Hudson, Elizabeth Jane. Breast Cancer: Dietary Links to Gland and Tumor Development and Effects of Genistein and $\alpha$-Lactalbumin on Primary Tumors in vitro (Under the direction of Dr. Brenda Alston-Mills.)

Nutrients found in foods have been shown to affect the occurrence of some types of cancer whereby they modulate hormone secretion, and have short-term impacts on reproductive organs and long-term impacts on the development of tumors in those tissues. Many types of food and nutrients in those foods have been shown to contribute by being a causative factor in tumor cell development. However, there are several foods that may have an opposite effect by serving as a source of cancer prevention or treatment. The whey milk protein, $\alpha$-lactalbumin ($\alpha$-LA) has been shown to inhibit growth of certain mammary tumor cell lines in vitro. Soy contains the isoflavone genistein, which in some cases, has also been shown to contribute to the prevention and occurrence of breast cancer. Cell lines provide some information but little work has been done using primary mammary tumor tissue. Some foods, i.e. soy, enhance the developmental process of the mammary gland resulting in accelerated maturity, leaving the gland less susceptible to tumor development. The objectives of this study were: 1) to determine the implications of nutrients in soy, skim, and casein-based diets at enhancing the maturity of the mammary gland in vivo and as a result the effects on tumorigenesis, and 2) to determine the effectiveness of $\alpha$-LA or genistein in inhibiting further growth of primary mammary tumor tissue cultured in vitro for 48hrs. The end result could lead to future treatments or dietary recommendations that may reduce incidence of breast cancer.

Mammary gland tissue from 21 female rats fed either a soy, skim, or casein-based diet were collected at d52 and analyzed for cell proliferation by proliferating cell nuclear antigen (PCNA) immunohistochemistry assay and amounts of the extracellular matrix protein, tenascin (TN), also by immunohistochemistry. The level of cell proliferation is linked to the maturity of the gland. A high level of cell proliferation is associated with an immature mammary gland, which is more susceptible to tumor development, and a low level of cell proliferation is associated with a more mature gland and
less susceptible to tumor development. The carcinogen, 7,12-dimethylbenz-(a)anthracene (DMBA), which induces mammary tumor development, was administered to the remaining 20 rats at d 53. Once tumors developed, both mammary gland and tumor tissue were collected and analyzed for PCNA values and amounts of TN present. In addition, tumor samples were also cultured for 48hr with 0, 10, 50, 100, 500, or 1000 ng/ml of α-LA or 0, 1, 10, 50, or 100µg/ml of genistein.

Results showed diet to significantly affect the stage of development for mammary gland tissue in this study. The casein-fed animals had much higher levels of PCNA than found in mammary gland tissue of soy or skim-fed animals from the pre-DMBA group. A significant difference was also found between rats fed soy, skim or casein based diets with respect to tumor incidence. The soy group had more tumors to develop than the casein or skim groups. After a 48hr culture of tumor samples with α-LA or genistein, no significant differences were found between the treatments or among treatment concentrations. However, an overall significant negative correlation between PCNA and TN levels in tumor tissue post culture was found.
Breast Cancer:
Dietary Links to Gland and Tumor Development and
Effects of Genistein and α-Lactalbumin on Primary Tumors in vitro

by

Elizabeth Jane Hudson

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North Carolina State University
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APPROVED BY:

____________________________________________________________________
Co-Chair of Advisory Committee

____________________________________________________________________
Chair of Advisory Committee
BIOGRAPHY

Elizabeth Jane Hudson was born March 4, 1974 in Forest City, North Carolina. She graduated from Rutherfordton-Spindale High School in 1992 and entered Isothermal Community College in the fall of 1992. She received an Associate of Science degree in Pre-Med in 1994. The fall of 1994 she entered Salem College and received a Bachelor of Science degree in Biology in 1996.

The author was employed at Wake Forest University-Baptist Medical Center from 1996 until 2000. During this time she worked on several biomedical research projects using macaque monkeys as the research model. In the fall of 2000, she entered Graduate School at North Carolina State University as a graduate research and teaching assistant where she pursued a Master of Science degree in Nutrition.
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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast Cancer</td>
<td>1</td>
</tr>
<tr>
<td>Mammary Gland Development</td>
<td>6</td>
</tr>
<tr>
<td>Development Stages</td>
<td>10</td>
</tr>
<tr>
<td>Embryonic – Human</td>
<td>11</td>
</tr>
<tr>
<td>Embryonic – Rodent</td>
<td>13</td>
</tr>
<tr>
<td>Neonatal</td>
<td>13</td>
</tr>
<tr>
<td>Puberty – Human</td>
<td>14</td>
</tr>
<tr>
<td>Puberty – Rodent</td>
<td>16</td>
</tr>
<tr>
<td>Sexual Maturity – Rodent</td>
<td>17</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>27</td>
</tr>
<tr>
<td>Diet and Breast Cancer</td>
<td>31</td>
</tr>
<tr>
<td>Major Dietary Components</td>
<td>32</td>
</tr>
<tr>
<td>Alcohol Consumption</td>
<td>32</td>
</tr>
<tr>
<td>Dietary Fat</td>
<td>32</td>
</tr>
<tr>
<td>Dairy Products: Milk</td>
<td>37</td>
</tr>
<tr>
<td>Micronutrients</td>
<td>38</td>
</tr>
<tr>
<td>Glucosinolates</td>
<td>39</td>
</tr>
<tr>
<td>Genistein</td>
<td>42</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>44</td>
</tr>
<tr>
<td>Soy Consumption, Genistein and Decreased Breast Cancer Incidence</td>
<td>45</td>
</tr>
<tr>
<td>Soy Consumption and Genistein</td>
<td>46</td>
</tr>
<tr>
<td>Timing of Exposure to Genistein</td>
<td>48</td>
</tr>
<tr>
<td>Estrogen Comparison</td>
<td>48</td>
</tr>
<tr>
<td>Estrogenic and Non-Estrogenic Properties of Genistein in vivo</td>
<td>51</td>
</tr>
<tr>
<td>In vitro Anticarcinogenic Effects of Genistein</td>
<td>51</td>
</tr>
<tr>
<td>Effects of Genistein on Mammary Tumorigenesis</td>
<td>55</td>
</tr>
<tr>
<td>Mechanisms of Action</td>
<td>56</td>
</tr>
<tr>
<td>Estrogen Receptors</td>
<td>57</td>
</tr>
<tr>
<td>Alteration of Estrogen Metabolism</td>
<td>58</td>
</tr>
<tr>
<td>Signal Transduction</td>
<td>61</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>62</td>
</tr>
<tr>
<td>Whey Proteins</td>
<td>63</td>
</tr>
<tr>
<td>Anti-carcinogenic Activity</td>
<td>63</td>
</tr>
<tr>
<td>Mechanism of Action</td>
<td>65</td>
</tr>
<tr>
<td>Regulatory Factors</td>
<td>66</td>
</tr>
<tr>
<td>The Extracellular Matrix</td>
<td>66</td>
</tr>
<tr>
<td>Extracellular Matrix Components</td>
<td>68</td>
</tr>
<tr>
<td>Integrin Receptors</td>
<td>68</td>
</tr>
<tr>
<td>Growth Factors</td>
<td>69</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>69</td>
</tr>
<tr>
<td>Transforming Growth Factor-α</td>
<td>70</td>
</tr>
<tr>
<td>Transforming Growth Factor-β</td>
<td>71</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Estimated new breast cancer cases and deaths in women by age, United States, 2001 ....5</td>
</tr>
<tr>
<td>2. Non-significant data ..................................................................................................................103</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Female Breast Cancer--incidence and death rates, by race, United States, 1994-1998</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>Developmental stages of the mammary gland</td>
<td>7</td>
</tr>
<tr>
<td>3.</td>
<td>Human mammary gland structure</td>
<td>8</td>
</tr>
<tr>
<td>4.</td>
<td>Juvenile mouse mammary gland ducts and TEBs</td>
<td>9</td>
</tr>
<tr>
<td>5.</td>
<td>Mouse mammary gland development from birth to pregnancy</td>
<td>12</td>
</tr>
<tr>
<td>6.</td>
<td>Human breast development at puberty</td>
<td>15</td>
</tr>
<tr>
<td>7.</td>
<td>Human breast TDLU</td>
<td>18</td>
</tr>
<tr>
<td>8.</td>
<td>Human breast after menstruation</td>
<td>19</td>
</tr>
<tr>
<td>9.</td>
<td>Human mammary gland TEBs, AB, lobules, and ducts</td>
<td>20</td>
</tr>
<tr>
<td>10.</td>
<td>Comparison of human TDLUs in the nulliparous and parous breast</td>
<td>21</td>
</tr>
<tr>
<td>11.</td>
<td>Comparison of human TDLUs at early menopause and in the elderly</td>
<td>22</td>
</tr>
<tr>
<td>12.</td>
<td>Mouse virgin mammary gland</td>
<td>23</td>
</tr>
<tr>
<td>13.</td>
<td>TEB structure</td>
<td>24</td>
</tr>
<tr>
<td>14.</td>
<td>Mammary gland branching patterns</td>
<td>25</td>
</tr>
<tr>
<td>15.</td>
<td>Comparison of nulliparous and parous mouse mammary gland</td>
<td>28</td>
</tr>
<tr>
<td>16.</td>
<td>Comparison of TDLU and LA units</td>
<td>29</td>
</tr>
<tr>
<td>17.</td>
<td>Mammary gland Involution</td>
<td>30</td>
</tr>
<tr>
<td>18.</td>
<td>Correlation between % calories as fat and breast cancer mortality</td>
<td>34</td>
</tr>
<tr>
<td>19.</td>
<td>Conversion of glucosinolate to isothiocyanate</td>
<td>40</td>
</tr>
<tr>
<td>20.</td>
<td>Role of phase 2 enzymes</td>
<td>41</td>
</tr>
<tr>
<td>21.</td>
<td>Comparison of endogenous estrogens to dietary isoflavones</td>
<td>43</td>
</tr>
<tr>
<td>22.</td>
<td>Structure of genistein, diadzein, and glycitein</td>
<td>47</td>
</tr>
<tr>
<td>23.</td>
<td>Comparison of equol to estradiol</td>
<td>53</td>
</tr>
<tr>
<td>24.</td>
<td>Distribution of estrogen receptors</td>
<td>54</td>
</tr>
<tr>
<td>25.</td>
<td>Estrogen metabolism</td>
<td>60</td>
</tr>
<tr>
<td>26.</td>
<td>Integration of growth and cell adhesion pathways in mammary cells</td>
<td>67</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

µg.............................................................. microgram
α-LA.......................................................... alpha-lactalbumin
µm.............................................................. micrometer
AB ............................................................... alveolar buds
ACS ............................................................ American Cancer Society
AICR ........................................................... American Institute of Cancer Research
cm............................................................. centimeter
CO₂........................................................... carbon dioxide
d............................................................ Day
DAB ............................................................ Diaminobenzide
DCIS ........................................................... Ductal carcinoma in situ
dl............................................................... de-ionized
DMBA......................................................... 7,12-dimethylbenz(a)anthracene
DMEM......................................................... Dulbecco’s modified eagle media
ECM............................................................ extracellular matrix
EGF ............................................................ epidermal growth factor
ER ............................................................... estrogen receptor
FBS ............................................................ fetal bovine serum
FDA ........................................................... Food and Drug Administration
g ............................................................. gram
H-α-LA ......................................................... Human alpha-lactalbumin
HAAs........................................................... Heterocyclic aromatic amines
hr............................................................... hour
IU .............................................................. International Unit
l ................................................................. liter
LAB ............................................................ Lactic acid bacteria
LA .............................................................. Lobulo-alveolar
MAL ........................................................... Multimeric-α-LA
mg............................................................ milligram
MIA ........................................................... Mammary inhibitory activity
min ............................................................ minute
ml ............................................................. milliliter
mm ............................................................. millimeter
M ............................................................... molar
NaCl ............................................................ sodium chloride
ng ............................................................. nanogram
PCNA.......................................................... Proliferating Cell Nuclear Antigen
SEER .......................................................... Surveillance, Epidemiology and End Result
TBS ............................................................ Tris buffered saline
TDLU .......................................................... terminal ductle-lobular unit
TD .............................................................. Terminal duct
TEB ............................................................ terminal end bud
TGF-α .......................................................... transforming growth factor-alpha
TGF-β .......................................................... transforming growth factor-beta
TN ............................................................. tenascin
Breast Cancer

According to the American Cancer Society, excluding cancers of the skin, breast cancer is currently the most common cancer among women in the United States accounting for one of every three cancers diagnosed. Since 1940, incidence rates of invasive breast cancer show three distinct phases. A steady increase of about 1% per year occurred between 1940 and 1980, possibly due to a gradual increase in underlying risk factors, such as delayed childbearing and having fewer children. Between 1980 and 1987, incidence rate increased by about 4% per year, mainly due to a greater capability of detecting smaller tumors during mammography screening. In this time frame, the number of small tumors <2.0cm more than doubled and incidence of tumors >3.0cm decreased by 27% (Garfinkel, 1994). Between 1987 and 1998, incidence rates of breast cancer have increased by 0.5% per year with the decline in incidence rate possibly attributed to increased detection and earlier diagnosis from mammography screening (Howe, 2001). In situ, or early stage, breast cancer incidence rates have also increased over the past 25 years. This is largely attributed to detection of ductal carcinoma in situ (DCIS) with mammography screening, which is directly linked to large increases in DCIS incidence rates since 1982. DCIS accounted for 51% of the in situ breast cancers diagnosed from 1994 to 1998 among women in the NCI Surveillance, Epidemiology and End Result (SEER) program areas. Within the period of 1973 and 1998, incidence rates of DCIS increased 6 times faster than incidence rates of invasive breast cancer (Ernster, 1996), attributing the difference in incidence rates to be a result of early detection and not a true increase in occurrence.

Breast cancer mortality rates were relatively stable between 1950 and the late 1980’s, but since then there has been a decrease. For all races combined between 1989 and 1995, mortality rates from breast cancer decreased by 1.6% annually and continued to decrease by 3.4% annually between 1995 and 1998 (Howe, 2001). The decline is a result of both improvements in breast cancer treatments and mammography screening.

Based on the current life expectancy for women in the United States, in 2001 an estimated one in 9 women would develop breast cancer. The risk was one out of 14 in 1960. In 2001, an estimated 192,200 new cases of female invasive cancer and 47,100 cases of female in situ breast cancer would be diagnosed and 40,200 women would die from the disease (Table 1). Although
incidence is low, 1,500 male cases were projected to be diagnosed in 2001, and 400 men would die from the disease (Greenlee, 2001).

The survival rate from breast cancer is influenced by several factors. The time from diagnosis is considered where survival is 86% within the first five years after diagnosis, 76% after 10 years, 58% after 15 years and 53% after 20 years (Ries, 2001). Other factors include age at the time of diagnosis, where it is speculated that younger women have lower survival rates because their tumors may be more aggressive and less responsive to hormonal treatment (Marcus, 1994), stage of the cancer at diagnosis, ethnicity (Ries, 2001), and socioeconomic factors (Eley, 1994).

There are several risk factors consistently associated with an increased risk of breast cancer. Breast cancer incidence increases with age and is the leading overall cause of cancer death in women between the ages of 40 and 59 within the United States (Figure 1). Within this age group, 77% of new cases and 84% of breast cancer deaths reported between 1994 -1998 occurred in women ages 50 and older. In addition to age, risk increases if a woman has a family history of breast cancer, begins menses at an earlier age (<12 years), experiences late menopause (>55) (Morabia and Costanza, 1999), or uses hormone replacement therapy (Pike and Ross, 2000). Breast cancer risk is in part associated with higher serum levels of endogenous hormones, in particular that of 17β-estradiol (Bernstein et al., 1990).

Endogenous estrogens are metabolized into estrone, followed by 17β-estradiol and sometimes further metabolized into estriol. The metabolite, 17β-estradiol regulates breast cell proliferation, which may potentially promote cancer cell growth. Metabolism of 17β-estradiol occurs mainly in the liver, to 2-, 4-, and 16α-hydroxylated estrogens, all having different capacities to influence mammary tumorigenesis. 2-Hydroxylated estrogens operate as anticarcinogens, unlike 4- and 16α-hydroxylated estrogens, which may act to enhance cancer development.

All women are at risk for developing breast cancer, but the ages at menarche and menopause are particularly important because greater lifelong exposure to 17β-estradiol is associated with increased risk. This explains why an earlier age at menses (<12 years), later ages at menopause (>55) (Morabia and Costanza, 1999) and hormone replacement therapy (Pike and Ross, 2000), albeit unchangeable, are considered to be risk factors. Other risk factors are modifiable,
including alcohol consumption, using hormone replacement therapy (Pike and Ross, 2000), obesity after menopause, childless or had the first child after age 30. However, over 70% of cases occur in women who have no identifiable risk factors.

All of these risk factors lead to the question of whether breast cancer can be prevented. Currently there is not a way to prevent breast cancer. Clinical research has provided women at very high risk with a way to further reduce their risk of breast cancer by using the FDA approved anti-estrogen drug tamoxifen. Although, many of the risk factors are uncontrollable and drugs are not always the preferred choice, a woman’s best strategy is to increase physical activity, eat healthy, reduce alcohol intake, and avoid obesity, all of which may help reduce her overall risk of breast cancer and possibly many other cancers. Additionally, there are several foods that are suggested to possibly prevent breast cancer. In this study, we specifically studied soy and skim milk based diets to see if cancer incidence was affected in vivo. In addition, the micronutrients α-lactalbumin, found in milk, and genistein, found in soybeans, were studied in vitro to detect quantifiable changes in primary tumors as a result of their presence.

The estimated risk of developing breast cancer for an individual can be determined at the web sites of Harvard Center for Cancer Prevention (http://www.yourcancerrisk.harvard.edu/) and National Cancer Institute (http://bora.nci.nih.gov/brc/).
Figure 1

Female Breast Cancer—Age-specific incidence and death rates, by race, United States, 1994-1998

(Source: American Cancer Society, Surveillance Research, 2001)
Table 1

Estimated new breast cancer cases and deaths in women by age, United States, 2001.

(Source: American Cancer Society, Surveillance Research, 2001)
Mammary Gland Development

The mammalian mammary gland is one of the most unique and fascinating organs because of its developmental processes. Maturation of the gland is a progressive and uneven process with the structure being dependant on the developmental stage of the female (Figure 2). Structural components of the mammary gland are similar among all mammalian species, but especially between humans and rodents, making the rodent mammary gland a suitable alternative model for breast cancer research (Schmeichel et al., 1998).

Mammary Gland Structure

In humans and rodents, the mammary gland is a complex structure populated by fibroblasts, adipocytes, epithelial cells and cells of the vasculature (Schmeichel et al., 1998). Specific structure of the gland involves ducts and tubules with ligaments for support. The breast lies on the rib cage with muscles aiding in attachment (Figure 3). For all mammalian species, the mammary gland exists as a community of epithelial cells that interact adhesively with myoepithelial cells and components of the immediately surrounding extracellular matrix (ECM) and stroma (Pitelka et al., 1973). The gland can be considered as two main components, the parenchyma that includes epithelial and myoepithelial cells, and the stroma. The parenchyma is a system of branching ducts and terminal end buds (TEBs) composed of an external discontinuous layer of myoepithelial cells resting on the basement membrane and an internal layer of epithelial cells, which line the lumen. Each branching duct of the parenchyma is connected to the nipple by a single primary duct. The stroma is comprised of multiple cellular and acellular components. Of the cellular components, adipocytes are the most abundant cell type, followed by fibroblasts, blood cells, endothelial cells, and nerve cells. The acellular portion includes fibrous and non-fibrous collagens, proteoglycans, and glycoproteins. All of the stromal constituents provide mechanical support to the tissue in addition to forming a developmentally active ECM at the epithelial-stromal boundary (Streuli and Haslam, 1998) (Figure 4).
Developmental stages of the mammary gland. The adult female mammary gland experiences recurrent cycles of regulated growth, differentiation, and apoptosis, with estrogen and progesterone playing a central role in this process. The cycles that occur in the mammary gland are divided into stages of puberty, pregnancy, lactation, and involution. During each stage, the structure and type of lobules change within the gland, reviewed in (Russo and Russo, 1998).

Source: (Hansen and Bissell, 2000)
Figure 3

Human mammary gland structure consists of ducts and lobules supported by ligaments.

Source: (Pelton, 1995)
Ducts and TEBs in the juvenile mouse mammary gland show the epithelial-stromal interface.

(a). Mammary ductal system in a 5-week-old nulliparous mouse. End buds (large arrows) identify growing ducts. End buds along the right side of the gland are in various stages of regression. Blunt-tipped branches (small arrows) mark growth quiescent.

(b). Longitudinal section through a TEB and duct. Large arrows show a fibrous connective tissue sheath. Small arrows show the extracellular matrix-basal lamina complex at the epithelial-stromal interface.

Source: (Silberstein, 2001)
The ECM is the microenvironment surrounding the ductal system made up of a complex meshwork containing glycoproteins and proteoglycans found within the stroma. These extracellular components exist within dense fibrous tissue and adipose, collectively known as the mammary fat pad portion of the stroma. For the extending ducts of the parenchyma, the ECM and fat pad provides a frame for support and a substrate for growth. The constituents of the stromal area in the virgin mammary gland are affected by growth hormones and estrogen to promote parenchymal growth (Kleinberg, 1997). Specifically, the ECM is key for normal growth of the gland where it actively contributes in cross-talk occurring with luminal epithelial cells of the ducts and TEBs. This cross-talk regulates growth for branching ducts within the mammary fat pad and involves endocrine hormones, growth factors, epithelial cells and ECM components (Bissell, 1999). All of these factors assist in controlling functional differentiation of the cells (Forsyth, 1986; Streuli and Haslam, 1998; Topper and Freeman, 1980).

