THE EFFECT OF DIETARY GENISTEIN ON BREAST CANCER METASTASIS

BY

CHUNG YAN FUNG

THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Food Science and Human Nutrition in the Graduate College of the University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Advisors:

Professor William G. Helferich, Chair
Associate Professor Nicki Engeseth
Assistant Professor Hong Chen
ABSTRACT

The dietary isoflavone supplements are now perceived as safe and a natural alternative to hormone replacement therapy (HRT) and are recently being consumed by postmenopausal women. However, as weak estrogens, isoflavones may have the potential to alter breast cancer (BC) growth and metastasis which is also more common in the postmenopausal age group. In this study, we investigated the role of the main soy isoflavone, genistein, on the metastasis of 4T1 murine BC cells implanted in ovariectomized (OVX) BALB/c mice (OVX mice mimic the postmenopausal age group). I hypothesized that weakly estrogenic genistein will enhance BC metastasis based on our previous studies. This study was conducted in two treatment groups: control group consisting of animals on the AIN-93G control diet and genistein group consisting of animals on the control diet + 750ppm genistein; which can provide physiologically relevant plasma concentrations to what is observed in humans consuming isoflavone-containing diets.

In this thesis, three investigations were conducted: 1) to apply bioluminescence imaging (BLI) techniques to monitor the tumor progression in vivo of the two dietary groups; 2) to determine the effect of genistein on the tumor burden of the India ink-stained lungs, one of the common metastatic sites; 3) to evaluate the effect of genistein supplementation on overall tumor colonies and tumor areas of both macro- and micro- metastases in the lung samples with Hematoxylin and Eosin (H and E) staining.
We demonstrated that 750ppm dietary genistein did not promote BC metastasis to the lungs in BLI compared to the control group. No significant difference in lung tumor burden was observed between the two groups with India ink and H and E staining techniques.

In conclusion, this appropriate preclinical postmenopausal animal model and the BLI system allow us to investigate the impact of genistein on BC metastasis under low plasma estradiol concentrations environment. Results from this study suggest that consumption of products containing genistein in a physiologically relevant level of a soy diet did not show any effect on breast tumor metastasis in vivo.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Prof. William G. Helferich, for his guidance and support throughout my Master’s study. With his patience and advice, I solved many problems and completed my entire course. With his consideration, I was given plenty of freedom to plan my schedule and arrange my time for experiments. Through our conversations and discussions, I got clearer directions and goals for my project. For these reasons, I would really like to express my gratitude to him, not just as an advisor, enriching my knowledge and providing me with guidance, but also as a considerate friend who always thought about the students.

In addition to Prof. Helferich, I would also like to thank his well-established and mature research team. Without their generous help and valuable advice, I would not have been able to complete all parts of the experiments smoothly and within the limited time available. A sincere thank you to Dr. Aashvini Belosay and Dr. Xujuan Yang, for their guidance since my first day in the laboratory; Mr. James Hartman for his technical help throughout my study; Ms. Eliana Rosales and Ms. Wenden Wang for sharing both the encouraging and frustrating experiences throughout the whole project, even though we were working in different fields of study.

Last but not least, I must thank my parents, siblings and fiancé, for their unconditional love and care during this challenging task. They all provided me with great emotional support and encouraged me to finish my Master’s study, especially this thesis research.
Table of Contents

List of Figures ................................................................................................................................. vi
List of Tables ................................................................................................................................. vii
List of Abbreviations ......................................................................................................................... viii
CHAPTER I INTRODUCTION ........................................................................................................... 1
CHAPTER II BACKGROUND ............................................................................................................... 4
   A. Breast cancer ................................................................................................................................. 4
   B. Isoflavones .................................................................................................................................... 15
   C. Imaging techniques for cancer research .................................................................................... 24
CHAPTER III METHODS ................................................................................................................... 26
   A. Materials ....................................................................................................................................... 26
   B. Animals and diets .......................................................................................................................... 26
   C. 4T1 cell culture ............................................................................................................................ 27
   D. Tail vein injection of 4T1 cells ..................................................................................................... 28
   E. Bioluminescence imaging ........................................................................................................... 28
   F. Analysis of uterine wet weights ................................................................................................. 29
   G. India ink staining of animal lungs .............................................................................................. 30
   H. Hematoxylin and Eosin staining of lung samples ..................................................................... 30
   I. Statistical analysis ....................................................................................................................... 31
CHAPTER IV RESULTS ....................................................................................................................... 32
   A. Effect of genistein on animal weight ........................................................................................... 32
   B. Effect of genistein on uterine wet weight .................................................................................. 32
   C. Bioluminescence imaging analysis ............................................................................................. 33
   D. India ink staining analysis ......................................................................................................... 35
   E. Hematoxylin and Eosin staining analysis ................................................................................. 35
CHAPTER V DISCUSSION .................................................................................................................. 51
CHAPTER VI CONCLUSIONS AND FUTURE DIRECTIONS ............................................................ 60
   A. Conclusions ................................................................................................................................. 60
   B. Future directions .......................................................................................................................... 62
REFERENCES ....................................................................................................................................... 64
List of Figures

Figure II-1: Cellular events in a metastatic process .................................................................13
Figure II-2: Structures of major soy isoflavones, genistein and estradiol .................................15
Figure II-3: Female BC incidence and mortality rates, 2002-2006 .............................................18
Figure IV-1: Animal weight throughout experimental period ....................................................38
Figure IV-2: Uterine wet weight of sample groups during necropsy .........................................39
Figure IV-3: Metastasis progression over time in control mouse number 11 ..............................41
Figure IV-4: Different locations of metastasis other than lungs in test mice ...............................42
Figure IV-5: BLI area results of two diet groups over time .......................................................43
Figure IV-6: BLI integrated density results of two diet groups over time .................................44
Figure IV-7: Appearance of original and India ink-infused lung sample specimens .................45
Figure IV-8: Tumor count on lung surface of sample groups .....................................................46
Figure IV-9: Macro-metastasis of sample lung tissue observed after H and E staining ...............47
Figure IV-10: Micro-metastasis of sample lung tissue observed after H and E staining .............48
Figure IV-11: Tumor count of sample groups observed after H and E staining .........................49
Figure IV-12: Tumor to lung area ratio of sample groups observed after H and E staining .......50
List of Tables

Table II-1: T, N, M staging of BC ................................................................. 6
Table IV-1: Metastasis occurrence rate of sample groups over test period......................40
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>BC</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomized</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescent imaging</td>
</tr>
<tr>
<td>H and E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>ERs</td>
<td>Estrogen receptors</td>
</tr>
<tr>
<td>EREs</td>
<td>Estrogen response elements</td>
</tr>
<tr>
<td>ER+</td>
<td>Estrogen-receptor-positive</td>
</tr>
<tr>
<td>ER-</td>
<td>Estrogen-receptor-negative</td>
</tr>
<tr>
<td>SERMs</td>
<td>Selective estrogen receptor modulators</td>
</tr>
<tr>
<td>AIs</td>
<td>Aromatase inhibitors</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge coupled device</td>
</tr>
<tr>
<td>FLI</td>
<td>Fluorescence imaging</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>FL</td>
<td>Firefly luciferase</td>
</tr>
<tr>
<td>AIN-93G</td>
<td>American Institute of Nutrition-93 Growth</td>
</tr>
<tr>
<td>TV</td>
<td>Tail vein</td>
</tr>
</tbody>
</table>
CHAPTER I   INTRODUCTION

BC is the most common type of cancer among women in the United States and accounts for one in four newly diagnosed female cancer cases. It was estimated that in 2009, 192,370 new cases of invasive BC would occur among American women, in addition to 62,280 new cases of in situ BC. BC is a progressive disease that begins in breast tissue which may then invade the surrounding tissue and later extend to other parts of the body (metastasis). While precise causes of the disease have not been fully understood, some factors are believed to contribute to the development of BC in women. One important risk factor is the lifetime exposure of women to estrogen, which is thought to promote cancer growth and development. Longer exposure of endogenous estrogen such as early menarche, late menopause or late first full-term pregnancy, is proved to be associated with higher risk of BC. The use of exogenous estrogen such as oral contraceptives or HRT, especially in combination with progestin, has been shown to increase BC incidence as well.

Since the 1960s, HRT has been used to treat menopausal symptoms resulting from declining hormonal levels, mainly estrogen and progesterone, during menopause. However, there have been concerns that long-term use of HRT may increase the risk of developing BC. As a result, women, especially those with BC, are not recommended to take HRT in order to avoid the risk of tumor development or recurrence. This has resulted in an increased use of isoflavones as an alternative therapy to relieve menopausal symptoms.
The most abundant dietary sources of isoflavones are soybeans and soy-based food products. Due to their plant-derived nature and their structural similarity with female hormone 17β-estradiol, isoflavones are commonly recognized as phytoestrogens which can bind to estrogen receptors (ERs) and exhibit estrogen-like properties. As a result, in recent years, dietary supplements containing soy isoflavones have been marketed as natural products for postmenopausal women to treat symptoms including night sweats, sleep difficulties, vaginal dryness and mood changes.

Furthermore, epidemiologic studies suggest that high soy intake is associated with lower risk of BC among Asian women and early exposure to isoflavones has shown protective effect against later development of BC. These observations have led to the perception that it is safe and beneficial to consume soy isoflavones for BC patients.

