Can Modulation of Mammary Gland Development by Dietary Factors Support Breast Cancer Prevention?

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Introduction

The mammary gland is one of the few organs which mainly develops after birth, and can repeatedly undergo cycles of growth, functional differentiation, and regression (involution). Regulation of mammary gland growth and differentiation is unique and complex and involves multiple steroid and peptide hormones and growth factors. Unraveling the developmental regulation is important in understanding breast cancer etiology and managing its prevention, as well as in establishing effective treatment strategies.

Mammary Gland Development and Breast Cancer in Human and Rodents

Mammary gland morphogenesis and histology are substantially similar between women, rats, and mice. The functional part of the mammary gland in human is the ‘terminal ductal lobular unit’, TDLU (also called LOB), and in rat and mouse the ‘lobuloalveolar unit’ (LA), or the terminal end buds (TEBs) and alveolar buds [1–3] (fig. 1). These structures have similar epithelial cells (luminal and myoepithelial), and they are the major hormone-sensitive areas of the mammary epithelium in all species studied so far.

Most mammary cancers in women as well as in rats and mice originate from the luminal epithelial cells lin-
Mammary cancers are classified according to their histopathology, and on the basis of their growth requirements, i.e. hormone-dependent and hormone-independent (humans [4], mice [6], and the references therein). In most strains of mice, mammary cancers are hormone-independent at the time of detection, while in rats, almost all are hormone-dependent, and humans have both phenotypes. However, as regards the etiology of both hormone-independent and hormone-dependent types, ovarian and pituitary hormones are essential in stimulating luminal mammary epithelial cell proliferation and the early development of the tumors [4].

**Key Stages and Hormones in Mammary Gland Development**

The development of the mammary gland can be divided into distinct stages: (1) embryonic and prepuberty, (2) puberty, (3) pregnancy and lactation, and (4) involution. The contribution of ovarian steroids and pituitary peptides to mammary gland development and differentiation has been well documented [7]. The major steroids are estrogens (E) and progestins (P), the effects of which are mediated by their respective receptors, E through two distinct estrogen receptor (ER) subtypes, ERα and ERβ, and P through two isoforms of the progesterone receptor (PR), PR-A and PR-B.

During embryogenesis the primary sprout, an invagination of epidermis, penetrates and grows into the mesenchyme, i.e. the future mammary fat pad of the adult gland [8]. From birth until the onset of puberty, mammary ducts continue to grow and branch slowly at a pace similar to the overall growth of the body. Epidermal
growth factor receptor (EGFR) and ERα in the mammary stroma are required for growth of the epithelium during this period [9, 10], when the epithelial cells themselves are devoid of ERα, revealing the importance of paracrine signaling and parenchymal-mesenchymal crosstalk in the mammary gland development.

Puberty – A Period of Major Changes in the Mammary Gland

The onset of puberty initiates major changes in the female mammary gland, both in the gene expression [11] and morphology [7]. The two ovarian hormones, E and P, acting through their respective receptors, stimulate allometric growth and morphogenesis of the gland [7, 12]. The ductal elongation and branching are driven by highly proliferative structures called terminal end buds (TEBs) at the tips of the growing ducts [13, 14] (fig. 1). Besides active cell proliferation, active cell death also takes place in the TEBs, leading to lumen formation. At this stage, both ER subtypes are found in a number of epithelial cells of ducts and alveoli, and stromal cells. ERα seems to be indispensable for normal ductal elongation [15], while ERβ appears to play a more subtle, but distinct role in the organization and adhesion of the epithelial cells, apparent in the pregnant and lactating gland [16]. Similarly, epithelial PRs are needed for normal ductal side-branching, and the development of lobuloalveolar structures of the mammary gland [17, 18, 99].

Recently, a new mechanism of regulation of ER and PR activity in the mammary gland was proven by skillful studies on specific ER- and PR-interacting proteins, the repressor of ER activity (REA) and steroid receptor co-activator-2 (SRC-2), respectively. As tools, two new mouse models were created that were deficient either of REA [19] or SRC-2 (the latter abrogated only in the PR-expressing cell lineages [20]). The results showed that REA counterbalances E-induced, and SRC-2 enables P-induced transcriptional activity of the corresponding receptor, thereby creating an additional level of regulation to control nuclear receptor activity and tissue specificity [21].

