

**THERAPEUTIC AND CHEMOPREVENTIVE POTENTIAL OF LUTEOLIN
AGAINST GROWTH AND METASTASIS OF BREAST CANCER**

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**THERAPEUTIC AND CHEMOPREVENTIVE POTENTIAL OF LUTEOLIN
AGAINST GROWTH AND METASTASIS OF BREAST CANCER**

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In loving memory of, Edward R Fieseler.

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LIST OF ABBREVIATIONS

Commonly used abbreviations found throughout this dissertation:

ACS	American Cancer Society
AKT	Protein Kinase B
ALDH	Aldehyde Dehydrogenase
ANOVA	Analysis of Variance
AR	Androgen Receptor
BCA	Bicinchoninic Acid
BCC	Breast Cancer Cell
BCL	B-cell Lymphoma, a Regulator of Apoptosis
BRCA	Breast Cancer, Early Onset (Gene)
CD24	Cluster of Differentiation 24
CD31	Platelet Endothelial Cell Adhesion Molecule
CD44	Cluster of Differentiation 44
CDK	Cyclin-dependent Kinase
CK	Cytokeratin
CSC	Cancer Stem Cell-like
DCC	Dextran-coated Charcoal
DCIS	Ductal Carcinoma In Situ
DMBA	7, 12-Dimethylbenz(a)anthracene
DMSO	Dimethyl Sulfoxide
E2	Estrogen
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay

EMT	Epithelial to Mesenchymal Transition
ER	Estrogen Receptor
FACS	Fluorescent-activated Cell Sorting
GEM	Genetically engineered mouse
GR	Glucocorticoid Receptor
HER2	Human Epidermal Growth Factor Receptor 2
HIF-1 α	Hypoxia-inducible Factor
HRT	Hormone Replacement Therapy
IgG	Immunoglobulin G
IHC	Immunohistochemistry
i.p.	Intraperitoneal
i.v.	Intravenous
KDR	Kinase Insert Domain Receptor (aka VEGFR2)
KDR-ab	KDR-antibody
Ki67	Nuclear Protein Associated with Proliferation
LU	Luteolin
MAPK	Mitogen-activate Protein Kinase
MET	Mesenchymal to Epithelial Transition
MHT	Menopausal Hormone Therapy
miRNA	MicroRNA
MMP	Matrix Metalloproteinase
MNU	N-methyl-N-nitrosourea
MPA	Medroxyprogesterone Acetate
NE	Norethindrone
NG	Norgestrel
NF-kB	Nuclear Factor-Kappa Beta

P	Progestin
P4	Progesterone
p53	Tumor Suppressor Protein P53 (TP53)
PDTX	Patient Derived Tumor Xenograft
PDGF	Platelet-derived Growth Factor
PDGFR	Platelet-derived Growth Factor Receptor
PI3K	Phosphoinositide 3-Kinase
PR	Progesterone Receptor
PRE	Progesterone Response Element
RANKL	Receptor of Activated Nuclear Kappa-Beta Ligand
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RU-486	Mifepristone, an Antiprogestin
SP-1	Specificity Protein 1 (Transcription Factor)
STAT3	Signal Transducer and Activator of Transcription 3
TNBC	Triple-negative Breast Cancer
TNF	Tumor Necrosis Factor
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VEGF	Vascular Endothelial Growth Factor
VEGFA	Vascular Endothelial Growth Factor A
VEGFA-ab	VEGF antibody
VEGFR	Vascular Endothelial Growth Factor Receptor
WHI	Women's Health Initiative

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DISSERTATION ABSTRACT

Breast cancer is the second leading cause of cancer-related death in older women. Many postmenopausal women undergo hormone replacement therapy (HRT) to alleviate the symptoms of menopause such as nausea and decreased libido. Unfortunately however, combination HRT, which contains both progestin and estrogen, is associated with an increased risk of breast cancer compared with women who receive estrogen alone. Recent studies implicate the progestin component of HRT as being most likely responsible for elevated breast cancer risk and increased mortality. Studies from our laboratory show that vascularization of mammary tumors is increased by progesterone, which is endogenous, and synthetic progestins, which induce the production of vascular endothelial growth factor (VEGF) by tumor cells. VEGF is potently angiogenic and its synthesis by breast cancer cells is associated with tumor expansion and increased mortality. Progestin-dependent tumor progression can be managed by synthetic anti-progestins such as RU-486; however, such compounds have serious side-effects due to cross reactivity with other steroid receptors, limiting their use. In the studies described herein we examined the ability of luteolin (LU), a naturally-occurring flavonoid found commonly in fruits and vegetables, to both prevent the onset of breast cancer, and inhibit breast tumor growth and metastasis.

In order to examine the preventative potential of LU we administered the flavonoid intraperitoneally (i.p.) in a progestin (P)-accelerated dimethylbenz(a)anthracene (DMBA)-induced rat model of mammary tumorigenesis. The ability of LU to prevent tumorigenesis was dose-dependent. The administration of both low and high doses of LU reduced tumor incidence, delayed the onset of tumor formation and decreased the number of large tumors formed. In contrast, the intermediate dose only prevented established tumors from expanding, i.e. minimized tumor volume. Immunohistochemical (IHC) analysis of mammary tissue showed that LU exerts suppressive effects on VEGF production that are long-lasting, as demonstrated by disruption of angiogenesis and intratumoral vascularization, which was further supported by CD31 analysis that determines blood vessel density.

In order to ascertain its therapeutic potential we examined the ability of LU to squelch P-induced VEGF production, a key component of neovascularization, proliferation, and metastasis. LU reduced both P-dependent VEGF mRNA synthesis and protein secretion in hormone-dependent human breast cancer cells, effects that occurred at non-apoptotic doses. This suggests that LU-mediated VEGF suppression precedes loss of cell viability. With this in mind we assessed the effects of LU in a P-dependent breast cancer xenograft nude mouse model and determined that the flavonoid effectively blocked the growth of P-dependent T47-D xenograft tumors. IHC revealed a dramatic reduction in VEGF and a moderate decrease in intratumoral blood vessels. Additional in vitro studies provided evidence suggesting that LU diminishes P-dependent stem cell-like enrichment, which likely explains the observed rapid decline in tumor volume. Progestins not only increase the risk of breast cancer in postmenopausal women but have also been implicated in driving 'stemness', an indicator of metastatic potential. Furthermore, hormone-driven breast cancer may escape its hormone dependency through receptor loss and compartmentalization, potentially progressing to more

aggressive, hormone-independent types of metastatic breast cancer. In order to address such a situation we employed a hormone-independent xenograft model of lung metastasis in which triple-negative breast cancer cells were injected via tail vein and allowed to circulate to distant lung capillaries. Two triple-negative breast cancer cell lines were used (MDA-MB-435 and MDA-MB-231 (4175) LM2 cells). LU significantly reduced the formation of lung colonies arising from both cell lines, with minimal to no animal toxicity. We next investigated the mechanism behind suppression of lung metastasis by LU and determined in vitro that its effects may be explained, at least in part, by reduced migration of triple-negative cells arising as a result of VEGF loss and consequently decreased VEGF signal propagation.

Overall, the studies reported in this thesis provide compelling evidence that LU possesses important chemopreventative and therapeutic properties which might be harnessed to combat metastatic breast cancer. Further studies are required to capitalize on these findings and to better understand the mechanism(s) by which LU suppresses the pathologic effects of progestins on breast tumor initiation and growth, as well as how the flavonoid reduces metastasis. The prospect of using LU as a chemopreventative agent is especially exciting and additional animal studies will be required to determine whether orally ingested LU produces effective circulating levels. The ultimate goal is the use of LU to both prevent and combat breast cancer in women. If this is to be achieved, clinical trials will be essential as a means of determining the appropriate dose and route of application.

STATEMENT OF SIGNIFICANCE AND OVERALL HYPOTHESIS

In the United States breast cancer is the second leading cause of cancer related death in women, with over 200,000 new cases and 40,000 deaths annually [1]. Seventy percent of all breast cancers express estrogen and progesterone receptors (ER and PR) [2], which promote tumor growth and metastasis [3-6]. Expression of ER and PR however, creates potential drug targets. Many post-menopausal women choose to undergo hormone replacement therapy (HRT), which alleviates the symptoms of post-menopause such as nausea, mood disorders, etc. Women without a uterus undergo HRT that contains only estrogen. However, women with an intact uterus are given an HRT regimen that contains a combination of both estrogen and progestin, since the progestin component reduces the risk of endometrial cancers that can arise as a consequence of estrogen-induced uterine cell proliferation [7]. Clinical trials and studies show that post-menopausal women undergoing combination HRT have an increased risk of developing breast cancer compared with those receiving estrogen alone [8-12]. Currently, an estimated 1.6 million post-menopausal women undergo combined HRT in the United States [13] and recent reports support the notion that it is the progestin component that is responsible for a higher incidence of breast cancer and related mortality in these women [2]. Moreover, current studies further bolster this concept by showing that progestins cause proliferation of breast epithelial cells in vivo [14, 15]. Since HRT dramatically improves the quality of life in these women, there is an urgent need for novel non-toxic compounds which can be administered concurrently with HRT to prevent the proliferative effects of progestins.