**Stages of Mammary Gland Development**

Across all mammalian species, mammary glands progress through distinctly similar stages of development. Initial development begins in utero but it is still immature at birth. After birth, the immature gland undergoes further growth and differentiation during the first two years of life. Ending the two-year postnatal period, the gland undergoes involutional changes similar to that seen in the breast of postmenopausal women (Anbazhagan et al., 1991). Continued growth from the postnatal period and prior to peri-puberty is isometric, only occurring at a maintenance level. The peri-pubertal stage follows, but is short-lived by the onset of puberty. Puberty begins with allometric growth, influenced by endocrine hormones, and is marked by the commencement of ductal elongation and branching that leads to rapid development of the mammary tree (Russo, 1987). The endocrine hormones, especially estrogen, cause the gland to exhibit a burst of growth. Afterwards, the gland reaches another developmental plateau during which minimal growth occurs by renewal and involution with each menstrual or estrous cycle. Finally, sexual maturity occurs with continued branching and TEB formation extending into the mammary fat pad. The gland only achieves a complete and final stage of maturity with full cell differentiation and ducts filling the entire fat pad.
through hormonal stimuli induced by pregnancy, parturition, and lactation (Forsyth, 1986; Russo, 1987; Topper and Freeman, 1980) (Figure 5).

**Embryonic Mammary Development: Human**

Embryonic development of the human mammary gland occurs similarly to the rodent, although more slowly. As reviewed by Dawson (1934) and Bassler (Bassler, 1970), embryological studies of the human mammary gland determined mammary epithelial cells to arise from a single ectodermal bud, which later forms into clusters and representing the areolus of each breast (Russo, 1987). A milk streak first appears during the 4th week of embryonic development and becomes the milk line during the 5th and 6th weeks (Dawson, 1934). The mammary ducts invade the underlying stroma during the 7th and 8th week, forming the mammary disc. Epithelial buds sprout from the ducts between the 10th and 12th weeks and is followed by an indentation in the 12th and 13th week that forms epithelial buds with notches at the epithelial-stromal border. Between the 13th and 20th week, branching of the ducts occurs resulting in 15-25 solid cords that will give rise to multiple openings at the nipple (Dabelow, 1957). These solid cords become open, canal-like tubes by apoptosis of the central epithelial cells (Raynaud, 1960). The peri-ductal stroma of the 32-week-old human fetus has a loose appearance where in the full term infant the rudimentary lobular structures are surrounded by a dense stroma (Naccarato, 2000). In the later stages of gestation, the TEBs develop into lobulo-alveolar structures and mammary epithelial cells appear to be secretory (Vorherr, 1974).

Functional receptors for a variety of hormones are present in the mammary glands of fetal and neonatal rodents and humans, allowing them to be responsive to maternal steroid hormones and lactogens both in vivo and in vitro. At birth the ducts of the newborn contain "witch's milk", a milk-like secretion that is expressible and comparable to the colostrum of the mother (Bloom, 1968). The secretory activity is not a specialized function of the newborn, but rather, part of the generalized response of mammary epithelium to maternal hormonal levels (Hiba et al., 1977). Secretory activity of the human newborn gland subsides within 3-4 weeks.
Figure 5
Mammary development in the mouse from birth to pregnancy.

Source: (Muller, 2001)
**Embryonic Mammary Development : Rodent**

Embryonic development of the mammary gland is a similar process in all species. Initial growth begins with localized ectodermal thickenings within the milk line that extends down both sides of the body from the neck to inguinal region of the developing fetus (Anderson, 1978). Small buds, or primitive nipples, form through elevation of an epidermal mammary crest and milk line. In rodents, the mammary buds form between embryonic days 10 and 11, followed by very little cell proliferation with the exception of around embryonic day 13 when each bud increases in size as a result of cell migration, causing a concentration of epithelial cells within the epidermis. At embryonic day 14, the first, slightly denser mesenchyme, orients around the epithelial buds and is made up of several concentric layers of fibroblasts. Deposition of lipids in the primitive mammary fat pad begins at embryonic day 16, and at embryonic day 17, a less-dense mesenchyme composed of preadipocytes begins to proliferate. The less dense mesenchyme will become the mammary fat pad, which appears as a distinct deposition of white adipose tissue at birth. The primary sprout of the mammary gland pushes through the dense mesenchyme in response to insulin and aldosterone causing penetration into the primitive mammary fat pad (Sakakura, 1987). Proliferation of the buds occur from embryonic day 16 to birth, around embryonic day 21, leading to the formation of a small ductal tree consisting of at least 15 branches stemming from a single duct attached to the nipple (Balinsky, 1950). In the newborn rodent, the mammary gland contains branching cords of epithelial cells within three main lactiferous ducts, connected and opening into the nipple by the single primary duct. These ducts are partially canalized and exhibit branching of the main ducts up to a third or fourth generation level. The ducts become fully canalized with ductal ends terminating into small TEBs holding an extensive proliferative ability (Daniel, 1987).

**Neonatal Mammary Development : Human and Rodent**

Epithelium of the mammary gland extends into the mammary fat pad as a progression of branching ducts. The ductal systems within the mammary glands of the human are more elaborate than that of the rodent. Human ducts have a more segmented structure with outlets from five to ten lactiferous ducts collecting in the nipple. These ducts extend in a more radial pattern, forming distinct
triangular lobes each with an entirely separate ductal system, collagenous stroma, and separate from adjacent lobes. In contrast, the mammary gland of the rodent typically terminates in a single lactiferous duct or sinus area that forms five to ten secondary ducts. The ductal system of the rodent grows into the mammary fat pad with a more linear pattern than that of humans.

Neonatal mammary glands of all mammalian species typically have only rudimentary ducts with small TEB-like ends that regress shortly after birth. Through the neonatal and peri-pubertal stages, mammary gland development in both human and rodent is rather dormant. During this period, the gland continues a maintenance level of growth, keeping pace with general growth of the body until the onset of puberty (Tanner, 1962). Growth at puberty occurs from branching TEBs and new end buds form through lateral branching from mature ducts for all mammalian species. Both TEBs and new end buds of lateral branching contain undifferentiated "stem" cells. The terminal ends of the ducts give rise to functional milk producing lobules of the mammary gland.

**Puberty Mammary Development and Differentiation: Human**

Epithelial proliferation within the human mammary gland is initiated by the onset of puberty at 10-12 years of age. The mammary gland begins to show growth activity in both the epithelium and the surrounding stroma. Glandular increase is due to lipid deposition within the mammary fat pad and division of small bundles of primary and secondary ducts (Russo, 1987). The main site of proliferation is a TEB-like structure where elongation of the ductal tree occurs and simultaneously undergoes dichotomous and sympodial branching (Figure 6). The ducts grow, divide, and comprise the functional portion of the human mammary gland, termed the "terminal ductal lobular unit" (TDLU) (Russo, 1990). The TDLUs are major hormone sensitive areas of mammary epithelium (Russo and Russo, 1998) and are the origin site for most mammary cancers because they contain the major proliferative stem cell populations thought to be most sensitive to somatic cell mutation (Russo, 1990). The TDLU responds to estrogen and progesterone by synthesizing DNA during the luteal phase. Mammary development induced by ovarian hormones during a menstrual cycle never fully returns to the starting point of the preceding cycle.
Human breast development at puberty. At or during onset, the ducts grow and divide in a dichotomous and sympodial basis ending in TEBs. n, nipple.

Source: (Russo, 1987)
As a result, each ovulatory cycle slightly fosters mammary development with new budding of structures, which continues until about age 35 (Vorherr, 1974). The human TDLU comprises a small unit resembling a cluster of grapes at the end of a stem, encased in a loose intra-lobular connective tissue surrounded by denser extra-lobular connective tissue (Figure 7). After the first menses, each individual terminal duct lobule gives rise to an average of 11 surrounding ductules with alveolar buds clustered to form lobule type 1 or virginal lobule (Russo and Russo, 1998) (Figure 8). Type 1 lobule formation is confined to the female breast and occurs within 1-2 years after the onset of the first menstrual period. The transition from lobule type 1, to type 2, to type 3 is a gradual process of sprouting new alveolar buds. The buds in lobules type 2 and 3 are called ductules; they increase in number from approximately 11 in the lobule type 1 to 47 and 80 in lobules type 2 and type 3, respectively (Figure 9). Lobule type 1 is typically found in the breast of nulliparous young women, whereas lobules type 2 and types 3 are more often found in the glands of parous women (Figure 10). However, the breasts of some parous women and that of women with a history of abortion also contain lobules type 1 and type 2, and they are occasionally found in the breast tissue of older women. Full differentiation of the mammary gland is a gradual process taking many years, and if pregnancy does not occur, it is never attained (Russo, 1987). With the repetitive cycling of human menstruation, the TDLU develops early and regresses in the post-menopausal years (Figure 11). Since TDLUs contain an abundance of proliferative undifferentiated stem cells, women without complete post-menopausal regression appear to be more prone to cancer.

**Puberty Mammary Development and Differentiation: Rodent**

Development of the rodent mammary gland and the human mammary gland is relatively similar. Much of the knowledge learned about the human breast thus far has been based on some human research, but mainly from rodent studies. Further discussion of mammary development will focus solely on that of the rodent. Onset of puberty within the rodent occurs initially at day 21 and extending through day 55 with ductal elongation and bifurcation occurring in the distal third of the gland, which contains long ducts ending in prominent TEBs. The number of TEBs is maximal at this stage. The terminus of the virgin mouse mammary gland is generally composed of an unbranched or
minimally branched ductule with a single TEB that may be blunt or globular (Figure 12). The TEBs are lined with multiple layers of epithelium containing undifferentiated stem cells and body cells. Undifferentiated stem cells, known as cap cells, are located on the basal lamina and have the highest proliferation of any cell population in the mammary gland (Daniel, 1987). Body cells are organized in multicellular layers within the central cavity of the TEB and are the focal area for apoptosis (Figure 13). Both cap cells and body cells are progenitors of the myoepithelium and ductal epithelium, respectively (Knight and Peaker, 1982).

Proliferation of the TEB is regulated by hormones and locally-acting growth factors including estrogen and progesterone, growth hormone (GH), epidermal growth factor (EGF), transforming growth factor alpha (TGFα), transforming growth factor beta (TGFβ) and insulin-like growth factor-1 (IGF-1) (Daniel and Robinson, 1992; Humphreys et al., 1997; Ruan et al., 1992; Silberstein and Daniel, 1982; Silberstein et al., 1994; Walden et al., 1998). All of these hormone and growth factors exert direct or indirect proliferative or inhibitory effects on the cells of the TEB by activating the intercellular signaling pathways. The proliferative capacity of the TEB is the source of rapid growth in the distal portions of the ductal tree during puberty, which permit ductal elongation and dynamic changes in the gland.

Along with proliferative activity in the TEB, programmed cell death (apoptosis) occurs in the TEB primarily in the body cells. Apoptosis typically occurs adjacent to or within a few cell layers of the developing ductal lumen and is rarely observed in cap cells, but it does occur. Apoptosis appears to complement proliferation within the endbud, which is suggestive by the close pattern of cell death and cell survival. The regulation of apoptosis demonstrates that it is integral in normal ductal development of the mammary gland.

**Sexual Maturity Mammary Development: Rodent**

Mammary gland development progresses with age by successive dichotomous branching of new ducts separated by less than a 90-degree angle, sprouting of side buds, elongation of existing ducts, and cleavage of TEBs into two or more small alveolar buds (ABs) (Figure 14).
Figure 7

Terminal ductal lobular unit (TDLU) within the human breast. TDLUs grow, divide and comprise the functional portion of the mammary gland. The human TDLU resembles a cluster of grapes at the end of a stalk.

Source: (Brisken, 2002)
Human breast development. After the first menstruation, the first lobular structures appear (lobules type 1); composed of alveolar buds (AB). Some branches end in TEBs or terminal ducts. n, nipple.

Source: (Russo, 1987)
Figure 9

Human mammary gland. Terminal end bud (TEB); alveolar buds (AB); Lobules types 1, 2, and 3 (Lob 1, Lob 2, and Lob 3); and ducts.

Source: (Russo, 1987)
Figure 10

Functional TDLU of the human breast.
A) Fully developed nulliparous. B) Fully developed parous.

Source: (Cardiff and Wellings, 1999)
Figure 11

Functional TDLU of the human breast.

A) Early menopausal  B) Elderly

Source: (Cardiff and Wellings, 1999)
Figure 12

Mammary gland of a 6 week old virgin mouse. The ductal system contains end buds, but no alveoli.

Source: (Cardiff and Wellings, 1999)
Figure 13

A typical mammary TEB. Adipocytes (a) lie on top of cap cells at the tip (left). Fibrocytes (f) comprise the connective tissue around the neck region. The basal lamina (bl) differentiates and is continuous with myoepithelial cells (mc) in the neck region. Mitosis is seen in the cap and body cells (bd).

Source: (Daniel, 1987)
Branching patterns in the mammary gland. Two types can be distinguished: (1) dichotomous branching, and (2) "sprouting" morphogenesis giving rise to side branches and alveoli.

Source: (Brisken, 2002)
All of the progressive growth is aimed directly into the fat pad and continues until the fat pad is filled with a “simple ductal system”. Once the duct system has filled the fat pad, the cap cell layer disappears, then TEBs and side buds form AB as a result of differentiation (Brisken, 2002; Russo, 1989a). The mammary epithelium mediates ductile morphogenesis and possibly alveolar differentiation through cell-cell and cell-ECM signaling with all components of the stroma, including the fat pad and ECM. By weeks 10-12 of age, many TEBs have progressed to ABs, however a large number of TEBs in the virgin fail to differentiate upon nearing the edge of the fat pad. These TEBs regress to form small, finger-shaped terminal ducts (TD). The TDs do not undergo further morphological changes until pregnancy occurs. Figure 15 shows the underdeveloped ductal system within a virgin mouse and then the gland of a non-pregnant parous mouse.

The rodent gland progresses gradually from puberty until eventual completion in the adult. In the sexually mature adult virgin, the mammary parenchyma is composed of a highly organized system of ducts with TEBs that have the potential to become functional units capable of milk production. These functional units within the rodent mammary gland are referred to as lobulo-alveolar (LA) units that are comprised of TEBs and side buds (Russo, 1990). The mouse develops LA structures when stimulated by growth factors, estrogen and progesterone and during pregnancy. The rodent LA units are hormone responsive and comparable to TDLUs of the human mammary gland with appearance of the fully developed rat LA units identical to human TDLUs (Figure 16).

In the sexually mature rodent and human, branching of the gland continues proximal to the nipple, in the form of buds, as a result of ovarian hormone secretion. Hormones released with each recurrent estrous/menstrual cycle and during pregnancy elicit ductal sprouting or bud formation. Subsets of mitotically quiescent cells lining the ductal lumen initiate proliferation, generating a sprout through alveologenesis. Elongation occurs with some sprouts as they propel themselves into the fat pad at a right angle and others remain as small sprouted buds that elongate slightly while remaining a sac-like alveolus. Over several cycles of branching morphogenesis, the ducts that form through sprouting and successive elongation form end buds and cease to grow. These ducts are referred to as side branches and are typically spaced regularly along the originating duct, whereas alveolar budding seems to occur more randomly. Side branches are defined as buds that grow out over their
diameter and form end buds. The remaining ABs tend to cleave into two smaller alveoli, or form alveolar clusters, from one budding site, which is similar to what occurs at the end of dichotomous branching (Brisken, 2002).

**Pregnancy: Mammary Development During and After**

During pregnancy of all species, the mammary gland fills the entire fat pad with the elongating side branches and alveoli buds or clusters. The mammary alveoli start to form larger alveolar lobules in early pregnancy. During the second half of pregnancy, a rapid increase in the number and size of alveoli occurs resulting in the development of fully differentiated, secretory lobules (Pitelka et al., 1973) *(Figure 10b)*. Changes are accompanied by cellular differentiation leading to the development of secretory epithelial cells that are capable of synthesizing and secreting milk proteins for lactation. Continued development and differentiation occurs throughout pregnancy and lactation. After weaning, the mammary gland regresses and death of most of the functional cells occurs. This process leaves the gland similar in appearance to that of the pre-pregnancy gland but never the same (Furth, 1999) *(Figure 17)*. The early phases of involution are characterized by widespread apoptosis with fragmentation of nuclei, disintegration of cytoplasmic organelles and formation of "apoptotic bodies". Involution after weaning, like in normal development, generally occurs unevenly within in the gland. The fully regressed mammary gland may retain some of this unevenness as well as remnants of the preceding pregnancy.
Comparison of a nulliparous and parous mouse mammary gland.

A. A portion of the mammary gland of a parous, non-pregnant mouse. Parous mammary gland has scattered alveoli not present in the gland of the virgin animal. There are four hyperplastic alveolar nodules visible (HAN).

B. A mouse virgin, nulliparous, mammary gland with ductal end buds and no alveoli.

Source: (Cardiff and Wellings, 1999)
Figure 16

A. Subgross image of a human mammary duct with multiple TDLUs
B. Subgross image of a mouse mammary duct with multiple LA units.

Source: (Cardiff and Wellings, 1999)
Advancement of the mammary gland through involution is regulated by progressive gain of death signals and loss of survival factors followed by initiation of tissue remodeling.

Source: (Furth, 1999)
Diet and Breast Cancer

A wealth of medical research continues with the hope of discovering better ways to treat or prevent all cancers. For breast cancer, researchers and medical doctors still do not know its underlying biological mechanisms or an infallible means of preventing the disease. Hormonal factors along with reproductive history are known to modulate the risk of breast cancer, except these factors hardly have preventative potential. Unlike hormonal factors and reproductive history, diet is modifiable, but the relation of diet to breast cancer is not clearly established. However, the technology available to date is enough to greatly reduce high cancer incidence with dietary factors, which comprises a large portion of the research.

The foods we consume contain components that play a central role in either causing or protecting against many forms of cancer, including breast cancer. Epidemiological studies recommend consumption of dark green and cruciferous vegetables, tomatoes and citrus fruits to provide a reduction in cancer incidence. Advice given to individuals from the report, Food, Nutrition, and the Prevention of Cancer: A Global Perspective, provides the following recommendations of diet and lifestyle: choose a predominantly plant-based diet rich in vegetables and fruit, avoid being over- or underweight; maintain moderate activity level; eat 400-800g of vegetables and 600-800g of grains and legumes per day; minimal to no alcohol consumption; eat only 80g or less of red meat daily; limit consumption of fatty; salty and charred foods; and do not smoke or chew tobacco. Based on these guidelines, staying physically active and maintaining a nutritious diet and a healthy weight can reduce cancer risk by 30 to 40%. Following the recommended dietary choices with no use of tobacco has the potential to reduce cancer risk by 60 to 70% while simply eating more fruits and vegetables could alone reduce cancer incidence by more than 20% (AICR (American Institute of Cancer Research), 1997).

Currently, it still is not completely clear how much of the apparent risk reduction can be attributed to the nutrient or non-nutrient components of certain foods, and to what extent indirect effects, including parallel reduction in fat intake, increased fiber intake and vitamin and carotenoid intake, may be responsible for this protection. Epidemiologists believe 75 to 80% of human cancers are related to “non-genetic” factors, which provides evidence that poor diets may contribute to the
development of many cancers (Doll, 1992). A more current estimate (Willett and Trichopoulos, 1996) suggests that diet is responsible for 20 to 33% of all cancers occurring in highly developed countries.

Some migration studies provide strong indications that diet plays a role in the incidence of breast cancer. Migrant studies by Zeigler et al. (1993) found breast cancer incidence was not substantially changed for immigrants themselves, but it was changed for the second- and third-generations. The apparent delay in incidence may indicate childhood lifestyle factors such as diet to be a stronger influence on breast cancer risk than adult lifestyle factors.

The interaction of diet and breast cancer risk can be divided into two distinct categories. First being the effects of some major dietary components including alcohol use, dietary fat intake, and milk and other dairy product consumption. The second category involves the role of micronutrients, such as isothiocyanates in cruciferous vegetables, genistein in soy foods and $\alpha$-lactalbumin in milk, all acting as anti-carcinogenic agents.

**Major Dietary Components**

**Alcohol Consumption**

A positive dose-response correlation between alcohol consumption and breast cancer risk has been observed in premenopausal but not in postmenopausal women. An 11% increase in the risk of breast cancer was attributed to the daily consumption of one alcoholic drink when compared to nondrinkers (Longnecker, 1994). The consumption of alcohol risk poses a dietary conflict, because there are increasing reports that support moderate alcohol consumption provide cardioprotective effects (Rimm, 1996). Alcohol consumption is higher among Caucasian-American women (56%), the average consumption to be at least 6 times per year higher than for African-American (44%) and Latino (47%) women (Patterson, 1995). It is not known if the higher intake of alcohol among Caucasian-American women may partially explain their higher rate of breast cancer.
**Dietary Fat**

Breast cancer research in years past was primarily focused on the possible effects of fat intake within the diet. An increase in total fat intake may boost production of carcinogenic estrogens and other intestinally produced carcinogens, increase the total amount of body fat, and lower the age of menarche. Experimental studies in rodents dating back to the 1940s pointed to the ability of a high-fat diet to promote the development of mammary tumors, with the possibility that certain fats may be particularly effective promoters. Further research, especially for postmenopausal women, has specifically linked the percentage of calories from dietary fat, the type of fat consumed, and the total grams of fat in the diet to an increase in risk (Ewertz, 1990). Typically a diet with daily consumption of a large number of fat grams is associated with a large daily consumption of calories. Based on this knowledge, it is safe to conclude that breast cancer risk is related to the combined elements of dietary fat percentage, total grams of fat, and total calories consumed (Figure 18).

Differences in dietary fat consumption and the specific fatty acid content of these fats may partially explain international variations in breast cancer rates. The intake in specific fatty acids, specifically the ratio between n-6 and n-3 polyunsaturated fatty acids, may be connected to breast tumor incidence. An increasing ratio of omega-3 fatty acids to total omega-6 fatty acids in adipose tissue has been associated with a trend toward a decreasing risk of breast cancer (Simonsen, 1998). Increases in breast cancer incidence and mortality rates have been reported in several Asian countries. Along with these increases include a noticeable change in dietary habits of women in urban areas of Asia, one involving an increase in the proportion of total energy consumed from fat. In the 1950’s fat comprised only \( \approx 8\% \) of the typical Asian diet, whereas in the late 1980’s fat comprised \( \approx 32\% \) of their diets and is close to reaching the typical US proportion of \( \approx 35\% \) (Kodama et al., 1991). The increase in fat consumption by Asian women, and yet still a lower incidence of breast cancer compared to women in Western countries, may support evidence suggesting that isoflavones within the Asian plant-based diet could possibly function to provide a decreased risk of breast cancer.
Figure 18

Correlation between % calories as fat and breast cancer mortality.