However, this safety assumption may not be correct. Since isoflavones are weak estrogens, this makes them an effective treatment for relieving menopausal symptoms but also a potential risk to hormone-related diseases such as BC. Genistein, a predominant isoflavone found in soy, acts as an estrogen agonist at physiological dosages. It has been demonstrated that genistein stimulates the growth of estrogen-dependent BC cells in both laboratory and animal studies. In addition, genistein has been shown to interfere with BC treatments using tamoxifen or aromatase inhibitors in animals. Furthermore, it has been discovered recently that genistein exerts estrogen-like systemic effects on the induction of BC metastasis. All of this evidence raises serious safety concerns about consumption of isoflavones by women with BC.
With the increasing popularity of the use of isoflavone supplements by American women and the safety concerns of consumption of phytoestrogens, it is important to understand the potential effects of isoflavones on BC, which is one of the leading diseases among women in the US. The overall goal of my thesis research is to determine the role of dietary genistein in BC metastasis, the most important aspect affecting BC survival. My overall hypothesis was that genistein will promote development of BC metastatic disease due to its estrogenic potential. Following are the specific objectives and the hypotheses of the research presented in this thesis.

Objective 1: to evaluate the effect of dietary genistein on BC progression by applying BLI techniques to monitor tumor progression in animal models implanted with 4T1 murine BC cell line via tail vein injection. My hypothesis was that genistein enhances BC metastasis.

Objective 2: to evaluate the effect of dietary genistein on metastatic lung tumor burden using India ink staining techniques to aid visual counting of tumor nodules on lung surface. My hypothesis was that genistein enhances metastatic tumor burden on lung surfaces.

Objective 3: to evaluate the effect of dietary genistein on micro-metastasis in lung tissue with the use of H and E staining. My hypothesis was that genistein enhances both macro- and micro- metastasis to the lungs.
CHAPTER II  BACKGROUND

A. Breast Cancer

1. Breast cancer overview

BC is the most common form of cancer among American women, excluding skin cancers, accounting for nearly 1 in 4 cases of cancers diagnosed in US women\(^1\). The chance of developing invasive BC at some time in a woman’s life is about 1 in 8 (12%) and the chance that BC will be responsible for a woman’s death is about 1 in 35 (3%). The American Cancer Society estimates that in 2009, approximately 192,370 new cases of invasive BC will be diagnosed among women, as well as an estimated 62,280 additional cases of \textit{in situ} BC (the non-invasive and the earliest form of BC)\(^1\). In 2009, approximately 40,170 will die from BC in the US\(^1\). This makes BC the second leading cause of cancer death among women living in the US, exceeded only by lung cancers.

Approximately 1,910 cases of BC are expected to occur among men in 2009, accounting for about 1% of all BCs and 440 men will die from BC\(^1\). Although BC in men is rare, the incidence rate has increased annually from 1975 to 2006. The reasons for the increase are still unknown. This shows that BC occurs not only in women but increasingly in men. Furthermore, men tend to delay diagnosis and therefore are more likely than women to be diagnosed with advanced cases of the disease, leading to poorer chance of survival\(^13\).
2. Breast cancer stages

BC is a progressive disease that begins in the breast tissue, which is made up of glands for milk production (lobules) and tubes that connect lobules and carry milk to nipples (ducts). The remainder of the breast is made up of fatty, connective and lymphatic tissue\(^1\). Cancers originate from lobules and ducts are known as lobular carcinomas and ductal carcinomas respectively. There are many different types of BC, with different stages (spread), aggressiveness and genetic makeup, and survival varies greatly depending on these factors\(^14\). Most masses are benign and hence are not life-threatening. Some BCs are called \textit{in situ} since they are confined within the lobules or ducts of the breast tissue. Most cancerous breast tumors are invasive or infiltrating in which they invade the surrounding tissue of the breast and spread to the other parts of the body.

Seriousness of BC is highly influenced by the stage of the disease, that is, the extent or spread of the cancer. The American Joint Committee on Cancer (AJCC) has a staging system for BC using information of tumor size and its spread within the breast and nearby organs (T), lymph node involvement (N) and presence of distant metastasis (M)\(^15\). A stage of 0, I, II, III or IV is assigned to describe the extent of cancer growth once the T, N and M are determined, with stage 0 being an early stage and stage IV being the most advanced stage\(^16\). Different stages of BC are described in Table II-1.
<table>
<thead>
<tr>
<th>Stage</th>
<th>T, N, M Criteria Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Carcinoma <em>in situ</em> (non-invasive cancer) – Lobular carcinoma <em>in situ</em> (LCIS) and Ductal carcinoma <em>in situ</em> (DCIS).</td>
</tr>
<tr>
<td>I</td>
<td>The tumor is no larger than 2 cm in diameter and has not spread beyond the breast.</td>
</tr>
<tr>
<td>II</td>
<td>The tumor is no more than 2 cm across and has spread to the axillary lymph nodes (the underarm lymph nodes); or the tumor is between 2 and 5 cm and has spread to the axillary lymph nodes; or the tumor is larger than 5 cm and has not spread to the axillary lymph nodes.</td>
</tr>
<tr>
<td>IIIA</td>
<td>The tumor is smaller than 5 cm and has spread to the axillary lymph nodes; or the tumor is larger than 5 cm across and has spread to the axillary lymph nodes.</td>
</tr>
<tr>
<td>IIIB</td>
<td>The tumor has grown into the chest wall or the skin of the breast; or has spread to the lymph nodes behind the breastbone.</td>
</tr>
<tr>
<td>IIIC</td>
<td>The tumor has spread to the lymph nodes behind the breastbone and the axillary lymph nodes or to the lymph nodes under or above the collarbone.</td>
</tr>
<tr>
<td>IV</td>
<td>Distant metastatic cancer spread to other parts of the body.</td>
</tr>
</tbody>
</table>

*Table II-1: T, N, M staging of BC*
**3. Risk factors of breast cancer**

While the specific cause of BC is unclear, several factors have been known to increase the risk of developing the disease\(^1\). Apart from being female\(^{18}\), the biggest risk factor is increasing age\(^{19}\). The probability of developing invasive female BC in the next 10 years is 3.4% at the age of 60 compared to 0.44% at the age of 30\(^1\). Currently, a woman living in the US has a 12%, or a 1 in 8, lifetime risk of being diagnosed with BC. Back in the 1970s, the lifetime risk of being diagnosed with BC was 1 in 11. This increase in the likelihood of being diagnosed with BC may be due to the longer average life span of women.

Other important risk factors include family history of the disease especially in a first-degree relative\(^{20}\) and genetic predisposition such as inherited mutations or alternations in the BC susceptibility genes of BRCA1 and BRCA2\(^{21}\). Instead of a 12% lifetime risk of BC, women with one of these genes have an increased risk of approximately 60%\(^{22}\).

High breast tissue density (a mammographic indicator of the amount of glandular tissue relative to fatty tissue in the breast)\(^{23}\), high bone mineral density in postmenopausal women\(^{24}\), biopsy-confirmed atypical hyperplasia and high-dose radiation to chest have been shown as the clinical risk factors to have a strong independent relationship with the development of BC.

Hormonal factors are also associated with increased BC risk due to the effects of reproductive hormones on cancer cell proliferation and DNA damage as well as promotion of cancer growth. These include early menarche (<12 years old) and late
menopause (>55 years old)\textsuperscript{25}, older age at first-full term pregnancy (>30 years old), lack of childbearing or breastfeeding\textsuperscript{26}, postmenopausal obesity and use of oral contraceptives. Recent use of HRT with combined estrogen and progestin therapy has shown to increase BC risk as well, with higher risk associated with longer use\textsuperscript{27,28}. One study found that current users of the combination of estrogen and progestin menopausal hormones have a relatively increased risk of developing BC of 26\%\textsuperscript{29}.

The actions of estrogen on breast and cancer cells are believed to be mainly through ERs that reside in cell nucleus as transcription factors. Upon binding of estrogen and other co-activators, ER modulates cellular gene transcription in target tissues\textsuperscript{30}. Briefly, estrogen binds to the ligand-binding domain of ER which triggers a spontaneous dimerization and leads to a subsequent interaction of dimer with the estrogen response elements (EREs) in target genes. The ligand-receptor complex recruits co-activator proteins to initiate gene transcription and ultimately regulates cellular response in target tissues. In the mammary gland, ER-mediated genes are associated with cell proliferation, differentiation and survival\textsuperscript{31}. Through the receptor-mediated process, estrogen promotes proliferation and differentiation of normal breast epithelium, as well as initiation and progression of malignant tumors. However, estrogens are not the only compounds capable of interacting with ER and eliciting a response. Estrogen agonists and antagonists can also bind to ER to activate and inhibit estrogenic activities, respectively.
Obesity, as well as weight gain during adulthood, increases the chance of postmenopausal BC\textsuperscript{32}. A recent study showed that women who gained 55 pounds or more after age 18 had almost 50% greater risk of BC compared with those who maintained their weight. A gain of 22 pounds or more after menopause was associated with an increased risk of 18%, whereas losing at least 22 pounds after menopause and maintaining the weight loss was associated with 57% lower BC risk \textsuperscript{33}. In postmenopausal women, circulating estrogen is primarily produced in fat tissue. Thus, having more fat tissue increases estrogen levels and the likelihood of developing BC. Given the large percentage of women in the US who are overweight or obese, strategies to maintain a healthy body weight are important to reduce the risk of both developing and dying from BC. As a result, there is a growing evidence of the modest protective effect of physical activity on BC due to its effect on body mass, hormones and energy balance\textsuperscript{1}.