However, it has been observed in several studies on human, mouse and rat that the mammary epithelial cells which express ERα and PR are not the ones that divide [22], indicating that they act via paracrine and/or juxtacrine (via cell membrane component/connection bound) growth factors, stimulating the nearby or immediately adjacent cells to divide [18]. During pubertal growth the action of E via ER(s) is mediated, at least partly, through growth hormone (GH) and/or insulin-like growth factor (IGF-I) [23, 24], and blocking of IGF-I action by a GH-antagonist treatment hinders pubertal but not adult mammary gland growth [25]. Several other signaling molecules and growth factors have also been identified as potential mediators of E and P (for a review, see e.g. Anderson and Clarke [26]).

In mouse, the mammary ducts have completely reached the periphery of the mammary fat pad in 10–12 weeks. At this point the TEBs transform to terminal ductal structures with low mitotic activity. In human, full ductal growth and branching morphogenesis is a gradual process and takes several years. This appears to be the period when the mammary gland has the highest number of stem cells, and is also most vulnerable to cancer-initiating events (discussed below). Transient alveolar development and functional differentiation occurs during each estrous cycle with the cycling levels of E and P. Breast epithelium of normally cycling women exhibits its maximal proliferative activity during the luteal phase, when progesterone levels are the highest, but estrogen levels are 2- to 3-fold lower than those observed during the follicular phase [27]. As a consequence, the TDLUs become more elaborate in terms of the number of alveoli [26, 28], which increases both the epithelial cell number and surface area. Similarly, in mice, E and P stimulate cell proliferation and formation of small side branches [7, 17], a peak occurring in the late proestrus and estrus phase, which then, unlike in humans, all regress by an increase in apoptosis in diestrus.

Completion of Mammary Gland Development in the Pregnancy-Induced Terminal Differentiation

Terminal differentiation of the mammary gland, however, is only attained by the first full-term pregnancy; cell differentiation becomes dominant from mid-pregnancy as the gland enters into the secretory initiation phase [29]. It is the result of a complex interaction of ovarian, pituitary and placental hormones, which in turn induce inhibition of cell proliferation, downregulation of ERα and PR, activation of differentiation-specific genes, and the expression of extracellular matrix proteins, as well as the milk proteins [30, 31]. Several different signaling molecules and negative and positive regulators have been shown to be important in the alveologenesis and terminal differentiation of the mammary gland. After cessation of lactation/nursing, the gland undergoes major changes, apoptosis and tissue remodeling, that restore morphology closely similar to that of a nulliparous gland. (See below, the hypotheses on the pregnancy-induced changes in the mammary stem cells, linked to protection against breast cancer.)
Breast Cancer Risk Factors and the Role of Steroid Hormones

A woman’s reproductive history is the strongest and most consistent breast cancer risk factor if genetic background and age are excluded [32]. Reproductive history associated with increased risk includes early menarche, late menopause, and nulliparity, or late age at first childbirth. An early age of menarche translates into earlier hormone exposure (E and P) and breast epithelial growth, a late menopause increases the number of total years of hormone exposure, and, thus, the cumulative lifetime exposure to reproductive hormones. First full-term pregnancy at an early age induces an early terminal differentiation of the breast epithelium. According to the Nurses’ Health Study [32] the adverse effects of an early age at menarche, and an extended time (>14 years) between menarche and first pregnancy are associated with an increased risk of breast cancer (the first with both ER- and PR-positive and -negative breast cancer, and the latter with receptor-negative breast cancer only [32, 33]). A comprehensive summary of recent clinical studies [34] concluded somewhat differently that an early age at menarche, nulliparity, and delayed childbearing are all associated with an increased risk for receptor-positive but not receptor-negative breast cancer: On the other hand, a full-term pregnancy at an early age (<20 years of age) confers a 2-fold lifelong protection from receptor-positive breast cancer risk [33, 35], and is observed in women of all ethnic groups with the exception of mutation carriers of high-penetrance breast cancer susceptibility genes [36].

At an individual level, variability of the genes regulating the key steroidogenetic and drug-metabolizing enzymes (e.g. CYP enzymes) as well as of the local steroidogenesis (in particular, aromatization) may contribute to breast cancer risk [3]. Furthermore, the mechanisms behind some of these hormonal (protective or risk increasing) effects may not all be related to E and P acting through their respective receptors [37].

The ultimate proof, however, of the central role of estrogens (during the early developmental period) in breast cancer etiology has come from the unfortunate experiment on pregnant women treated with a synthetic estrogen (diethylstilbestrol) to prevent an imminent miscarriage. The data collected to date show that the in utero exposure to diethylstilbestrol has significantly increased the risk of breast cancer in women at age 50 years or older, the corresponding excess of breast cancers being 200% [38].