Source: (Carroll, 1991)
Although the direct link between dietary fats to breast cancer is still highly debated, an indirect link may exist through estrogen. Some studies have found that lowering dietary fat lowers estrogen levels in the blood. Also, a high fat diet is associated with early menarche, which coexists with an elevated estrogen level, and contributes to an increased risk for breast cancer (Merzensch, 1993). An enormous amount of research has provided compelling evidence that circulating estrogens influence breast cancer risk (Hakansson et al., 1998). This knowledge has prompted studies that focus on the influence of dietary intervention on endogenous estrogen concentrations (Wu et al., 1999) and also reported reductions in serum estradiol among pre-menopausal and post-menopausal women as a result of dietary fat reduction. Some researchers found correlations between total fat intake and serum estrogen levels along with a correlation between monounsaturated fats and serum estrogens (Nagata et al., 2000).

Meat consumption, which serves as a source of dietary fat and animal protein, may be linked to increased breast cancer risk due to carcinogens, such as heterocyclic aromatic amines (HAAs). In particular, HAA’s produced during food preparation of red and fried meats (Ronco, 1996), are linked to breast cancer risk. Heterocyclic aromatic amines are produced during the cooking process of foods with high creatine, free amino acid, and sugar content, where it appears in the form of browned or charred surfaces on cooked muscle meats and fish. Ingestion of HAAs through alimentation may be involved in an increased risk of mammary gland and colon cancer development. The mutagenic potential by HAA is very high and multi-site tumors have been induced in rodents and non-human primates in colon cancer studies (Wakabayashi et al., 1992). HAAs have also induced mammary gland tumors in rat models as well (Snyderwine, 1994). De Stefani (De Stefani, 1997) found significant increasing trends between high levels of HAA exposure and breast cancer incidence. A variance in HAA intake between ethnic groups has been shown with the highest being in African-Americans, intermediate in Caucasians, and the lowest in Latinos and Asians (Patterson, 1995).

Consumption of fermented dairy foods, specifically probiotics, may modify the concentrations of intestinal flora. Probiotics are of interest among diet factors that could contribute to a decrease in human mammary gland and colon cancer potentially linked to HAAs. *In vitro* and *in vivo* studies have shown probiotics, specifically several lactic acid bacteria (LAB), to be among the diet factors that
could contribute to a decreased risk of human mammary and colon cancer. Lactic acid bacteria are present in fermented milk and demonstrate anti-mutagenic and anti-carcinogenic properties. There were protective effects from fermented milk and LAB (*Lactobacillus*, *Bifidobacterium* and *Streptococcus thermophilus*) against chemically induced colon carcinogenesis in rats found by Rao *et al.* (Rao *et al.*, 1999). An inhibition of colon tumor (100%), liver cancer (80%) and lowered multiplicity of tumors in F344 male rats fed a diet supplemented with 0.5% lyophilized *Bifidobacterium longum* was found in 1993 (Reddy and Rivenson, 1993). Within the same study, female rats, also supplemented with 0.5% lyophilized *Bifidobacterium longum*, suppressed mammary carcinogenesis 50% and inhibited mammary tumor multiplicity. A more recent study evaluated the prevention potential of fermented milk LAB *B. animalis* and *S. thermophilus* on HAA-induced colon carcinogenesis in rats (Tavan *et al.*, 2002). Their results demonstrated milk having an early protective effect against carcinogenesis with the effect being more pronounced in the case of milk fermented by LAB. Another study found significantly lower consumption of fermented milk products among 133 incident breast cancer cases as compared to 289 population controls (Vantveer *et al.*, 1989). Evidence from this study also suggested that a high consumption of fermented milk products might have a protective effect on the risk of breast cancer, which supports the previously presented hypothesis that fermented milk products may protect against breast cancer. Possible mechanisms for LAB include an interference with enterohepatic circulation or stimulation of immunological activity, but evidence to support either mechanism remains inconclusive. Although data has been presented in favor of fermented milk and some cheese products, no significant relationship was observed between consumption of milk and breast cancer.

Any possible links between breast cancer and fat intake continue to be highly debated. Contradictory to epidemiologic studies that indicate a risk from dietary fats, no correlation between dietary fat consumption and breast cancer risk was found (Holmes *et al.*, 1999). In 2000, researchers involved in a pooled analysis of cohort studies, concerning fat consumption and breast cancer risk, concluded that lowering the total intake of fat in midlife is unlikely to reduce the risk of breast cancer substantially (Hunter *et al.*, 1996).
**Dairy Products: Milk**

A large portion of current research has been directed specifically toward consumption of dairy products, which provides much of the fat intake in many Western countries. Dairy consumption also yields conflicting results regarding breast cancer incidence. Specifically, the role of milk is the most controversial of dairy products with some studies suggesting that milk is a risk factor and others suggesting milk consumption to be protective against breast cancer. The association between milk consumption and breast cancer mortality may be of essential biological significance. Many components in milk, such as growth factors and fatty acids, are hypothesized to play a role in breast cancer incidence.

Comparisons among earlier studies have suggested that milk consumption may contribute to the initiation or development of breast cancer. An earlier study measured and partially controlled the effects of dietary patterns, levels of wealth, reproduction patterns, genetic predisposition, and social and cultural patterns by examining the relationship between milk consumption and breast cancer mortality only within the United States (Gaskill et al., 1979). Results did show within the United States, age-adjusted breast cancer mortality correlates positively with the consumption of milk, butter, and total milk fat beyond childhood. It is suggested that the positive association between milk consumption and breast cancer mortality may be specifically related to milk fat and independent of other dietary and demographic variables. Only in a few cases, when milk demand was simultaneously controlled for certain combinations of the dietary and demographic variables, did the strength and significance of its association with breast cancer mortality weaken appreciably. The possibility still remains that these results could be interpreted as an indication that milk consumption is no more than a marker for a high-risk lifestyle which also includes a late first pregnancy, high income, living in a city, or eating a diet high in protein, fat, and/or calories.

The risk of early-onset breast cancer related to diet during adolescence was analyzed (Potischman et al., 1998). The participants of this study were from three geographical regions covered by cancer registries: Atlanta, GA; Seattle/Puget Sound, WA; and central New Jersey. Case patients, n=1647, were newly diagnosed with breast cancer, and control subjects (n=1501) were chosen randomly. During an interview, each subject was asked to recall the frequency of
consumption and portion size of 29 key food items, including whole milk, ice cream, butter and cheeses, at ages 12-13 years. Comparison of high versus low quartiles of consumption suggested a reduced risk associated with high consumption of fruits and vegetables, although this finding was not significant. Also, the intake of animal fat, high-fat foods, high-fat snacks and desserts, or dairy products during adolescence had no apparent influence on breast cancer risk. Overall, the data provided by Potischman provides no evidence for a strong influence of dietary intakes during adolescence on risk of early-onset breast cancer.

A more recent population–based study (Hjartaker et al., 2001) examined milk consumption of individuals, both as a child and as an adult, and any possible association with breast cancer incidence. These researchers reported 317 incident cases of breast cancer within the cohort study of 48,884 premenopausal Norwegian women following a mean follow-up time of 6.2 years. Milk consumption as a child was negatively associated with subsequent breast cancer among the young women (34-39 years), but not among older ones (40-49 years). Adult milk consumption was negatively related to breast cancer incidence after adjustment for age, reproductive and hormonal factors, body mass index, education, physical activity, and alcohol consumption. Their analyses based on the type of milk and milk fat consumed did not reveal any clear associations. A combination of childhood and adult milk consumption produced a clear negative trend in breast cancer incidence with increasing milk consumption. No significant association between only childhood milk consumption and breast cancer incidence was found in this study. However, an inverse dose-response association between milk consumption of a population consuming large amounts of milk and premenopausal breast cancer incidence was found.

**Micronutrients**

Increasing evidence supports a variety of micronutrients within food that appear to have significant anti-cancer activity. Several micronutrients and hormones may act as protective agents against a wide variety of cancers including hormonally related cancers like breast cancer (Bradlow and Sepkovic, 2002). Micronutrients comprise a diverse group of compounds, ranging from indole derivatives, like indole-3-carbinol, and isothiocyanates, such as sulforaphane, both found as glucosinolates in cruciferous vegetables, catechins found in green tea, the isoflavone genistein in soy
foods, and α-lactalbumin within milk. A search to develop diet-based chemoprotection has increased as a result of the apparent association between consumption of micronutrients and cancer incidence.

**Glucosinolates**

A portion of research has focused on micronutrients within fruits and vegetables thought to be responsible for anti-cancer effects, which includes vitamin C, β-carotene and phytochemicals such as glucosinolates and lycopene. Cruciferous vegetables including broccoli, kale, cabbage, and cauliflower are especially rich in glucosinolates, which are converted by plant myrosinase and gastrointestinal microflora to isothiocyanates (Figure 19). Many isothiocyanates are potent inducers of phase 2 detoxification enzymes in mammalian tissue, with the most potent inducer of these enzymes found thus far being isothiocyanate sulforaphane, isolated from broccoli (Zhang et al., 1992). Phase 2-detoxification enzyme induction is a highly effective way of reducing susceptibility to carcinogens. Phase 2-detoxification enzymes (e.g. glutathione-S-transferase, quinone reductase, and epoxide hyrolase) inactivate reactive carcinogens by destroying their reactive centers or by conjugating them with endogenous ligands and facilitating their elimination from the body (Talalay, 1992) (Figure 20). Most all of the inducer activity from crucifers, functioning to activate the phase 2-detoxification enzymes, is due to isothiocyanates and the hydrolysis of glucosinolates by myrosinase. Overall, one study indicated that Americans are consuming more fruits and vegetables than in the past, but dark green and cruciferous vegetable consumption remains low (Johnston et al., 2000).
Conversion of glucosinolate to isothiocyanate by myrosinase enzymes

Source: (Fahey and Stephenson, 1999)
Role of Phase 2 enzymes in deactivation of reactive carcinogens. Susceptibility to carcinogen damage is controlled by balance between Phase 1 enzymes that activate and Phase 2 enzymes that detoxify carcinogens. (ROS = reactive oxygen species; Phase 1 enzymes are typically cytochromes P-450; Phase 2 enzymes are typically glutathione transferases).

Source: (Fahey and Stephenson, 1999)
Genistein

Isoflavones affect many hormone-dependent and hormone-independent conditions, including cancer, menopausal symptoms, cardiovascular disease and osteoporosis. An enormous amount of research has focused on isoflavones found in soy products. There are many other phytochemicals in soybeans, but isoflavones still seem to be the key phytochemical responsible for anti-cancer effects. Isoflavones are considered phytoestrogens found in plants, particularly in soybeans, and Walz determined isoflavones to be highly polar (water-soluble) glycosides ((Walz, 1931). Based on in vitro and animal studies, isoflavones found within soy foods are believed to be the active micronutrient causing the reduction in breast cancer incidence. A high dietary intake of soy foods has been associated with a reduction of breast cancer incidence in premenopausal women. Phytoestrogens are non-steroidal chemicals behaving in such a way that it mimics the functions of naturally occurring endogenous estrogens and compete with endogenous estrogens for estrogen receptor (ER) binding sites. In 1988, Setchell and Adlercreutz discovered the similarity in chemical structure of isoflavones to mammalian estrogens (Figure 21). One study found consumption of soy to significantly reduced 17β-estradiol, the key estrogen metabolite linked to breast cancer in premenopausal women (Lu et al., 1996). Results from a more recent study found soy isoflavones to lower circulating 17β-estradiol levels and increase levels of the protective, anti-carcinogenic metabolite 2-hydroxylated estrogen and in turn lowering the long-term risk for breast cancer (Lu et al., 2000a).

Low breast cancer mortality rates along with a high soy diet, found in Asian countries, has initiated breast cancer research that focuses on discovering the link between soy intake and cancer risk. Another study showed a reduced number of 7,12-dimethylbenz (a) anthracene (DMBA)-induced mammary tumors observed in rats fed soy-based diets (Barnes, 1990). An epidemiologic study in Singapore found a decrease of breast cancer risk in premenopausal women that had regular consumption of soy foods, but not in postmenopausal women (Lee et al., 1991). A Japanese study noted tofu intake to be associated with decreased risk of breast cancer in premenopausal women, but not in postmenopausal women (Hirose et al., 1995). A case-control study in the United States found tofu consumption to be protective in both premenopausal and postmenopausal Asian women (Wu et al., 1996).
Comparison of the chemical structures of endogenous estrogen with the dietary estrogen, isoflavones.

Source: (Setchell, 1998)
However, the protective effects seen by this study were of Asian women born in Asia who had migrated to the United States. The findings by Wu indicate that tofu, or soy product, intake may not per se be the protective determinant, but rather its consumption possibly reflects a protective lifestyle common to women of Asian ancestry born in Asia but not those born in the United States.

A proactive approach with early consumption of soy may interestingly be the key to these protective effects and explain the discrepancy in protection seen between Asian natives and those born in the United States that only have Asian descendants. Lamartiniere et al. (Lamartiniere et al., 1995a) suggested early exposure of soy by young girls could potentially reduce cancer risk later in life. This theory was tested in rodents and found that when rodents were given genistein, an isoflavone, subcutaneously during the neonatal and prepubertal growth stages, 7,12-dimethylbenz (a) anthracene (DMBA) -induced mammary tumors were inhibited and of those tumors that did develop the latency period was increased. The research by Lamartiniere and others may further explain and support the findings of Wu. Epidemiologic data is inconclusive with relatively little to support any association between soy intake and a decreased risk of postmenopausal breast cancer and only a limited amount of data suggests a correlation between soy intake and a decreased risk of premenopausal breast cancer. Ironic to all the data provided which support soy for reduction of breast cancer risk, there is also a concern that soy consumption could be detrimental to women with estrogen-sensitive breast cancer, or to women at a high risk of developing breast cancer. The relationship between soy intake and breast cancer risk is still undoubtedly one of the most confusing areas of soy research.

\textit{\textbf{α-Lactalbumin}}

In addition to the micronutrients found in milk that provide biological health benefits, protein components of milk are also being studied for potential anti-tumor properties. Epidemiological studies have shown that breastfeeding may protect the mother against breast cancer (Davis, 1988), supporting the hypothesis that milk contains molecules with anti-tumor activity. The milk proteins lactoferrin, β-lactoglobulin and α-lactalbumin (α-LA) are the most studied proteins that appear to possess alternate functions in addition to their biological roles making milk a nutritious fluid. In
particular, the whey protein, α-LA may also function as an effective growth regulator and anti-

α-Lactalbumin is produced and secreted by the mammary gland of all mammals and its
specific biological function is biosynthesis of the milk sugar lactose and as a result, any milk
containing this sugar contains α-LA (Jenness, 1979). It is a metalloprotein with a high affinity for Ca²⁺
and other divalent cations (Kronman et al., 1981), a molecular mass of 14kDa, and an isoelectric
point of 5.0 (Thompson et al., 1992). The presence of Ca²⁺ is essential for the folding and structural
stability of α-LA (Musci and Berliner, 1985).

Favorable anti-growth properties of α-LA may be linked to the production of glutathione,
which are involved in either regulation of cell proliferation, removal of free radicals or detoxification of
carcinogens. The specific mechanism by which α-LA operates may be through the glutathione anti-
oxidant enzyme glutathione-S-transferase. Studies have linked the methionine and cysteine sulfur
amino acid components α-LA as the causative factor that may aid in suppression or reduced tumor
incidence because these amino acids are required for glutathione production (McIntosh, 1995;
McIntosh and PK., 1998; Parodi, 1998).

**Soy Consumption, Genistein and Decreased Breast Cancer Incidence**

Populations consuming diets rich in soy, such as the Japanese, have low breast cancer
mortality rates compared to women in Western areas like the United States that consume a typical
Western diet (Ziegler et al., 1993). This observance, in conjunction with data showing weak
estrogens in soy can function as anti-estrogens, has prompted speculation that soy may reduce
breast cancer risk. Many researchers have investigated the possible link between ethnicity and
breast cancer incidence, predominantly focusing on Asian women because they have a relatively low
incidence of breast cancer. Asian diets are highly plant-based including many soy foods like tofu,
soymilk, green soybeans, soybean sprouts, miso, tofu skin, and fermented soybeans. Western diets
are typically low in grain and high in fat, dairy and red meat (Maskarinec and Meng, 2001). The
typical Asian diet is the opposite and is characterized by a high cereal, low fat, low dairy and low red
meat intake.
**Soy Consumption and Genistein**

Soy foods contain isoflavones, phyto-chemicals, which act as weak estrogens. Phyto-chemicals like diadzein and genistein are isoflavones possessing between $10^{-5}$ and $10^{-2}$ of the activity of 17β-estradiol on a molecular basis (Markiewicz et al., 1993). The precursor for diadzein and genistein originates primarily in soybeans, but also is found in whole grain cereals, seeds, berries and nuts. Typical isoflavone content within soy foods is 0.2 – 0.4 mg/g of fresh product and 2 - 4 mg/g protein (Murphy et al., 1999). The average daily dietary intake of isoflavones in Western populations is very minimal (<1 mg/d), whereas in Japan and China it ranges from 20-50mg/d (Nagata et al., 1998a). Physiologic effects in humans consuming soy foods are likely, because as a result of dietary supplementation isoflavone levels are 100–1000 times higher than endogenous estrogen levels (Probst-Hensch et al., 2000). Regular soy consumption caused plasma isoflavone levels to exceed normal plasma estradiol concentrations, which range between 40- 80 pg/ml (Setchell et al., 1984). Consumption of isoflavones ($\leq$ 100mg) within the diet yields serum isoflavone concentrations of 50-800ng/ml, similar to values found in Japanese people (Adlercreutz et al., 1993a). Following consumption of soybean isoflavonoids, metabolism results in the formation of estrogen-like phyto-estrogen compounds diadzein, genistein and glycitein, which are further metabolized to metabolites, including equol and p-ethylphenol (Axelson et al., 1984) (Figure 22). Phyto-estrogen metabolites circulating in the body can bind weakly to estrogen receptors in hormone-dependant tissues, like the breast, and provide potential anti-oxidant, anti-estrogenic, anti-mutagenic, anti-proliferative, transformation-inhibiting, and apoptosis-inducing effects. All of these effects make isoflavones strong candidates for being natural cancer preventing compounds.
Chemical structures of the soy isoflavones: **A**, genistein; **B**, diadzein; **C**, glycitein.

Source: (Barnes, 1997)
Timing of Exposure to Genistein

Some studies report the need for soy-rich diets to begin earlier in life, as in Asian cultures, to provide chemopreventative results (Fritz et al., 1998; Lamartiniere, 2000; Murrill et al., 1996). Anticarcinogenic effects are seen in Asian women who have eaten soy their entire life. It is not certain that the same beneficial results are attained if soy consumption begins later in life. Several investigators researched the protective benefits of genistein through in vivo studies where genistein was either given by injection or diet, all having very similar results. Pharmacological doses of genistein were given by injection to neonatal, 5mg/rat (Lamartiniere et al., 1995b), and prepubertal, 500µg/g body weight (Murrill et al., 1996), rats. Another study administered physiologic concentrations of genistein (25mg/g body weight) via the diet from conception to 21d postpartum, at which time total genistein concentrations in the serum were 1.8µmol/L (Fritz et al., 1998). After administration of 7,12-dimethylbenz (a) anthracene (DMBA) on postpartum d 50 within all of these studies, mammary tumorigenesis was reduced by ∼50%. DMBA-induced mammary cancer and tumor multiplicity were significantly reduced in an additional study in which ∼1mg/kg body weight of genistein was administered to prepubertal rats between day 7 and 20 (Hilakivi-Clarke et al., 1999). Genistein given to neonatal and prepubertal rats altered their mammary gland development and rendered the adult rat less susceptible to chemically induced mammary cancer (Lamartiniere et al., 1998a).

Russo and Russo proposed a mechanism for the protective effects of early genistein exposure (Russo and Russo, 1978). Early exposure to genistein may stimulate cell proliferation and differentiation of TEBs in the mammary gland resulting in enhanced mammary gland maturation by way of lobule formation. TEBs are the least differentiated and the most susceptible of the mammary structures to carcinogenic action, whereas lobules are the most differentiated and least susceptible to carcinogenesis. Proliferation to form more lobular structures is a basic and protective mechanism against chemical carcinogenesis. One study supported this theory by determining that genistein exposed rats had higher numbers of differentiated alveolar buds, and fewer terminal ducts in DMBA-treated mammary glands (Hilakivi-Clarke et al., 1999). Protective effects obtained by early exposure to genistein are similar to those resulting from exposure to estrogen and progesterone during
pregnancy at an early age. These findings are consistent with the observation that early full-term pregnancy reduces the risk of breast cancer, because pregnancy also initiates gland differentiation in a similar way.

Dietary effects of genistein, at 250mg/kg, on mammary and prostate cancers in rats have been studied (Lamartiniere, 2002). Rats treated during prepubertal and combined prepubertal/adult phases of development had mammary cancer chemoprevention whereas those treated after the prenatal stage or upon reaching adulthood did not. In the prostate, genistein also reduced cancer incidence and down-regulated androgen receptor, estrogen receptor-\(\alpha\) (ER-\(\alpha\)), epidermal growth factor receptor (EGF-R), insulin-like growth factor-I (I-LGF), and extracellular signal-regulated kinase1. Their study reinforces the idea that early exposure to genistein is a key factor for cancer prevention. They determined that genistein only had anti-carcinogenic effects when given to adult rats if the adults had been exposed to genistein early in life and the chemoprevention occurred possibly through regulation of specific sex-steroid receptors and growth factor signaling pathways.