In recent years, research has indicated the impact of diet and other behaviors on BC. These additional risk factors include a high-fat diet\textsuperscript{34}, alcohol intake\textsuperscript{35} and environmental factors such as tobacco use and radiation\textsuperscript{36}. Recent studies have reported that even low to moderate alcohol consumption (3-14 drinks per week) is associated with a slight increase in the risk of BC by increasing estrogen and androgen levels in a dose-dependent manner\textsuperscript{37,38}. Although radiation from mammography is a low dose, the cumulative effect can also cause cancer\textsuperscript{39}.
4. Treatments of breast cancer

To treat BC, most women will have some type of surgery which is often combined with other treatments such as radiation therapy and/or systemic therapy (biologic therapy, chemotherapy and/or hormone therapy). BC surgery is to remove the cancer from the breast and to assess the stage of the disease and/or to apply partial or total mastectomy. Radiotherapy is given after surgery to the region of tumor bed, to destroy microscopic tumors that may have escaped surgery. Radiation can reduce the risk of recurrence by 50-66% when delivered in the correct dose\textsuperscript{36}.

Adjuvant systemic treatments (biologic therapy, chemotherapy and hormone therapy) can be used to destroy remaining cancer cells after surgery or to reduce tumor size before surgery. Anti-cancer drugs are injected into a vein or given by mouth in which they travel through the bloodstream to all parts of the body. For metastatic BC, since the removal of all cancer cells by surgery is not possible, systemic therapies are the main treatment approaches.

One of the biologic therapies is the use of herceptin (trastuzumab), which is a monoclonal antibody that directly targets the growth-promoting HER2/neu protein that is overproduced in approximately 15% to 30% of breast tumors and offers survival benefit for some women with metastatic BC\textsuperscript{40}. Trastuzumab, however, is expensive, and approximately 2% of patients suffer significant heart damage from this treatment.
Research has established that, in most cases, combinations of drugs are more effective than just one drug alone for BC treatment when applying chemotherapy. It is most effective with full dose and completion of the drug cycle in a timely manner. These drugs may also be used to shrink cancer that has metastasized.

BC is commonly divided into two subtypes based on the presence of ERs in tumor, estrogen-receptor-positive (ER+) cancer and estrogen-receptor-negative (ER-) cancer. According to National Cancer Institute, approximately two thirds of BC cases are ER+.

The last systemic therapy is hormone therapy, which can be given to women with ER+ BC to block the effects of estrogen. It usually involves the use of selective estrogen receptor modulators (SERMs) or aromatase inhibitors (AIs). Both classes of drugs treat BC by blocking the actions of estrogen on tumor cells, but the effect comes through two different mechanisms. SERMs, such as tamoxifen, act as estrogen antagonists by competing with estrogen for ER binding. Tamoxifen has been used for more than 30 years as a treatment for some BCs and it is proved effective in both pre-menopausal and postmenopausal patients with estrogen-dependent BCs. However, concerns regarding tamoxifen-associated side effects, including endocrine resistance and endometrial cancer, have lead to a reduction in its usage. AIs such as letrozole, anastrozole and exemestane, work by inhibiting the peripheral synthesis of estrogen by the enzyme aromatase which converts androgens to estrogens. AIs are generally not used to treat breast cancer in pre-menopausal women since most of the circulating estrogen is produced by the ovaries, not by conversion of androgens to estrogens, blocking the enzyme aromatase does not significantly decrease the production of
estrogen and hence may not be effective in breast cancer treatment. While in postmenopausal women, AIs have proven to be more effective in preventing cancer recurrence with early-stage BC and have shown improved survival and fewer side effects than those associated with tamoxifen. However, because AIs completely deplete postmenopausal woman of estrogen, they can cause osteoporosis, bone fractures and other musculoskeletal symptoms.

BC treatments can result in a variety of short-term and long-term side effects that affect quality of life, including psychological distress, hormonal symptoms and fatigue.

5. Breast cancer metastasis

BC causes more than 40,000 deaths in women each year. The majority of these deaths are caused by metastasis at distant organs rather than primary cancer in mammary gland. About 6% of BC patients are in stage IV of distant metastasis at the time of disease diagnosis and up to 30% of patients diagnosed in earlier stages develop distant metastasis eventually. Distant metastasis is often associated with increased mortality. In the US, a 5-year relative survival rate for localized BC is 98% and for regionally spread cancer is 84% but for metastatic BC is only 27%.

In recent years, some important advances have been made in understanding the molecular mechanisms underlying BC progression and metastasis. A series of cellular events are required for the metastatic process (Figure II-1). First, new blood supply needs to form around primary tumor to provide an escape route for cancer cells. This
process is called angiogenesis. Through these newly developed blood vessels, cancer cells invade into the circulatory system. Some cells may also enter the circulation indirectly through the lymphatic system. A small part of circulating cancer cells survive in the bloodstream and then extravasate into distal organs, colonize and grow into a metastatic lesion with or without a period of latency\textsuperscript{50}. When cancer cells spread and form a new tumor in a different organ, the new tumor is termed as a metastatic tumor. The cells in the metastatic tumor come from the original tumor. This means, if BC spreads to the lungs, the metastatic tumor in the lungs is made up of cancerous breast cells. When viewed under a microscope, metastatic BC cells generally look the same as cancer cells in the breast.

\textbf{Figure II-1: Cellular events in a metastatic process}\textsuperscript{51}

Whether metastases develop depends on the complex interaction of many tumor cell factors, including the type of cancer, the degree of differentiation of the tumor cells, the
location and how long the cancer has been present, the lymph node involvement, the absence of hormone receptor and vascular invasion as well as other incompletely understood factors. The patterns of metastasis are also linked to hormone receptor status. ER+ cancer tends to metastasize to bone and reproductive organs, while ER-tumors favor viscera such as lungs. Cancer cells can spread to almost any region of the body. The most common sites for BC metastasis are bone, lung, liver and brain. Metastases may occur at a single organ but more likely develop at multiple locations. The two most popular sites for the metastasis of BC cells are in bone and the lungs, which can be found in approximately 70% of patients at the time of death.

When cancer has metastasized, it may be treated with chemotherapy, radiation therapy, biological therapy, hormone therapy, surgery, cryosurgery or a combination of these. The choice of treatment generally depends on the type of primary cancer, the size and location of the metastasis, also the age and general health of the patient. Currently, metastatic cancer is considered incurable. Treatments, although relatively toxic, mainly focus on alleviating symptoms and improving quality of life of patients. Systemic adjuvant therapies may involve chemotherapy, tamoxifen or AIs for ER+ cancer, and trastuzumab for HER2/neu positive cancer. Bevacizumab, an antibody targeting VEGF, may be used for metastasis treatment due to its inhibitory effect on angiogenesis.
B. Isoflavones

1. Isoflavone overview

Phytoestrogens are a group of plant-derived compounds that have a similar chemical structure with female hormone 17β-estradiol. Major phytoestrogens include isoflavones, lignans, coumestans and stilbenes, with the last two classes being less abundant in the diet. Isoflavones are the most common form of phytoestrogens. The basic structural feature of isoflavone compounds is shown in Figure II-2. The flavone nucleus is composed of two benzene rings linked through a heterocyclic pyrane ring\(^{57}\).

![Figure II-2: Structures of major soy isoflavones, genistein and estradiol](image)

Due to this structural similarity, isoflavones have the ability to bind to ERs\(^ {58}\) and therefore act as estrogen agonists to exert weak estrogenic effects\(^ {59}\). The estrogenic activity of isoflavones is generally \(10^2\)-\(10^5\) weaker than estradiol. Genistein, one type of isoflavone, binds to ERs with an affinity approximately 100-fold less than that of estradiol\(^ {60}\). Nevertheless, plasma isoflavone concentration can be \(10^2\)-\(10^3\) higher than endogenous estradiol following a soy-rich diet\(^ {61}\). Adlercreutz \textit{et al.}\(^ {62}\) have found that the plasma level of genistein in people having a soy-rich diet was 1-5 µM after...
metabolism and excretion. A recent report from India also revealed an adequate circulating level of genistein after a single dose of soy extract\textsuperscript{63}.

Isoflavones are present in a variety of plants such as legumes and clovers, but the most abundant dietary sources of isoflavones are soybeans and soy-based food products\textsuperscript{64}. Three main isoflavones are genistein, daidzein and glycitein, which account for approximately 50-55\%, 40-45\% and 5-10\% of the total isoflavones content in soy respectively\textsuperscript{65}. Naturally occurring isoflavones are in their glycoside inactive forms as genistin, daidzin and glycitin. Upon consumption, the conjugated compounds are metabolized by intestinal bacteria to produce active aglycone form of isoflavones\textsuperscript{66}.

The consumption of isoflavones varies between Eastern and Western countries\textsuperscript{67}. Among Asian populations such as Chinese and Japanese, between 25-50 mg of isoflavones are typically consumed daily. However, the intake of isoflavones in the US and Europe is significantly lower, with the average intake being less than 1 mg per day. In addition, the forms of isoflavones taken by the two cultures are also different. It is more common in Asian diets to include traditional non-fermented soy foods (tofu, soybeans and soy milk), fermented soy foods (miso and natto) or other soy products (fried, dried and pressed soy products). In contrast, in Western populations, Asian soy foods are rarely consumed but isoflavones are attained mainly in pure or enriched forms of soy protein isolates or dietary supplements together with small quantities from legumes, sprouts and some other vegetables. It has been shown that 10 mg of isoflavones everyday, obtained in a standard serving of tofu, may have lasting beneficial
effects against BC development in Asian women\textsuperscript{68}. In parallel, relatively high levels of soy isoflavones have been found in serum, urine and prostatic fluid of Asian men who consume a soy-rich diet which possibly contribute in lowering the incidence of prostate cancer\textsuperscript{69}. Hence, it is believed that the decreased risk of localized prostate cancer was also associated with soy product and isoflavone consumption\textsuperscript{70}.