Findings from our laboratory and others show that progestins accelerate tumor progression by increasing levels of vascular endothelial growth factor (VEGF), a potent angiogenic agent [16-21]. The development of new blood vessels (angiogenesis) is

essential for tumor survival, growth and metastasis [22-24] and is associated with increased mortality in individuals with breast carcinomas [25]. Progestin-dependent tumor progression can be controlled by synthetic anti-progestins such as RU-486; however, such compounds have serious side-effects due to cross reactivity with other steroid receptors, limiting their use [26, 27]. Consequently it is essential that we develop new and novel compounds which can be used therapeutically to oppose the proliferative effects of progestins in mammary cells.

Studies in our laboratory have focused on a number of different phytochemicals as potential chemo-preventive and/or -therapeutic compounds since such compounds are naturally available and are non-toxic. Luteolin [2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one], a naturally-occurring plant flavonoid, has previously been shown to effectively inhibit the proliferation of several different types of cancer cell in a non-toxic manner [13, 28, 29].

Studies herein investigate luteolin activity in vitro using T47-D and BT-474 human breast cancer cell lines and in vivo using progestin-dependent models of mammary cancer. T47-D and BT-474 cells express ER and PR and therefore respond to estrogen and progestins; both cell types also express mutant p53 which is a prerequisite for the induction of progestin-dependent VEGF [20]. The in vivo therapeutic and preventive effects of luteolin are examined in a progestin-dependent xenograft mouse model, as well as, a triple-negative breast cancer metastasis xenograft model derived from human breast cancer cells, and a DMBA-induced [7,12-dimethylbenz(a)anthracene] rat mammary tumor model. The studies described in this dissertation are designed to elucidate the biological properties of luteolin and determine its capacity as an effective naturally-occurring non-toxic compound which could be used safely to prevent and/or treat the progression of progestin-dependent breast disease. Studies reported in this

dissertation test the hypothesis that ***luteolin inhibits growth and metastasis of progestin-dependent and triple-negative breast cancer cells.*** The following specific aims are addressed to prove or disprove this hypothesis:

Specific Aim 1: Determine the preventive potential of luteolin in HRT using the progestin-accelerated DMBA-induced model of mammary carcinogenesis.

Specific Aim 2: Determine whether luteolin inhibits the production of VEGF in response to both naturally-occurring and synthetic progestins in human breast cancer cells and the ability of luteolin to suppress progestin-dependent tumor growth in xenografts.

Specific Aim 3: Characterize the anti-metastatic potential of luteolin using triple-negative breast cancer models.

Positive outcomes from these studies will support the use of luteolin as a pharmacological compound which exhibits low toxicity and can be used both therapeutically and in a preventive capacity to combat growth and metastasis of breast cancer.

CHAPTER 1: REVIEW OF LITERATURE

This dissertation will focus on progestin-responsive breast cancer and the capacity of luteolin as a potential chemo-preventive and chemotherapeutic nutraceutical. Initially I will provide a general description of breast cancer and its clinical prevalence, followed by a deliberation on the connection between hormone replacement therapy (HRT) and increased breast cancer risk, particularly in postmenopausal women. I will then argue, based on the literature and findings from our laboratory, that it is the progestin component of combination HRT that is responsible for the increased risk of breast cancer. A synopsis of the progesterone receptor and its ligands, both naturally-occurring and synthetic, will be followed by a review of our current understanding of the role played by progestins in breast cancer. I will focus on the effect progestins have on angiogenesis, and their role in the acquisition of stem cell-like characteristics and breast cancer cell metastasis. Finally I will review some models of breast cancer, chiefly focusing on cell culture and rodent models used to elucidate progestin-responsive effects. The advantages, disadvantages and clinical relevance of each model will be described. Lastly, I will give a general overview of natural dietary compounds which may be used to prevent or treat progestin-dependent breast cancer. The previously reported effects of luteolin, a plant-derived flavonoid which has been shown to have anti-cancer properties against a variety of different cancers, will be highlighted.

1.1 Breast Cancer Epidemiology

In general, cancer is considered an affliction of the aging whose onset is determined randomly. Environmental and genetic factors however, likely affect the chance that an individual will develop a particular cancer [30]. In 2011 the United States Agency for Healthcare Research and Quality estimated the direct medical costs of cancer to be \$88.7 billion, a significant economic burden to the nation. Breast cancer is the second

leading cause of cancer-related death in women. Worldwide over 500,000 women died due to breast cancer in 2011, with 1.7 million new cases in 2012 [31]. A common misconception is that breast cancer is a disease that occurs predominantly in affluent countries. In fact about 50% of all cases and 58% of all breast cancer-related deaths occur in lesser developed countries [32]. Not surprisingly, in most developed nations, survival rates are 80% or above; in middle- to low-income countries, those rates hover between 40 and 60%, respectively [33]. Reduced survival rates in developing countries are likely due to shortages of healthcare, lack of public awareness and infrequent screening, all of which result in most cases being diagnosed at a late stage. In the United States, breast cancer is the second most frequently diagnosed form of the disease in women, after cancers of the skin. In 2015, the American Cancer Society (ACS) estimates that there will be 234,190 newly diagnosed cases of breast cancer, with approximately 40,730 deaths, accounting for 28.6% of all new cases [34].

Worldwide breast cancer incidence has been on the rise in lower risk nations such as Japan [35] and other Asian countries, Sweden [36], and England and Wales [37]. The rise in the incidence of breast cancer in European countries is likely due to the increased use of mammograms, which can detect early disease. However, in Asian populations the trend of increasing breast cancer incidence is likely explained by a variety of factors, including growing economies, increased women in the workforce, and changes in nutrition and life-style that affect the age of menarche and fertility. In such nations, the risk-factor profile now approaches that of Western populations.

1.1.1 Inherent and Extrinsic Risk Factors Associated with Breast Cancer

The life-long risk of a woman developing breast cancer is about 12% [38], a number based on numerous risk factors, a few of which are summarized in **Table 1.1**. Some risk

Table 1.1. Breast Cancer Risk Factors.