Genistein has also been studied in humans for possible early exposure effects. Most studies have focused on native Asians, Asian immigrants and descendants of Asian immigrants due to an apparent decrease of cancer protection from the second or third generation of the immigrants. Ziegler found Asian-American women that were born in the United States had a breast cancer risk higher than Asian-American women born in Eastern countries of China and Japan (Ziegler et al., 1993). Of the Asian immigrants descendents, risk was determined by whether their grandparents, particularly the grandmothers, were born in the Eastern countries or in the United States. Risk was 50% higher for Asian-American women with all grandparents born in the United States than for women with all grandparents born in Eastern countries. Ziegler projected exposure to lifestyles in the United States affected the risk for breast cancer for Asian immigrants over the remainder of their lifetime. More recent studies by Maskarinec and Meng determined that exposure to phyto-estrogens prior to adolescence is important for breast development and also for reduced breast cancer risk later in life (Maskarinec and Meng, 2001). Adult Asians who immigrate to Western countries do still maintain a lesser incidence of breast cancer development, but an increased risk of breast cancer does appear for their Asian-American descendants after two or more generations.
The noticeable loss of protection for Asian immigrants is still not well understood, however the loss does point to dietary effects. Recent data found breast cancer incidence rates to be 30.8 cases/100,000 women in Japan compared to 78.3 and 97.1 cases/100,000 for Hawaiian women of Japanese and American descent, respectively (Maskarinec et al., 2002). This study also found that risk increased over 2 or 3 generations for Asians who immigrated to Hawaii, suggesting that environmental factors, like diet, may be involved in the loss of protection. Maskarinec further investigated the possible link between diet and modulation of breast density, a possible marker for breast cancer risk. Mammographic density patterns refer to the distribution of fat, connective and epithelial tissue within the healthy female breast. The study involved a comparison of mammographic densities between three groups of women, native Japanese, Hawaiian women of Japanese decent, and women of American decent, all being at different levels of cancer risk. Breast size among women of Japanese ancestry was similar in Hawaii and Japan but much smaller than that of Caucasian-American women. Dense areas within the breast were smallest among women in Japan, followed by Japanese women in Hawaii, with the largest dense areas found among Caucasian-American women. Percent densities were greater in Japanese women than Caucasian-American women, possibly due to larger breast size in Caucasians. Although, percent densities among Japanese women were significantly lower for those living in Japan than among third- and fourth-generation Japanese immigrants in Hawaii. Totally, results show that size of the total breast differs primarily by ethnicity, and size of the dense areas differs by place of residence. Mammographic density is a useful indicator of breast cancer for American-Caucasian women in the United States. However, relative density is not the best risk marker for Japanese women since they have distinctly different physical proportions. Due to this difference, specific size of the dense areas still represents the extent of breast tissue at risk and may be a better indicator of breast cancer risk across ethnic groups than is percent densities (Maskarinec et al., 2002). The possibility exists that differences in hormonal patterns may cause the differences in mammographic densities between Japanese and American-Caucasians. Asian women have been found to have lower levels of circulating estrogens than Caucasian women (Macmahon et al., 1971).
Comparison of Genistein to Estrogen

Lower levels of estrogen in Asian women may be in response to dietary soy. Phyto-estrogens act as weak estrogens. Since they are heterocyclic phenols similar in structure to natural estrogens they demonstrate estrogenic ability and are particularly important with respect to breast cancer. Estrogen plays an important role in normal mammary gland development and is also believed to be a significant factor in tumorigenesis. Length of exposure to estrogen seems to increase risk; therefore certain anti-estrogens such as the drug tamoxifen and the isoflavone genistein (the main isoflavone in soybeans), can decrease risk. Genistein may suppress or inhibit normal estrogen secretion or normal estrogenic activities in estrogen-responsive tissues, such as in the breast. Because of these functions, genistein may reduce breast cancer risk even though it is actually less estrogenic to breast tissue than other tissue (Wu et al., 1998). The decreased risk is seen in animal models and in vitro experiments where isoflavones appear to act as anti-estrogens, with anti-estrogen effects mediated by competition with endogenous estrogen metabolite 17β-estradiol for estrogen receptors (Cassidy, 1996).

Estrogenic and Non-Estrogenic Properties of Genistein In vivo

Structures of isoflavone metabolite equol and endogenous estrogen estradiol are very similar and when overlaid, they appear to be superimposed (Figure 23). As a result of this similarity, isoflavones can bind to estrogen receptors and affect estrogen-regulated gene products (Markiewicz et al., 1993). Isoflavones are tissue selective and may have very different estrogen-like effects in different tissues. For example, estrogenic effects by isoflavones are seen in coronary vessels but not in other tissues like endometrium. This may be partially due to higher binding affinity of isoflavones for estrogen receptor-β (ERβ) compared with estrogen receptor-α (ERα) (Kuiper et al., 1997). Distribution of these receptors among different tissues may result in tissue selectivity of isoflavones (Cassidy and Faughnan, 2000) (Figure 24).

Isoflavones, particularly genistein, appear to display dual functions. When placed in a high-estrogen environment, such as in premenopausal women, genistein appears to antagonize and inhibit estrogen actions, and in a low-estrogen environment, such as in postmenopausal women, it has
estrogenic effects. Dual effects of genistein are possibly dependent on competing estrogen concentrations and physiological factors such as receptor number, occupancy, and location. Several \textit{in vitro} studies have assessed the estrogenic and antiestrogenic potential of genistein with many of them concluding that genistein functions mainly as an estrogen agonist, providing the tissue with similar effects presented by estrogen (Dees et al., 1997; Zava and Duwe, 1997). Contradicting results found genistein to function dually as an estrogen agonist and antagonist (Miodini et al., 1999).

The first \textit{in vivo} study showing genistein to possess anti-estrogenic activity was published in 1966 (Folman and Pope, 1966). In their study, genistein was injected subcutaneously into female mice where it inhibited estrone stimulation of uterine growth. More recent studies have validated genistein functioning as either an agonist or antagonist. One study monitored uterine weights in ovariectomized mice that were fed a soy diet and either received or did not receive synthetic estrogen. Uterine weights increased in ovariectomized mice that were not given a synthetic estrogen but decreased in mice given the synthetic estrogen (Makela et al., 1995). Other studies have found no definitive evidence suggesting genistein exerts anti-estrogenic effects in humans. However, a recent example of \textit{in vivo} anti-estrogenic activity was found in adult, surgically postmenopausal female macaques (Foth and Cline, 1998). The consumption of an isoflavone-rich soy protein inhibited stimulatory effects of estrogen on mammary cell proliferation.
Figure 23

Comparison of isoflavone metabolite equol structure to estradiol.

Source: (Setchell and Cassidy, 1999)
Figure 24

Anatomical distribution of estrogen receptors, ERβ and ERα.

Source: (Setchell and Cassidy, 1999)
*In vitro* Anti-Carcinogen Effects of Genistein

Endogenous estrogens have been found to have a dual, dose-dependent effect on mammary tumor induction and growth. Large doses of estrogen inhibit tumor development and suppress growth of established tumors, whereas lower, more physiological doses of estrogen stimulate tumor growth. Like estrogen, genistein has been found to have both estrogenic and anti-estrogenic effects at varying concentrations (Jordan, 1990).

Early *in vitro* studies found that genistein stimulated growth of estrogen-receptor positive MCF-7 human tumor cells by acting through the estrogen receptor sites (Martin et al., 1978). Others suggested that the stimulation rather than inhibition found in past studies was dose-dependent and different doses of genistein resulted in either growth inhibition or stimulation within the same breast cancer cells (Molteni et al., 1995). More recently, multiple *in vitro* studies have found genistein, at concentrations <10 µmol/L, to stimulate growth of estrogen-receptor positive MCF-7 human tumor cells (Hsieh et al., 1998; Miodini et al., 1999; Zava and Duwe, 1997).

Genistein also inhibits *in vitro* growth of hormone-independent cancer cells including: colon (Kuo, 1996); skin (Rauth et al., 1997); hormone-dependent cancer cells within such tissues as the breast with genistein values between ~10 to 50 µmol/L (Peterson and Barnes, 1991); prostate (Peterson and Barnes, 1993). Metastatic activity in breast (Scholar and Toews, 1994) and prostate (Santibanez et al., 1997) tissue has also been inhibited by genistein.

The significance of cancer effects of genistein within *in vitro* studies has been questioned. It is uncertain that *in vivo* serum concentrations of free genistein would reach the low micromolar range needed for inhibitory effects seen *in vitro*, even in people consuming abundant amounts of soy (Adlercreutz and Mazur, 1997). Genistein may actually be more potent *in vivo* than *in vitro* and yield similar results as found with *in vitro* studies, just at lower, more physiological concentrations. Dietary genistein at 0.25mg/g has been found to have an anti-cancer effect in rats despite free genistein concentrations in serum and tissue of only 18.4nmol/L and 17.5pmol/g, respectively (Dalu et al., 1998).

A direct effect of genistein on angiogenesis within tumors through inhibition of endothelial cell proliferation has also been tested. The inhibition of angiogenesis may attribute to the delay in tumor
appearance and may account for antimetastatic activity (Weidner et al., 1991). *In vitro*, genistein gave half-maximal inhibitory effects on angiogenesis at a concentration of 10 µmol/L (Fotsis et al., 1997). An *in vivo* study also reported inhibition of angiogenesis in mice injected subcutaneously with genistein at doses of 0.1, 0.2, or 0.5-mg/kg body weight (Shao et al., 1998). The inhibition of tumor growth and in turn angiogenesis was dose-dependent with 40% reduction in tumor volume at the maximum concentration of 0.5 mg/kg.

**Effects of Genistein on Mammary Tumorigenesis**

Development of chemically induced tumors has been studied *in vivo* to determine the effects of different soy products. Not all reports agree, but the data from several studies showed a 25 – 50% reduction in tumor incidence, compared to control rats when the typical protein, casein, was replaced with soy protein in the diet (Barnes, 1990; Hakkak, 2000). Other studies showed weaker protective effects where soy protein reduced chemically induced tumor incidence relative to casein, but inhibition was only about half of that reported for whey protein (Hakkak, 2000). Biochanin A, the precursor for genistein, also resulted in inhibitory effects with a significant decrease of tumor multiplicity when given at a low dose of 10mg/kg and a decrease in tumor incidence when given at a higher dose of 50mg/kg (Gotoh et al., 1998).

Tumor progression has also been studied, primarily for the benefit of breast cancer survivors. These studies focus on prevention of further tumor development in cancer survivors. In one study, rats were fed a casein-based diet and then injected with a mammary carcinogen. When the first tumors grew, they were surgically removed as to mimic a similar process taken in women cancer patients. At this point, half of the rats remained on the casein-based diet while the other half began a soy isolate diet. The soy rats developed significantly fewer tumors and histopathological analysis found tumors to be more aggressive in the casein-fed rats than in the soy-fed rats (Hawrylewicz et al., 1995).

Opposite findings were reported that may make breast cancer survivors want to avoid soy completely (Hsieh et al., 1998). In ovariectomized athymic mice, stimulated growth of subcutaneously implanted MCF-7 cells were reported after the mice were given dietary genistein
Another study using a similar experimental design found genistein at various amounts (15, 150 and 300µg/g) within isolated soy protein to stimulate tumor growth in a dose-dependent manner (Allred et al., 2001).

Further research, with respect to dosage and reproductive status of the female, supports possible dual effects of genistein. Some researchers reported tumor inhibition in intact nude mice fed estrogen pellets when given the equivalent of 900µg/g of genistein in the diet (Shao et al., 1998), which is similar to the 750µg/g doses used by other researchers (Hsieh et al., 1998). These studies help validate genistein being estrogenic and exerting a proliferative effect on breast tissue when in a low estrogen environment, similar to postmenopausal women and breast cancer patients with chemotherapy induced menopause, and anti-estrogenic in a high estrogen environment existing in premenopausal women. All of these findings are important, as they indicate that the advice given to women concerning dietary soy supplementation should depend on their own personal reproductive status.

Mechanisms of Action for Genistein

There have been several proposed mechanisms of action for genistein. Some of these methods include estrogen receptors β and α, alteration of estrogen metabolism and through signal transduction.

Estrogen Receptors

Early studies focused on the anti-estrogenic activity, finding genistein to have the ability to bind in vitro to sheep uterine estrogen receptors (ER) and human breast cancer estrogen receptors (Martin et al., 1978; Shutt and Cox, 1972). Both studies suggested the chemopreventative function presented by genistein was possibly the result of interference at the ER level in the tumor-promoting effect of estrogen. However, it seems that genistein exhibits both estrogenic and anti-estrogenic effects depending on the tissue type in which it binds and the reproductive state of the female. This may be a direct result of two different estrogen receptors, ERβ and ERα. Some researchers believed ERβ to be easier to antagonize than ERα (Pike et al., 1999). Opposing data indicated genistein to be
a potent agonist for ERβ (An et al., 2001). The conflicting transcriptional activity of estrogens and isoflavones results not only from different binding affinities, but also their ability to take on co-regulators and trigger transcriptional functions of ERβ and ERα. Within breast carcinogenesis, determining of the role of ERβ may be a critical link for understanding the effects of genistein intake on breast cancer risk.

Since endogenous estrogen metabolite, 17β-Estradiol, and genistein metabolite, equol, have similar structures, they compete for binding with ERβ and ERα. 17β-Estradiol is the most abundant estrogen metabolite and an important breast mitogen shown to increase epithelial cell proliferation. Therefore, blockage of estrogen receptors with weak estrogen-like compounds, like genistein, prevents endogenous estrogens from binding to receptor sites within the tissue and as a result lessens the tissues exposure to 17β-estradiol. Most genistein circulates the plasma conjugated to glucuronic acid, although the binding is weaker than that of estrogen to serum proteins. This may benefit genistein, in its competition with estrogen, by increasing the availability of genistein for binding.

In addition to competition, genistein has been shown to down-regulate estrogen receptors (Sathyamoorthy and Wang, 1997). Fritz et al. studied responses of genistein in the rat prostate and also found genistein to down-regulate the expression of ERβ and ERα using 25mg or 250mg/kg of genistein in the diet of the pregnant females for neonatal exposure, and 250mg or 1000mg of genistein/kg for exposure in the adult males (Fritz et al., 2002). These concentrations were chosen as comparable concentrations to those found in humans on a soy diet.

**Alteration of Estrogen Metabolism**

Estrogen is metabolized first into estrone, then to 17β-estradiol in the metabolic pathway and is further metabolized into estriol (Figure 25). The metabolite 17β-estradiol is an important factor to consider in breast cancer studies since its regulation of breast cell proliferation could potentially lead to the promotion of breast cancer cell growth. The metabolism of 17β-estradiol occurs mainly in the liver to 2-, 4-, and 16α-hydroxylated estrogens, all having different capacities to influence mammary
tumorigenesis (Schneider et al., 1982). 2-Hydroxylated estrogens are suggested to be anti-
carcinogens, unlike 4- and 16α-hydroxylated estrogens, which may act carcinogenic and enhance
cancer development (Bradlow et al., 1996; Telang et al., 1992).
Figure 25

Metabolism of estrogen, E1, estrone; E2, 17β-estradiol; E3, estriol

Source: (Lu et al., 2001)
It has been suggested that higher levels of 4- and 16α-hydroxylated estrogens and lower levels of 2-hydroxylated estrogens are a risk indicator for breast cancer. A study by Lu et al. found that consumption of soy for one month significantly reduced 17β-estradiol and progesterone in premenopausal women (Lu et al., 1996). A follow up study hypothesized that consumption of a soy diet containing weak estrogens genistein and diadzein may alter the metabolism of 17β-estradiol to 2- and 16α-hydroxylated estrogens. The study provided 113-202mg/day of isoflavones through the diet, and urine samples were collected during the testing period to determine excreted amounts of diadzein (11.6-39.2 mg/day) and genistein (2.9-18.2 mg/day). Over the testing period, isoflavone consumption increased the urinary excretion of 2-hydroxyestrone from 11.6 ± 2.06 to 17.0 ± 2.96 nmol/12-h (P=0.03), but there was no change in excretion of 16α-hydroxyestrone (Lu et al., 2000a). This study suggested that soy isoflavones do increase the metabolism of the protective estrogen metabolite 2-hydroxylated estrogens in women.

**Signal Transduction**

Despite all of these possible anti-estrogenic mechanisms, direct interaction with an estrogen receptor does not have to occur in order for isoflavones to show hormonal and anti-estrogenic effects. Genistein also influences signal transduction by inhibiting enzymatic action. Recently, research has focused on the ability of genistein to inhibit the activity of key enzymes, such as tyrosine protein kinases (TPK) and their receptors (receptor tyrosine kinases -RTK), and mitogen-activated protein (MAP) kinase (Thorburn and Thorburn, 1994). Genistein specifically inhibits TPK by competing with ATP rather than with the protein substrate, indicating the anti-proliferative effect is as a result of interference in the tyrosine kinase cascade activated by mitogens (Akiyama et al., 1987). Many oncogenes produce protein products such as intracellular proteins that catalyze tyrosine phosphorylation or membrane-bound receptors with tyrosine kinase activity (Hunter, 1984).

Peterson and Barnes proposed that genistein prevents the initiation of cancer cells rather than inhibiting the growth of already existing cancer cells (Peterson and Barnes, 1991). The mechanisms for genistein in this scenario is also thought to result from inhibiting the actions of enzymes involved in signal transduction, like tyrosine kinase. Tyrosine kinase enzymes play an...
important role in cell proliferation by down-regulating receptors for epidermal growth factor (EGF) (Dalu et al., 1998) and possibly insulin-like growth factor-1 receptors (I-LGF-1). Both of these growth factors are associated with breast cancer oncogene expression (Lecam, 1991; Lehtola et al., 1992). Genistein is a specific inhibitor of tyrosine autophosphorylation of the epidermal growth factor receptor (EGF-R), preventing release of EGF (Kim et al., 1998). Genistein directly stimulates EGF-receptor in prepubertal rats resulting in enhanced mammary gland differentiation. The differentiated cells are then programmed for a down-regulated EGF-signaling pathway in TEBs of adult mammary glands. Decreased EGF-receptor expression during carcinogen exposure may possibly explain the anti-tumor effects observed after early exposure to genistein (Brown et al., 1998).

*In vitro* studies have shown that isoflavones inhibit the activity of enzymes involved in estrogen metabolism as well. Isoflavones inhibit the 17β-oxidoreduction of estrogens (Makela et al., 1998). The enzyme, 17β-oxidoreductases, is present in target tissues of estrogen and converts the weak estrogen estrone into the more potent form, estradiol. The inhibition of this enzyme occurs at relatively low micromolar concentrations of isoflavone and is important at the tissue level since locally generated estrogens are thought to be the driving force behind estrogen-sensitive mammary tumors.

Genistein also alters signal transduction by influencing cellular factors that control the growth of cells (Constantinou et al., 1990), such as transforming growth factor beta-1 (TGF-β1), which is thought to cause inhibition of cancer cell growth (Constantinou and Huberman, 1995). Genistein has been found to increase the *in vitro* concentrations of TGF-β1 (Peterson et al., 1998) and others suggested the mechanism of genistein to involve, or possibly require, TGF-β1 signaling (Kim et al., 1998). Modification of cellular molecules that control signaling, growth, and death may also explain the dual effects of genistein, where low, more physiological concentrations at <10μmol/L stimulate growth and higher concentrations from 10 to 50μmol/L inhibit growth.

**α-Lactalbumin**

Milk is not only a nutrient source, but also considered a biological fluid. Milk contains a wide variety of molecules with anti-microbial activity, antibodies to bacterial, viral and protozoal antigens (Redhead et al., 1990); bacteriocidal molecules including lysozyme and lactoferrin (Lonnerdal, 1985);
fatty acids that lyse bacteria and viral particles (Sarkar et al., 1973); and glycoconjugates that inhibit bacterial adherence to epithelial cells (Svanborg, 1991). These components function to protect the breast–fed infant from infection caused by a broad range of mucosal pathogens.

**Whey Proteins**

Milk also contains a diverse mixture of proteins, particularly in the whey portion that remain after the casein fraction is precipitated out that provide autocrine/paracrine regulation of growth and differentiation of the mammary gland. The growth regulatory capabilities of the milk proteins have been further studied for their effectiveness as an anti-carcinogen. The effects of mixed proteins in regular mouse chow, casein, and whey proteins on carcinogen-induced mice to determine if any of the different diets affected colon tumor incidence was compared (Papenburg et al., 1990). The most tumors developed in the chow fed mice and 31 and 77% fewer tumors in the casein and whey protein fed mice, respectively. Tumor area was different as well with whey, casein, and chow fed animals having ≈ 40, 90, 160 mm² of tumor area. The whey protein diet appeared to be an effective anti-carcinogen against the development of chemically induced colon tumors of mice and produced smaller tumors.