It is believed that due to the high daily intake of soy isoflavones as an important dietary component for centuries, there are lower incidences of certain chronic diseases such as heart disease and cancer in Asian populations. In particular, epidemiologic studies have associated high soy intake with many health benefits, such as lowering incidence of cancers especially breast, prostate and colon cancers, protection from cardiovascular diseases\textsuperscript{71}, prevention of osteoporosis and attenuation of menopausal symptoms\textsuperscript{72}. Soybeans contain numerous biologically active compounds that may be important in BC. Although it is not clear which component of soy is the most responsible for its overall effects, isoflavones are believed to be important for these health benefits\textsuperscript{73,74}.

Laboratory research backed by epidemiological studies emancipating from the last few decades have provided convincing evidence that isoflavones in soy-rich foods contribute to relatively lower rates of BCs in Asian countries such as China and Japan than in Western populations (Figure II-3). On average, 1 in 8 women in the US develop BC compared to 1 in 30 women in Japan. Furthermore, the incidence of BC in migrant Japanese women to the US increases in the first and second generations of offspring after settlement in the US\textsuperscript{75}. Epidemiologic studies attribute the protective effect against
BC in Asian women to lifestyle, environmental and dietary factors, especially the high consumption of soy-contained foods\textsuperscript{76}. Increased soy food intake was associated with reduced risk of BC among pre-menopausal women\textsuperscript{77}, postmenopausal women\textsuperscript{78} and also BC survivors\textsuperscript{79}. In addition, protection against BC can be lost with a few generations after Asian immigrants are exposed to Western lifestyles\textsuperscript{80,81,82}. This observation further suggests that lifestyle and eating habit, in particular soy and vegetable intake, may play an important role in BC risk.

Figure II-3: Female BC incidence and mortality rates, 2002-2006 \textsuperscript{1}

Based on epidemiological findings of the relationship between soy and BC, numerous studies have been conducted to investigate the role of soy isoflavones in cancer
development. Clinical and preclinical laboratory animal and in vitro studies have demonstrated the hormonal activity of dietary isoflavones. It has been proposed that isoflavones, due to their estrogenic activities, are responsible for the chemoprotective effect of soy against BC^83.

2. Genistein and breast cancer

Isoflavone components found within dietary supplements are biochanin A, genistin, genistein, formononetin, daidzin and daidzein^84. In these products, genistein (4,5,7-trihydroxyisoflavone) is typically the isoflavone in the highest concentration^85. Because of its structural similarity to 17β-estradiol, genistein has shown to compete with 17β-estradiol in ER binding assays. Kuiper et al. ^86 reported that genistein binds to both ERs, with higher affinity to ER-β. The binding affinity of genistein for ER-α was 4%, and for ER-β was 87%, compared to estradiol. Thus, it is hypothesized that, by its affinity and competition with estrogen in interacting with ERs, genistein blocks the binding of more potent estrogen and also affects estrogen metabolism, thereby exerting a potential favorable role in the prevention of hormone related cancers.

Genistein, the predominant isoflavone found in plant food such as soybean which comprises a significant portion of Asian diet, has shown to inhibit carcinogenesis in animal models, via modulation of genes that are related to the control of cell cycle and apoptosis. Genistein inhibits activation of NF-κB and Akt signaling pathways, both of which are known to maintain a homeostatic balance between cell survival and apoptosis^33. Moreover, genistein antagonizes estrogen- and androgen-mediated
signaling pathways in the processes of carcinogenesis. Furthermore, genistein has been found to have antioxidant properties and shown to be a potent inhibitor of angiogenesis and metastasis. Taken together, both \textit{in vitro} and \textit{in vivo} studies have clearly shown that genistein is a promising agent for cancer chemoprevention and an adjunct to cancer therapy.

It has been reported that early exposure to reproductive hormones reduces the incidence of BC in women\textsuperscript{87} and the preventive effect on BC following early exposure to isoflavones in childhood or adolescence has been observed in some epidemiologic studies\textsuperscript{4,5}. Lamartiniere \textit{et al}. demonstrated that pre-pubertal exposure to genistein before administration of the carcinogen DMBA (dimethylbenzanethrene) was protective against mammary cancer in rats\textsuperscript{88,89}. This may due to the stimulatory effect of genistein on mammary gland cell maturation\textsuperscript{90}. Nagasawa \textit{et al}. suggest that genistein acts as an estrogen agonist to enhance mammary gland differentiation and the differentiated cells undergo less proliferation and therefore are less likely to progress through cancer process\textsuperscript{91} and this may be the mechanism responsible for the chemopreventive effects of genistein in the rat DMBA mammary carcinogenesis model\textsuperscript{92}.

However, not all studies have reported such protection against tumor development. Approximately two thirds of BCs are ER+ and isoflavones may adversely affect these tumors by exerting estrogenic and proliferation-inducing effects. Additionally, it is observed that the timing of exposure, the dosage applied and the form administrated are important issues that determine the outcome of isoflavone consumption on BC.
The protective effect of genistein against later development of BC may not apply to existing mammary tumors. Both laboratory and animal studies have shown that genistein acts as an estrogen agonist to stimulate the growth of ER+ BC cells\textsuperscript{93}. \textit{In vitro}, it has been demonstrated that genistein enhanced the growth of estrogen-dependent human BC cells at concentration as low as 10 nM and achieved proliferative effects similar to those of 1 nM estradiol with concentration of 100 nM. Expression of the estrogen-responsive gene \textit{pS2} was also induced in ER+ cell lines in response to treatment with a concentration of genistein as low as 1 µM\textsuperscript{65}. \textit{In vivo}, Ju \textit{et al.} showed that physiological dosages of genistein stimulated MCF-7 tumor growth in athymic mice in a dose-dependent manner\textsuperscript{94}. Thus, whether genistein acts as a chemopreventive agent or as a stimulatory agent to tumor growth is likely dependent on the timing of genistein administration. Furthermore, several animal studies have demonstrated that dietary genistein can interfere with BC treatments by negating or overwhelming the inhibitory effects of anti-estrogen tamoxifen or aromatase inhibitor letrozole\textsuperscript{10,11,12}, which further confirmed that genistein acts estrogenically via binding with the ERs.

Dosage of genistein used may also pose different effects on BC. High concentrations of genistein (>20 µM) inhibit the growth of estrogen-dependent human BC cells \textit{in vitro}\textsuperscript{95}. This anti-proliferative effect of genistein may be through its blocking effect of the cell cycle at G\textsubscript{2}-M phase and this inhibits tyrosine phosphorylation\textsuperscript{96,97,98}. However, it is unlikely that the concentration required to inhibit MCF-7 cell growth can be achieved \textit{in vivo} and hence such high concentrations in blood are not likely to be obtained from dietary exposure of genistein in humans as well\textsuperscript{99}. Even if the conjugated form is
biologically active, these levels are below the high concentrations required *in vitro* to produce a dose-dependent inhibition in tumor cell growth. While on the other hand, the low concentrations (0.1 to 1 µM) are easily achieved from typical consumption of dietary isoflavones among American women\textsuperscript{100}. Unfortunately, these physiologically relevant levels appear to be stimulatory to tumor development as documented previously.

There is some evidence suggesting that the effect of isoflavones on BC may also depend on the hormonal status of a woman. Case-control studies often found that soy is more protective against BC in pre-menopausal women than in postmenopausal women\textsuperscript{101}. Animal studies conducted on intact animals found that soy protein isolate containing genistein had no effect on mammary tumor growth\textsuperscript{102}, while studies using OVX mice observed tumor-stimulating effects from dietary genistein\textsuperscript{66}. This data suggests that in the presence of higher circulating estradiol levels, genistein may have insignificant impact on BC growth and development. However, when endogenous estradiol concentrations are low, genistein may be capable of stimulating tumor growth. This is an important issue because majority of BC cases are diagnosed in postmenopausal women whose endogenous estrogen levels are naturally very low and their body cells are hypersensitive to exogenous estrogens and estrogen agonists such as genistein. Consequentially, the estrogenic activity of these weak estrogens, isoflavones, may be more significant in postmenopausal women.

In addition, the sources of isoflavones may also have an impact on BC. In Asian countries, most isoflavones come from traditional soy diets, such as tofu and miso.
However, the intake of soy-based food remains low in the US, while consuming isolated soy isoflavones in pure form as in supplements or food products such as soy protein isolates are more popular approaches to increase isoflavone intake\textsuperscript{103}. An animal study conducted by Allred \textit{et al.} demonstrated that soy flour did not stimulate MCF-7 mammary tumors, while isoflavone extracts and pure forms did promote tumor growth\textsuperscript{104}. This result suggests that consuming less-processed soy foods instead of purified forms may be safer to women with BC.

There is still no conclusive clinical trial available to confirm the effect of isoflavones in humans. Some studies showed that exposure to various doses of soy isoflavones did not reduce mammographic density in pre-menopausal\textsuperscript{105} and postmenopausal women\textsuperscript{106} while some studies found that soy supplementation stimulated mammary gland cell proliferation in healthy women\textsuperscript{107,108}. Petrakis \textit{et al.} demonstrated that consumption of soy protein isolate had stimulatory effects on breast tissue of pre-menopausal women\textsuperscript{109}. These results suggest that soy isoflavones might increase BC risk in humans and hence bring serious concerns on the safety of consumption by women with BC.
C. Imaging techniques for cancer research

Biomedical research is often limited by the difficulty of gathering data from animals in a real-time longitudinal manner. The advances of molecular imaging technology have expanded the limits of animal research and allow cancer biologists to collect physiological and pathological information at multiple time points without sacrificing the animals. Using this approach, animals can act as their own control, which will produce more accurate and valuable information than that from various samples. As a result, non-invasive in vivo imaging in animal research has a clear advantage when monitoring a biological progressive process, such as cancer metastasis\textsuperscript{110}. The main techniques used in animal research include Positron Emission Tomography (PET), Computed Tomography (CT), Magnetic Resonance Imaging (MRI) and optical imaging using fluorescence or bioluminescence\textsuperscript{111}.