Intrinsic Risk Factors	Explanation
Gender	The main risk factor for breast cancer is simply being a woman. Although men are susceptible to breast cancer the disease is approximately 100 times more common in women than men, which is likely due to significant levels of estrogen and progesterone produced by women.
Aging	Increasing age increases the risk of breast cancer. Epidemiological studies show that 1 out of 8 women who develop invasive breast cancer are younger than 45, while about two-thirds of invasive breast cancers occur in women older than 55.
Genetic Risk Factors	Hereditary – 5-10% of all breast cancers. 1. Principal genes BRCA1 and BRCA2 a. Tumor suppressor genes that prevent abnormal cell growth. Mutations increase breast cancer by 55% and 45%, respectively. Usually occurs in younger women. 2. Other important genes associated with breast cancer: a. TP53 – involved in apoptosis, genomic stability, and inhibition of angiogenesis. b. PTEN – regulates cell growth.
History	1. Familial – a nuclear family member with breast cancer increases risk by 2-fold a. 85% of women who have breast cancer do not have a previous familial history of the disease. 2. Personal – Having had unilateral breast cancer increases the risk of occurrence in the unaffected breast 3-4 fold.
Ethnicity	1. White women are more at risk of developing breast cancer than African-American women. 2. Asian, Hispanic, and Native American women are at a lower risk of developing cancer.
Breast Tissue Density	Women with dense breast tissue, i.e. higher glandular and fibrous tissue and less adipose tissue have a ~ 2-fold increased risk of breast cancer. This may be partly due to the sensitivity of a mammogram being inversely proportional to breast tissue density.
Benign Breast Conditions	1. Non-proliferative lesions – minor increased risk of breast cancer, e.g. fibrotic cysts. 2. Proliferative lesions without atypia – can increase breast cancer risk up to ~2-fold, e.g. fibroadenoma, sclerosing adenosis, papillomatosis, radial scars. 3. Proliferative lesions with atypia – may increase risk up to ~5 fold, e.g. atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia (ALH).
Lobular Carcinoma	A noninvasive breast carcinoma that is associated with a 7- to 11-fold increased risk of developing invasive breast cancer.
Menstrual Periods	There is a slightly increased risk of breast cancer in women who start menstruating early (age 12) and enter menopause late (> age 55).
Radiation Exposure	Increased risk of breast cancer is directly related to exposure to chest radiation (e.g. chemotherapy for another cancer) and indirectly proportional to age, i.e. exposure to radiation in adolescence is associated with the highest increased risk of breast cancer while exposure after the age of 40 shows no significantly increased risk.
Diethylstilbestrol (DES) Exposure	DES was prescribed to pregnant women between the 1940s and 1970s because it was thought to reduce miscarriages. Women who have taken DES, and their progeny, are slightly more likely to develop breast cancer.
Extrinsic Risk Factors	Explanation
Parity	Women who have not had children by 30 years of age are at a slightly higher overall risk of developing breast cancer.
Birth Control	1. Oral contraceptives – Women using oral contraceptives are at a higher risk of breast cancer, but this risk diminishes upon cessation and eventually becomes insignificant after 10 years. 2. Depot-medroxyprogesterone acetate (DMPA) – a few studies implicate DMPA in increasing the risk of breast cancer, but this risk disappears 5 years after DMPA cessation.
Menopause Hormone Therapy	Used to alleviate the symptoms of menopause and help prevent osteoporosis. For women with an intact uterus, combined hormone therapy is prescribed to counteract the proliferative effect estrogen has on the uterus, leading to an increased risk of uterine cancer. Women who have had a hysterectomy are prescribed estrogen alone. Combined hormone replacement therapy in post-menopausal women greatly increases the risk of breast cancer, metastasis, and morbidity.
Lactation	Prolonged breastfeeding for 1-2 years is associated with a decreased risk of breast cancer.
Alcohol	Consumption of alcohol is associated with an increased risk of breast cancer and is dependent on the amount of alcohol consumed. 2-5 drinks per day increases overall risk 1.5 times.
Overweight or Obesity	Overweight postmenopausal women have an increased risk of breast cancer. When ovarian endogenous estrogen production ceases, the majority of estrogen comes from adipose tissue. Increased levels of estrogen and insulin are associated with an increased risk of breast cancer.
Physical Activity	Physical activity in the form of moderate exercise reduces breast cancer risk. The Women's Health Initiative found that as little as 2 ½ hours per week of brisk walking could reduce a woman's risk by 18%.

factors are inherent and have to do with non-modifiable traits like gender (being a woman is the number one risk factor), ethnicity, breast tissue density and familial or personal history. However, there are other risk factors that are potentially modifiable. These include changes in life-style, such as minimizing obesity and its associated pathologies (especially in postmenopausal women), ceasing hormonal birth control and reducing alcohol consumption. Some of the more recent and/or controversial risk factors such as environmental chemicals, tobacco and night work will not be discussed.

Inherent risk factors that are associated with increased breast cancer include breast tissue density (increased glandular tissue relative to adipose), type 2 diabetes, ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (LCIS); with the latter two being examples of noninvasive aberrant cell growth. Previous familial history of breast cancer also increases the risk 2-fold, though only 15% of all new cases have a first-degree relative (mother, sister) who have had the disease. Multiple cases of breast cancer in a single family are often linked to genetic alterations in BRCA1 (breast cancer type 1, early onset) and BRCA2 (breast cancer type 2, early onset). BRCA1 and BRCA2 are tumor suppressor proteins, which normally protect cells from aberrant alterations, repairing DNA damage and maintaining genomic stability. A mutation in these genes is considered a loss of function and increases the overall chance of developing cancer, though this is one of many steps that are necessary for cancer to take hold. Taken together, mutations in BRCA1 and BRCA2 account for 20-25% of all hereditary based instances of breast cancer [39], representing approximately 5-10% of all cases [40]. BRCA1 and BRCA2 associated breast cancers tend to develop in women that are younger than their nonhereditary counterparts and only one mutant copy of the gene needs to be present to increase the overall risk by 55-65% by the age of 70 [41, 42]. Inherited mutations like BRCA1 and BRCA2 are rare in the general population; however,

other more common variations may impart lesser or indirect increases in breast cancer risk.

Potentially modifiable factors associated with an increased risk of breast cancer include weight gain and obesity, especially in postmenopausal women whose adipose production of estradiol is noticeably elevated. As discussed earlier, combination HRT containing both estrogen and progestin also presents a significant risk factor for postmenopausal women (see below). Factors such as weight gain after menarche, hormonal birth control, physical inactivity and alcohol consumption, increase the risk of breast cancer in younger women. Recent research suggests that long-term smoking, especially in women who start the habit before their first pregnancy, increases the risk of breast cancer compared with their non-smoking counterparts. While many of these factors are modifiable, the focus of this dissertation will be on the risks posed by HRT to postmenopausal women.

1.2 Early Detection and Treatment

Numerous studies show that early detection of breast cancer saves lives and increases available treatment options. There are various ways to screen for breast cancer, from simple self-examinations to more complex clinical breast exams and mammography, the latter of which is sensitive enough to detect asymptomatic breast cancer lesions. However, the vast majority (95%) of women with an abnormal mammogram do not have cancer. Suspicious lesions are typically biopsied and classified, as will be discussed below. Women at high risk of breast cancer, e.g. those carrying a BRCA1 and BRCA2 mutant, are recommended to receive an annual mammogram, together with magnetic resonance imaging (MRI) screening beginning at age 30. Those in the general population who are not deemed high risk are encouraged to receive regular mammograms starting at 40 years of age.

When a woman receives the diagnosis that she does indeed have breast cancer, many options are available. These depend on an individual's hormone profile, as well as cancer characteristics, such as tumor size and whether or not metastasis has occurred. Also important are patient preferences; treatment usually involves breast conserving surgery, as opposed to mastectomy, which is an option in severe cases, along with adjunctive chemotherapy. Studies show that early, noninvasive detection of breast cancer results in levels of long-term survival that are comparable in women given a mastectomy and those treated with breast-conserving surgery and adjunctive therapy. In either case, underarm lymph nodes are usually surgically removed and evaluated for metastatic lesions. Treatments may involve radiation, chemotherapy, hormone replacement, and/or target therapy, the latter of which is employed in women with breast cancers that overexpress human epidermal growth factor receptor 2 (HER2/neu). 61% of noninvasive breast cancer cases have a 99% 5-year relative survival rate, whereas survival rates drop to 85% and 25% respectively for those with breast cancer that has spread to a regional or distant site. Survival rates for all stages of breast cancer are as follows: 5 years-89%, 10 years-84%, 15 years-78%. Black women have a lower rate of survival at all stages [34]. Overall, early detection of breast cancer increases the efficacy of chemotherapeutic options and patient disease-free survival.

1.3 Breast Cancer Classification

Breast cancer is a heterogeneous malady that is typically categorized based on several characteristics and criteria and which is often treated in a patient-specific manner. The major clinical criteria for classifying breast cancer are the 18 different histopathological subtypes, tumor grades 1-3 (well, moderately and poorly differentiated), stage, and hormone and/or genetic profile [43, 44]. Traditionally, breast cancer is categorized into two different classes; luminal cancers that represent 85% of all breast cancer cases, and