Thus, reprogramming of the mammary gland upon pregnancy or short-term hormonal treatment must be non-reversible to explain the persistent, lifelong protective effects. The key question is: Is the reprogramming of the mammary gland caused by persistent changes in the hormonal environment or permanent biochemical alterations in the mammary epithelia – or both? [see 30, and the hypotheses below]. Based on preclinical studies, it has been concluded that the altered genotype of a transformed cell is irreversible. However, the expression of the transformed phenotype requires further genetic and/or epigenetic changes, which are considered to be reversible [42]. So far, it has not been fully established whether estrogens (or their metabolites) act as endogenous carcinogens or as growth promoters of the transformed cells, or both [3].

**Take-home message of the results from preclinical and clinical studies** [also reviewed in 40, 41]

Risk increasing factors:
- Long duration of E and P in adolescence and adulthood
- High in utero E

Risk reducing factors:
- Short duration of pregnancy levels of hormones during early reproductive years

**Mammary Stem Cells and Cancer Stem Cells: New Findings and New Hypotheses**

The existence of mammary stem cells and their committed progenitor cells was first demonstrated by limiting dilution experiments of mouse mammary epithelial cells and transplants with limited developmental capacity, respectively [43]. Mammary epithelial stem cells are multipotent, exhibit properties of self-renewal (ca. 7–10 divisions) and may exist either as long-lived non-dividing cells, or as proliferating-differentiating cells [44]. A conclusive proof of the presence and importance of stem cells in the mammary gland was recently obtained in a study...
demonstrating that the mammary stem cell is capable of producing a renewable and complete mammary epithelium in mouse [45]. Thus, it is hypothesized that, analogously to the hematopoietic system, a primary mammary epithelial stem cell gives rise to a hierarchy of epithelial progenitor cell lineages to ultimately produce all the different cells found in the mammary epithelium [46, 47; see the hypotheses below, and 48].

Women in their adolescence have the highest susceptibility to breast cancer instigating development, which appears to be the period when the mammary gland has the highest number of stem cells [49]. Proliferation of subpopulations of epithelial cells within the TDLU (of women) or the TEB and LA (of mice and rats) are believed to include the stem cells from which the ductal as well as lobular premalignant breast lesions ultimately arise (ductal hyperplasia, atypical lobular hyperplasia and lobular carcinoma in situ, respectively). It has been suggested that these represent the precursors from which breast cancers arise, based on the observation of bilateral risks and frequent multifocality [5]. Since reproductive history has a major impact on breast tumorigenesis, it is reasonable to assume that pregnancy and lactation have enduring effects on cancer susceptibility of these cancer-initiating multipotent stem (or progenitor) cells.

Hypotheses on Pregnancy-Induced Changes in the Mammary Gland and Stem Cells

Several hypotheses have been presented of the role of reproductive hormones and mammary stem cells in breast carcinogenesis:

(1) The developmental fate hypothesis by Medina [40] suggests that at the critical period in adolescence the hormonal milieu of pregnancy affects the developmental fate of a subset of mammary epithelial cells and their progeny, resulting in persistent differences in the molecular pathways between the epithelial cells of hormone-treated (parous) and mature virgin (nulliparous) mammary glands. These changes in turn affect (block) the proliferative responses to carcinogen challenge. Several putative mediators of the molecular pathways have been suggested, including tumor suppressor proteins p53, mdm2, and p21, which in turn may be affected by alterations in genes regulating chromatin remodeling [40].

(2) The pregnancy-specific stem cells hypothesis [50] was enhanced by the new techniques utilizing a cre-lox conditional activation of reporter genes, which led to the finding of a new epithelial progenitor, specific for mammary secretory epithelium in postlactation female mice [43]. These pregnancy-induced mammary epithelial cells (PI-MECs) originate from the differentiating cells during the first pregnancy and lactation cycle, and have stem cell-like features, such as self-renewal, and the ability to produce progeny with diverse cellular fates. PI-MECs do not undergo apoptosis during postlactational remodeling but persist throughout the remainder of the female’s life [50]. A possible explanation for the pregnancy-induced refractoriness to carcinogenesis comes from the observation that PI-MECs appear to adopt the strategy of selective segregation of their template DNA strands during cell division and self-renewal (in the mammary transplants) and, thus, protect themselves from mutation and cancer risk [51]. Yet another important piece of information was added when Booth and Smith [52] were able to show that a small number of mammary epithelial cells with stem cell-like characteristics express both ERα and PR, the expression of which is sensitive to E, P, and prolactin treatment.