In our study, one of the optical imaging techniques was employed. To create images in optical imaging, an agent that can be detected through fluorescence or luminescence is administrated to the animals and a highly sensitive charge coupled device (CCD) camera is required to detect the light from visible to near-infrared range.

In fluorescence imaging (FLI), a colored protein, such as green fluorescent protein (GFP), is transfected into the cells so that they can be visualized from inside the body. This optical technique produces excellent images with structures near the surface but is limited to organs deep in the body\textsuperscript{112}.
In BLI, which was used in our experiment to follow BC progression, the light emitted is originated from a substrate-enzyme reaction\textsuperscript{113}. This can be done by tagging the cells with luciferase photon protein isolated from fireflies and prior to imaging, delivered to the animals with the firefly luciferin substrate in order to carry out the reaction which will emit light.

There are many advantages of employing BLI for animal research: 1) the use of luciferase as optical indicators allows detection of low levels of light signal because of its low noise background in live mammalian cells and tissues\textsuperscript{114}; 2) the substrate-enzyme reaction allows expression of luciferase only in the labeled cells and tissues which increases the specificity of the imaging. 3) BLI is a small and convenient unit that can complete the measurement in a short period of time, which makes it practical to image approximately 60 animals in one day. All of these advantages make BLI an attractive non-invasive imaging modality for animal and cancer research.

However, on the other hand, there are some limitations of BLI in BC metastasis research: 1) optical imaging is based on 2D image and hence results in poor spatial resolution\textsuperscript{115}; 2) light emitted from the enzymatic reaction can be attenuated by different organs and tissues, which is why metastasis in lung areas is always more easily detected than in liver areas in the imaging\textsuperscript{116}; 3) it is difficult to distinguish anatomic structures in the images because of the overlapping of light emitted from adjacent areas\textsuperscript{117}. All of these disadvantages make BLI a semi-quantitative method for cancer metastasis detection.
CHAPTER III  METHODS

A. Materials
Murine 4T1 BC cells, labeled with firefly luciferase (FL), were kindly provided by Dr. David Piwnica-Worms from Washington University (St. Louis, MO). Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 1% glutamine and Heat Inactivated Fetal Bovine Serum (HI-FBS) were purchased from the Cell Media Facility of the school of Chemistry Sciences, at the University of Illinois at Urbana-Champaign (UIUC). Penicillin/streptomycin and trypsin/EDTA were purchased from Invitrogen (Carlsbad, CA). Matrigel\textsuperscript{TM} matrix was purchased from BD Biosciences (San Jose, CA). Hanks balanced salt solution was purchased from Sigma-Aldrich (St Louis, MO). Laboratory animal diets were purchased from Research Diets (Brunswick, NJ). GEN was purchased from Indofine Chemical Company (Hillsborough, NJ). D-luciferin potassium salt was purchased from Regis Technologies (Morton Grove, IL). India ink was purchased from Sanford (Bellwood, IL).

B. Animals and diets
Thirty three 4-to-6-week old female OVX BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were ovariectomized at 21-days of age by the vendor and were allowed 1 week to recover after delivery. The animals were single caged in a controlled environment and were maintained under the standard light-dark cycle with artificial light (12-hour light and 12-hour dark) and were allowed unrestricted access to water and modified AIN-93G diet. All the housing and animal care was
carried out under approved animal study protocols by the Institutional Animal Care and Uses Committee (IACUC) of UIUC. During the study, the mice body weights were monitored and recorded weekly. Uterine wet weights were measured after the mice were sacrificed at the end of the study.

American Institute of Nutrition-93 Growth (AIN-93G) semi-purified diet was selected as a base diet for control mice as it has been established to meet all the nutritional requirements of mice\textsuperscript{118}. All the mice were fed AIN-93G diet throughout the study. One concern for the use of a commercial diet is the presence of phytoestrogens from plant sources such as soybean meal. Protein in the AIN-93G diet is derived from casein only and soybean oil was substituted with corn oil as a sole fat source; thus, the potential interference of phytoestrogens in soy-based protein and fat supplements is eliminated.

### C. 4T1 cell culture

The murine metastatic mammary carcinoma 4T1 cells are a transplantable tumor cell line\textsuperscript{119,120}. The 4T1-FL cells were maintained in DMEM supplemented with 10% HI-FBS, 1% glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.1% fungizone and incubated at 37°C with 5% CO\textsubscript{2} and 95% humidified air as a monolayer culture in 100 mm x 20 mm tissue culture polystyrene plates. Cells were harvested at 70-80% confluence by washing two times with 1X PBS followed by trypsinization with trypsin-EDTA. Cells were counted using a hemocytometer (Fisher Scientific, Pittsburgh, PA) and diluted in Hanks balanced salt solution to a concentration of ~5000 cells/100 μL before tail vein injection to the mice.
D. Tail vein injection of 4T1 cells

In this study, tail vein (TV) metastasis model was used. To make the mouse tail vein easier to locate for injection, the mice were placed in a mouse restrainer and the lateral tail veins were dilated by warming the tails in 42°C water for 3 min. 5000 4T1 cells in 100 µL Hanks balanced salt solution were injected intravenously into the lateral tail vein of each mouse. Dietary treatments were started on the same day. Mice were randomly divided into two groups in which they were fed with either AIN-93G as the control diet or AIN-93G plus 750 parts per million (ppm) genistein. Mice were also weighed once a week to monitor their weight difference in the two diets. To detect and monitor tumor metastasis, BLI was conducted on all of the mice routinely throughout the study (2 times a week) and the final imaging was performed prior to sacrifice. The study lasted 3 weeks after the tail vein injection. A thorough necropsy was conducted to verify metastasis at the time of sacrificing the mice. The lungs from all mice were infused with India ink to evaluate lung metastasis. All the protocols used in these experiments were approved by the IACUC at UIUC.

E. Bioluminescence imaging

BLI was carried out using a custom built imaging system assembled by Stanford Photonics (Palo Alto, CA) which included a photon-tight box, bright field illumination, warming plate and a dual micro-channel plate intensified charge coupled device (CCD) camera (Mega 10-Z with cathode cooling). Mice were injected intraperitoneally with 15 mg/mL D-luciferin solution in PBS was freshly made before each time of imaging. The amount of D-luciferin for each mouse was determined by body weight according to the
equation: Dosage (µL) = 10 x body weight (g). Three minutes after administration of D-luciferin, which is the time for optimal luciferase-luciferin activity, the mice were anesthetized using isoflurane/O₂ gas from a vaporizer and placed face-up in a height-adjustable tray in the imaging system for a whole body scan. General anesthesia was maintained through an inlet tube during the scanning process. Photon emission was accumulated for a 3-minute period using the imaging software Piper Control (Stanford Photonics, Palo Alto, CA) under movie mode which provided continuous recording of discrete 66 imaging frames and allowed analyzing and processing images at any time point during the 3 minutes. Bioluminescence was visualized as a pseudocolor scale: blue, the lowest photon flux; green, the highest photon flux which indicates that the imaging summation memory within Piper Control has reached full 16 depth. Acquired images were post-processed using the software ImageJ (NIH, Bethesda, MD) and Photoshop Elements (Adobe, San Jose, CA) software to combine background image (mouse body) with imaging data (tumor). Metastasis was semi-quantified using ImageJ based on two parameters, the bioluminescent area and the integrated density of the metastases. The images were set as 8-bit and the threshold for measurement was set at 50 (maximum 256).

F. Analysis of uterine wet weights
During necropsy, the mice were sacrificed and the uteri were removed and all connective and fat tissues were cleaned for assessment of wet weights. The uterine wet weight for each animal was measured and recorded.
G. India ink staining of animal lungs

The animal was placed on its back after being euthanized by CO$_2$. The rib cage was cut open to expose the lungs and an incision was made on the neck to expose its trachea carefully. Two mL of India ink solution (85% India ink / 15% dd H$_2$O) was slowly infused into the lungs through the trachea by a 25-gauge needle. The infused lung samples were kept in Fekete’s solution (900mL 70% ethanol / 90mL 37% formaldehyde / 15mL 91% acetic acid) for destaining. The tumor nodules do not absorb India ink, which results in the normal lung tissue staining black while the tumor nodules remain white. White tumor nodules were counted blindly by 3 individuals and the numbers were recorded and averaged as the tumor count on the lungs for each of the animals. This is a well accepted method for determining tumor load on the lungs. Lung samples were then further processed for the H and E staining to look for micro-metastases inside the lung tissue.

H. Hematoxylin and Eosin staining of lung samples

After tumor counting of the India ink-infused lungs, samples were dissected carefully. Two sections were vertically cut from the right lobe and another two sections were cut from the lowest lobe of the left side of the lungs. Matching locations were chosen for all the lung samples. To prepare the staining sections, the 4 lung sections were embedded in a paraffin block and the block was sliced into 5 $\mu$m sections and 3 sections per lung sample were randomly chosen to be mounted on the microscope slides. The slides were then stained by the conventional H and E staining and observed under the AxioSkop 40 microscope (Carl Zeiss, Thornwood, NY) for both macro- and micro-
metastasis. The pictures of the regions of interest (ROI) were taken and transferred to a computer screen using Axio Cam HRc (Carl Zeiss, Thornwood, NY). Metastasis was quantified by counting the tumor colonies in the lung tissue per section and measuring tumor areas using AxioVision AC software (Carl Zeiss, Thornwood, NY). Tumor area data are shown as parts per thousand of the tumor areas in the whole lung tissue per section. All slides were measured in a blind fashion without knowledge of the treatment groups.