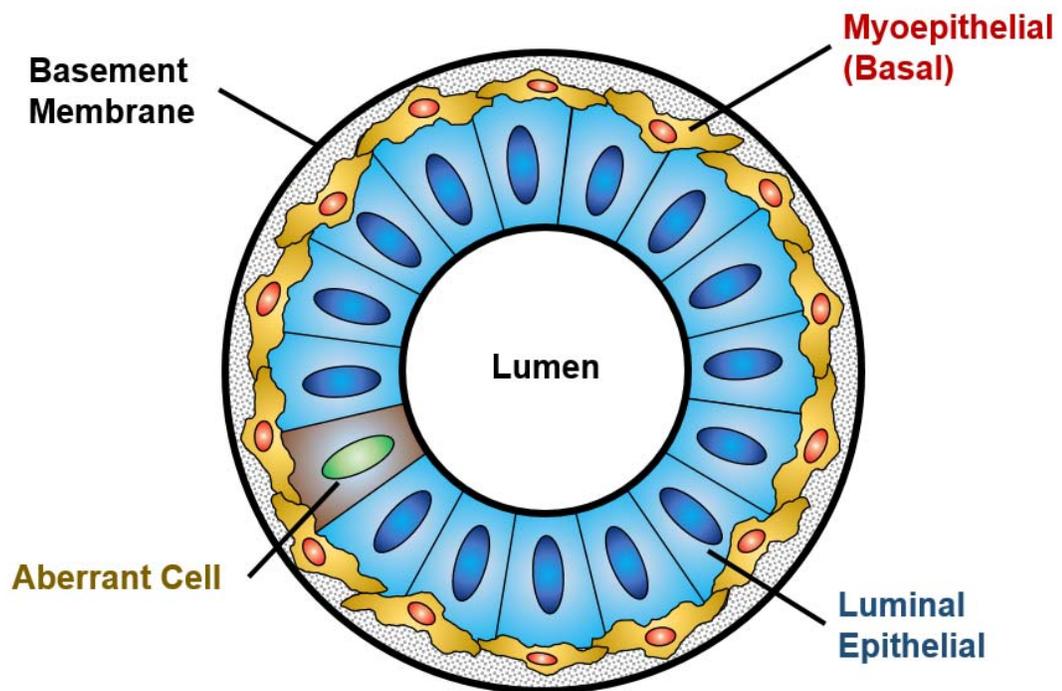


Figure 1.1. Anatomy of human mammary duct. Simple diagram showing the main ductal layers: basement membrane, basal-layer, and luminal-layer. The aberrant cell represents a luminal precancerous cell.

basal, which make up 5-15% [43, 45] (please see ductal diagram **Figure 1.1**).

Ultimately, the luminal and basal classes are expanded into four classifications (clinical prevalence): luminal A (low Ki67, a marker of cell proliferation: 40%), luminal B (high Ki67: 20%), triple negative/basal-like (TNBC: 12-20%), and HER2 type (10-15%). As a point of distinction between the two luminal classes, luminal B tumors are typically more prevalent in younger women and have a poorer prognosis [46-48], which is likely due to their higher proliferative index.

The four breast cancer classifications described above are based on the presence or absence of specific receptors and genetic markers. For instance, if the cancer contains either the progesterone receptor (PR) or estrogen receptor (ER) they are considered hormone receptor-positive. Hormone receptor-positive cancers typically occur in postmenopausal women and have a more favorable prognosis than their hormone receptor-negative counterparts, which lack both ER and PR. Hormone receptor-negative cancers tend to grow more rapidly and are more common in premenopausal women. A recent report in which 466 breast cancer tumors from 463 patients were examined, found that the vast majority of luminal A and luminal B breast cancers are ER and PR positive, with the HER2 receptor evenly split [49]. The clinical relevance of these subtypes is summarized in **Table 1.2**. Interestingly, about 6-10% of all breast cancers are classified as “normal-like” [50, 51]. Such tumors have a good prognosis and are typically very small. These tumors do not fall into the distinct molecular classes described above, but this is probably due to inadequate tissue for sampling. Further molecular profiling has revealed that a vast number of mutant genes are associated with breast cancer. These include: tp53, PIK3CA, ERBB2, GATA3, and MAP3K; as well as other characteristic stem cell-like markers such as CK5+ [52], CD24-/CD44+ [53] and ALDH+ [54, 55], discussed in further detail below.

Table 1.2. Clinical Relevance of Breast Cancer Classification*

Molecular Classification (Prevalence)	Clinical Receptor Profile (%)	Cell Line (e.g.)	Hormone Profile	Type	Origin
Luminal A (40%)	ER+ (96) PR+ (90) HER2+ (6)	T47-D ^{1,2}	ER+, PR+, HER2-	IDC	PE
Luminal B (20%)	ER+ (99) PR+ (77) HER2+ (16)	BT-474 ^{1,3}	ER+, PR+, HER2+	IDC	P
HER2/neu (15-20%)	ER+ (53) PR+ (36) HER2+ (67)	SKBR3 ¹	ER-, PR-, HER2+	AC	PE
Basal-like (10-15%)	ER+ (14) PR+ (9) HER2+ (2)	MDA-MB-231 ^{1,4,5}	ER-, PR-, HER2-	AC	PE
All Tumors (~100%)	ER+ (72) PR+ (65) HER2+ (15)				

AC, adenocarcinoma; ER, estrogen receptor; HER2, human epidermal growth factor receptor-2; IDC, invasive ductal carcinoma; P, primary breast; PE, pleural effusion; PR, progesterone receptor

*Based on:

Neve, R. M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F. L., Fevr, T., ... & Gray, J. W. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer cell*, 10(6), 515-527.

Cancer Genome Atlas Network. (2012). Comprehensive molecular portraits of human breast tumours. *Nature*, 490(7418), 61-70.

Literature Cited:

¹Engel LW, Young NA: 1978. Human breast carcinoma in continuous culture: a review. *Cancer Res* 38: 4327-4339.

²Keydar I, Chen L, Karby S, Weiss FR, Delarea J, Radu M, Chaitcik S, Brenner HJ. 1979. Establishment and characterization of a cell line of human breast carcinoma origin. *Eur J Cancer* 15: 659-670.

³Lasfargues EY, Coutinho WG, Redfield ES. 1978. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. *J Natl Cancer Inst* 61: 967-978.

⁴Cailleau R, Olivé M, Cruciger QV 1978. Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In vitro* 14: 911-915.

⁵Cailleau R, Young R, Olivé M, Reeves Jr WJ 1974. Breast tumor cell lines from pleural effusions. *J Natl Cancer Inst* 53: 661-674.

1.4 Hormone Replacement Therapy in Breast Cancer

Hormone replacement therapy (HRT) is commonly used to supplement growth hormones and combat allergies, as well as being administered to young women as a contraceptive, and given to postmenopausal women to alleviate the symptoms of menopause. HRT also includes estrogen modifiers (tamoxifen), aromatase inhibitors (letrozole, anastrozole, or exemestane), and/or ovarian ablation; all of which are used to combat breast cancer. For example, women with early stage breast cancer who test positive for hormone receptors (as described above) benefit from hormonal therapy treatment, i.e. tamoxifen, for at least 5 years, whereas postmenopausal women are typically treated with an aromatase inhibitor in addition to, or instead of, tamoxifen. For women with cancers that over-express the growth-promoting protein HER2, several target therapies are available. Henceforth, for the purpose of this dissertation the use of HRT will specifically refer to women taking synthetic estrogens and/or progestins to combat postmenopausal symptoms.

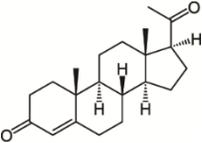
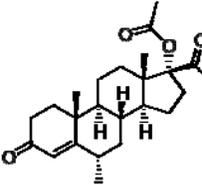
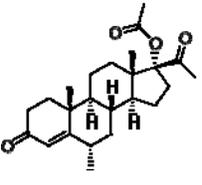
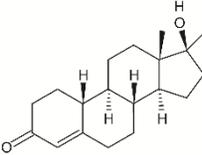
For decades, women have been prescribed HRT to combat postmenopausal symptoms, which include hot flashes, mood swings, night sweats, insomnia, decreased libido and dementia [56]. In the past it was widely regarded that HRT not only helped alleviate such symptoms, but also imparted health benefits. This however is no longer believed to be the case. Typical HRT regimens include a synthetic estrogen, which is prescribed to alleviate postmenopausal symptom. Unfortunately though, estrogens promote endometrial proliferation, which can lead to uterine cancer; to circumvent this possibility a progestin component is co-administered to counteract the proliferative effects of estrogen [7]. HRT, and specifically its progestin component, came under scrutiny following findings published by the Women's Health Initiative (WHI) and others. These clinical studies show that post-menopausal women undergoing HRT containing both

estrogen and progestin have an increased risk of developing breast cancer compared with those given estrogen alone [8-12]. Recent evidence shows that taking combined HRT increases the risk of breast cancer slightly each year [57] and that there is an additive effect over time which nearly doubles the risk of breast cancer after five or more years [58]. Fortunately, the risk of breast cancer, which has been ascribed to the progestin component of combined HRT, rapidly diminishes when treatment stops [59]. Clinical trials conducted as part of The Women's Health Initiative (WHI) were halted early due to the minimal health benefit and increased breast cancer risk (**Table 1.3**) in women taking progestins [11]. As a consequence, HRT has since declined, though an estimated 1.6 million women currently undergo combined HRT in the United States [13]. Indirect epidemiological evidence also supports the increased risk of breast cancer associated with combined HRT. For example, following publication of the findings of the WHI in 2002, by the following year breast cancer incidence had dropped by 7% in white women, a statistic that can be largely attributed to reductions in HRT [34]. More recently, researchers found a direct correlation between declines in DCIS and invasive breast cancer and reduced numbers of women undergoing HRT [60, 61]. As a result of these findings, although HRT is approved by the U.S. Food and Drug Administration (FDA) for short-term use in postmenopausal women, it is recommended that women use the minimum dose for the shortest period possible [57].