The new techniques enabling work on normal human breast epithelial cells, both in vitro [53], and recently also in vivo (see below) [54, 55] have led to results confirming the existence of mammary stem cells in the human breast epithelium, similar to those found in the mouse and rat mammary gland. In line with the above hypotheses, the differentiation hypothesis [49] is also based on the now generally accepted view that the TDLU (called the LOB1 by Russo et al. [3, 49]), which is the most undifferentiated structure in the breast of young nulliparous women (fig. 1), is the site of origin of ductal carcinomas. According to the differentiation hypothesis, the LOB1 are biologically different in nulliparous and parous women: 'stem cells 1' (in the LOB1 of nulliparous women) being susceptible to neoplastic transformation, while 'stem cells 2' (in the LOB1 of early parous women) being refractory to neoplastic transformation, after having acquired 'a changed genomic signature' [49]. Also, the ability of the LOB1 cells to metabolize estrogens or repair genotoxic damage may be different in parous and nulliparous women [3].

Studies on human mammary epithelium have also been enhanced by a new technique where mammary fat pads of immunodeficient mice were first humanized by human mammary stromal cells, after which it was possible to successfully transplant and experiment on human mammary epithelium in vivo [54]. The fact that the mouse mammary fat pad needed to be humanized with human mammary stromal cells also underlines the importance of epithelial-stromal interactions, and paracrine signaling in the mammary stem cell growth, and further strengthens the concept of a stem cell ‘niche’ [56] driving the mammary gland growth.
Molecular Mechanisms Behind the Pregnancy-Associated Protection against Breast Cancer

Recently, by using microarray expression profiling, Chodosh et al. [57] have identified gene expression changes associated with persistent pregnancy-induced protection against mammary tumors in mice, as well as in rats of several strains which have a hormone-induced protection against mammary carcinogenesis but a different intrinsic susceptibility to it [58]. Chodosh et al. [58] describe a common genetic signature of 70 genes that were conserved among the two species and the several (rat) strains, and thus, are more likely to contribute causally. The described signature includes persistent downregulation of multiple genes encoding growth factors, as well as persistent upregulation of a growth-inhibitory molecule, transforming growth factor-β, and several of its transcriptional targets [58]. Based on these results, Chodosh et al. [58] conclude that parity results in a persistent increase in the differentiated state of the mammary gland, including changes in the extracellular environment and stromal-epithelial interactions, as well as in the hematopoietic cell types resident within the mammary gland.

These new data on rodents have been strengthened by important findings on normal human breast tissue obtained from reduction mammoplasties. Balogh et al. [59] and Verlinden et al. [60] have analyzed global gene expression profiles of the epithelium and the stroma of normal breast tissues from both parous and nulliparous women. Similarly to rodent studies, several genes were identified encoding for growth-promoting, cytoskeletal and extracellular matrix proteins that are highly expressed in the nulliparous mammary gland but are lost after pregnancy [60]. One striking example of these genes is the small breast epithelial mucin, which is almost completely downregulated upon a first full-term pregnancy but is known to be expressed in more than 90% of invasie ductal carcinomas of the breast [60].

Taken together, the above results on the central role of stem cells in breast cancer initiation, and the differential gene expression profiles of parous vs. nulliparous mammary gland define a developmental state of the gland that is refractory to carcinogenesis, and suggest new and more detailed hypotheses on the mechanisms by which endogenous (and exogenous) hormones may modulate breast cancer risk.

Diet – A Strategy for Lowering Breast Cancer Risk?

As discussed above, the timing and the dose of exposure to reproductive hormones seems to be critical for breast cancer susceptibility. Because pregnancy at a young age could not be advised as a means for breast cancer prevention, there is a great need for better strategies population-wide, and especially for high-risk groups. Since dietary factors are estimated to account for approximately 40% of all cancers in Western countries (estimated by the WHO [61]), and even higher, up to 60% of the cancers of women in the countries with high breast cancer incidence rates (estimated by the Finnish cancer organizations [62]), finding out the mechanisms by which specific dietary factors can modulate mammary gland growth during its vulnerable stages, and consequently, also breast cancer risk, is highly relevant and urgent.