**Anti-Carcinogenic Activity of α-Lactalbumin**

Along with its role in lactose synthesis, several research groups have studied the specific role of the whey protein α-LA as a growth regulator and effective anti-carcinogen. Numerous growth stimulating factors in milk have been found (Bano et al., 1985), and along with polypeptide growth inhibitors for the regulation of cell proliferation (Bohmer et al., 1985; Wang and Hsu, 1986). These stimulatory and inhibitory factors can be isolated from mammary gland tissue and milk. Lactating bovine mammary gland was the source of mammary derived growth inhibitor (MDGI), which prevents proliferation of cultured mammary epithelial cells in a dose-dependent, non-toxic and reversible manner (Bohmer et al., 1985). Bovine milk whey has been added to cultures of both human mammary cancer cells (MCF-7) and human prostate cancer cells (PC-3), observing significant decreases in cell growth, especially in the mammary tumor cells (Bourtourault, 1991).
A growth inhibitor termed mammary inhibitory activity (MIA), which was fractioned from human milk has been studied (Thompson et al., 1992). It has a molecular mass of 14kDa, an isoelectric point of 5.0, and following amino acid analyses and partial amino acid acid sequencing revealed MIA to be human H-\(\alpha\)-LA. \(\alpha\)-Lactalbumin has been isolated from several mammalian species; this along with chemically modified \(\alpha\)-LA have been compared to MIA and evaluated for their affect on mammary cell growth in culture. Upon comparison, the active portion of MIA and \(\alpha\)-LA appear to be one and the same. \(\alpha\)-Lactalbumin appears to not only be the end product of mammary cell differentiation and a regulator of lactose metabolism, but also possibly a physiologically relevant growth inhibitor. Immortalized human (AIN4) and neoplastic (MCF-7) mammary cell lines showed inhibition of cell growth from 40 to 80% when exposed to MIA at concentrations from 5 to 10 ng/ml, but human fibroblast cells were not inhibited (Thompson et al., 1992). Although MIA/H-\(\alpha\)-LA have similar properties with another inhibitor, MDGI factor, found in bovine milk, sequence analysis does distinguish MIA/H-\(\alpha\)-LA from MDGI. Other reports corroborate with the work of Thompson; also found \(\alpha\)-LA to significantly decrease cell proliferation of cultured bovine mammary cells using \(\alpha\)-LA concentrations from 0 to 625 \(\mu\)g/ml (Rejman, 1992). Alston-Mills and Hepler et al. (1998) also found \(\alpha\)-LA to inhibit cell growth.

Monomeric \(\alpha\)-LA is the form naturally secreted by the mammary epithelium and is the major whey protein of human milk. More recent data showed that human and bovine milk contain a multimeric \(\alpha\)-LA (MAL), from the casein fraction, functioning as a potent Ca\(^{2+}\) -elevating, apoptosis-inducing agent, although not yet characterized (Hakansson et al., 1995). Native monomeric \(\alpha\)-LA from human whey milk did not have the same apoptotic ability an seen by the multimeric form from the casein fraction. When MAL was added to several mammalian cell cultures, apoptosis was induced in tumor cells and immature cells, but not in mature healthy cells (Hakansson et al., 1995). The multimeric forms were isolated and found to be apoptosis-inducing fractions of \(\alpha\)-LA contains oligomers that have undergone a conformational change toward a molten globule-like state, or also referred to as a stable folding variant of \(\alpha\)-LA (Svensson et al., 1999). The mechanisms of apoptosis induction and the molecular basis for the difference in susceptibility between tumor cells and healthy
cells have not been defined for MAL. Some data shows that the release of cytochrome c and activation of the caspase (cysteine-containing aspartate-specific proteases) cascade are early events seen during apoptosis initiated by MAL (Kohler et al., 1999). Co-localization of MAL with mitochondria suggests that MAL may induce apoptosis via a direct interaction with the mitochondria, triggering the release of mitochondrial cytochrome c into the cytosol and in turn activation of the caspase cascade.

**Mechanism of Action for α-Lactalbumin**

The high amounts of sulfur amino acids cysteine and methionine within the whey protein are required for the synthesis of glutathione. Glutathione, the most important intracellular antioxidant, performs functions that may be associated with anti-carcinogenesis, such as regulation of cell proliferation and immune response. Glutathione is a substrate for selenium-dependent glutathione peroxidase and glutathione-S-transferase, therefore it may act in conjunction with enzyme-associated mechanisms or independently (Parodi, 2001).

There are several researchers who think that the mechanism by which milk acts is through these enzymes, especially glutathione-S-transferase, which are involved in either removal of free radicals or detoxification of carcinogens. Studies have linked these amino acid components in the whey proteins that may suppression or reduced tumor incidence (McIntosh, 1995; McIntosh and PK., 1998; Parodi, 1998). One of the specific proteins in the whey fraction of milk to yield favorable anti-growth properties may be that of α-LA by production of glutathione.
The Extracellular Matrix

Molecular characterization of human breast epithelium found cell shape, tissue structure and overall cell behavior to depend on the molecular associations that mediate cell-cell and cell-extracellular matrix (ECM) interactions (Eaton and Simons, 1995). Several research groups in the 1970’s have studied the importance of the ECM (Emerman, 1979; Pitelka, 1980). Also in the 1970s, Mina Bissell, a cell biologist at Berkeley, elucidated the need for cell-cell and cell-ECM interactions. During the in vitro study to determine the behavior of healthy and malignant cells in culture, all of the cells became morphologically and functionally undifferentiated shortly after being removed from the living organism. The loss of differentiation implied an important relationship, or cross-talk, existed between the development of cells and their surrounding ECM, possibly through signals within the ECM that may coordinate further signaling between the cells (Bissell et al., 1982). (Figure 26)

In order for cells to establish and maintain tissue-specific functions, there must be the maintenance of homeostasis between the cells and their surrounding microenvironment. A positive disturbance to the homeostatic state may be induced by normal growth changes, such as embryogenesis or wound healing. While epithelial cell-ECM interactions mediate normal cell behavior, disruptions in these interactions have been implicated in cancer progression, reviewed by (Alford, 1996). This mechanism has been referred to as the “dynamic reciprocity” feedback mechanism (Bissell et al., 1982) and has currently been described as the mechanism to be coupled with regulation of cellular differentiation, proliferation and survival (Roskelley, 1995). Altered signaling within cell-cell and cell-ECM interactions may cause abnormal intracellular oncogene expression or the loss of a suppressor gene expression. Negative disturbances may affect the composition of the ECM and result in abnormal cellular behavior, potentially leading to malignant tumor progression based on the ECM theory proposed by Bissell (Schmeichel et al., 1998). The theory suggests that a direct link exists between the development of breast cancer and the molecular proteins within the surrounding ECM.
Figure 26

Integration of growth and adhesion pathways in mammary epithelial cells. Cell shape, cell-cell interactions and the extracellular matrix, affect how mammary epithelial cells sense and respond to external stimuli. When cells are spread and grown as monolayers, their adhesion and growth pathways are depicted as linear processes. With a basement membrane, mammary cells respond to either growth or adhesion signals as an integrated degree of reciprocal cross-interaction between the various signaling pathways.

Source: (Weaver and Bissell, 1999)
Breast cancer development is usually characterized by the loss of differentiation, disorganization of cellular structure and alteration in the amounts of ECM glycoprotein constituents. As long as cellular and tissue structure are maintained, tumor development can be suppressed despite the presence of cancer-inducing genes. Throughout the life of the adult female, there is a constant ebb and flow of hormones that influence the microenvironment around cells. As a result, by unknown mechanisms, the ECM is altered and tumors begin to develop.

**Extracellular Matrix Components**

Qualitative and quantitative changes of ECM components, such as integrin receptors, cell adhesion molecules, growth factors and steroid hormone-signaling pathways, have been involved in cancer progression, reviewed by (Alford, 1996). Transformed mammary epithelial cells also secrete additional amounts of proteolytic enzymes, including plasminogen activator and collagenase. These proteinases may allow malignant cells to move through connective tissue spaces and blood vessel walls during metastatic spread of cancer (Rifkin, 1979).

**Integrin Receptors**

Integrins are a family of transmembrane heterodimeric receptors that are involved in the cross-talk between cells and the ECM. They are located on the basolateral surfaces of epithelial cells where they function as receptors for glycoproteins, such as tenascin, fibronectin and collagen, within the ECM. The extracellular portion of the receptor binds to specific ligands of the ECM. The binding triggers changes in the intracellular portion of the receptor, altering their interaction with ECM molecules that regulate cell adhesion, growth and migration. At the same time, signals generated inside the cell can alter the activation state of some integrins, affecting their affinity for their extracellular ligands. Integrins are able to signal across the membrane in both directions, inside out and outside in.

Integrin receptors on mammary cell surfaces facilitate cell adhesion to the surrounding ECM by regulating tyrosine kinase autophosphorylation within signal transduction pathways (Clark and Brugge, 1995). Overall cell shape and structural stability are provided by integrin-mediated cell-ECM
interactions through the association of the integrin receptors with intracellular cytoskeleton components. Normal cellular behavior is dependant on these interactions (Schmeichel et al., 1998) and modification in integrin receptor expression has accompanied the development of malignant tumors (Koukoulis, 1991).

There are several known subunits of integrin receptors that exist in normal human breast epithelium. At least two β-subunits, β₁ and β₄, exist along with four different α-subunits, α₁, α₂, α₃, and α₆ (Adams, 1993). Tumor cells appear to express high levels of β₁ integrin, which promotes growth and may contribute to tumorigenesis (Schmeichel et al., 1998; Weaver et al., 1997). These observations led ECM research groups to hypothesize that not all tumor cells necessarily lose their ability to respond to ECM-generated signals through integrin receptors, but instead may have changes in the level of signaling that, if attenuated, could lead to normal cell behavior. As a result of integrins dual functioning, integrin receptors have become targets for study of the progression of cancer in human breast (Schmeichel et al., 1998; Weaver et al., 1997).

**Growth Factors**

**Epidermal growth factor**

Epidermal growth factor (EGF) is a transmembrane protein with several cysteine residues, which form disulfide bonds that contribute to its activity level (Plaut, 1993). The receptors of EGF (EGFR) are on the cell surfaces and belong to a group of closely related receptor tyrosine kinases (RTKs). Ligands for EGFR, such as EGF and transforming growth factor-α (TGF-α), are produced from within a cell and follow through with either autocrine or paracrine EGFR activation (Massague, 1993). The binding or either EGF or TGF-α ligands to the EGFRs causes them to dimerize and become tyrosine kinase phosphorylated. This process activates signals within and between cells to regulate and stimulate cellular growth (Schlessinger, 1992). Epidermal growth factor works in concert with estrogen and progesterone hormones. The activation and resulting signals that are produced from EGFR binding are important mediators for initiation of a cascade of signals that follow to stimulate growth and development of luminal cells of the mammary gland ductal system and to carry
out further cell processes (Coleman, 1990; Hackel, 1999). Most breast cancers discovered to date have originated from luminal epithelial cell layers (Schmeichel et al., 1998).

Not only does EGF have an affect on mammary luminal epithelial cells, but it may also influence human breast cancer cells. Cell proliferation was increased within a culture of MCF-7 mammary cells when EGF was added as compared to cells without the addition of EGF (Fitzpatrick, 1984). Further research has supported the connection between EGF and breast cancer through deregulation of the controlled signaling network by receptor overexpression, autocrine ligand stimulation or mutations has been linked to breast cancer (Earp, 1995).

**Transforming Growth Factors**

Transforming growth factors belong to a large family of cytokines that function to stimulate or inhibit growth of both normal mammary gland epithelium and tumor cells by diverse signal-generating cell-surface receptors (Forsyth, 1989).

**Transforming Growth Factor-α**

Transforming growth factor alpha (TGF-α) and growth factor EGF share the same receptor site of EGFR, causing competition between the receptors (Massague, 1983). Transforming growth factor-α is similar in structure to EGF as they both belong to the same family of peptides (Lawrence, 1985) and contain cysteine residues that form disulfide bridges that are important for their function. Because of their similar structure and apparent competition for the same type of receptor, TGF-α activates similar signaling cascades as EGF does for stimulation of cellular growth. Transforming growth factor-α primarily affects the cells of the proliferating cap cell layer in the TEBs of the mammary gland where they promote cell growth (Moses, 1981). The mammary glands of virgin mice were exposed to TGF-α through implanted pellets. Lobulo-alveolar development was stimulated at a smaller dose of EGF with no hormonal priming of the tissue (Vonderhaar, 1987).

Several studies indicate that human cancer cells produce and release TGF-α and have functional EGFRs for TGF-α (Dickenson et al., 1986; Halper, 1983). Dickenson et al. observed the stimulation of TGF-α production by estrogen in human breast cancer cell lines and concluded that
TGF-α may play an influential role in neoplastic cell stimulation (Dickenson et al., 1986). Over expression of TGF-α in transgenic mice have reduced levels of post-lactational involution and retain secretory cells that continue to express milk proteins several days after the end of lactation (Smith, 1995). This suggests that over expression of TGF-α may function in being anti-apoptotic by promoting the survival of alveolar cells in the normal post-lactating human mammary gland.

**Transforming Growth Factor-β**

There are three known isoforms of transforming growth factor β (TGF-β) produced in vivo, TGF-β1, TGF-β2, and TGF-β3. The specific physiologic role of each TGF-β isoform remains poorly understood. Transforming growth factor-β1 serves as the prototype for the large family of TGF-β cytokines, where it functions in control of cell proliferation, differentiation, motility, and apoptosis (Roberts, 1993). In general, the TGF-β family of polypeptide growth factors has been shown to regulate cellular processes in most tissues (Massague, 1998).

TGF-β has been found to regulate normal mammary epithelial growth and ductal development (Daniel and Robinson, 1992), as well as functioning during inflammation and tissue repair (Kingsley, 1994). During mammary gland development, and during and after lactation in the adult gland, TGF-β potentially inhibits cell proliferation and regression in the lobules and ducts (Arteaga, 1996).

There has also been a functional role of TGF-β found in the development and progression of breast tumors (Wakefield, 1991). In breast cancer, TGF-β appears to play a dual role compared to the role seen in normal mammary gland development. In early stages of the tumor development, TGF-β, as a result of its antiproliferative functions, acts as a tumor suppressor as it inhibits the outgrowth of tumors within the gland. In the later stages of tumor development, cancer cells increase secretion of TGF-β and appear to promote tumor progression (Reiss, 1997). The apparent change in the regulatory role of TGF-β during tumorigenesis may be a result of altered tumor cell responsiveness. Malignant breast carcinoma cells seem to loose most or all sensitivity to TGFβ-induced growth inhibition, while tumor cells derived from early stages of tumor development are
usually inhibited by TGF-β. This loss of antiproliferative responsiveness may attribute to cancer progression (Fynan, 1993).

The antiproliferative response to TGF-β depends on a signaling pathway that is initiated by the ligand-activated TGF-β receptor complex on the cell surface and is transduced into the nucleus by SMAD proteins acting as signaling mediators (Massague, 1998). The SMAD protein is an acronym for referring to both the *Drosophila* gene *Mad* (Sekelsky, 1995) and the *Mad* homologues called *sma* (Savage, 1996). Many of the homologues were described and named SMADs (for *SMA/MAD* related). In this signaling pathway, TGF-β binds to a specific pair of serine/threonine kinase receptors, leading to the type I receptor to dimerize with the type II receptor that initiates signaling by phosphorylation and activation of the type I receptor. *(Figure 27)* The activated TGF-β receptor then phosphorylates a specific subset of SMAD proteins, which subsequently advances into the nucleus. During movement toward the nucleus, the phosphorylated SMAD protein subset becomes associated with another related SMAD protein. Once in the nucleus, SMAD proteins form functional transcription complexes in association with DNA binding factors, coactivators, or corepressors (Derynck et al., 1998). *(Figure 28)* Transforming growth factor-β may contribute to enhancing tumor cell motility, invasiveness and the capacity of the developing tumor to metastasize (Welch, 1990).
In the signaling pathway, TGF-β binds to a specific pair of serine/threonine kinase receptors, leading to the type I receptor to dimerize with the type II receptor that initiates signaling by phosphorylation and the activated type I receptor.

(Source: mammary.nih.gov)
Various molecular mechanisms that can contribute to negative regulation of SMAD signaling and thereby to a loss of transforming growth factor-induced growth inhibition.

Source: (Kretzchmar, 2000)
Some studies support the possibility that loss of TGF-β receptor sensitivity and thereby apoptosis, may play a central role in breast tumorigenesis (Kalkhoven, 1995; Koli, 1997).

TGF-β1 is known to stimulate fibroblastic cells to attain smooth muscle (SM) cell features resulting in the formation of myofibroblasts, which are observed during wound healing and organ fibrosis, reviewed by (Grinnell, 1994). Myofibroblasts are also typical components of the stroma reaction to epithelial tumors where they secrete proteolytic enzymes and growth factors that may activate cancer cell invasive behavior, reviewed by (Ronnov-Jessen et al., 1996). Myofibroblasts, and in turn TGF-β1, have been associated with the pathogenesis of fibrotic diseases, where they are responsible for tissue retraction and overproduction of ECM components, specifically collagen and fibronectin (Roberts, 1986).

**Fibronectin**

Fibronectin (FN) is another widely distributed glycoprotein found in plasma and in the ECM where it is involved in cellular migration during development and cell adhesion during wound healing (Kurkinen, 1980). It is a dimer made up of two long subunits joined by a pair of disulphide bonds. Individual areas within the dimer are specialized for binding to a particular molecule or cell. (Figure 29) A possible association between TGF-β1 and myofibroblast formation through fibronectin has been determined (Serini et al., 1998). Fibronectin may interact with an unknown cell surface receptor that in turn may transduce signals initiated by TGF--β1. This action appears to be a crucial intermediary step for the induction of myofibroblastic features, such as cell adhesive collagen expression.
Fibronectin is a dimer made up of two long subunits joined by a pair of disulphide bonds. Individual areas within the dimer are specialized for binding to a particular molecule or cell.

(Source: http://www.teaching-biomed.man.ac.uk/lawson/Fibronec.HTM)
Tenascin

Tenascin (TN) is a significant ECM glycoprotein that is selectively present during embryogenesis. It is typically expressed in the ECM surrounding fetal rat mammary glands (Kratochwil, 1969), developing hair follicles, feather, scales, review by (Sengel, 1983), and teeth (Thesleff, 1981). Similar interactions are found in adult tissue where it is expressed during maintenance of normal structure and function, wound healing, and also in abnormal development of neoplastic tissue (Sakakura, 1983). Tenascin seems to function by initiating cell proliferation and migration (Mackie, 1988a), cell adhesion (Vacca, 1996) as well as in remodeling of the ECM (Erickson, 1988). The complete physiological role of TN in vivo has remained ambiguous and TN seems to be regulated in a stage and tissue dependent manner (Erickson, 1988). It has been proposed that dual expression of TN, with expression occurring in embryonic breast development, as well as during malignant tumor development in the mammary gland of the adult animal (Chiquet-Ehrismann, 1986).

Tenascin in Normal Mammary Tissue

Tenascin expression seems to decrease in the postnatal period to such an extent that it was once thought to be absent from the normal adult human mammary gland (Mackie, 1987). Contradicting research focused on TN distribution in the normal adult mammary gland in relation to the menstrual cycle (Ferguson, 1990). They found TN to be present in a cyclical manner within the normal adult mammary gland basement membrane and in the layer of fibroblasts around ductules. The distribution and quantity of TN changed during the menstrual cycle showing a constant increase with each progressive week. During the luteal phase of the menstrual cycle, estrogen levels are dropping and TN expression appeared to be maximal. They suggested TN could possibly mediate the hormonal control of mammary epithelial cell behavior based on its ebb and flow with the menstrual cycle. Similarly, TN has been found to be noticeably present in normal adult breast tissue (Borsi, 1992; Koukoulis, 1991).

During pregnancy, TN is expressed by the fetal and maternal stroma at initiation sites of tissue formation, myometrium, within the blood vessels of the uterine wall, and during cell
differentiation (Damsky, 1992). When TN expression was examined during lactation, minimal to no TN was present (Jones, 1995). In contrast, TN was notably expressed during involution, a stage characterized by the degradation of the ECM. This appears to support the ECM remodeling function of TN.

Tenascin has poor adhesive properties (Chiquet-Ehrismann, 1988) that may reduce cell-matrix adhesion thereby supporting cell migration from the highly proliferating TEB into the adjacent ductules. This allows the apoptotic cells from the luminal area to move to the duct area where they undergo phagocytosis. This process of epithelial migration supports the maintenance of cell proliferation and cell death and is also found during wound healing (Mackie, 1988b). Cyclical changes in TN possibly facilitate ductule cell migration and may alter cell shape, polarization, secretory, and cell differentiation (Mackie, 1987b).

The synthesis of TN remains unclear. Several in vitro research studies have found EGF, TGF-α, and TGF-β1, either alone or in combination, stimulate the synthesis of TN in embryonic mouse palatal mesenchymal cells (Dixon, 1990; Sharpe, 1990). Estrogen receptors are found on the mammary epithelial cells, which are target cells for estrogen in the mammary gland. The epithelium produces growth factors, TGF-α and TGF-β1, under estrogentic hormonal control, which in turn affects the cells either in an autocrine or paracrine manner. The production of growth factors then affects TN production by the stromal cells while in turn, TN affects epithelial morphology and function (Inaguma, 1988).

**Tenascin in Tumor Tissue**

Ferguson et al. studied the presence of TN in relation to different types of mammary carcinoma (Ferguson, 1990). Tenascin was associated with mammary carcinoma tissue, but its distribution changes between carcinoma-in-situ and invasive ductal carcinoma types of cancer (Ferguson, 1990). Some research has found TN to also be noticeably present in benign fibrocystic tumors (Borsi, 1992; Koukoulis, 1991).

Malignant epithelium produces excessive amounts of TGF-α and TGF-β (Salomon, 1984), which may act on the stromal fibroblasts to produce increased ECM molecules such as TN. The
excess of growth factors produced may have a mitogenic affect on the tumor cells, especially TN because it contains a similar structure to EGF within the molecule. This may allow TN to mimic the mitogenic effects of EGF and increase cell proliferation (Chiquet-Ehrismann, 1986).

Tenascin also acts as a weak adhesion molecule and changes in the number of TN molecules have been implicated in cancer progression (Schmeichel et al., 1998). Increased amounts of TN at the tumor epithelial-stromal interface may promote cell rounding and decrease adhesiveness. A loss in cell-cell adhesion and an increase in cell motility are required for development of invasive tumors and metastasis. This action facilitates the detachment of tumor cells from the epithelial mass and invasion of the stroma, which may lead to metastasis (Inaguma, 1988). There appear to be other ECM molecules like fibronectin that are present in excessive amounts in malignant stroma. The increased production of fibronectin has been linked to a decreased metastatic potential (Christensen, 1988). Tenascin has been found in the stroma of a metastatic cell line (Inaguma, 1988), which may link TN to metastatic potential. Tenascin appears to antagonize the cell binding activity of fibronectin and as a result influencing tumor progression through stimulated cell migration (Chiquet-Ehrismann, 1988). Most of these proposed functions of TN have been questioned based on the finding of Talts et al. (1999). They found the absence of TN in mice to have very little effect on the spontaneous development of mammary tumors and metastasis into the lungs.