1.4.1 Progestogens

Progestogens are a class of steroid hormone that actively binds to PR and includes naturally occurring, endogenous progesterone (P4) and synthetic progestins such as medroxyprogesterone acetate (MPA). Synthetic progestins were developed to overcome the poor oral absorption of progesterone and to maintain stable blood levels of the hormone [62]. They are commonly used as contraceptives. Progestins may differ

Table 1.3. Progestogen Pharmacokinetics*

Progestogen	Structure	Half-Life	Off-targets	Common Use
Progesterone (P4)		<10 min	anti -androgenic (±) -estrogenic -mineralocorticoid	endogenous steroid
Medroxyprogesterone acetate (MPA)		40-60 h	androgenic (±) anti-estrogenic glucocorticoid	oral contraceptive and HRT
Norgestrel (NG) Levonorgestrel (D-NG)		5-14 h	androgenic anti-estrogenic	morning after pill
Norethindrone (NE)		7 h	androgenic anti-estrogenic estrogenic	oral contraceptive

*Based on:

Garefalakis M and Hickey M. Role of androgens, progestins and tibolone in the treatment of menopausal symptoms: a review of the clinical evidence. *Clinical Interventions in Aging* 2008; 3(1): 1-8.

only slightly in their structure, but exhibit widely varying degrees of potency, clinical effectiveness and endocrine profiles [63]. **Table 1.3** highlights the pharmacokinetic differences in progestogens used in this dissertation. Such differences, for example causing the off-target activation of glucocorticoid receptors, highlight the difficulty in extrapolating findings from one progestin to another.

1.4.2 Progesterone Receptor (PR)

Progestins mediate their physiological actions via the progesterone receptor (PR), a ligand-activated transcription factor in the nuclear hormone superfamily [64, 65]. Ligand-occupied PR is activated and binds to DNA [66], where it recruits cofactors in order to activate or “squench” transcriptional machinery [67, 68]. At the same time, unoccupied PR forms complexes with heat shock proteins (hsp) 90 and 70 and other accessory proteins that maintain the receptor in an inactive state [69]. PR-mediated transcriptional regulation is dependent upon two isoforms, progesterone receptors A and B (PRA and PRB) [70-72], and their possible interaction with other transcriptional factors such as SP1 and FOXO1 [73]. PRA and PRB are transcribed from the same gene. They contain conserved steroid binding domains, but differ structurally, in that PRB has an N-terminus that is 164 amino acids larger than PRA [69, 74, 75]. In normal breast tissue the two isoforms are expressed at similar levels; however, in breast tumors PRA may become the predominant form [76]. Interestingly though, during mammary gland development, the two isoforms of PR are naturally differentially expressed [77]. PRA and PRB have overlapping but distinct transcriptional activities [72]. PRA is important for proper ovarian function, whereas PRB is necessary for appropriate mammary function [78]. Intracellular PRB is implicated in cross-talk between progesterone and the epidermal growth factor receptor (EGFR), leading to sustained phosphorylation of ERK [79], a prolific secondary messenger. All in all, PR activation can directly regulate

transcription via the classical progesterone response element (PRE) pathway, c-Src/MAPK mediated PR-SP1 tethering, or indirectly through MAPK activation via a c-Src kinase-dependent pathway [80, 81].

Current conventional adjuvant breast cancer therapies involve the use of anti-estrogens and aromatase inhibitors [82, 83]. At the cellular level, estrogen stimulates PR synthesis [5], cell proliferation [6], metastasis [3], and promotes angiogenesis [4]. While the role of estrogen is well characterized in breast cancer, the role played by progesterone is poorly understood and definitive data describing progesterone effects is overshadowed by data relating to the effects of estrogen [84]. Nevertheless, PR measurements are used as a prognostic indicator when deciding appropriate breast cancer treatment [85]. Since estrogen regulates PR expression [75, 86], and there is evidence linking progestins to increased breast cancer pathogenesis, PR is a rational target through which to treat breast tumors. For example, when progesterone is preponderant, the anti-progestin RU-486 is most effective and has been shown to effectively inhibit the production of VEGF in breast cancer models [26, 27]. However, synthetic anti-progestins are generally toxic and disrupt homeostasis by cross-reacting with other steroid receptors [87], making their clinical use of limited value.

1.4.3 Progestins and Breast Cancer

The role of progestins in breast cancer is controversial and there has been much debate on whether they protect against, or actively promote the disease. As described previously, there is overwhelming clinical evidence that combined HRT increases the incidence of breast cancer in postmenopausal women [9-11, 88]. This may be due to increased proliferation of breast epithelium occurring in response to combination HRT [11]. However, the issue is to some degree clouded by contradictory clinical evidence showing that progesterone may be protective. In a clinical study by Fiodart *et al.* (1998)

it was reported that a 2-week topical administration of estradiol increased cell proliferation, a situation that was reversed by co-administration of progesterone [89]. This study was a small 40 person cohort containing 10 patients in the groups given placebo and estradiol, leaving 13 and 7 patients respectively in the progesterone and combined groups. Considering the small cohort size, topical versus systemic administration, and short (2 week) duration it is difficult to correlate these findings with those obtained from women taking long-term orally administered systemic estrogen plus progestin HRT, especially considering the varying targets of progestogens (**Table 1.3**). Schumhammer *et al.* (2013) examined endogenous sex hormones in premenopausal women and found that, except for high levels of free testosterone, endogenous sex steroids are not associated with increased breast cancer risk [90]. It has been reported that women taking oral contraceptives have an increased breast cancer risk [8], though the progestogen components of the various prescription regimens were not defined, making the oral contraceptive study difficult to interpret. Studies from France report that using natural progesterone instead of synthetic progestins in HRT does not increase breast cancer risk [91, 92]. Women who go through early menarche and those who give birth before the age of 20 have a reduced risk of breast cancer [93], though whether or not progesterone endows a protective effect in these cases is not known. Taking all these different types of studies into account, and given the diverse pharmacological profile of various progestins [94], it does appear that the type of progestogen used is extremely important in determining breast cancer risk.

In a post-mortem study of women with no history of breast cancer, it was found that approximately 30% of women in their 40s had precancerous breast lesions or DCIS [95]. Considering that the relative lifetime risk of a woman developing breast cancer is 12% [38], these findings suggest that the majority of breast lesions and tumors are not

clinically relevant. That being said, evidence from both in vitro and in vivo studies suggest that progestins may stimulate dormant lesions into becoming malignant in postmenopausal women [17, 19, 96]. Bring in Swedish trials where women who previously had breast cancer and were given HRT again developed breast cancer within 1-2 yrs). To sum up, we still have a lot to learn regarding the role of progestogens in breast cancer. However, it is becoming increasingly clear that they do increase breast cancer risk, though in a manner that is highly dependent on a variety of factors, such as age, exposure, dose, route of administration and type of progestogen given.

1.4.4 Targeting Progestin-induced Effects for Chemoprevention

Epidemiological studies support the notion that progestins are an important factor in the etiology of breast cancer. In particular, the progestin component of combination HRT is implicated in the increased incidence of the disease in postmenopausal women. A plausible scenario is that progestins act in concert with estrogen in a paracrine/autocrine fashion to stimulate otherwise dormant lesions and thereby increase breast cancer incidence, a mechanism that is highly conceivable given that PR modulates downstream effectors in the mammary gland such as cyclin D1 (encoded by *CCND1*), WNT4 and receptor activator of nuclear factor- κ B (NF- κ B) ligand i.e. RANKL. In the normal mammary gland, PR activated WNT4 and RANKL signaling stimulates neighboring hormone-negative mammary cells in a proliferative manner [97-99]. Intriguingly, studies show that long-term stimulation of PR, though not short-term, induce a cyclin D1-dependent increase in hormone-responsive cells resulting in cell-intrinsic proliferation. When blocked in *Ccd1^{-/-}* mice, only intrinsic proliferation is affected; paracrine-driven proliferation remains unblocked [97]. Disruption of PR-dependent paracrine proliferative-driven actions occurs when RANKL signaling is interfered with, either genetically or

pharmacologically. This effectively suppresses progestin-accelerated tumorigenesis [100, 101].