Dietary Phytoestrogens, Naturally Occurring Modulators of Steroid Hormone Action

Diet contains multiple naturally occurring phenolic compounds, which potentially can act as steroid action modulators. Phytoestrogens, which include flavonoids, tannins, stilbenoids, and lignans, are regarded as such compounds. Experimental studies in rodents suggest that consumption of these compounds during specific stages of ontogenesis may affect mammary gland development and, consequently, the risk of breast cancer later in life [63, 64]. However, epidemiological studies in women are inconclusive [65].

Phytoestrogens are ubiquitous in edible plants, and they are consumed as a part of the regular diet. Therefore, people are exposed to these compounds at different developmental stages throughout the life. The types of ingested phytoestrogens differ depending on the diet. Isoflavones, such as genistein (fig. 2), are obtained mainly from soy protein-containing foods, while lignans are more ubiquitous and are found in many seeds, grains, vegetables and berries. Flaxseed is one of the richest dietary sources of plant lignans containing up to 0.2% (w/w) of its major lignan, secoisolariciresinol diglycoside (SDG; fig. 2). When ingested, many dietary phytoestrogens, such as plant isoflavones and some of the plant lignans, can be absorbed in significant amounts both in humans [66, 67] and in experimental animals [68]. Moreover, some plant isoflavones and lignans can be further transformed into mammalian metabolites, such as equol and enterolactone, respectively, by intestinal microbiota of both humans and animals [69, 70]. In serum, these compounds and their metabolites have been found in mi-
cromolar concentrations [66, 67, 71], i.e. in over 100-fold higher concentrations than endogenous estrogens, suggesting that, similar to endogenous estrogens, they may affect the development of steroid hormone target tissues, such as the mammary gland.

In in vitro studies, phytoestrogens, especially isoflavones, have been shown to act similarly to endogenous estrogens, although with lower potency. Isoflavones bind to both ERα and ERβ, induce the expression of estrogen-responsive genes, and stimulate the growth of estrogen-sensitive breast cancer cells [72–75].

**Isoflavones and Lignans Modulate Mammary Gland Development**

It has been shown in several preclinical studies that phytoestrogens can affect mammary gland development (tables 1, 2). However, because of the specific nature of the mammary gland development (embryonal-prepubertal vs. pubertal), and terminal differentiation (upon pregnancy only), also the effects of phytoestrogens are likely to vary depending on the timing and extent of the exposure. The effects of phytoestrogens on mammary gland development have been investigated mainly in rats and mice (tables 1 and 2, respectively). In rats, perinatal or postnatal administration of soy protein- or genistein-containing diet to dams during pregnancy and lactation [76, 77] or by injection of genistein to female rat pups prior to weaning [78], in doses relevant to human exposure, have been demonstrated to reduce the number of TEBs at the age of 50 days (postnatal day (PND) 50), i.e. at the time the rat mammary gland is known to be most susceptible to carcinogens, due to the highly proliferating TEBs [79]. Therefore, reduction of the number of TEBs (before and at PND 50) results in a reduced number of carcinogen-induced mammary tumors in rats. Similarly to soy protein and isoflavones, administration of flaxseed or plant lignan SDG in diet to dams during pregnancy and lactation, or during lactation only, first increased the number of TEBs at PND 21 of the pups [80], but later, at PND 50, reduced their number [80–82], which can be interpreted as an enhancement of the development and earlier maturation of the mammary gland. Furthermore, feeding of SDG- or genistein-rich diets to prepubertal rats increased the number of differentiated lobuloalveolar structures in the mammary gland in adulthood [80, 81]. Similar effects have also been demonstrated in female mice after short-term postnatal exposure to isoflavones [83, 84]. These findings on phytoestrogens, i.e. soy isoflavones and flaxseed lignans, concur with the observed responses to estrogens. Administration of estradiol to rats postnatally has been shown to reduce the number of TEBs and increase the number of alveolar structures at PND 50 [85, 86], indicating enhanced maturation of the mammary gland.