Despite the expression of TN in stroma of primary and metastatic tumors, as well as its recently discovered presence in the normal mammary gland, the complete function of TN remains questionable. One commonality that does remain with the expression of TN is that it seems to usually be expressed during periods of growth.
Methods of Study

The mechanisms of normal mammary gland development and initiation of tumor growth within humans are generally studied through experimental animal models that exhibit the same complex regulatory interactions. Zwieten determined several criteria that the ideal animal model for studying in vitro carcinogenesis should have in order to closely mimic the initiation of human breast cancer (Zwieten, 1984). There should be establishment of similar factors found in the host for initiation of tumorigenesis, mimicking of tumorigenic responses received under specific age and reproductive conditions, and determination of the response of tumors after chemotherapy.

Rodents do not meet all of the criteria for the perfect model, but they are the most widely used because they have a short life span and gestation period, along with similar development and differentiation of the mammary gland compared to that of the human. Because of ethical concerns with using human subjects in biological research, the use of rodent mammary glands as a model allows normal development of the human breast and initiation of breast cancer to be better understood in the absence of human data (Schmeichel et al., 1998).

Mammary gland tissue of the rodent is also highly susceptible to chemically induced tumor growth and shows a pathogenesis very similar to that which occurs in humans. These factors make the rodent mammary gland a unique target for determining initiation and growth of breast cancer. Comparative studies with the development of the human breast and the pathogenesis of breast cancer within the human and rodent have contributed to validate rodent-to-human data (Russo and Russo, 1996).

It has not been definitively established what type of information is necessary for human risk assessment, but understanding the way normal and malignant cells interact with each other and with the surrounding extracellular matrix are relationships that show importance for prevention or treatment of breast cancer (Hansen and Bissell, 2000).
Hypothesis

Our hypothesis is that the soy and skim diets will accelerate the maturity of rodent mammary gland tissue and as a result decrease the tumor incidence of rats in these diet groups. Based on past research, we believe that the protein $\alpha$-lactalbumin, in the skim diet, and the soy protein of the soy diet were the factors that may alter the mammary gland and reduce the development of tumors. Additionally, we propose that human $\alpha$-lactalbumin and genistein, possibly at a particular concentration, will have some affect on the growth of primary tumor tissue.
Experiment
Introduction

α-Lactalbumin, a milk protein, has been shown to inhibit growth of certain mammary tumor cell lines (Thompson et al., 1992). The soy isoflavone genistein, in some cases, has also been shown to contribute in preventing the occurrence of breast cancer in rats (Fritz et al., 1998; Lamartiniere et al., 1995a). Cell lines provide some information and have been the main model of in vitro studies thus far. Relatively little work has been done using primary mammary tumor tissue. Studying the effects of these two proteins on mammary gland and primary tumor development in an in vivo system allows for a better understanding of what may actually occur in the human breast development and maturation, as well as with tumor development based on dietary consumption. It is suggested that some foods, like soy, may enhance the developmental process of the mammary gland in Asian women, causing the gland to mature earlier. This leaves the gland less susceptible to tumor development and may explain why lower breast cancer rates in women are found in Asian countries compared to breast cancer rates in the United States (Ziegler et al., 1993). Other foods such as skim milk contain the whey milk protein α-lactalbumin, which has mainly been shown to inhibit cell proliferation, may also accelerate the developmental process of the mammary gland.

Further in vitro analysis using genistein and human α-lactalbumin at various concentrations (Alston-Mills, 1998; Fritz et al., 1998) on primary tumor tissue may provide valuable knowledge about these proteins for future drug development. Very little research has been done with primary tumor tissue and with human α-lactalbumin.

The present investigation was designed with the following objectives:

1. To determine the implications of nutrients in soy, skim, and casein-based diets at enhancing the maturity of the mammary gland in vivo and as a result the effects on tumorigenesis;
2. To determine the effects of human α-LA or genistein on primary mammary tumor tissue cultured in vitro for 48hrs.
Materials and Methods

Experiment:

Materials

1. Dulbecco’s Modified Eagle Media (Gibco, Grand Island, NY)
2. Human α-Lactalbumin (Sigma, St. Louis, MO)
3. Genistein (Sigma, St. Louis, MO)
4. 6-well culture plate (Becton Dickinson & Co., Franklin Lakes, NJ)
5. Penicillin/Streptomycin (Gibco BRL, Grand Island, NY)
6. Fetal Bovine Serum (Gibco BRL, Grand Island, NY)
7. Lens Tissue-105 (Whatman International Ltd, Maidstone, England)
8. Vectastain Elite ABC kit (Vector, Burlingame, CA)
10. 7,12-dimethylbenz(a)antracene (DMBA) (Sigma Chemicals, St. Louis, MO)

Methods

Animals

All forty-one female Sprague Dawley rats were derived from the International Genetic Standard Crl: CD (SD) IGS BR stock and obtained from Charles River Laboratories, Raleigh, NC. The rats were housed in the Biological Resources Facility at North Carolina State University with water and food available ad libitum. The housing environment for the animals was maintained at a range temperature of 70-75°F, 30-45% humidity and a 12-hr light/12-hr dark cycle. Northeastern Products Corp., Warrensburg, NY, provided the bedding material, BETA CHIP.

Rats were separated into three diet-based treatment groups, soy, skim, and casein, provided by Dyets Inc., Bethlehem, PA. Group-1 received Soy based AIN-93G diet, group-2 received Skim Milk Powder based Modified AIN-93G diet, and group-3 served as the dietary control and received Modified AIN-93G (Casein) diet. All diets met 1995 NRC dietary requirements determined for rats and the diets were balanced for protein and energy (appendix I).

Carcinogen Administration

At day 53, the female rats were treated intragastrically with 15mg of 7,12-dimethylbenz(a)antracene (DMBA) (Sigma Chemicals, St. Louis, MO) dissolved in
0.5ml sesame seed oil (30mg/ml). Tumors were expected to begin development after an initial incubation period of approximately 3 weeks.

**Mammary Gland and Tumor Tissue Collection**

From each diet group a total of 7 rats were euthanized by CO$_2$ asphyxiation, in accordance with North Carolina State University animal protocols and procedures. Four rats on day 51 and 3 rats on day 52 were euthanized and portions of both abdominal and inguinal mammary glands were surgically removed by sterile procedure and rinsed with .85% saline. Mammary gland samples used for determining cell proliferation (PCNA) were stored in 2% formalin at 4°C and tissue samples used to assess levels of tenascin (TN) were immediately snap-frozen in liquid N$_2$ and stored at -80°C. All samples were kept in those conditions until later immunohistochemical analyses were completed.

The remaining rats (n=6 to 7) for each diet group were given DMBA. As the tumors developed, reaching 1.5cm, the rat was euthanized via CO$_2$ asphyxiation. Samples of abdominal and inguinal mammary gland tissue, in addition to tumor tissue, were surgically removed by sterile procedure and rinsed with .85% saline. Mammary gland tissue samples were prepared and stored for later immunohistochemical analyses as previously described. Tissue of tumors >1.5cm were cut in half, with one portion being used for tissue culture and the other portion used for immunohistochemical analysis. Tumor tissue intended for culture was immediately submerged in Dulbecco’s Modified Eagle Medium (DMEM) media and kept in a 37°C humidified incubator prior to preparation for culture. The remaining portion of tumor tissue intended for immunohistochemical analyses was stored either in 2% formalin at 4°C for determination of PCNA or immediately snap-frozen in liquid N$_2$ and stored at -80°C to assess levels of TN.

**Blood Collection**

The maximal amount of blood was collected from each rat at sacrifice. The serum was removed and used for determining estradiol concentrations through a radioimmunoassay (RIA).
**Tissue Culture Procedures**

Under sterile procedures in a laminar flow hood, the tumor portions were chopped into 1mm³ pieces and placed on siliconized lens paper, which served as a matrix for growth. Siliconized lens paper was prepared by washing in ethyl ether 3x, 95% alcohol 3x and deionized water 3x followed by soaking in Prosil 25 for 30 minutes (appendix II) according to the methods of Vonderhaar et al. (1991). Two six-well (35mm diameter) culture plates were used per tumor for each treatment group of human α-LA or genistein. At least 3 tumor tissue pieces were placed in each of 11 different wells. This was done in duplicate for each treatment dosage. For the tissue culture, 3ml of DMEM media containing high glucose, L-glutamine, pyridoxine hydrochloride, 110mg/L sodium pyruvate, and without sodium bicarbonate was used. Ten percent fetal bovine serum (FBS), 100 IU/ml of penicillin, 100µg/ml of streptomycin, and 3.7g/L of sodium bicarbonate were added to the media. Appropriate amounts of human α-LA or genistein were added to the media to yield the correct treatment dosage. Treatment group dosages were as follows: human α-LA 1) culture control without α-LA, 2) 1000ng/ml, 3) 500ng/ml, 4) 100ng/ml, 5) 50ng/ml, 6) 10ng/ml, and for genistein: 1) culture control without genistein, 2) 100µg/ml, 3) 50µg/ml, 4) 10µg/ml, 5) 1µg/ml. Cultures were incubated in a water-jacketed incubator at 37°C with 6% CO₂. After 48 hours the cultures were terminated. Representative samples of each treatment dosage were fixed in either 2% formalin and stored at 4°C or snap-frozen and stored at -80°C for immunohistochemical analyses as described above.

**Proliferating Cell Nuclear Antigen Immunohistochemistry**

**Tissue Preparation**

Cell proliferation values of both mammary gland and tumor tissue were determined by measuring the expression of PCNA. Sections of both mammary gland and tumor were analyzed using immunohistochemical labeling for detection of PCNA. Mammary gland and tumor specimens were fixed in 2% formalin buffer and stored at 4°C. Tissue specimens were paraffin embedded and sectioned at 4µm.
**Assay Procedure**

The sections were mounted on gelatin coated glass slides, deparaffinized and then rehydrated. Slides were then washed for 5 min in 0.05M TBS buffer twice (For 1L: 6.057g TRIS, 2.922g NaCl. Dissolve NaCl in 500ml dH2O, and then add TRIS. Fill to 950ml with dH2O and stir to dissolve, pH to 7.6. Fill to 1L with dH2O.), then immunostaining was performed using the Vectastain Elite Avidin and Biotinylated horseradish peroxidase macromolecular Complex (ABC) kit (Vector, Burlingame, CA) with primary antibody incubation for 1h at a 1:100 dilution and secondary antibody incubation for 30 min at a 1:100 dilution. These dilutions were chosen based on the protocol from Vector, which manufactures the antibodies. The immunostaining protocol used monoclonal primary (#NCL-PCNA) and biotinylated secondary (#BA-2001) antibodies (Vector, Burlingame, CA). DAB was the chosen chromogen, and Haematoxylin counterstain was used (appendix III).

**Tenascin Immunohistochemistry**

**Tissue Preparation**

The expression of tenascin was measured in both mammary gland and tumor tissue. Sections of mammary gland and tumor tissue were analyzed using immunohistochemical labeling for detection of TN. Mammary gland and tumor specimens were snap-frozen in liquid N2 and stored at -80°C. Frozen tissue samples were embedded in OCT embedding media (Triangle Biomedical Sciences, Durham, NC), cryostat sectioned at 4µm and placed on pre-gelatin coated slides and stored at -20°C.

**Assay Procedure**

Frozen slides with tissue samples were warmed to room temperature then washed for 5 min in 0.05M TBS buffer twice (as previously described), then incubated at 37°C for 20 min with normal horse serum (Vector, Burlingame, CA) blocking agent at a 3:200 dilution as suggested by Vector. Using the Avidin-biotin-complex (ABC) method, sections were then incubated with the primary antibody for 1h at a 1:100 dilution and secondary antibody incubation for 30 min at a 1:100 dilution as suggested by Vector. Immunostaining was performed using the Vectastain Elite Avidin and
Biotinylated horseradish peroxidase macromolecular Complex (ABC) kit (Vector, Burlingame, CA), following the suggested protocol from Vector. The immunostaining protocol used monoclonal primary (#NCL-Tenascin) and biotinylated secondary (#BA-2001) antibodies (Vector, Burlingame, CA). NovaRED was the chosen chromogen, and Haematoxylin counterstain was used (appendix IV).

**Assay Staining**

TN was stained with NovaRED, appearing red and distributed throughout the stroma or parenchyma of the tissue. PCNA was observed as DAB stained brown cells primarily in the epithelium of the ductal and end bud areas.

**Scoring of both TN and PCNA**

All immunostained sections were examined under 40x using a light microscope. Therefore, the images were consistent is size and scoring was uniform. At least 4 images were taken of each tissue sample and scored, with the scores averaged to reach a final quantitative value for proliferation within that tissue. The same was done for scoring the TN images, only a percent score was recorded for TN present and the percent scores were averaged to reach a final quantitative value for TN expression within that tissue (appendix V).

**Statistics**

Data was analyzed using the Mixed Procedure program in SAS. Comparisons were made using least squares means ± standard error. The Pearson Correlation Coefficient program in SAS was used for determining correlations between two groups of values. It is an index that ranges from -1.0 to 1.0 inclusive and reflects the extent of a linear relationship between two data sets.
Results

In vivo

Mammary Gland

Overall comparisons were made of mammary tissue between animals of pre-DMBA and DMBA-treated rats with respect to amounts of tenascin and the degree of cell proliferation as represented by TN and PCNA assays, respectively. For both pre-DMBA and DMBA-treated rats, values of TN were consistently higher than values for cell proliferation (PCNA). (Figure 30) A statistically significant difference between PCNA and TN present in mammary tissue of rats from both pre-DMBA (p<0.01) and DMBA (p<0.01) groups which supports the observed relationship. In addition, the levels of TN within mammary tissue of the pre-DMBA rats compared to that of the DMBA rats is also statistically different (p<0.01). Likewise, a statistical difference was found for levels of PCNA in mammary tissue between the pre-DMBA rats and DMBA-treated rats. Images from TN and PCNA assays demonstrate the statistically determined relationship. (Figures 31 A-D)

An overall significant difference (p<0.01) was found between diets of pre-DMBA or DMBA-treated rats with respect to PCNA levels in the mammary gland. (Figure 32) A significant difference (p<0.01) was found between PCNA levels of casein-fed rats of the pre-DMBA and DMBA-treated rats. In the mammary gland of pre-DMBA rats, PCNA of the casein-fed rats was significantly (p<0.01) higher compared to the skim-fed rats and significantly higher (p<0.01) compared to the soy-fed rats. There were no significant differences found between the pre-DMBA skim and soy-fed diets or between DMBA-treated casein, skim or soy-fed rats. Images of mammary tissue labeled with PCNA demonstrate these observations. (Figures 33 A-F)

No significant differences were found between diets of pre-DMBA or DMBA-treated rats with respect to TN levels in mammary gland tissue. (Figure 34) Considering the effects of specific diets on PCNA (Figure 32) and TN (Figure 34) levels separately supported the previous finding (Figure 30) where TN levels were consistently higher than PCNA levels. Images of mammary tissue labeled with TN demonstrate this observation. (Figure 35 A-F)
Mean values: TN (79.01±5.1) and PCNA (22.29±2.1) pre-DMBA rats
TN (43.63±5.6) and PCNA (7.97±2.3) DMBA-treated rats

Different letters represent significant differences found between:
- TN and PCNA of pre-DBMA and DMBA-treated rats (p<0.01);
- TN of pre-DMBA and DMBA-treated rats (p<0.01);
- PCNA of pre-DBMA and DMBA-treated rats (p<0.01)
Mammary Gland

TN vs PCNA

(Figure 30)
Figure 31

Images of mammary gland tissue

A) TN localization in pre-DMBA rats; mean TN (79.01±5.1)

B) PCNA present in pre-DMBA rats; mean PCNA (22.29±2.1)

C) TN localization in DMBA-treated rats; mean TN (43.63±5.6)

D) PCNA present in DMBA-treated rats; mean PCNA (7.97±2.3)

Arrows point to stromal localization of TN or proliferating epithelial cells within the duct of PCNA stained tissue
(Figure 31)
Figure 32

*Mammary gland PCNA of pre-DMBA and DMBA rats, by diet*

Significant overall diet by treatment effect on PCNA (p<0.01).

Different letters represent significances found between:
- Casein pre-DMBA (35.03±3.6) and skim pre-DMBA (16.26±3.6) rats (p<0.01);
- Casein pre-DMBA (35.03±3.6) and soy pre-DMBA (15.57±3.7) rats (p<0.01);
- Casein pre-DMBA (35.03±3.6) and casein DMBA rats (5.56±4.2)

Comparisons of dietary effects were made specifically within a treatment group (pre-DMBA or DMBA) or between like diets.
Mammary Gland
PCNA vs. Diet

(Figure 32)
Figure 33

Images of mammary gland PCNA by diet

A). Pre-DMBA, casein-fed rats; mean (35.03±3.6)
B). Pre-DMBA, skim-fed rats; (16.26±3.6)
C). Pre-DMBA, soy-fed rats; mean (15.57±3.7)
D). DMBA-treated, casein-fed rats; mean (5.56±4.2)
E). DMBA-treated, skim-fed rats; mean (8.52±3.9)
F). DMBA-treated, soy-fed rats; mean (9.78±3.6)

Arrows point to proliferating cells within mammary gland tissue
(Figure 33)
Figure 34

Mammary gland TN of pre-DMBA and DMBA rats, by diet

An overall diet by treatment (pre-DMBA and DMBA) effect on TN was determined to be insignificant. Therefore, any apparent differences among diets within and between treatment groups are considered inconclusive.
Mammary Gland
TN vs. Diet

(Figure 34)
Figure 35

Images of mammary gland TN by diet

A). Pre-DMBA, casein-fed rats; mean (77.08±5.9)
B). Pre-DMBA, skim-fed rats; mean (90.69±5.9)
C). Pre-DMBA, soy-fed rats; mean (68.26±5.6)
D). DMBA-treated, casein-fed rats; mean (28.75±13.4)
E). DMBA-treated, skim-fed rats; mean (54.70±12.2)
F). DMBA-treated, soy-fed rats; mean (47.29±11.6)

Arrows in figures A, B, and D have stromal localization. Arrows in figures C, E, and F have both stromal and epithelial localization.
**Body Weight gain**

Statistical calculation of mean body weight gained for all rats at sacrifice was compared between each diet and of both pre-DMBA and DMBA treatment groups. There were no differences determined in weight gain (Table 2)

**Estradiol**

Overall comparisons of serum estradiol concentrations between rats of different diets within both pre-DMBA and DMBA treatment groups were statistically compared with no statistical differences found (Table 2)

**Tumor incidence**

The numbers of tumors or, tumor incidence, in each rat of the DMBA-treated group were compared among the soy, casein and skim diets. Different dietary treatments did show a significant effect on tumor incidence (p<0.05). The soy-fed DMBA treated animals had significantly (p<0.05) more tumors (n=45) than those fed the casein diet (n=12) as well as those fed the skim diet (n=11) (p<0.05). Tumor incidence between the skim-fed and the casein-fed groups were similar. (Figure 36) The incidence of mammary tumors within these rats did not affect the levels of PCNA or TN within the tumor tissue. Images of mammary gland tissue labeled with TN or PCNA show the observed levels of TN or PCNA, which supports the lack of significance. (Figure 37 A-F)

**Tumor weights**

For each dietary group, the mean weight of tumors in each animal treated with DMBA was determined. The mean tumor weights for soy-fed rats, 0.52g, and skim-fed rats, 0.48g, were similar while the mean tumor weight for casein-fed rats, 0.86g, was higher. (Figure 38) The overall weight of mammary gland tumors within rats fed different diets did not affect the values for PCNA or TN. Comparisons of mean tumor weight and mean tumor incidence were done with respect to dietary effects. Only the soy-fed group showed a significant difference between mean tumor incidence and mean tumor weight (p<0.01). (Figure 38)
Table 2

Additional data

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Figure 36

Dietary effects on primary mammary tumor incidence

Different letters represent significances between:

Soy (6.42±1.15) and casein (2.4±1.37) rats (p<0.05);
Soy (6.42±1.15) and skim (1.83±1.25) rats (p<0.05)

Bars with same letters in the graph represent no significance.
Tumor Incidence vs. Diet

(Figure 36)
Figure 37

*Images of mammary tumor incidence with respect to PCNA and TN levels*

A). High incidence PCNA (n=13)  
B). High incidence TN (n=13)  
C). Moderate incidence PCNA (n=6)  
D). Moderate incidence TN (n=6)  
E). Low incidence PCNA (n=1)  
F). Low incidence TN (n=1)

**PCNA:** Arrows point to proliferating epithelial cells  
**TN:** Arrows point to localization of tenascin
Figure 38

Comparison of dietary effects on mean tumor incidence and mean total weights of primary mammary tumors

Different letters represent significances found between:

Mean tumor incidence (6.42±1.15) and mean tumor weight (0.52±0.15) of soy-fed rats (p<0.01).