I. Statistical analysis

Nonparametric statistical tests were performed for the independent and not normally distributed data from BLI, India ink staining and H and E staining in the experiment. All results were analyzed with the independent two-group Wilcoxon Rank Sum Test using the SAS program (SAS, Cary, NC). Error bars on all graphs are representative of the standard error of the mean.
CHAPTER IV RESULTS

A. Effect of genistein on animal weight
Animal weights were recorded once a week throughout the study starting from week 0 of tail vein cell injection until week 4 before the sacrifice of the mice. Figure IV-1 shows that body weights of the mice were slightly increasing throughout the study in both groups but there was no significant difference between the two diet groups on each day of measurement. It indicated that genistein diet did not affect the growth of the animals with metastatic BC during the experimental period.

B. Effect of genistein on uterine wet weight
Uterotrophic effects are directly affected by an estrogen receptor-mediated mechanism, especially by ER-α agonists and antagonists\textsuperscript{121}. Internal organs including the uterus were collected upon sacrifice of the mice on Day 21. The uterus was removed and all of the connective and fat tissues were cleaned during necropsy to minimize inaccurate measurement. Then the uterine wet weight was measured by an accurate balance in milligrams (mg). The average uterine wet weights of the control group and genistein group were 9.89 ± 1.0 mg and 9.86 ± 0.9 mg respectively, as shown in Figure IV-2. The genistein diet did not affect the uterine wet weight significantly compared to the control diet, proving that, although genistein has a similar structure to estrogen, it does not have as great an estrogenic effect on the uterine weight.
C. Bioluminescence imaging analysis

Twice-a-week BLI provided longitudinal monitoring of the metastasis progression. This technique is non-invasive, convenient and time-saving. It involves an enzymatic reaction between luciferase which is engineered into the tumor cells and the substrate luciferin, which is usually administrated intraperitoneally and distributed rapidly through the whole body, passing all blood-tissue barriers, including brain and placenta. Then the light emitted from the reaction is captured by a CCD camera. With the bioluminescence images, three parameters can be analyzed and used to reflect the metastatic tumor spread and severity. First, the metastasis occurrence rate was determined. Then metastatic tumor area and integrated density of the images were measured. All bioluminescence images were measured with the same threshold.

1. Metastasis occurrence rate

A mouse was counted as having metastasis when there was fluorescence light captured in the image regardless of the light intensity and the size of the metastases. The metastasis occurrence rate was expressed as the number of metastasis occurrences over sample size and the percentages were also calculated. BLI was carried out on Day 2, 6, 9, 13, 16 and 21 for both groups. On Day 21, one mouse was found dead in the genistein group.

The data for the number of mice showing metastasis is summarized in Table IV-1. The metastasis progression showed a gradually increasing growth pattern. There was no visual metastasis on BLI, even with increased contrast, until Day 9 of the study.
However, the metastasis appeared and increased as the experiment proceeded for both groups. From Day 9 to Day 13, the animals showing metastasis increased from ~20% to ~80% in both groups. Also, once the metastasis was detected by BLI, it kept spreading and the light emitted from the enzymatic reaction captured by the camera became increasingly stronger (Figure IV-3). After that, the rate remained high for both groups but genistein group showed decreased rate towards the end of the study. On the last imaging day (Day 21), control and genistein groups had ~88% and ~71% of metastasis occurrence rate, respectively.

Throughout the study, the BLI results indicated that genistein treatment resulted in decreased metastasis occurrence rate compared to that of the control group. However the difference on imaging was not significant indicating that the dietary genistein did not alter the development of metastasis to the lungs.

From imaging pictures, it was observed that metastasis occurred not only in the lungs but also in other organs including the lymph node, abdomen, leg and pelvis as shown in Figure IV-4.

2. Area and integrated density of metastases

After counting the number of mice showing metastasis, we measured the area and integrated density of the metastatic tumors using ImageJ at a setting of 8-bit and threshold 50. This is only a semi-quantitative measurement giving a general idea of the BC metastasis. The images from BLI were analyzed from Day 2 to Day 21. Results of
the BLI area and integrated density on each day of imaging with the two groups of mice are summarized in Figure IV-5 and Figure IV-6, respectively. Both parameters showed similar trends. There is no metastasis observed for the first 2 days of imaging. From Day 9 onwards, the metastasis progression was gradually increasing until it reached the highest level on the day before mice sacrifice. Same as the result of the metastasis occurrence rate above, the genistein group showed less metastasis in each day of measurement with metastasis progressing more slowly throughout the experiment compared to the control group. However, with the use of the Wilcoxon Rank Sum Test for statistics, there is no significant difference between the two groups, in terms of the measured area and integrated density of the light captured from the BLI.

D. India ink staining analysis

India ink staining was conducted to count visible tumor nodules on the surface of lung samples. Compared to the original lung sample, the use of India ink infusion provides an easier visual observation of tumors on the lung surface as demonstrated in Figure IV-7. On average, 1.95 ± 0.49 white tumor nodules were counted in the genistein group, compared to 2.15 ± 0.57 of the control group (Figure IV-8). There is no significant difference in the number of tumors on the lung surface between the two groups. Genistein group did not show any effect on the metastatic tumor burden.

E. Hematoxylin and Eosin staining analysis

H and E staining was carried out to quantify micro-metastasis inside the lungs in order to evaluate the severity of tumor burden. Two parameters were analyzed for the H and
E stained lung tissues, counting of both the macro- and micro-tumor colonies and measuring of the tumor area of the observed metastases.

1. **Tumor colonies of lung samples**

After India ink staining, the normal lung cells were black in color while the dense tumor cells appeared in reddish purple color as a consequent of the H and E staining. In this way, we could easily distinguish normal and tumor cells under the microscope. H and E can also detect micro-metastasis which cannot be observed with India ink staining. We observed large tumor areas in the lung tissue from mice with their India ink-infused lungs also showing white tumor nodules on the surface. In addition to the macro-metastasis, we counted small tumor areas (micro-metastasis) as well. The representative images of H and E stained macro-metastasis and micro-metastasis are shown in Figure IV-9 and Figure IV-10 respectively. Both macro- and micro metastasis were added together and expressed as the total tumor counted in each of the lung samples and used to compare the effects of the two diet groups. On average, 4.20 ± 0.99 tumor colonies were observed in the genistein animals while 4.31 ± 1.14 tumor colonies were counted in the control animals. This trend of having less metastatic lung tumors observed in the genistein group was consistent with both the results of BLI and that of India ink. However, again, the data did not show any significant difference between the two groups. The results of tumor count after H and E staining is shown in Figure IV-11.
2. Tumor areas of lung samples

Pictures of the tumor colonies were taken with the tumor cells on the background of the India ink-stained normal cells. Then the area of all the tumor colonies in a lung sample was measured. To compare the effect of the two diets on metastasis, the ratio of the measured tumor area to the whole lung area was used. In Figure IV-12, which displays the ratios of each individual animal and the mean ratio for both groups, it shows that the control group had relatively lower tumor area/lung area ratios with its average tumor area ratio smaller than that of the genistein group. Although this result was opposite to the previous experimental approaches, again the increase in the genistein group was not significant compared to the control group.
Figure IV-1: Animal weight throughout experimental period

Measurement was carried out once a week. Results were expressed as mean ± standard error. Control group with n = 16 while genistein group with n = 14/15. Animals’ weights increased slightly for both groups throughout the duration of the study with no significant difference between the two groups on each day of measurement. Week 0 was the injection week and the mice diet was switched to AIN-93G regular diet or genistein diet and maintained throughout the study until the mice were sacrificed after 3 weeks.
Results were expressed as mean ± standard error. Control group with n = 16 while genistein group with n = 14. There was no statistical difference in the average uterine wet weights measured between the genistein group (9.89 ± 1.0 mg) and the control group (9.86 ± 0.9 mg).

Figure IV-2: Uterine wet weight of sample groups during necropsy
<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>Genistein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>0 / 5 (0%)</td>
<td>0 / 5 (0%)</td>
</tr>
<tr>
<td>Day 6</td>
<td>1 / 7 (14.29%)</td>
<td>1 / 6 (16.67%)</td>
</tr>
<tr>
<td>Day 9</td>
<td>4 / 16 (25.00%)</td>
<td>3 / 15 (20.00%)</td>
</tr>
<tr>
<td>Day 13</td>
<td>13 / 16 (81.25%)</td>
<td>12 / 15 (80.00%)</td>
</tr>
<tr>
<td>Day 16</td>
<td>14 / 16 (87.50%)</td>
<td>11 / 15 (73.33%)</td>
</tr>
<tr>
<td>Day 21</td>
<td>14 / 16 (87.50%)</td>
<td>10 / 14 (71.43%)</td>
</tr>
</tbody>
</table>

**Table IV-1: Metastasis occurrence rate of sample groups over test period**

Results were expressed as the number of metastasis occurrences over the sample size with the percentage occurrence calculated in blanks. On Day 2 and 6, half of the entire population of the two groups was imaged for metastasis. While on Day 9, 13, 16 and 21, all the mice were undergoing BLI and one mouse was found dead in the genistein group on Day 21. The rate increased rapidly from Day 9 to Day 13 and stayed high towards the end of the study with the control group showing relatively higher number and percentage of metastasis.
The bioluminescence images were taken from Day 2 to Day 21. For Day 2, Day 6 and Day 9, there was no visual metastasis from BLI. The metastasis was first detected on Day 13. It kept spreading and the light emitted from the enzymatic reaction captured by the camera became increasingly stronger until saturation at the end of the study.
Other organs including the lymph node (control mouse #19), abdomen (genistein mouse #32), leg (control mouse #3) and pelvis (genistein mouse #16) had shown metastasis on the bioluminescence images. Those metastases were also measured and counted as the overall metastasis in the animals.
Results were expressed as mean ± standard error. Control group had n = 16 while genistein group had n = 14/15. No sign of metastasis for the first 2 days of imaging. Trend of increasing metastasis from Day 9 to Day 21 observed for both groups with the control group showing more metastasis on each day of measurement and its level increased more rapidly than that of the genistein group. No significant difference in metastasis area between the two groups.
Figure IV-6: BLI integrated density results of two diet groups over time

Results were expressed as mean ± standard error. Control group had n = 16 while genistein group had n = 14/15. It showed similar trend with the result of the BLI area. Similarly, the integrated density of the control group was not significantly different than that of the genistein group.
India ink infusion resulted in white tumor nodules on the black background of India ink-infused normal lung tissue. It enhanced the visual counting of the tumor burden on the lungs and it also increased the volume of the lung and hardened the lung tissue for easier dissection in the following H and E staining.