**Postmenopause Is Another Period of Vulnerability for the Mammary Gland**

In addition to prepubertal and early reproductive years, another period of vulnerability of the postnatal mammary gland is the time of low endogenous estrogen milieu of the body, i.e. postmenopause. During this time, exogenous E and phytoestrogen exposure can be critical in terms of stimulation of possible precancerous and malignant foci in the breast. Ovariectomized (OVX) rodents and OVX primates are generally used as animal models mimicking the lack of ovarian hormone production of postmenopausal women. Administration of soy extract or purified soy isoflavone genistein to rats inhibited the OVX-induced regression of mammary gland (i.e. had an estrogenic effect [87, 88]), while combined administration of genistein with estradiol (E2) showed no attenuation of E2-stimulated mammary gland morphogenesis (i.e. no antiestrogenic effect detected). These results indicate that in the adult OVX rats, administration of isoflavones from 3 weeks to 3 months resulted in morphological responses similar to those caused by endogenous estrogens in the mammary gland. However, E2 and phytoestrogen effects on mammary gland ERs also varied in the adults, as in the peripubertal animals discussed above. In OVX rats, administration of isoflavone-containing soy extract, unlike E2, reduced the expression of epithelial and stromal ERβ in the mammary glands while no changes in epithelial or stromal ERα, PR or Ki-67 immunoreactivity were observed after soy extract administration compared to control OVX rats [87].

![Chemical structures of the plant lignan secoisolariciresinol diglycoside (SDG) and the soy isoflavone genistein.](image-url)
Primates are generally considered to mimic the hormonal responses of women better than rodents. However, no results on the developmental effects of phytoestrogens are available, and only very limited number of studies have been performed in adult primates. In adult female OVX macaques, administration of soy isoflavones in diet for 1–6 months did not result in any significant estrogen-like changes in the mammary gland morphology or in the proliferation markers [89, 90]. These studies imply, however, that the responses to phytoestrogens may differ between rodent and primate mammary glands. Whether the OVX primate models predict the dietary phytoestrogen responses in postmenopausal women better than rodent models, still remains open.

Table 1. Phytoestrogen effects on mammary gland development in rats

<table>
<thead>
<tr>
<th>Rats</th>
<th>Phytoestrogen</th>
<th>Exposure time</th>
<th>Effect on mammary gland</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>Prepubertal exposure</strong></td>
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<tr>
<td>Perinatal and postnatal administration</td>
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<tr>
<td>SD, females</td>
<td>250 ppm genistein in diet</td>
<td>GD 0 – PND 21</td>
<td>At PND 21 and 50, reduced number and proliferation of TEBs</td>
<td>76</td>
</tr>
<tr>
<td>SD, females</td>
<td>10% flaxseed in diet or 1.5 mg SDG/rat p.o.</td>
<td>GD 0 – PND 21 or 50</td>
<td>At PND 50, reduced number of TEBs with both treatments. Flaxseed increased the number of alveolar buds, while SDG reduced the numbers</td>
<td>81</td>
</tr>
<tr>
<td>SD, males and females</td>
<td>Genistein 5, 25, 100, 250, 625, or 1,250 ppm in diet</td>
<td>GD 7 – PND 50</td>
<td>At PND 50, increased ductal and alveolar hyperplasia in females (625 and 1,250 ppm) and in males (25 ppm and higher)</td>
<td>94</td>
</tr>
<tr>
<td>SD, females</td>
<td>20% soy protein isolate in diet</td>
<td>GD 4 – PND 21, 33 or 50</td>
<td>At PND 50, reduced number of TEBs with increased expression of PR. No change in ERs or lobuloalveolar PRs</td>
<td>77</td>
</tr>
<tr>
<td>SD, males and females</td>
<td>300 or 800 ppm genistein in diet</td>
<td>GD 1 – PND 22</td>
<td>At PND 22, no effect with 300 ppm. With 800 ppm increased ductal branching in both sexes. Females: no effect on mammary epithelial area, number of TEBs or lateral buds. Males: increased proliferation of mammary epithelial and ERs, PR and IGF-1R immunostaining</td>
<td>95</td>
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<tr>
<td><strong>Postnatal administration</strong></td>
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<tr>
<td>SD, females</td>
<td>Genistein 50 μg/pup s.c. (0.25–3.3 mg/kg)</td>
<td>PND 7–20</td>
<td>At PND 50, reduced size of mammary epithelial area, reduced number of TEBs, increased density of lobuloalveolar structures, BRCA 1 mRNA upregulated</td>
<td>78</td>
</tr>
<tr>
<td>SD, females</td>
<td>10% flaxseed or 200 ppm SDG in diet</td>
<td>PND 1–21</td>
<td>At PND 21, increased number of TEBs, terminal ducts, epithelial cell proliferation and EGFR, and stromal fibroblast EGF. At PND 49–51, reduced number of lobular, and reduced expression of EGFR and EGF</td>
<td>80</td>
</tr>
<tr>
<td>SD, females</td>
<td>10% flaxseed in diet</td>
<td>PND 1–21 or 50</td>
<td>At PND 50, reduced number of TEBs and increased number of alveolar buds</td>
<td>82</td>
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<tr>
<td><strong>Peripubertal exposure</strong></td>
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<tr>
<td>SD, females</td>
<td>Genistein 375 or 750 ppm in diet</td>
<td>PND 31–45</td>
<td>No effects on lobuloalveolar and ductal development</td>
<td>88</td>
</tr>
<tr>
<td>SD, females</td>
<td>10% flaxseed in diet</td>
<td>PND 21–50</td>
<td>No effect on development of mammary gland structures</td>
<td>81</td>
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<tr>
<td><strong>Exposure in adulthood</strong></td>
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<tr>
<td>SD, OVX females</td>
<td>Genistein 750 ppm in diet</td>
<td>3 weeks</td>
<td>Inhibition of OVX-induced mammary gland regression</td>
<td>88</td>
</tr>
<tr>
<td>SD, OVX females</td>
<td>Genistein 53 mg/day in diet</td>
<td>3 months</td>
<td>Increased lobuloalveolar growth and nuclear expression of PCNA</td>
<td>87</td>
</tr>
<tr>
<td>SD, OVX females</td>
<td>Soy extract 100 mg/kg p.o.</td>
<td>6 weeks</td>
<td>Attenuation of OVX-induced glandular atrophy, reduction in ERβ. No change in ERs, PR or Ki-67</td>
<td>96</td>
</tr>
</tbody>
</table>