There were no other significant differences found.
Tumor incidence and weight (g)

(Figure 38)
A positive (0.88), significant (p<0.01) correlation between total tumor weight and total tumor incidence was determined through the Pearson Correlation Coefficient. (Figure 39)

**Immunohistochemistry**

**Tumor In vivo**

Dietary effects on mammary tumors were analyzed with respect to either PCNA or TN and no differences were found. (Figure 40) Images show PCNA in tumor tissue for each diet compared to images showing levels of TN in tumor tissue support the results. (Figure 41 A-F) There were no correlations between PCNA and TN levels within mammary tumors. (Figure 42)

**Tumor: In vitro**

Tumor samples were placed in culture for 48hr with either α-LA or genistein treatments. There was a significant negative correlation (p<0.01) between PCNA and TN values in all tumor samples, without consideration of diet or treatment in culture. (Figure 43) An overall effect of α-LA and genistein treatments in culture were compared with respect to levels of PCNA in mammary tumor tissue and sorted by diet. (Figure 44) Specific concentrations of α-LA and genistein were not considered in this analysis. There were no significant differences found among any of the diets or culture treatments. Images of tumor tissue from each diet with either α-LA or genistein culture treatments support the statistical conclusion. (Figure 45 A-F) A similar analysis was made for TN levels in mammary tumors post-culture. (Figure 46) The overall effects of α-LA and genistein treatments were compared with respect to levels of TN and sorted by diet. No significant differences were found. Images of tumor tissue from each diet with either α-LA or genistein culture treatment are support the insignificance. (Figure 47 A-F)
Figure 39

Correlation between total tumor incidence and total tumor weight for all rats in each diet group

There was a significant (p <0.01) positive correlation (0.88) found between total tumor incidence and total tumor weight.
Tumor weight (g) and Tumor incidence (n)

\[ R^2 = 0.7433 \]

(Figure 39)
There were no significant dietary effects on PCNA levels and TN expression in tumor tissue (p>0.05).
Figure 40

Tumor
Diet vs. PCNA and TN

PCNA (n) and TN (%)

(Figure 40)
Figure 41

Images of PCNA and TN in mammary tumor before culture

A). Skim-fed rat, PCNA mean (25.35±7.0)
B). Soy-fed rat, PCNA mean (16.74±5.0)
C). Casein-fed rat, PCNA mean (13.57±5.4)
D). Skim-fed rat, TN mean (36.8±13.1)
E). Soy-fed rat, TN mean (36.3±9.3)
F). Casein-fed, TN mean (27.9±10.2)

PCNA: Arrows point to proliferating epithelial cells
TN: Arrows point to localization of tenascin
Figure 42

**Correlation between TN expression and PCNA of primary mammary tumors prior to tissue culture**

There was no significant correlation found between TN and PCNA (p>0.05).
Pre-culture tumor
PCNA vs. TN (p0.65)

$R^2 = 0.0177$

(Figure 42)
Correlation between TN expression and PCNA of primary mammary tumor after culture

There was a significant (p <0.05) negative correlation (-0.52) found between TN and PCNA of tumor tissue in vitro.

Separate analyses also found negative correlations for tumor samples treated with α-LA or genistein, however the p-values were not significant (p>0.05).
Tumor in vitro
PCNA vs. TN (p<0.0122)

(Figure 43)
Figure 44

In vitro effects of α-LA or genistein on cell proliferation of primary mammary tumor

PCNA values at each concentration were averaged together for each diet.

There were no significant differences in PCNA levels after culture with α-LA or genistein for any of the diets groups.
Tumor in vitro
PCNA vs. a-LA and Genistein

(Figure 44)
Figure 45

Images of mammary tumors post-culture

A). Soy-fed rats, α-LA, PCNA mean (24.31±5.5)
B). Casein-fed rats, α-LA, PCNA mean (18.61±7.7)
C). Skim-fed rats, α-LA, PCNA mean (6.07±9.5)
D). Soy-fed rats, genistein, PCNA mean (23.73±5.5)
E). Casein-fed rats, genistein, PCNA mean (16.88±7.7)
F). Skim-fed rats, genistein, PCNA mean (19.35±9.5)
(Figure 45)
In vitro effects of α-LA or genistein on TN levels in primary mammary tumor

TN values at each concentration were averaged together for each diet.

There were no significant differences in TN levels after culture with α-LA or genistein for any of the diets groups.
Tumor in vitro
TN vs. a-LA and Genistein

(Figure 46)
Figure 47

Images of mammary tumor post-culture

A). Skim-fed rats, α-LA, TN mean (27.67±12.6)
B). Soy-fed rats, α-LA, TN mean (18.31±7.3)
C). Casein-fed rats, α-LA, TN mean (13.78±10.3)
D). Skim-fed rats, genistein, TN mean (35.10±12.6)
E). Soy-fed rats, genistein, TN mean (13.6±7.3)
F). Casein-fed rats, genistein, TN mean (17.4±10.3)
(Figure 47)
Specific effects of supplementation with human α-LA at 0, 10, 50, 100, 500, and 1000ng/ml or genistein at 0, 1, 10, 50, and 100µg/ml during the 48hr tissue culture were compared. Levels of PCNA and TN amounts present within mammary tumor samples were analyzed and sorted by the diets, which were fed both prior and during tumor development. There was no significant concentration with diet effects for either α-LA (Figure 48) or genistein (Figure 50) treatments on PCNA levels within tumor samples in vitro. In figure 48, PCNA levels in tumor tissue treated with α-LA, specifically in the skim-fed group showed a slight, but still insignificant, dietary effect. High statistical variances precluded significant dietary differences and with an overall statistical p-value of 0.22, these observations were determined to be inconclusive. There was a low tumor number for the skim group and therefore there were fewer numbers to work with. This may possibly contribute to the observed PCNA in tumors of the skim group. Although no significance was found, there appears to be a pattern that exists between the diet groups. At each α-LA concentration, a consistent trend shows the lowest mean PCNA levels were observed in tumors from rats having consumed a skim-based diet and the highest levels were observed for rats fed a soy-based diet. The PCNA values for rats fed casein were between those observed in animals fed skim and soy. This pattern is consistent with tumor incidence. (Figure 36) PCNA levels in tumor tissue treated with genistein also show an inconclusive pattern among the treatment concentrations. (Figure 50) Consistently across concentrations of genistein, tumor PCNA levels from soy-fed rats were higher than PCNA levels of tumors from the casein-fed rats. The PCNA levels within tumors from skim-fed rats consistently increased from 0 to 50µg/ml and then decrease when treated with 100µg/ml. Similar to the PCNA results, there were no concentration with diet effects by either α-LA or genistein on TN expression in mammary tumors post-culture. (Figures 49 & 51) No particularly consistent pattern within TN levels appeared graphically with the exception of tumor samples treated with 10, 50 and 100ng/ml of α-LA. (Figure 49) Within those treatment concentrations, TN amounts in tumors from skim fed rats were consistently higher than tumors from soy and casein-fed rats, which were consistently lower. A similar inconclusive pattern appeared with TN amounts in genistein treated tumor samples. (Figure 51) The amounts of TN present in tumors from skim-fed rats were higher than those from soy and casein-fed rats in samples treated with 0, 1, 10 and 50µg/ml of genistein.
Figure 48

*In vitro* effects of α-LA on PCNA levels in primary mammary tumors by diet

There were no significant effects of diet and α-LA concentration on PCNA in tumor tissue.
Tumor in vitro
[a-LA] ng/ml and PCNA

(Figure 48)
Figure 49

*In vitro effects of α-LA on TN levels in primary mammary tumors by diet*

There were no significant effects of diet and α-LA concentration on TN in tumor tissue.
Tumor in vitro
[a-LA] ng/ml and TN

(Figure 49)
Figure 50

*In vitro effects of genistein on PCNA levels in primary mammary tumors by diet*

There were no significant effects of diet by genistein concentrations on PCNA in tumor tissue.
Tumor in vitro
[gen] ug/ml and PCNA

(Figure 50)
Figure 51

*In vitro* effects of genistein on TN levels in primary mammary tumors by diet

There were no significant effects of diet by genistein concentrations on TN in tumor tissue.
Tumor in vitro [gen] ug/ml and TN

(Figure 51)
**Discussion**

This study was an extension of previous research within our laboratory showing differences in tumor incidence for rats fed different diets. The rats from an earlier study were fed a soy, casein, whey, or skim-based diet. The skim- and whey-based diets contained the milk protein $\alpha$-LA and had the lowest tumor incidence compared to all other diets in the study. A skim-based diet is one of the focal points within our current study.

The focus of this research was to determine how different dietary regimens of casein, skim or soy affected cell proliferation and differentiation of mammary epithelium and the presence of the extracellular matrix protein tenascin. The dietary regimens were fed during weaning at day 21 to day 55 and continued after administration of the carcinogen, DMBA. Dietary effects were observed on tumor incidence as well as on cell proliferation levels and the presence of tenascin within primary mammary gland tumors. *In vitro*, we observed the extent to which human $\alpha$-LA and genistein treatments may affect cell proliferation and the presence of tenascin.

**In vivo Analysis:**

It is generally known that proliferating cell nuclear antigen (PCNA) is a cell cycle antigen that is maximally elevated in late G1 and S phases of proliferating cells and positively correlates with levels of cellular proliferation and DNA synthesis. In essence, PCNA also is a reflection of tissue growth and progression along with serving as a possible representation for the level of maturity within mammary gland tissue. Stimulation of cell proliferation and differentiation within the TEBs of the mammary gland by extrinsic factors, like diet, may result in enhanced mammary gland maturation (Russo and Russo, 1978). As a result the gland is less susceptible to carcinogenesis by way of having more mature TEBs. Measuring the expression of proliferating cell nuclear antigen using the PCNA assay is an indicator of cell proliferation and a possible prognostic marker for malignancy determination (Robbins, 1987). Likewise, TN is distributed throughout the stroma within the normal mammary gland as a potential mediator in hormonal control of epithelial cell behavior (Ferguson, 1990). Previous research suggested TN to be a good marker for epithelial malignancy because of its stromal distribution (Chiquet-Ehrismann, 1986). Tenascin is also associated with anti-adhesion of
normal mammary epithelial and malignant tumor cells that may aid in cell migration during metastasis (Chiquet-Ehrismann, 1988).

**Normal Mammary Gland**

The results from our study found levels of TN and PCNA within normal mammary gland tissue of pre-DMBA treated rats to be significantly higher than the levels found in mammary gland tissue of rats in the DMBA group that had existing mammary tumors (Figure 30). Actual images show levels of PCNA and distribution of TN found within mammary gland tissue (Figure 31 A-F). The distribution of TN is roughly 4x greater than levels of PCNA within normal mammary glands of pre-DMBA treated rats. A similar observation was found within the mammary glands of DMBA-treated rats. One consideration is age of the rat at the time of tissue collection. Age typically reflects developmental status of the gland. The mammary gland tissue from rats within the pre-DMBA group was collected close to the onset of puberty at 52 days of age. At this age the gland tissue is actively proliferating and the presence of undifferentiated TEBs is maximal (Russo, 1987) (Figure 31B). TEBs are the source of rapid growth for the mammary gland during puberty. This supports the data that we observed with higher values of PCNA within the mammary gland of pre-DMBA rats, which is indicative of rapid growth, compared to the levels of PCNA we found within the mammary gland of DMBA-treated rats.

The DMBA-treated rats were given a carcinogen at d53, a time when the TEBs are maximal and still undifferentiated, yielding the gland highly susceptible to tumor development (Russo, 1987). Mammary tissue samples were later collected from the DMBA-treated rats in conjunction with tumor tissue. At the time of tissue collection, the sexually mature rats ranged in age from 101 to 150 days. By 84 days of age, the TEBs within the sexually mature virgin rat regress to form small finger-shaped terminal ducts. No further morphologic changes appear within the mammary gland until pregnancy occurs (Brisken, 2002; Moon, 1969). The collection of mammary gland tissue for DMBA-treated rats occurred when the rats were mature and estrous cycling was established. Also, during this time there is typically minimal cell proliferation occurring (Figure 31D). This supports the lower levels of PCNA that were observed within the DMBA-treated animals.
Angiogenesis is also a consideration for the difference seen in the gland tissue for pre-DMBA and DMBA-treated rats. Angiogenesis may exist between the mammary gland of DMBA-treated rats and the tumors that exist in those glands. The ECM provides necessary signaling with the luminal epithelial cells of the TEBs for normal growth and development (Bissell, 1999). Knowing this allows the speculation that if a component within the ECM becomes inactive or too active, then the regulation of normal growth for the gland tissue becomes skewed and carcinogenesis may occur and in turn may support neoplastic growth. Components of the ECM surrounding the mammary gland tissue may be unregulated in such a way that it supports the growth of developing tumors and eventual metastasis, as the tumors assume a parasitic role within the host. The engagement of a parasitic type relationship by the tumor with the surrounding ECM may leave the gland deprived of normal regulation and less capable of proliferating, hence the lower levels of PCNA within the mammary gland of DMBA-treated rats (Figures 30 & 31D).

In addition, the presence or absence of tumors within mammary glands may also provide explanation for some differences between pre-DMBA and DMBA-treated rats. Our study found TN to be higher in the mammary gland of pre-DMBA rats compared to the mammary gland of DMBA-treated rats (Figures 30, 31A & C). TN appears to antagonize fibronectin and function to dissolve cell adhesion as it aids in the migration of apoptotic mammary epithelial cells out of the luminal portion of the duct for phagocytosis (Chiquet-Ehrismann, 1988). Our findings suggests the presence of tumors may lower TN levels in the mammary gland and may support TN antagonizing the cell binding activity of fibronectin. In the mammary gland of DMBA-treated rats, the levels of TN may be lower because the glands are not at a similarly high proliferative state, as mentioned with respect to the age of the rats. Also, the active site for TN may be shifted from the mammary gland to the tumor for support of cell migration and metastasis, resulting in TN functioning more in the tumors than in the gland itself. This is what may decrease the amount of TN available for functioning within the mammary gland, which coincides with the lower levels of TN we found in the pre-DMBA mammary gland (Figure 30). Tenascin may also be higher in order to facilitate the migration of cells during mammary gland fat pad invasion, which is influenced by anti-adhesion of the cells. Periods of high proliferation in TEBs are associated with cell apoptosis in order to maintain equilibrium between cell
proliferation and cell death, which would also result in higher levels of TN present. The mammary gland tissue from pre-DMBA rats was collected during high proliferation in the TEB. As a result, TN is likely higher in the mammary tissue of pre-DMBA rats because it functions to assist in removal of apoptotic, dying cells. Because TN has anti-adhesion properties to rid TEBs of dying cells and possibly facilitate in gland maturation, the levels of TN would likely be higher when the TEBs are in an active state of growth showing high PCNA, as we found in the pre-DMBA rats. This also may explain the relationship between PCNA and TN within both the normal and tumor filled mammary glands (Figure 30). In both gland tissues, cell proliferation was present along with higher levels of TN that may assist in maintaining a regulated cycle of cell growth and death.

A significant diet effect on the levels of PCNA between mammary gland tissues of rats from pre-DMBA and DMBA-treated rats was found (Figure 32). Extrinsic factors, such as diet, may advance the developmental process of the mammary gland, allowing the gland to differentiate earlier. This leaves the gland with more mature TEBs and as a result the gland becomes less susceptible to tumor growth at an earlier age (Russo, 1989a). As mentioned, collection of mammary gland tissue for the pre-DMBA rats occurred when the TEBs were maximal and cell proliferation within the buds would typically be the highest (Russo, 1987). All diets in the DMBA-treated rats showed low cell proliferation, therefore it appears that once tumors are present, the diet has no, or very little, effect on PCNA within the gland (Figure 33 D-F). At that point the tumors appear to have taken control of the gland, causing the gland to be in a stagnant proliferative state. Casein-fed rats served as the dietary control. The casein pre-DMBA rats in our research had significantly high levels of PCNA (35.03) compared to the PCNA level found in DMBA-treated, casein-fed rats (5.56). The higher levels of PCNA in the glands of casein-fed, pre-DMBA rats suggest that the epithelium of the gland was proliferating rapidly (Figure 33A) and did not have a marked advancement in TEB maturity. The gland of pre-DMBA treated skim and soy rats had lower levels of PCNA, which is indicative of early gland maturation with increased TEB cell differentiation that ultimately leaves the gland less susceptible to tumor development (Figure 33B,C). Therefore, the lower level of PCNA found in skim and soy rat glands may indicate the existence of factors within the diets that cause advance mammary gland development, which would be supported by a lower tumor incidence.
We found no significant dietary effects with TN expression in mammary tissue of pre-DMBA and DMBA-treated animals (Figure 34). The levels of TN for each diet are lower in the DMBA treated rats, as observed in figure 35 D-F, than levels found in mammary tissue of each diet in the pre-DMBA group, as observed in figure 35 A-C. The difference in TN levels may be due to the existence of tumors in the gland. TN activity may be shifted from within the mammary gland and to the tumor for support of cell migration and metastasis. Based on figure 34, it appears that levels of TN would be statistically different for the same diet between treatment groups, i.e. the soy pre-DMBA group compared to the soy DMBA group with respect to TN expression. However, any visible graphical differences among diets within or between the pre-DMBA and DMBA treatment groups are statistically inconclusive. Although inconclusive with dietary effects, as shown in figure 30, there appears to be an overall lower level of TN in DMBA treated rats compared to pre-DMBA rats.

Tumor Incidence and Diet

Tumor incidence was measured for rats all three diets (Figure 36). The casein diet, being the control diet, was initially thought to have the least protective effect on the mammary gland for prevention of tumor development. However, that is not what we observed. The soy-fed animals actually had a total of 45 tumors, which was the highest tumor incidence among the diets. The tumor incidence for our soy diet did not support the previously found benefits of soy consumption (Lamartiniere et al., 1995a). They suggested early exposure to genistein, a soy isoflavone, could result in enhanced gland maturity by accelerating the cell differentiation within the TEBs and as a result lowering the levels of PCNA. This leaves the adult gland less susceptible to chemically induced mammary cancer. The high number of tumors to develop, which contradicts their findings may reflect of the absence of genistein in our soy diet.

Tumor incidence was significantly lower for the casein group compared to the soy group. The casein group developed a total of 12 tumors, which was close to that of the skim group with a total of 11 tumors (Figure 36). Based on our findings, we are unsure of the casein component that may have achieved the lower tumor incidence. We speculate the decrease in tumors to be a result of a degradation by-product of casein. This finding is contrary to earlier trials in our laboratory, which
showed casein-fed animals to have high tumor incidence. However, other studies did find casein to have a lowering effect on tumor incidence that supports our findings (Papenburg et al., 1990).

The skim diet yielded a low tumor incidence, which was consistent with results from our previous unpublished data. The unpublished data also found that $\alpha$-LA within the diet could be detected in the blood stream as well (Alston-Mills, unpublished data). The skim-fed group had a total of 11 tumors with over half of them being $<0.1$ grams (Figure 36). The skim diet contained several proteins, but particularly that of $\alpha$-LA, which, as previously mentioned, has effective growth regulator and anti-carcinogenic capabilities (Rejman, 1992; Thompson et al., 1992). Having $\alpha$-LA in the skim diet may help explain the low level of tumor incidence and stimulatory effects to proliferate and mature the gland of skim rats. A growth inhibitor termed mammary inhibitory activity (MIA) fractioned from human milk, was determined to be human $\alpha$-LA (H-$\alpha$-LA) (Thompson et al., 1992). They found an inhibition of cell proliferation in immortalized human (AIN4; 80%) and neoplastic (MCF-7; 40%) mammary cell lines when exposed to MIA at concentrations from 5 to 10ng/ml. These previous studies support our findings of lower PCNA levels within the mammary gland of animals fed a skim diet, which contained $\alpha$-LA protein. In addition to $\alpha$-LA possibly functioning to inhibit cell growth, $\alpha$-LA may also facilitate gland maturation. The skim rats fed a skim diet had low levels of PCNA, which would be expected if the gland had already reached a more mature state. The lower levels of PCNA along with low tumor incidence in rats from the skim group supports the theory that $\alpha$-LA from the diet enhanced gland maturation. A recent study in our lab found mice fed a skim diet or a skim with an $\alpha$-LA additive had more mature gland development based on whole mount observations and TEB counts (Alston-Mills, unpublished data 2003).

Other studies have found a whey diet containing $\alpha$-LA to provide anti-cancer results in chemically induced tumors in rats (Papenburg et al., 1990). The whey diet contains $\alpha$-LA that may function to enhance growth and differentiation of the mammary gland, which adds clarity and support to our results. Levels of endogenous $\alpha$-LA are typically high during lactation. We were giving exogenous $\alpha$-LA, by diet, by way of skim milk, which may have mimicked values found during lactation, which is the state of terminal structural and functional differentiation. If, according to
Papenburg et al. (Papenburg et al., 1990), α-LA has developmental altering capabilities through facilitation of structural differentiation, then the migration and fat pad invasion would occur at an earlier age as a result of the diet. This adds even more clarity as we found the α-LA containing skim diet given to pre-DMBA rats had a low level of PCNA, suggesting accelerated gland development and differentiation (Figure 36).

The decreased incidence of tumors in both the casein and skim fed rats agrees with the findings of Papenburg et al. (Papenburg et al., 1990) where they compared mixed proteins in regular mouse chow, casein, and whey proteins on carcinogen-induced mice to determine if any of the different diets affected colon tumor development. The chow fed mice developed the most tumors and fewer tumors developed in the casein and whey protein fed mice. The whey protein diet appeared to be an effective anti-carcinogen against the development of chemically induced colon tumors, suggesting that the whey diet altered the developmental process of the mammary gland.

We also analyzed the number of tumors present to determine if the incidence had any affect on levels of PCNA and TN between the tumors for each rat. The quantity of PCNA within a rat having a total of 13 tumors was not significantly different from the levels found within rats that developed only 6 tumors or even only 1 tumor and we determined that despite the number of tumors a rat may develop, the levels of PCNA and TN would remain similar within each tumor. (Figure 37 A,C, & E). We found the same to be true with no statistical difference in measured levels of TN expression (Figure 37 B, D, & F).

In our study, the mean body weight gained for all rats was similar with no significant differences based on diet (Table 2). Therefore, the dietary effects that were found are not a result of some rats consuming more of their diet than other rats. Also, there were no differences found between serum estradiol levels for rats of each diet (Table 2). High levels of serum estradiol are associated with human breast cancer risk (Bernstein et al., 1990), but this study showed no correlation to the level of tumor incidence and high or low levels of estrogen. A comparison of the mean total tumor incidence to the mean total tumor weight did show a significant difference for tumors of soy-fed animals, having a lot of small tumors (Figure 38). Animals of the casein diet had fewer (n=12), but larger tumors that weighed on average of 0.9g. The larger size of tumors that develop in
casein rats negates any previous benefits found through a low tumor incidence with the consumption of a casein-based diet. The tumors from the skim (n=11) and soy (n=45) fed animals, regardless of the tumor incidence, weighed on average around 0.5g. This provides support for the benefits of consuming a soy-based diet because even though tumors may develop, soy consumption may keep the tumors smaller. The consumption of the skim-based diet not only reduced the number of tumors that developed, but the average size was much smaller as well. The small size of tumors in the skim-fed rats may also be attributed to growth regulation of $\alpha$-LA.