Figure IV-7: Appearance of original and India ink-infused lung sample specimens
After India ink staining, white tumor nodules on the lung samples were counted independently by 3 individuals who were blind to the study design. Results were expressed as mean ± standard error. Control group with n = 16 and genistein group with n = 14. On average, 2.15 ± 0.57 tumors were counted on the lung surface of the control group compared to 1.95 ± 0.49 tumors in the genistein group. The genistein diet administrated after cancer cell injection showed no significant effect on the tumor burden observed in the animal model compared to that of the control group.
Figure IV-9: Macro-metastasis of sample lung tissue observed after H and E staining

Large tumor areas stained with H and E on the black background of the India ink-stained normal lung tissue. Each area was counted as one tumor colony and its area was measured.
Figure IV-10: Micro-metastasis of sample lung tissue observed after H and E staining

Arrows indicate much smaller tumor areas within the lung tissue compared to the macro-metastasis. Each area was counted as one tumor colony and its area was measured.
Both macro- and micro-metastasis were counted and added together for each slide of lung tissues and average was taken from all the animal samples for each group. Control group with $n = 16$ and genistein group with $n = 14$. Results were expressed as mean ± SEM. On average, control group had $4.31 ± 1.14$ tumor colonies on the lung tissue compared to $4.20 ± 0.99$ tumor colonies from the genistein group. Although the control group showed slightly more tumor colonies than that of the genistein group, there is no significant difference in the number of tumor developed between the two groups. The results were similar to those of the India ink tumor counting.
Figure IV-12: Tumor to lung area ratio of sample groups observed after H and E staining

Control group with $n = 16$ while genistein group with $n = 14$. For each of the lung samples, both the tumor area and the whole lung area on the slide were measured and the results were expressed as:

$$\frac{\text{Tumor Area}}{\text{Lung Area}}$$

Ratio of Individual Animal –

- (Control group)
- (Genistein group)

Control group mean ratio –

Genistein group mean ratio –

Control group showed lower tumor area/lung area ratios and its average ratio was also smaller than that of the genistein group but the differences were not statistically significant when analyzed using the Wilcoxon Rank Sum Test.
CHAPTER V  DISCUSSION

BC is the most commonly diagnosed and the second most common cause of cancer death among women after lung cancer in the US. BC causes more than 40,000 deaths in women each year, the majority of which are due to metastasis to distant organs\textsuperscript{122}. As a result, cancer researchers have begun to pay more attention to the mechanisms of metastasis and the potential therapies targeting this process.

It is extremely difficult to treat the disease once cancer has metastasized and our results of BLI images also showed that once the metastasis was detected by BLI, it kept spreading and the light captured from the tumor became increasingly stronger (Figure IV-3). Hence, treatments that influence the development of metastasis may potentially affect overall BC survival.

BC rates increase with age and \textasciitilde75\% of cancer cases occur in women age 50 or older\textsuperscript{1} and \textasciitilde70\% are estrogen-dependent\textsuperscript{123}. Because of the significantly reduced ovarian function of postmenopausal women, the estrogen levels in their body reduce drastically. During menopause, the ovaries stop producing estrogen. Circulating estrogen levels in postmenopausal women range from 20–150 pM\textsuperscript{124}, which is much lower than the estrogen levels observed in pre-menopausal women (700-3500 pM at pre-ovulation)\textsuperscript{125}. Since estrogens are essential for normal functions of the female reproductive system, such as breast development and menstrual cycle, the decreased hormonal levels result in a variety of symptoms, including vasomotor symptoms (hot flashes and night sweats), vaginal symptoms (dryness, discomfort, itching and dyspareunia) and neurological
symptoms (trouble sleeping, depression, anxiety, memory loss and headache). One of the harsh consequences of low estrogens is severe osteoporosis. That is one of the main reasons why HRT became popular to replace the failing estrogen levels in the body in order to relieve these symptoms traditionally.

However, connection between estrogen and BC was first recognized in 1896 when a Scottish doctor George Beatson showed regression of mammary tumors with ovariectomy of pre-menopausal women with advanced BC. More recent epidemiologic data observe higher BC risk in women with prolonged period of estrogen exposure. Elevated serum and urine estrogen concentrations have been observed in postmenopausal BC patients. All these observations suggest excessive endogenous hormones, especially estrogens, may increase the chance of developing BC. In addition, exogenous hormone use can raise circulating estrogen levels in the body and affect BC risk as well. Both HRT and oral contraceptives have been reported to increase the risk of the disease. Women’s Health Initiative, a large randomized clinical trial, reported that a 5-year treatment of estrogen plus progestin increased the risk of BC and heart disease in healthy postmenopausal women. Furthermore, study has shown that ER+ BC incidence decreased following the drop of HRT use that began in 2002 and the decrease is particularly significant among women aged 50-69 years, in whom HRT use is the most common. All these evidences indicate that estrogen plays an important role in the growth and development of BC, especially in postmenopausal women.
As a result, many oncologists do not recommend HRT to BC patients due to the concern that it may also increase the risk of metastatic BC. Therefore, in the US, more and more of these women tend to self-medicate with dietary supplements containing soy isoflavones as an alternative therapy to relieve menopausal symptoms and improve bone health. Approximately 30% of Americans are using at least one complementary and alternative medicine (CAM) such as dietary and herbal supplements per year, typically for chronic rather than acute medical conditions\textsuperscript{134}. In addition, a recent review showed that 45% of BC patients used CAM (including soy isoflavones) not prescribed by physicians\textsuperscript{135, 136}. In the last decade, phytoestrogen-containing products have increased in the market about 4-fold\textsuperscript{137}.

Soy isoflavones are assumed to be a safe alternative to HRT with the perception that these phytoestrogens are without the risks associated with HRT since they are from natural products and soy has been proven to have many health benefits based on some epidemiologic studies. However, consumption of whole soy foods may have different effects than that of isolated soy isoflavones and hence isoflavones, in pure form, as in supplements, may pose some health concerns to metastatic BC patients.

The role that isoflavones play in BC is unclear. Genistein, being the major isoflavone in soybeans, acts as an estrogen agonist at physiologically relevant dosages\textsuperscript{138}. Some reports indicate that genistein can prevent the development of BC. Studies published over the last 5 years have demonstrated that exposure to dietary genistein before puberty reduces the number of chemically induced mammary tumors formed in female
Sprague Dawley rats. Whereas others show that genistein stimulates the growth of existing estrogen-dependent tumors.

The objective of the current study was to evaluate the effect of dietary genistein, being the most abundant and active component in soybeans, on BC metastasis using 4T1 inoculated OVX BALB/c mouse model which mimics postmenopausal women with metastatic BC. This research is important because genistein exists in high concentration in isoflavone-containing foods or supplements that may be consumed by many women including BC survivors for alleviating menopausal symptoms. Our animal model injected with 4T1 cell line is a syngeneic model which mimics postmenopausal women with a highly metastatic BC and genistein was administrated after cancer onset. Hence, the results from this study assist in the investigation of the effects of genistein on BC metastasis and the safety concerns of consuming dietary isoflavone supplements by BC patients to treat menopausal symptoms.

There are several reasons of using 4T1 cancer cell: 1) it is a murine cell line originated from a single mouse mammary tumor by Miller in 1983\textsuperscript{139,140}, which is from the same species of the experimental animals. This can eliminate the use of immuno-compromised nude mice if human cells are used which may not be an ideal model to study metastasis when considering the interaction between tumor cells and the host animal; 2) it can develop spontaneous metastasis in a similar manner to that observed in a clinical environment; 3) the luciferase genes stably integrated \textsuperscript{141} allow the quantification of 4T1 cells \textit{in vivo} using BLI, which is a fast, convenient, sensitive and
non-invasive method of detecting and monitoring the spread and severity of tumor metastases in real-time. Previously, our team conducted an *in vitro* assay to evaluate the luciferase activity of 4T1 cells. The intensity of light from BLI was positively correlated with the number of 4T1 cells, indicating that it is an effective technique to monitor tumor growth.