EGF = Epidermal growth factor; EGFR = epidermal growth factor receptor; ER = estrogen receptor; GD = gestational day; IGF-1R = insulin-like growth factor 1 receptor; OVX = ovariectomized; PND = postnatal day; PR = progesterone receptor; SD = Sprague-Dawley; SDG = secoisolariciresinol diglucoside; TEB = terminal end bud.
Studies on the cellular and molecular mechanisms behind the observed morphological changes are in rapid progress, and have been conducted mostly in young animals. Based on recent results, epigenetic modifications of the genome have been suggested as possible mechanisms by which phytoestrogens could modify gene expression and cellular functions [reviewed in 91]. Similar changes in global gene expression profiles have been observed in the mammary gland after prepubertal estradiol and genistein exposure [83]. Thus, some phytoestrogens may enhance the development and maturation of the (prepubertal) mammary gland the same way as endogenous estrogens do.

The effect of phytoestrogens on the expression of mammary gland ERs has been actively studied, but the results so far have been controversial. The cells in the rat TEBs and LOBs (equivalent to TDLUs) express both ER$\alpha$ and ER$\beta$ [77, 78]. However, consumption of soy protein isolate-containing diet (rich e.g. in genistein) by rat dams during pregnancy and lactation and by their female pups throughout their prenatal and postnatal life until their adolescence (until PND 50) did not induce any alteration in the ER$\alpha$ or ER$\beta$ of the TEB and LOB cells, or PRs of LOB cells [77].

In contrast to the above result obtained with soy protein-containing diet, exposure of female rats to pure genistein during prepuberty resulted in a higher number of epithelial cells expressing ER$\alpha$ and ER$\beta$ in the postpubertal mammary glands (at the age of 8 weeks [78]). These findings would indicate that despite the similarities in the morphological

**Table 2. Phytoestrogen effects on mammary gland development in mice**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Phytoestrogen</th>
<th>Exposure time</th>
<th>Effect on mammary gland</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>In utero exposure</td>
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<tr>
<td>CD-1, females</td>
<td>Genistein 20 $\mu$g/dam s.c.</td>
<td>GD 15–20</td>
<td>At PND 50, increased density of TEBs and reduced epithelial differentiation in female offspring</td>
<td>100</td>
</tr>
<tr>
<td>Prepubertal exposure</td>
<td>Soy isoflavone mixture (Prevastein) 270 ppm in diet</td>
<td>GD 0 – PND 21</td>
<td>At PND 28, increased branching morphogenesis</td>
<td>83</td>
</tr>
<tr>
<td>Postnatal administration</td>
<td>Genistein 0.1, 0.5, 2.5, or 10 mg/kg p.o.</td>
<td>GD 12 – PND 21</td>
<td>At PND 49, no effects on epithelial tree lengths, area, number of TEBs, or density of alveolar buds</td>
<td>97</td>
</tr>
<tr>
<td>CD-1, females</td>
<td>Genistein 10 mg/kg s.c.</td>
<td>PND 15–18</td>
<td>No change in mammary gland growth at the age of 4, 8, or 24 weeks</td>
<td>98</td>
</tr>
<tr>
<td>FVB/N, females</td>
<td>Soy isoflavone mixture (Prevastein) 270 ppm in diet</td>
<td>PND 21–28</td>
<td>At PND 42–43, transient reduction in the number of TEBs</td>
<td>83</td>
</tr>
<tr>
<td>CD-1, females</td>
<td>Genistein 0.5, 5, or 50 mg/kg s.c.</td>
<td>PND 1–5</td>
<td>At 5 weeks, reduced number of branch points (5 and 50 mg/kg), increased expression of PR (0.5 and 5 mg/kg) At 5 and 6 weeks, reduced expression of ER$\alpha$ At 6 weeks, enhanced ductal elongation (0.5 and 5 mg/kg), reduced number of TEBs (5 and 50 mg/kg)</td>
<td>84</td>
</tr>
</tbody>
</table>