Studies designed to elucidate the mechanism(s) underlying combination HRT-related breast cancer show that the progestin component clearly plays a role by exerting its actions in a variety of different ways [8, 17, 102-105]. MPA, a commonly prescribed component of HRT, has been implicated in reactivating breast cancer stem cell subpopulations in hormone-responsive cell lines [103, 104, 106]. Progestins are also believed to act in concert with RANKL to increase cellular proliferation [100, 101].

Production and secretion of vascular endothelial growth factor (VEGF), a potent mitogen that is an essential component of neovascularization, has also been shown to be induced by a variety of progestins [17]. This subject will be described in more detail below. All in all it is becoming increasingly apparent that progestins promote a microenvironment conducive to tumor growth and metastasis. In order to combat progestin-related pathologies of the breast we must exploit specific targets such as PR and downstream signal propagators and develop novel chemotherapeutic therapies to both prevent the development of HRT-related cancers, and reverse already existing disease. With this in mind the focus of this dissertation will be progestin-induced angiogenesis, cancer stem cell-like enrichment, and metastasis.

1.4.4.1 Progestin-induced Vascular Endothelial Growth Factor (VEGF)

Neovascularization is essential for tumor progression and metastasis [22-24].

Angiogenesis is regulated by many factors. VEGF is one such factor which is transcriptionally controlled by hypoxia-inducible factor (HIF-1 α), a master regulator of VEGF synthesis [107]. In the progestin-dependent breast cancer model, PR binds to the progesterone response element (PRE) which is located upstream of the HIF-1 α response element (HRE) on the VEGF promoter [107-109], causing an increase in

VEGF production in hormone-responsive breast cancer cells [17]. This phenomenon was subsequently confirmed in vivo in two different models; a progestin-responsive xenograft model and a model of progestin-accelerated DMBA-induced breast cancer, discussed in more detail below [96, 104]. Wu et al. (2004) reported that progestin-dependent VEGF induction is preferentially mediated through PRB, and that inhibiting either PI3-kinase or the transcription factor SP-1 effectively blocks progestin-induced VEGF production [109]. Furthermore the progestin-induced VEGF response is dependent on p53, likely due to p53-mediated transcriptional interference [20]. p53 can also suppress VEGF through SP-1 [110] and E2F [111] and previous reports show that VEGF production is SP-1 dependent [109]. Additional findings suggest that the down-regulation of PR (either directly or indirectly via suppression of ER) and/or HIF-1 α inhibition, effectively inhibits progestin-induced VEGF production [19, 109]. The physiological relevance behind progestin-induced up-regulation of VEGF is that it may stimulate the vasculature to produce new blood vessels, exert mitogenic effects on breast cancer cells themselves, and substantially increase levels of Bcl-2, an anti-apoptotic protein [112]. Taken together these studies demonstrate that both progesterone and synthetic progestins increase the synthesis and secretion of VEGF [20, 104], and that locally produced VEGF acts in a paracrine manner to stimulate endothelial and tumor epithelial cells [19]. Based on such findings, it is reasonable to speculate that elevated levels of VEGF, produced in response to progestins, are largely responsible for the increased incidence of breast cancer in women undergoing combination HRT. Such a scenario offers an intriguing array of possible alternative chemotherapeutic approaches.

1.4.4.2 Progestin-induced Cancer Stem Cell-like Cells

An emerging concept is that cancer stem cells, which are cells that possess the ability to give rise to all cell types in a particular cancer, are the main protagonists behind sustained growth and metastasis. In the normal breast, stem-cells are multipotent progenitor cells with limited lineage capabilities that competently form normal mammary tissue [45, 113, 114]. It is believed that either these stem cells themselves become cancer cells, or a progenitor cell becomes cancerous and dedifferentiates, gaining the capacity for self-renewal [115]. While the first inference may have some merit, the second is arguably more probable. Either way, cancer stem cell-like cells (CSCs) are inherently rare. That being said, new studies suggests that progestogens may enrich this CSC subpopulation [103, 116-118]. There is also evidence that progesterone enriches stem cells in the normal mammary glands [106, 116].

Recent findings suggests that breast cancer cell lines may contain CSCs [119]. Indeed, a number of markers that are characteristic of “stemness” have been used to identify CSCs. These include ALDH⁺⁺ (aldehyde dehydrogenase), CD44⁺/CD24⁻ (cluster of differentiation) and CK5⁺ (cytokeratin). In 2007, Ginestier et al. reported that ALDH⁺⁺ is a marker of CSC stemness and a predictor of poor clinical prognosis [55]. They further showed that high ALDH activity represents a breast cancer cell (BCC) subpopulation with self-renewal characteristics able to regenerate a heterogeneous tumor. Al-Hajj and others, showed the importance of the cell adhesion molecule CD44⁺/CD24⁻ as one of the first CSC markers [53]. They found that by isolating patient-derived tumor cells with the CD44⁺/CD24⁻ expression profile they could greatly increase tumorigenicity. Similarly Boecker et al. (2002) found differential patterns of CK expression between normal luminal and basal breast tissue. CK5⁺CK18⁺ and CK5⁺SMA⁺, respectively represented two distinct lineages [120]. They determined CK5⁺ to be a marker of oligopotent

stemness due to the absence of a dual CK18⁺SMA⁺ breast cell [121]. In subsequent studies CK5⁺ was validated as a CSC marker, and intriguingly found to coexist with CD44⁺; in T47-D xenografts 95% of CK5⁺ cells are CD44⁺ [103]. Furthermore, when ER⁺, PR⁺, and CK18⁺ BCC xenografts were exposed to progestins, enrichment of the subpopulation of CK5⁺ cancer stem cell-like cells occurred [52]. It has been proposed that progestins induce a shift from ER⁺,PR⁺,CK5⁺ luminal-like cells to ER⁻,PR⁻,CD44⁺,CK5⁺ basal-like CSC [103]. The mechanisms behind progestin-induced stemness are still elusive but several reports show that the process may involve the down-regulation of miR-29 [117] or miR-141 [118] and/or BCL6 inhibition [122]. If the progestin-induced switch to stemness is not reversible, it may lead to an expanded CSC pool, allowing for greater tumor proliferation and a potential increase in tumor incidence and morbidity. Epidemiological studies of combination HRT demonstrate morbidity rates that reflect this scenario.

1.4.4.3 Progestin-induced Metastasis

An emerging hypothesis is that progestins induce a stem cell-like state that facilitates the metastasis of cancer cells to distant organs, though scant data exists to prove the involvement of progestins in this process. Recent data obtained from several models of breast cancer do show that progestins increase metastatic potential. Lanari (2009) for example showed in a mouse model that progestins promote lymph node metastasis [123]. Similar observations have been made using in vivo xenograft models derived from BT-474 [124] and T47D [125] human breast cancer cells. In the latter model, it was reported that E2 + P4 significantly increased cell metastasis and that the metastatic cells contained a large ER⁻, PR⁻, CK5⁺ population, which is surprising considering that the parental luminal T47D cell line is ER⁺, PR⁺, CK5⁻. As previously described, CK5 and CD44 are CSC markers that are co-expressed in some hormone-responsive cell lines.

Interestingly a CD44 variant was reported to bestow increased metastatic potential to rat carcinoma cells [126]. Progestins are known to upregulate CD44 in hormone responsive BCCs [103], therefore it is highly likely that progestins do indeed promote metastasis.

The process of metastasis is complicated and involves multiple steps in which a cancer cell must undergo morphological and physiological changes. The capacity to undergo the epithelial-mesenchymal transition (EMT), which, in the case of breast cancer, involves the transformation of a luminal-like epithelial cell to a basal-like cell, is of particular interest. In BCCs, the EMT phenotype is strongly associated with CD44 up-regulation [127]. This phenotype, and the derived invasiveness of BCCs are inhibited by CD44-specific antibodies [128]. Takahashi et al. (2010) determined that the CD44 EMT transition commences through tumor necrosis factor-alpha (TNF α) mediated CD44 activation [129]. However this does not exclude the possibility of TGF β 1 mediated CD44 migration [130] and/or progestin-induced CD44 activation, as well as other mechanisms for progestin-induced metastasis. Recent epidemiological studies report that women taking combined HRT have a higher risk of developing metastatic breast cancer [61]. With this in mind, it is imperative that we further develop our understanding of progestin-induced metastasis.