Correlation analyses determined a significant positive correlation between tumor incidence and tumor weight (Figure 39). As the total number of tumors that develops within a rat increases, the total tumor weight within that rat increases.

**Tumor Pre-Culture**

Values of PCNA and expression of TN were similar and no significant dietary effects were found in tumors of animals fed the skim, soy or casein diets (Figure 40). Although values were generally similar and insignificant among the diets, it appears that tumors from skim-fed animals were proliferating somewhat more rapidly than those from animals fed a soy or casein diet (Figure 41 A-C). $\alpha$-LA within the skim diet is possibly the factor to stimulate the growth of tumor cells.

Although not significant, the tumors from rats fed a soy diet have a slightly lower level of PCNA than what we observed for the tumors from skim-fed rats. In past research, similar observances found genistein to be protective against breast cancer *in vivo* through the diet (Fritz et al., 1998; Lamartiniere, 2000; Lamartiniere et al., 1995a), but contrary results have been found with neoplastic sensitive tissue and cancer cell lines (Allred, 2001a). The tumors from the casein group had the lowest PCNA, which was unexpected. As previously mentioned, there may be a metabolite within the casein diet that is more effective at lowering PCNA within mammary gland tumors. Levels of TN present were generally similar and insignificant among the diets for pre-culture tumor tissue (Figures 40, 41 D-F).

Although no conclusive differences were observed among the diets for PCNA or TN, expression in pre-culture tumor TN levels were consistently higher than PCNA levels. A similar
pattern was observed in mammary gland of non-DMBA and DMBA-treated rats (Figures 32, 34, 40). We speculate that dietary influences on PCNA and TN expression on the mammary gland also influences the tumor tissue as well. We suggest that whatever effects the diet had on the mammary gland during the developmental stages of puberty is retained within the mammary epithelial cells that may be targets for transformation into tumor cells. Cells that are protected may not be transformed into cancerous cells. This is corroborated by milk studies with children drinking milk during developmental pre-puberty years (Hjartaker et al., 2001).

After tumor tissue was subjected to a 48hr culture with various concentrations of genistein or \( \alpha \)-LA, we observed a significant negative correlation between PCNA and TN expression (Figure 43). TN expression may be correlated with angiogenesis and tumor progression, periods of high cell proliferation (Vacca, 1996). For pre-culture tumor, we found TN to be present at high levels with lower PCNA. The highest levels of TN expression were found when PCNA was the lowest (Figure 43, 44 and 46). As previously mentioned, TN to be associated with cell anti-adhesion in tumors by antagonizing the activity of fibronectin and aiding in the promotion of cell migration with eventual metastasis (Chiquet-Ehrismann, 1988). TN was also found to be associated with ECM remodeling, which would also be linked to tumor establishment and progression (Erickson, 1988). These functions of TN support our findings of TN being high when PCNA was low in tumor tissue. Overall, it appears that the presence of TN had a controlling effect on PCNA values within mammary tumor tissue. Figures 44 and 46 show the inverse trend that exists with levels of TN and PCNA within tumor tissue post-culture. This is similar to the trend we found in tumor samples not subjected to culture, although the correlation was not significant (Figure 40).

**In vitro**

**Tumor Post-Culture**

We analyzed post-culture tumor tissue for the effects of human \( \alpha \)-LA or genistein on PCNA among the different diet groups (Figure 44). For this analysis, there was no consideration to specific concentrations of human \( \alpha \)-LA or genistein. No significant differences were found for tumors treated
with α-LA or genistein among any of the diet groups (Figure 45 A-F). This may be a result of small sample sizes and high variance, especially within the skim group.

Even though no statistical differences were found, there was a trend for decreased PCNA in tumors excised from rats fed a skim-based diet and cultured with α-LA when compared to tumors from rats fed a soy or casein-based diet (Figures 44, 45 A-C). As already discussed, the possible functioning of α-LA as a growth regulator and apoptosis inducer (Papenburg et al., 1990) may well explain why the skim diet has a lowering effect on PCNA within tumor tissue. The values of PCNA were much lower after being cultured with α-LA treatment than the levels of PCNA observed for the skim tumors prior to being cultured where the effects were only diet based, seen in figure 40, suggesting that levels of α-LA within the skim diet were effective at achieving a relatively low PCNA, but not as effective as the concentration used in culture.

As for the soy tumors, PCNA appeared to increase slightly after being cultured with α-LA or genistein compared to levels observed in tumors prior to culture, found in figure 40. Our soy diet may have an unknown protein, which may contribute to the lowering effect on PCNA in tumor samples not subjected to culture and maintaining that effect after culture in the soy tumors. Levels of PCNA in tumors from the casein-fed rats remained similar between pre- and post-culture tumors.

Post-culture tumor tissue was analyzed for the effects of α-LA or genistein on the levels of TN present among the different diet groups (Figure 46). For this analysis, there was no consideration to specific concentrations of α-LA or genistein. No significant differences were found among any of the dietary groups for tumors treated with α-LA or genistein (Figures 46 & 47 A-F). This may be a result of small sample sizes and high variance, especially within the skim group. Despite no statistical differences among diets or treatments, tumors from all diets had lower levels of TN present after the culture with α-LA or genistein compared to the levels prior to culture, seen in figure 40. The on exception was tumors from the skim diet treated with genistein. The TN levels appeared to remain similar to those of skim tumors prior to being cultured.

The effects of α-LA at specific concentrations among the different diets were analyzed to determine if a particular concentration significantly affected the level of PCNA and the presence of
tenascin within the tumor tissue. No significant differences for PCNA were found at any of the concentrations of \( \alpha \)-LA or genistein within each of the diet groups (Figure 48). The same was found for tenascin expression as well (Figure 49). However, several trends were observed. The tumors from rats fed a skim milk diet has the lowest level of PCNA, with the next lowest from the casein diet, and the highest in tumors from soy-fed animals. This was the same trend that was observed for the effects of \( \alpha \)-LA on PCNA without consideration to specific concentrations (Figure 44). Again, because of high variances that confound the effects on tumors in vitro, it is difficult to make definitive conclusions regarding the effects of \( \alpha \)-LA.

The negative correlation between PCNA and TN that was observed in Figure 43 is also apparent between PCNA and TN levels of tumors when specific concentrations of \( \alpha \)-LA are considered from Figures 48 & 49. The soy and casein tumors cultured with \( \alpha \)-LA have high levels of PCNA and low level of TN expression. Talts et al. (Talts, 1999) found the growth of primary and metastatic tumors to not be affected by the lack of tenascin. As a result, they suggested tenascin might have no function in cancer cell proliferation and migration. As originally thought our data for tumors of casein and soy diets are in agreement with Talts et al (1999). We also suggest that tenascin has little affect on tumor PCNA, as seen in tumors of animals fed a soy or casein-based diet and cultured with \( \alpha \)-LA.

The opposite is observed for skim tumors cultured with \( \alpha \)-LA, where relatively low PCNA is observed and much higher levels of TN are present, which contradicts the suggestion of Talts et al (1999). Although it is inconclusive, tumor samples of the skim diet cultured with 50ng/ml of \( \alpha \)-LA have a low level of PCNA and the highest level of TN present. The lowest level of PCNA was achieved by 100ng/ml of \( \alpha \)-LA with roughly half the level of TN expressed from 50ng/ml of \( \alpha \)-LA. According to our skim diet data, TN does appear to be capable of regulating the growth or metastasis of primary tumor cells within the skim group, which contradicts the suggestions of Talts et al (1999). Therefore, we suggest that TN may function to modulate tumor PCNA naturally to some extent as seen in the pre-culture tumor of other diets (Figure 40), but a greater level of reduced PCNA is achieved in conjunction with \( \alpha \)-LA treatment and a skim diet together. \( \alpha \)-LA appears to favor anti-
adhesion and as a result PCNA is lowered and allows for a higher TN. The protective function of α-LA, seen by low cell proliferation, is supported by α-LA favoring anti-adhesion. Alternatively, it may be the PCNA that modulates the expression of TN. The data are not clear which one is the modulator.

The effects of genistein at specific concentrations among the different diets were also analyzed for the determination of effects on PCNA and TN. No significant differences were found for genistein at any of the different concentrations for PCNA (Figure 50) or TN expression (Figure 51). However, several trends were also observed for tumor samples cultured with genistein. As was observed for the α-LA treatment during culture, a trend with TN being high when PCNA levels are low and vice versa is also observed with genistein cultured tumor samples. Again, this is noticed specifically with the tumor samples of rats fed a skim diet, possibly in conjunction with α-LA, and compared to the findings of Talts et al.(1999). We found a low level of PCNA for tumor cells from the skim diet cultured with genistein, but not as low as was found for those cultured with α-LA. Tenascin in our data does appear to have a partial role in cancer cell proliferation and migration. The levels of PCNA for the casein and skim tumors are relatively the same for most concentrations of genistein, but the skim tumors are typically higher (Figure 50). However, the TN expression for skim and casein tumors is drastically different (Figure 51). This observation in our data may support the findings of Talts et al. (1999). Even though the level of PCNA in tumors cultured with genistein from the skim and casein diets were similar, the levels of TN were different. This suggests that TN may have little or no affect on PCNA, because the same levels of PCNA were observed regardless to the amounts of TN present. This observation may also suggest that without the α-LA in the diet, like in the casein and soy groups, as effectively. TN may not function in tumor cell proliferation and migration. The levels of PCNA in the tumors cultured with genistein of the soy diet are high and TN is low (Figures 50 & 51). This same pattern was also observed for the soy and casein tumors cultured with α-LA (Figures 48 & 49). The levels of PCNA are slightly higher in the soy compared to that of the skim diet and the levels of TN are lower for soy compared to skim. This supports the suggestion of Talts et al. (1999).
**Overall Summary**

These findings have several important points. First, we have shown that diet does affect the growth and development of the mammary gland. Overall, it appears that the skim milk diet is the most effective in accelerating the growth and differentiation of the mammary gland. This was evident by the significantly low level of tumor incidence within rats fed the skim diet. Secondly, diet can influence the growth and development of mammary tumors. The tumors that did develop within the skim-fed rats were fewer in existence and smaller than other groups. This was good outcome, however, the small numbers and sizes of tumors that did develop in skim-fed rats hindered my data because of small sample sizes and an overall high variance. The soy diet appeared to have similar affects on tumor size, yielding small tumors. However, the soy diet had a much higher tumor incidence than the skim diet. Thirdly, the diet also appeared to affect the levels of PCNA compared to the levels of TN present within both mammary gland tissue and tumor tissue. Both soy and casein diets appeared to be less effective at lowering the levels of PCNA and increasing the levels on TN. The skim diet appeared to be more effective at lowering levels of PCNA and raising levels of TN. Fourth, from our data there is a link between the levels of PCNA and TN for all the diets. Fifth, the trend is that $\alpha$-LA in conjunction with a skim diet provides the lowest levels of PCNA and highest levels of TN in tumor tissue. We speculate that the best combination is consumption of a skim-based diet prior to tumor development and treatment of the tumors with around 50ng/ml of $\alpha$-LA post-tumor development. This combination within our study affected the greatest level of tumor cell arrest and lowered PCNA. And lastly, genistein did have some effective at lowering PCNA and increasing levels of TN. However, the contrast between PCNA and TN for casein, soy, or skim diets was not as prominent compared to the observed $\alpha$-LA treatment.
Appendix I

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<th>Ingredient</th>
<th>Modified AIN-93G kcal/gm</th>
<th>Soy based AIN-93G gram/kg</th>
<th>Skim milk powder based AIN-93G gram/kg</th>
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All diets met 1995 NRC dietary requirements determined for rats and the diets were balanced for protein and energy.
Appendix II

Siliconized Lens Paper

Reagents:
- Whatman 105 Lens Cleaning
- Silicone: Prosil-28 (Thomas Scientific #7805-S25)
- Ethyl ether
- 95% ethanol

Procedure:
- Wearing gloves and using forceps, separate individual sheets of lens paper and place them in a glass dish in such a way that they can be individually grasped later
- Wash 3x with ethyl ether (20-30ml each time); aspirate after each washing
- Wash 3x with 95% ethanol (20-30 minutes each); aspirate after each washing
- Wash 4x with deionized water 15 minutes
- Dry in a drying oven @ 37°C
- Separate each sheet
- Siliconize (Prosil-28) 1:100 with deionized water 15 minutes
- Wash 4x with 100ml deionized water 15 minutes
- Dry in a drying oven @ 37°C
- Cut to desired size
- Autoclave in a glass petri dish to sterilize
- Leave sealed in petri dish until ready to use
Appendix III

Proliferating cell nuclear antigen-Assay (PCNA)

Materials:

- 70% Ethanol
- 95% Ethanol
- 100% Ethanol
- Methyl Salicylate (Fisher Scientific, Pittsburgh, PA)
- Hemo-De (Fisher Scientific, Pittsburgh, PA)
- 0.05M TBS pH 7.6
- Horse serum (Vector, Burlingame, CA)
- Blocking reagent
- Primary antibody (#NCL-PCNA, Vector, Burlingame, CA)
- Biotinylated secondary antibody (#BA-2001, Vector, Burlingame, CA)
- Standard VECTASTAIN Elite ABC kit (Vector, Burlingame, CA)
- DAB substrate kit (Vector, Burlingame, CA)
- Vectamount™ mounting media (Vector, Burlingame, CA)
- Distilled water
- 37°C humidified chamber

Solutions: make all day of staining

**Vectastain Elite ABC Reagent™ kit:** ***make this first***

- Add 100µl of Reagent A to 5ml of 0.05M TBS buffer
- Then, add 100µl of Reagent B
- Mix immediately
- Stand for 30 minutes prior to use
- Total volume = 5.2ml

**Blocking Reagent:**

- Add 150µl of stock Horse Serum to 10ml of 0.05M TBS buffer
- Total volume = 10.15ml
- A 3:200 dilution
- 10.15ml will cover approximately 100 sections of tissue at 100µl per section

**Primary Antibody (1º Ab):**

- Add 100µl of 1º Ab to 10ml of 0.05M TBS buffer
- Total volume = 10.1ml
- A 1:100 dilution
- 10.1ml will cover approximately 100 sections of tissue at 100µl per section

**Biotinylated Secondary Antibody (2º Ab):**

- Add 100µl of 2º Ab to 10ml of 0.05M TBS buffer
- Then, add 150µl of stock Horse Serum
- Total volume = 10.25ml
- A 1:100 dilution
- 10.25ml will cover approximately 100 sections of tissue at 100µl per section

**DAB substrate kit:** **Prepare immediately to use**

- As instructed by Vector:
- To 5ml of distilled H₂O, add 2 drops of Buffer Stock Solution, mix well
• Add 4 drops of DAB Stock Solution, mix well
• Add 2 drops of Hydrogen Peroxide Solution, mix well
• If a gray-black stain is desired, add 2 drops of the Nickel Solution, mix well

**Tissue Preparation:**

• Deparaffinize tissue sections on slides in Hemo-De for 3 minutes, repeat in fresh Hemo-De
• Rehydrate tissue sections using an ethanol series. Dip slides into 100% EtOH for 3 minutes, 95% for 1 minute, and 70% ethanol for 1 minute. Rinse in distilled water for 5 minutes.
• Wash sections in 0.05M TBS buffer for 5 minutes, repeat
• Dry slide around the edges of each tissue section

**PCNA Assay:**

• Immediately cover tissue sections with blocking reagent and incubate for 20 minutes in a 37°C humidified chamber
• Shake off excess blocking reagent, do not wash slide. Dry slide around tissue
• Immediately cover sections with 1°Ab and incubate 1hr at room temperature (25°C)
• Wash sections in 0.05M TBS for 5 minutes, repeat
• Dry slide around tissue sections
• Immediately cover sections with the 2° Ab and incubate 30 minutes at room temperature (25°C)
• Wash sections in 0.05M TBS for 5 minutes, repeat
• Dry slide around tissue sections
• Mix DAB substrate according to manufacturers directions. Apply 500µl to each section and incubate at room temperature for 6-8 minutes. Staining time will vary
• Wash in distilled water for 5 minutes
• Counterstain with Haematoxylin for 15-20 seconds. Staining occurs quickly
• Wash slides under running tap water for 5 minutes
• Dehydrate slides through an ethanol series. 70% EtOH for 2 minutes, repeat with fresh 70% EtOH, 95% EtOH for 2 minutes, repeat using fresh 95% EtOH, and 100% EtOH for 2 minutes, repeat using fresh 100% EtOH.
• Clear slides with Hemo-De for 2 minutes, repeat with fresh Hemo-De
• Permanently mount slides using 1 drop of Vectamount mounting media. Apply a cover slip and store at room temperature
Appendix IV

Tenascin Immunohistochemistry

Reagents:

- 0.05M TBS pH 7.6
- Horse serum (Vector, Burlingame, CA)
- Blocking reagent
- Primary antibody (#NCL-Tenascin, Vector, Burlingame, CA)
- Biotinylated secondary antibody (#BA-2001, Vector, Burlingame, CA)
- Standard VECTASTAIN Elite ABC kit (Vector, Burlingame, CA)
- NovaRED substrate kit (Vector, Burlingame, CA)
- Vectamount™ mounting media (Vector, Burlingame, CA)
- Distilled water
- 37°C humidified chamber

Solutions: make all day of staining

** Vectastain Elite ABC Reagent™ kit: ***make this first***

- Add 100µl of Reagent A to 5ml of 0.05M TBS buffer
- Then, add 100µl of Reagent B
- Mix immediately
- Stand for 30 minutes prior to use
- Total volume = 5.2ml

Blocking Reagent:

- Add 150µl of stock Horse Serum to 10ml of 0.05M TBS buffer
- Total volume = 10.15ml
- A 3:200 dilution
- 10.15ml will cover approximately 100 sections of tissue at 100µl per section

Primary Antibody (1º Ab):

- Add 100µl of 1º Ab to 10ml of 0.05M TBS buffer
- Total volume = 10.1ml
- A 1:100 dilution
- 10.1ml will cover approximately 100 sections of tissue at 100µl per section

Biotinylated Secondary Antibody (2º Ab):

- Add 100µl of 2º Ab to 10ml of 0.05M TBS buffer
- Then, add 150µl of stock Horse Serum
- Total volume = 10.25ml
- A 1:100 dilution
- 10.1ml will cover approximately 100 sections of tissue at 100µl per section

** NovaRED substrate kit: Prepare immediately to use**

- As instructed by Vector:
  - To 5ml of distilled water, add 3 drops of Reagent 1, mix well
  - Add 2 drops of Reagent 2, mix well
  - Add 2 drops of Reagent 3, mix well
  - Add 2 drops of Hydrogen Peroxide Solution, mix well
**Tissue Preparation:**
- Use 4µm thick tissue sections cut with a cryostat at –30 to -35°C
- Bring slides with adhered tissue sections, stored at -20°C, to room temperature (25°C)

**Tenascin Assay:**
- Wash slides in 0.05M TBS buffer for 5minutes, repeat
- Dry slide around edge of tissue sections
- Immediately cover tissue sections with Blocking Reagent and incubate for 20 minutes in a 37°C humidified chamber
- Shake off excess blocking reagent, do not wash slide. Dry slide around tissue
- Immediately cover sections with 1°Ab and incubate 1hr at room temperature (25°C)
- Wash in 0.05M TBS buffer for 5 minutes, repeat
- Dry slide around tissue sections
- Immediately cover sections with 2° Ab and incubate for 30 minutes at room temperature (25°C)
- Wash in 0.05M TBS for 5 minutes, repeat
- Dry slide around tissue sections
- Immediately cover sections with Vectastain Elite ABC reagent and incubate for 30 minutes at room temperature (25°C)
- Wash in 0.05M TBS for 5 minutes, repeat
- Dry slide around tissue sections
- Mix NovaRED substrate according to manufacturers directions. Apply 500µl to each section and incubate at room temperature for 6-8 minutes. Staining time will vary
- Wash in distilled water for 5 minutes
- Counterstain with Haematoxylin for 15-20 seconds. Staining occurs quickly
- Wash slides under running tap water for 5 minutes
- Dehydrate slides through an ethanol series. 70% EtOH for 5 minutes, repeat with fresh 70% EtOH, 95% EtOH for 5 minutes, repeat using fresh 95% EtOH, and 100% EtOH for 5 minutes, repeat using fresh 100% EtOH.
- Clear slides with Hemo-De for 5 minutes, repeat with fresh Hemo-De
- Permanently mount slides using 1 drop of Vectamount mounting media. Apply a cover slip and store at room temperature
Appendix V

Scoring of Immunohistochemical Assays

For both PCNA and TN assays:

Tissue Preparation and Viewing:
- Tissue was prepared according to the protocol and sectioned
- 3 – 4 sections of one tissue sample were placed on each slide
- The assay was completed
- All sections of tissue on each slide were viewed using an Image Analysis light microscope
- 4 random images were taken per slide at 40x (at least one image per section on each slide)
- The images were saved and viewed/analyzed at a later time

Tissue Analysis:
- All images were viewed on a computer and analyzed
- The score given to each section of tissue per sample was recorded
- All recorded scores per tissue sample were averaged together to get one final score per tissue sample
- The final scores were then used for statistical analysis

PCNA
- This assay is a quantitative measure of cell proliferation. The proliferating cells are stained brown with DAB and all other cells are counterstained with haematoxylin (purple)
- The score for this assay was based on a visual numerical count of cells that were stained brown-positive for proliferating

TN
- This assay is qualitative, noting the presence or absence of TN within the tissue (stained red)
- Tenascin is mainly found in the extracellular matrix are in between cells within the tissue
- If TN was present, the amount of TN was quantified
- Since a “count” is not possible, a visual percentage was assigned in an attempt to quantify the amount of TN present in order for comparison


Koli, K., Ramsey, TT., Ko, Y., Dugger, TC., Brattain, MG. and Arteaga, C. (1997). Blockade of transforming growth factor-beta signaling does not abrogate antiestrogen-