GD = Gestational day; PND = postnatal day; TEB = terminal end bud; ER = estrogen receptor.

**Take-home message of the results from preclinical models**

- Prepuberty and postmenopause are the periods when the mammary gland is most vulnerable to the effects of dietary phytoestrogen
- Modulation of mammary gland development affects breast cancer susceptibility

**Cellular and Molecular Mechanisms of Phytoestrogen Action**

Studies on the cellular and molecular mechanisms behind the observed morphological changes are in rapid progress, and have been conducted mostly in young animals. Based on recent results, epigenetic modifications of the genome have been suggested as possible mechanisms by which phytoestrogens could modify gene expression and cellular functions [reviewed in 91]. Similar changes in global gene expression profiles have been observed in the mammary gland after prepubertal estradiol and genistein exposure [83]. Thus, some phytoestrogens may enhance the development and maturation of the (prepubertal) mammary gland the same way as endogenous estrogens do.
responses of the developing mammary gland after exposure to endogenous E and phytoestrogens, their mechanisms of action could be different. Moreover, the tissue levels of ERs and PRs do not tell about their activity.

As discussed above, another important level of regulation of ER and PR activity in the mammary gland occurs through ER- and PR-interacting proteins [21], the expression and activity of which could be targets of phytoestrogen action as well. Even more complexity to studies on phytoestrogens adds the fact that their metabolism is known to vary significantly depending on the compound, dose, form of administration (i.e. in food matrix or as an isolated compound), and route of administration (orally vs. subcutaneously). This is likely to affect significantly the measured responses of the target tissues, and, thus, could explain at least some of the variability in results of different studies.

Conclusions and Future Perspectives

The past few years have generated important new information and confirmed the existing knowledge about the etiology of breast cancer. The results point out the early onset of breast cancer-initiating events, and the sensitivity of such events to endogenous and exogenous hormones, thus defining the vulnerability phases of mammary gland development. The recent results on the mammary stem or progenitor cells as the sites of cancer initiation have substantially enhanced our understanding of breast cancer etiology. Furthermore, significant new information concerning the molecular mechanisms of breast cancer initiation and progression is evolving, such as the involvement of specific genes, regulation of their expression via genetic and epigenetic mechanisms, additional levels of regulation of steroid receptor activity through interacting proteins, as well as involvement of other signaling pathways and tissue interactions. At the same time it has become more evident that dietary and lifestyle factors also have a major impact on breast cancer risk, their ultimate targets in cells and tissues being, at least in part, the same mechanisms as with endogenous hormones. However, the current knowledge has not yet translated into a decreased prevalence of breast cancer. As an effort to better results, the 3rd International Conference on Recent Advances and Future Directions in Endocrine Manipulation of Breast Cancer [92] concluded that ’It may be possible to develop a short-term intervention strategy with an agonist SERM (‘selective estrogen receptor modulator’) that would be safe and tolerable preventive therapy for young women at increased risk for breast cancer.’ Instead of drug therapy of healthy women, or in addition to it, dietary strategies as well as specific functional foods containing estrogen action modulators (steroid action modulators, e.g. phytoestrogens) could offer a feasible tool for breast cancer prevention and improvement of adjuvant treatment [93].

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105 Booth BW, Smith GH: Estrogen receptor-α and progesterone receptor are expressed in label-retaining mammary epithelial cells that divide asymmetrically and retain their template DNA strands. Breast Cancer Res 2006;8:R49.


93 AICR: Diet and cancer: http://www.aicr.org/site/PageServer?pagename = dc_home


