed, lignans, and cancer. In: Thompson LU, Cunnane SC, Eds. Flaxseed in Human Nutrition (2nd ed.). Champaign, IL: AOCS Press, pp194–222, 2003.
21. Frazier AL, Ryan CT, Rockett H, Willett WC, Colditz GA. Adolescent diet and risk of breast cancer. Breast Cancer Res 5:R59–R64, 2003.
22. Ward WE, Jiang FO, Thompson LU. Exposure to flaxseed or purified lignan during lactation influences rat mammary gland structures. Nutr Cancer 37:69–74, 2000.
23. Chen J, Tan KP, Ward WE, Thompson LU. Exposure to flaxseed or its purified lignan during suckling inhibits chemically induced mammary tumorigenesis. Exp Biol Med (Maywood) 228:951–958, 2003.
24. Brown NM, Wang J, Cotroneo MS, Zhao YX, Lamartiniere CA. Prepubertal genistein treatment modulates TGF-α, EGF and EGF-receptor mRNAs and proteins in the rat mammary gland. Mol Cell Endocrinol 144:149–165, 1998.
25. Cotroneo MS, Wang J, Fritz WA, Eltoum I, Lamartiniere CA. Genistein action in the prepubertal mammary gland in a chemoprevention model. Carcinogenesis 23:1467–1474, 2002.
26. Imagawa W, Pedchenko VK, Helber J, Zhang H. Hormone/growth factor interactions mediating epithelial-stromal communication in mammary gland development and carcinogenesis. J Steroid Biochem Mol Biol 80:213–230, 2002.
27. Reeves PG, Nielsen FM, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1239–1251, 1993.
28. Thompson LU, Rickard SE, Orcheson LJ, Seidl MM. Flaxseed and its lignans and oil components reduce mammary tumor growth at a late stage of carcinogenesis. Carcinogenesis 17:1373–1376, 1996.
29. Canadian Council on Animal Care. Guide to the Care and Use of Experimental Animals. Ottawa: Canadian Council on Animal Care, 1984.
30. Foley J, Ton T, Maronpot R, Butterworth B, Goldsworthy TL. Proliferating cell nuclear antigen (PCNA): comparison to tritiated thymidine (^{3}H-TdR) as a marker of proliferating hepatocytes in rats. Environ Health Prospect 101:199–205, 1993.
31. Odum J, Pyrah ITG, Foster JR, Van Miller JP, Joiner RL, Ashby J. Comparative activities of p-nonylphenol and diethylstilbestrol in noble rat mammary gland and uterotrophic assays. Reg Toxicol Pharmacol 29:184–195, 1999.
32. Brown N, Lamartiniere CA. Xenoestrogens alter mammary gland differentiation and cell proliferation in the rat. Environ Health Perspect 103:708–713, 1995.
33. Harvey JM, Clark GM, Osborne CK, Allred D. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 17:1474–1481, 1999.
34. Chen J, Stavro M, Thompson LU. Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. Nutr Cancer 43:187–192, 2002.
35. Kleer CG, Wojno KJ, Fields K, Singleton TP. Detection of estrogen receptor of the breast using automated immunohistochemistry. Appl Immunohistochemistry Mol Morphol 7:103–107, 1999.
36. Ward WE, Chen J, Thompson LU. Exposure to flaxseed or its lignan during suckling only or continuous does not alter reproductive indices in male and female offspring. J Toxicol Environ Health A 64:567–577, 2001.
37. Hiroi H, Inoue S, Watanabe T, Orimo A, Momoeda M, Tsutsumi O, Muramatsu M. Differential immunolocalization of estrogen receptor α and β in rat ovary and uterus. J Mol Endocrinol 22:37–44, 1999.
38. Saunders PTK, Maguire SM, Gaughan J, Millar MR. Expression of oestrogen receptor β (ERβ) in multiple rat tissues visualized by immunohistochemistry. J Endocrinol 154:13–16, 1997.
39. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, Van der Saag PT, Van der Burg B, Gustaffson J-A. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252–4263, 1998.
40. Saarinen NM, Warri A, Makela SI, Eckerman C, Reunanen M, Ahotupa M, Salmi SM, Franke AA, Kangas L, Santti R. Hydroxymatairesinol, a novel enterolactone precursor with antitumor properties from coniferous tree (*Picea abies*). Nutr Cancer 36:207–216, 2000.
41. Saji S, Jensen EV, Nilsson S, Rylander T, Warner M, Gustafsson J-A. Estrogen receptors α and β in the rodent mammary gland. Proc Natl Acad Sci U S A 97:337–342, 2000.
42. Saji S, Sakaguchi H, Andersson S, Warner M, Gustafsson J-A. Quantitative analysis of estrogen receptor proteins in rat mammary gland. Endocrinology 142:3177–3186, 2001.
43. Forster C, Makela S, Warri A, Kietz S, Becker D, Hultenby K, Warner M, Gustafsson J-A. Involvement of estrogen receptor β in terminal differentiation of mammary gland epithelium. Proc Natl Acad Sci USA 99:15578–15583, 2002.
44. Coleman S, Silberstein GB, Daniel CW. Ductal morphogenesis in the mouse mammary gland: evidence supporting a role of epidermal growth factor. Dev Biol 127:304–315, 1988.
45. Stern DF. ErbBs in mammary development. Exp Cell Res 284:89–98, 2003.
46. Zhang Z, Teng CT. Methoxychlor stimulates the mouse lactoferrin gene promoter through a GC-rich element. Biochem Cell Biol 80:23–26, 2002.
47. Wiesen JF, Young P, Werb Z, Cunha GR. Signaling through the stromal epidermal growth factor is necessary for mammary ductal development. Development 126:335–344, 1999.
48. Sebastian J, Richards RG, Walker MP, Wiesen JF, Werb Z, Derynck R, Hom YK, Cunha GR, DiAugustine RP. Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. Cell Growth Diff 9:777–785, 1998.
49. Matsui Y, Halter JT, Holt JT, Hogan BL, Coffey RJ. Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. Cell 61:1147–1155, 1990.
50. Xie W, Paterson A, Chin E, Nabell L, Kudlow J. Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development. Mol Endocrinol 1:1766–1781, 1997.
51. Yang J, Yoshizawa K, Nandi S, Tsubura A. Protective effects of pregnancy and lactation against N-methyl-N-nitrosourea–induced mammary carcinomas in female Lewis rats. Carcinogenesis 20:623–628, 1999.
52. Thompson LU. Analysis and bioavailability of lignans. In: Thompson LU, Cunnane SC, Eds. Flaxseed in Human Nutrition (2nd ed.). Champaign, IL: AOCS Press, pp92–116, 2003.
53. Adlercreutz H, Bannwart C, Wahala K, Makela T, Brunow G, Hase T, Arosemena PJ, Kellis JT, Vickery LE. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J Steroid Biochem Mol Biol 44:147–153, 1993.