1.5 Breast Cancer Models

Cancer is a heterogeneous disease with several characteristic hallmarks. These include sustained proliferation, replicative immortality, angiogenesis, resistance to cell death, and invasion and metastasis [131]. The greatest challenge to be overcome when developing a model of breast cancer is simulating intratumoral phenotypic heterogeneity and its microenvironment. Consequently a wide variety of models have been developed to facilitate investigation of the diverse array of breast cancers facing investigators.

The most widely used models for studying preclinical breast cancer are the well-characterized breast cancer cell lines [132], which are derived from the four major classifications of breast cancer: luminal A, luminal B, HER2, and basal-like breast cancers, and represent both primary and distal carcinomas. It is important that BCCs provide an experimental system that mimics, as closely as possible, the situation in the developing tumor; consequently investigators must ask whether a particular BCC line maintains its original tumor characteristics.

The multistep carcinogenesis model, as applied to breast cancer, postulates that the disease arises through multiple somatic alterations leading to various subpopulations and divergent clonal expansions, a process that ultimately is at the core of the heterogeneous nature of breast cancer. Individually, BCCs are a snapshot of tumor development and lack the phenotypic 'mosaic' of their origins. However, taken together BCCs represent multiple stages of the disease, with varying molecular profiles (**Table 1.2**). Prolonged exposure to artificial conditions will likely modify characteristics of a particular BCC line. Patient-derived BCCs may therefore better represent the in vivo situation, though such studies are limited by access to tissue. Either way, BCCs maintained in cell culture do not necessarily exhibit the same characteristics as cells that make up a tumor. For example, BCC lines do not typically undergo spontaneous EMT, though EMT is inducible [133]. BCCs are useful tools for answering specific questions, such as whether RU-486 blocks progesterin-induced VEGF production in hormone-dependent cells. However, since such problems are context specific and do not account for cell-type interaction and overall tumor microenvironment, models of breast cancer were developed in animals to reproduce as closely as possible the situation in humans. Such models are classified into three major categories [134]: xenograft models,

genetically engineered mice (GEM), and extrinsically induced models (e.g. carcinogens, radiation, viruses).

Animal models provide a more holistic approach to the study of breast cancer since the stromal compartment and organizational components provide a naturalistic microenvironment for tumor development. However, xenografts are not without their own challenges. Tumorigenicity of human derived BCCs in mice depends on a number of factors such as cell injection with or without matrigel, hormone-dependency and injection site. BCCs injected into immune incompetent mice are able to grow at various sites, though implantation into the mammary fat pad is most appropriate for facilitating tumor development in an accurate microenvironment [135, 136]. Xenograft studies, especially those that are patient-derived (PDTX), are becoming more common in clinics as a test bed for providing personalized chemotherapeutic options. Xenograft models are used routinely as a basic research tool, though their applications are limited to context specific questions. For example, xenograft tumors are not typically heterogeneous in composition and their blood supply is via peripheral vessels. Secondly, xenografts lack an immune response, which may promote or inhibit spontaneous cancer. Lastly, while the mouse provides a subtle microenvironment for the growth of human BCCs, the precise interactions occurring between the mouse stromal compartment and human BCCs are of concern. For instance, there is mounting evidence that the microenvironment [137] and epithelial-stromal interactions [138] are essential in modulating tumorigenesis.

GEM and models of induced mammary carcinogenesis are widely used to study a wide variety of cancers. GEM models are designed to recapitulate genetic alterations, such as TP53, BRCA1, and HER2/ERBB2/NEU that are commonly found in human breast cancers [139, 140]. Models involving the induction of mammary carcinogenesis are

used to study the progression of breast cancer in response to a specific extrinsic factor. Models in this category have the advantage of producing a tumor microenvironment similar to that found in humans. However, histopathological characteristics of these models vary between mice and women, limiting their interpretation [141, 142]. For example, unlike humans, in which most mammary tumors are ER and PR-positive and hormone-dependent, the vast majority of those arising in either GEM or in mice exposed to extrinsic factors, are ER and PR-negative and therefore hormone-independent [143]. In other words, the situation in mice is molecularly opposite to that in humans [144]. Furthermore, patterns of metastasis differ between mice and women. In humans, breast cancer invades the surrounding tissues, enters the lymphatic system, and metastasizes predominantly to bone, brain and lungs. In stark contrast, mouse mammary cancer hematogenously disseminates principally to the lungs [132]. Overall, mice are excellent models for elucidating molecular pathways involved in cancer progression and testing various preventive/therapeutic approaches [141].

Rats are an alternative animal model used to study breast carcinogenesis. In general, rat physiology resembles that of humans more closely than the mouse. This is especially the case with regard to liver metabolism, but is also true for human mammary gland development and mammary carcinogenesis. Rats respond to chemically- induced mammary carcinogenesis by developing tumors that are histopathologically similar to humans, share comparable hormone receptor profiles and dependency, and develop genetic mutations that are also seen in humans [96, 145-147]. Furthermore, PR co-localization and isoform expression patterns between cells are similar in rat and human mammary gland [148]. H-ras is the primary instigator of DMBA-induced mammary carcinogenesis in mice [149] and rats [150]. However, the H-ras mutation does not readily occur in either primary or metastatic [151] human breast cancer [152-154],

limiting the applicability of carcinogen-induced rodent models to the human situation. Recent reports using BCC lines suggest that the frequency of RAS pathway mutations may be as high as 25% [155].

1.5.1 Studying Progestin-dependent Breast Cancer Using Animal Models

Most mouse models give rise to breast tumors that are hormone-independent, limiting their use as a means of studying the effects of HRT on breast cancer. The alternative, a rat model is more physiologically relevant to humans, except for the H-ras mutation. It is therefore essential to develop multiple in vivo approaches for testing breast cancer development and progression. Several rodent models for examining progestin-dependent mammary cancer will be described below.

Orthotopic xenotransplantation of human BCC in immune-compromised mice is one of the most common methods for testing various cancer therapies [156]. However, difficulty arises when assessing hormone-dependent tumors due to the experimental practice of using Matrigel, which is produced by mouse Engelbreth-Holm-Swarm sarcoma cells. Matrigel is a gelatinous protein mixture that forms a complex extracellular matrix for cell growth. Van Slooten and colleagues (1995) were among the first to establish a hormone-responsive xenograft using BT-474 BCCs in Matrigel supplemented with a slow release E2 pellet [157]. An inherent drawback to the use of Matrigel is that it contains growth factors such as transforming growth factor-beta (TGF- β) and EGF. As previously described, PRB may modulate EGFR, which is closely related to HER2/neu. Consequently the addition of Matrigel-containing EGF may potentially mask or manipulate a PR-dependent response.

Matrigel was the most reliable means of producing hormone-responsive xenografts until 2007, when Liang *et al.* developed a viable xenograft model without Matrigel [104]. In this model, immune-deficient mice are implanted subcutaneously (s.c.) with E2 pellets

prior to inoculation with human BCCs into mammary fat pads. After a period of rapid growth the nascent tumor begins to regress and become senescent, a phenomenon likely arising due to an inadequate microenvironment. Progestogen supplementation resuscitates BCCs, facilitating tumor growth and expansion compared with animals treated with E2 alone. Intriguingly, when the progesterone stimulus is removed, tumors rapidly regress, demonstrating that tumor rescue in the xenograft model is hormone-dependent, see **Figure 1.2** below [104]. As discussed earlier, progestins induce production of VEGF in hormone-responsive cells, exerting mitogenic actions on both the tumor vasculature and BCCs. Loss of P-induced VEGF production is likely an important contributory factor responsible for tumor regression. Further studies showed that the progestin norethindrone (NE) significantly increased metastasis to lymph nodes [124]. Such an effect is not normally observed in hormone-responsive xenografts [158]. Taking into account its many advantages, the hormone-responsive BCC xenograft model is a powerful tool for studying the activity of anti-progestins with a view to developing new methods of chemotherapy. The model is also valuable as a means by which to investigate the progression of disease into the lymphatic system.