The conventional tail vein injection approach we used can directly introduce cancer cells into the blood circulation of the animals and easily induce metastasis in organs that have a rich blood supply, such as lungs. We observed majority of lung metastasis from BLI together with few other metastatic locations (Figure IV-4) including lymph nodes, abdomen, legs and pelvis. However, one of its potential disadvantages is that there is no primary tumor established. Cancer metastasis usually involves the establishment of primary tumor, infiltration of tumor cells into the circulation and development of distal metastasis. Elimination of early steps in metastasis cascade may change the interpretation of the results. In some cases, lung metastasis developed from tail vein injection has been described as multiple primary tumors in the lungs because of the absence of primary tumors. Also, after tail vein injection, most of the tumor cells would be killed by the immune system, which caused a long latent period. Other metastasis models can be considered, such as the injection of cancer cells to mammary fat pad or bone marrow cavity of tibia. Mammary fat pad injection is more similar to human BC and includes all the necessary steps of disease progression but the fast growth of primary tumor will be problematic. With enriched blood supply and growth factors, bone is the most common site for BC metastasis. In addition, due to the development of
primary bone metastasis in the intra-tibia injection model, the development of lung tumors can be considered as secondary metastasis. Hence, in our future studies, we may consider using different injection models to elucidate the mechanism of different metastasis patterns caused by various injection routes and determine if the use of different cell administration routes would affect BC metastasis and the outcomes of different dietary treatments.

Since different concentration of isoflavones applied will alter their hormonal effects as discussed previously, it is important to focus on their effects at plasma concentrations that are relevant to what is observed in humans consuming isoflavone-containing diets. In our animal study, 750ppm of dietary genistein dosage had been chosen to evaluate its effect on BC metastasis. Based on our previous studies, this dosage provides approximately 1 to 2 µM plasma genistein concentration in mice, which is similar to the blood levels in women consuming soy supplement in the US\textsuperscript{142} and women consuming varying amounts of isoflavones from soy milk\textsuperscript{143}.

Once the two different dietary treatments (control and genistein) began, BLI was performed to detect metastasis and monitor tumorigenesis in which the metastasis occurrence rate and area could be recorded throughout the study (Table IV-1 and Figure IV-5). At the end of the study after sacrificing the animals, the metastatic tumor burden on the lungs was counted with India ink staining and macro- as well as micro-metastases were detected using H and E staining of the lung samples from both the genistein treated animals and control animals.
All of the data collected could give an overall view of the seriousness and extent of BC spread in the experimental animals due to the genistein diet. In general, mice in genistein diet group exhibited less lung metastasis than those in control group. The lungs from genistein treated mice showed less tumor nodules and smaller tumor areas (both macro-metastasis and micro-metastasis) than that of the control animals. However, the differences were not statistically significant and we can conclude that genistein has no estrogenic effect on breast cancer metastasis in this study.

Also, when observing the uterine wet weight of the two groups, there was no detectable uterotrophic effect by genistein as well. It may be due to the fact that genistein is only a weak estrogen agonist, which has a much lower affinity to ERs than that of estradiol and hence its estrogenic effect on uterus is much weaker. Also, since ER-α is dominant in breast and uterus and ER-β is preferentially expressed in heart, prostate and bone\(^{144}\), genistein, being shown to have a much higher affinity to ER-β than ER-α and an affinity to ER-α 100-1000 times lower than estrogen, is believed to pose less estrogenic effect on uterus. This may be beneficial in reducing the risk of endometrial cancer when consuming genistein.

Most of our previous studies were designed to evaluate the effect of soy isoflavones, being weak estrogens, on ER+ BC and the related treatments on their growth. However, in this study, 4T1 cells were chosen to represent the later stage BC cells. 4T1 cells are estrogen non-responsive and ER-α negative. Hence, the effect on lung metastasis by genistein was more likely to be indirect. Two recent studies have demonstrated that
estrogen promoted metastasis by influencing the host physiology and they suggested that estrogenic compounds may also affect metastasis indirectly\textsuperscript{145,146}. Since genistein is estrogenic and acts similar to estrogen, it is possible that genistein exerts similar systemic effects on the microenvironment for tumor to metastasize. Additionally, isoflavones have also been found to have non-hormonal activities independent of the ER\textsuperscript{147}. It has been suggested that soy isoflavones may influence BC risk via their anti-proliferative, anti-angiogenic, anti-oxidative and anti-inflammatory properties\textsuperscript{148}. Nevertheless, the specific actions of genistein on this process are still unclear. Although this study showed no effect of genistein influencing the overall tumor progression and tumor burden appeared on lungs, other possible mechanisms may involve angiogenesis, growth factors or other steps during the metastatic process.

Isoflavones possess diverse biological activities and potencies. These activities are often dependent upon the concentration and timing of administration of the isoflavones and the types of cancer cells. In regard to BC, isoflavones, specifically genistein, have paradoxical effects. For example, genistein has a stimulatory effect at lower concentrations and an inhibitory effect at higher concentrations and only minor changes in dose can dramatically alter its biological responses\textsuperscript{149}. Also, pre-pubertal exposure to genistein appears to be protective against the development of BC, but consumption of the phytoestrogen in either pure form or in soy protein isolate after the development of an estrogen-dependent BC may enhance tumor growth, while our study demonstrates that dietary genistein has no beneficial or adverse effect on lung metastasis of estrogen non-responsive BC \textit{in vivo}. 
Consumption of soy isoflavones as dietary supplements may have numerous health benefits such as reducing risk of cardiovascular disease, osteoporosis and alleviating menopausal symptoms but the biological activities of genistein may be different once a cancer exists\textsuperscript{150}. As a result, additional investigation into the biological activities and specific actions of genistein including effects on estrogen metabolizing enzymes, cell cycle, cell differentiation, proliferation, apoptosis, the inflammatory response and various other cell signaling pathways and the mechanisms involved in metastasis is needed in order to fully evaluate the biological relevance of experimental findings and to conclude if the consumption of genistein-containing products will be advisable and have no adverse effect on BC progression for the subgroup of postmenopausal women who have or are at high risk of developing BC.
A. Conclusions

Soy isoflavones have functional similarity to human estrogens and may protect against BC as a result of their anti-proliferative activity or increase risk as a result of their estrogen-like properties. Hence, in the current study, we examined the relationship between isoflavones supplementation and BC metastasis, the major cause of BC deaths among postmenopausal women. Since distant metastasis is associated with increased mortality, BC patients need to be cautious when consuming products containing phytoestrogens such as genistein for the treatment of menopausal symptoms.

The specific objective of the study presented in this thesis was to evaluate the effect of dietary genistein on BC metastasis using preclinical animal models implanted with 4T1 murine BC cells via 1) the application of BLI techniques to monitor tumor progression; 2) the use of India ink staining to estimate the metastatic tumor burden on the lung surface; 3) the analysis of H and E stained lung samples of micro-metastases.

BLI has proved to be a very effective technique for monitoring metastasis progression in vivo and real-time semi-quantitative data could be collected without sacrificing the animals throughout the experimental period. From the BLI data, we found that genistein diet did affect the metastasis occurrence rate during the study. There was no effect on BLI areas and integrated density of the metastasis areas with genistein supplementation.
Secondly, with the enhanced visual counting of the India ink-infused lungs, 750ppm of dietary genistein was found to have no significant effect on 4T1 tumor growth on the lungs.

The last experiment was designed to detect and count the micro-metastasis in mice on the two diet treatments. The impact of dietary genistein on the total lung metastasis count was not significant and the tumor area measured was not different for the genistein group from the control group. To conclude, there is no significant difference in tumor burden in the lung tissues between the two diet groups.

In summary, although we did not observe a beneficial effect of soy-derived isoflavones on BC metastasis, there was also no sign of adverse effects. Hence, from this study we can conclude that dietary genistein has no effect on BC metastasis and lung tumor burden in postmenopausal animal model when supplemented after cancer cell implantation.

It can be concluded that although genistein has an estrogen-like structure but it may not have a strong systemic effect as the endogenous estrogens on the microenvironment that is important for metastatic tumor development. However, on the other hand, genistein may act on the other pathways or mechanisms involved in metastasis for it is a complex and multi-factor disease. As a result, further BC metastasis research is needed to produce more consistent preclinical data in order to determine if the consumption of genistein is safe for BC survivors, especially for women in their
postmenopausal age group, for genistein is hypothesized to exert greater estrogenic effects in a low endogenous estrogen environment\(^1\). Future investigation of the effect of dietary genistein on BC metastasis and the understanding of the related mechanisms is important to help health professionals to make appropriate recommendations on isoflavones supplement consumption.

**B. Future directions**

Evaluation of metastatic occurrence and lung tumor burden is one way of representing BC metastatic stage. It is necessary to conduct more research to investigate the possible mechanisms or pathways on how isoflavones act to influence BC metastasis through the systemic effects on metastatic tumor development. The future directions of the research may include:

1) To evaluate the effects of dietary genistein on different possible cellular mechanism of BC metastasis including the regulation of cell cycle (proliferation) and programmed cell death (apoptosis). The ratio between proliferation and apoptosis regulates metastatic tumor growth and expansion of metastases. Some studies had demonstrated that dietary genistein significantly decreased the percentage of proliferating tumor cells and significantly increased apoptotic tumor cells;

2) To evaluate the effects of dietary genistein on BC metastasis when it is taken before cancer cell injection to investigate if it can act as a preventive measure to BC for postmenopausal women and if it will show more significant protective results since all of
the results in this study were not significant although genistein had shown to have lowered metastases and lung tumor burden compared to control. Hence, it is suspected that genistein may show more significant beneficial effects if taken before BCs exist in the body;

3) To vary other factors, for examples dosage of genistein, timing of administration and form or level of processing, to investigate the difference in effects on BC metastasis;

4) To evaluate the effects of dietary genistein on metastasis to other tissues using different administrative routes such as the mammary fat pad injection model or intra-tibia injection model for there may be some drawbacks of the tail vein injection model;

5) To evaluate other isoflavones compounds such as daidzein and equol and their effects on BC metastasis.

Some of these proposed studies are under investigations by the colleagues in our laboratory and we are looking forward to reporting our research findings in the near future.
REFERENCES