In addition to xenograft techniques, common inducible models are also available for the study of hormone-responsive breast cancer. While working with MPA as an inhibitor of invasively benign fibroblastic proliferations, i.e. desmoid tumors, Lanari et al. serendipitously discovered an MPA-induced mammary tumorigenesis model [159]. In this model, pellets containing 40 mg/kg MPA induce DCIS (ductal carcinoma *in situ*) in BALB/c mice after 52 weeks, with 80% incidence. Tumors are hormone dependent, express both ER/PR and metastasize to lymph [123]. Intriguingly, tumor incidence and hormone sensitivity is progestogen dependent [160]. The MPA-induced mammary

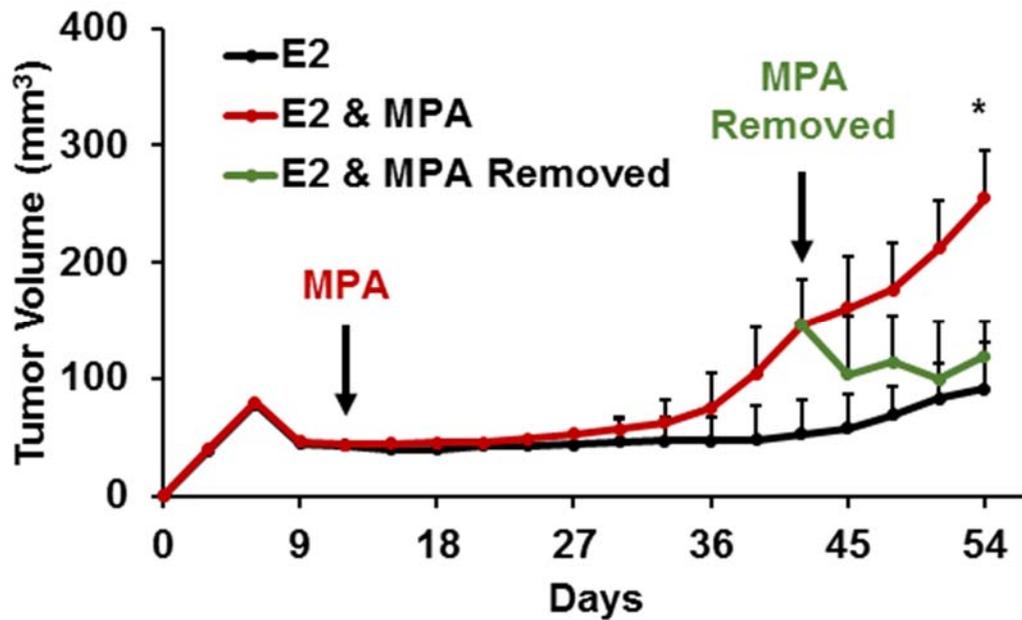


Figure 1.2. Progesterone promotes growth in BT-474 xenograft tumors. Progesterone pellets (P4) resuscitated tumor growth following a brief regression phase. Tumors in the estradiol (E2) group maintained a slow and steady growth that was significantly lower than tumors in the P4 group. Removal of the P4 pellet (P4-) caused tumor regression.

*Adapted from:

Liang Y, Besch-Williford C, Brekken RA, Hyder SM. Progestin-dependent progression of human breast tumor xenografts: a novel model for evaluating antitumor therapeutics. *Cancer research*. 2007;67:9929-36.

Table 1.4. MPA-induced Mammary Cancer Model Compared to Human Breast Cancer

MPA-induced Mammary Cancer	Human Breast Cancer
Mostly DCIS (ductal carcinoma <i>in situ</i>)	~78% DCIS ¹
Metastasizes to Lymph and Lungs	Metastasizes to Lymph, Bone, and Brain
ER+ and PR+	ER+ and PR+ > 70%
c-erbB2 ⁺	20-25% c-erbB2 ⁺
~20% tp53	18-25% tp53 ²
Responds to Endocrine Therapy	Responds to Endocrine Therapy
Progresses to Hormone Resistant Cancer	Progresses to Hormone Resistant Cancer

¹Glass AG, Lacey JV Jr., Carreon JD & Hoover RN 2007. Breast cancer incidence, 1980–2006: combined roles of menopausal hormone therapy, screening mammography, and estrogen receptor status. *Journal of the National Cancer Institute* 99 1152–1161.

²ALSner, J. et al. 2000. Heterogeneity in the clinical phenotype of TP53 mutations in breast cancer patients. *Clin Cancer Res* 6 (10), 3923.

carcinoma is an important model that has many similarities to human breast cancer that are not typically seen in mouse models (see **Table 1.4**). Having said this, an inherent weakness of this model, besides mammary gland development, is the long gestation period necessary for tumorigenesis to occur. This can present significant logistical and cost issues, though all things considered the MPA-induced mammary carcinoma model is an excellent tool for studying progestin-dependent effects on breast cancer.

Since rat and human mammary glands develop similarly [161], and demonstrate comparable patterns of PR expression [148], rats are a more suitable model than mice for studying progestin-responsive breast cancer. In the early 1960s Huggins et al began developing and characterizing a rat model using single administration of carcinogens, among which, 7,12-dimethylbenz(a)anthracene (DMBA) was extremely promising as a test agent [162]. A single bolus of 10-20 mg/kg DMBA, given orally immediately prior to pubescence when mammary tissue proliferation begins to accelerate, resulted in an incidence of 80-100% mammary carcinoma. DMBA initiates cancer by forming DMBA-DNA adducts. This damages DNA and leads to improper replication, which may then result in mutagenesis following ineffective DNA repair. In highly proliferative tissues, the odds of causing cancer increase, which is why DMBA is given at a prepubescent stage. DMBA is nonspecific and causes cancer at other sites. For example, dosing animals with DMBA also causes leukemia [163], which increases morbidity.

It was subsequently determined that tumors arising in response to DMBA are hormone-responsive; specifically being PR-responsive [164]. Furthermore, in a small study in which tumors were induced by 25 A'-methyl'-A'-nitrosourea (MNU), Chan et al found that the molecular signature of mammary tumors so induced were similar to low-to-intermediate grade ER positive human breast cancer [150]. In an early study using MPA, it was found that the progestin significantly increased the incidence of DMBA-

induced tumors in rats [161]. Pazos et. al. and Aldaz and colleagues demonstrated similar results in mice following tumor induction by MNU or DMBA [102, 165]. MPA accelerated tumor development and increased their incidence in both models. At the time, these reports were controversial because most tumors in mice are hormone-independent, and other investigators found that MPA protected against DMBA-induced mammary carcinogenesis. However, studies show that DMBA-induced mammary tumors abundantly express both ER and PR receptors and are hormone-dependent [166]. The importance of PR was later confirmed when it was shown that the anti-progestins RU-486 and ZK98299 inhibit DMBA-induced mammary carcinogenesis in both rats [167] and mice [168]. Discrepancies in the literature regarding the effects of MPA and other progestins in DMBA-induced mammary cancer in Sprague-Dawley rats likely arise due to a variety of factors that influence progestin activity. These include MPA dosage, since less than 5mg/kg MPA may be protective, whereas doses above this level may stimulate cancer development. The type of progestogen given is important, as is the period elapsing between DMBA administration and implantation of progestin pellet, since this affects tumor latency significantly. Additional factors include the age of the rats used, since there may be changes in mammary and tumor morphology at different ages, and even seasonal variations [96, 102, 161, 169].

Studies from our laboratory and others show that administration of a single 20 mg bolus of DMBA causes mammary cancer in 45-50 day old Sprague-Dawley rats and that subcutaneous implantation of long-term release progestin pellets accelerates tumorigenesis [96, 161]. We use this model routinely to evaluate the capacity of various naturally-occurring and synthetic compounds to prevent progestin-dependent mammary tumors since a number of recent studies show that certain dietary compounds such as

curcumin and apigenin, inhibit MPA-accelerated tumor growth in the DMBA model [170, 171].

The models described above enable researchers to study progestin-dependent breast cancer, such as that occurring in women undergoing HRT, in a variety of ways. The mouse xenograft model developed by Liang and colleagues [104] provides an excellent means by which to study the therapeutic effects of anti-progestins against both primary and distant (metastatic) tumors. The progestin-accelerated model of DMBA-induced mammary tumorigenesis gives us the opportunity to study both the preventative and therapeutic effects of anti-progestins. The latter rat model also has the advantage of being more physiologically and structurally relevant to the human situation.

1.5.2 Challenges in Researching Progestin-Effects on Breast Cancer

Research regarding the role played by PR in breast cancer is largely overshadowed by the mitogenic actions of estrogens. There is no doubt that ER plays a crucial role in hormone-responsive breast cancer. However, there is overwhelming evidence, both from epidemiological studies and preclinical research, to show that PR-related effects are important. The study of PR-dependent effects is challenging, since in both normal and tumor cells the receptor is regulated by estrogens [172]. Since PR can be transcriptionally regulated independently of ER [173], contrasting PR regulation presents unique challenges to the study of its effects in breast cancer. Moreover, ER-mediated expression of PR isoforms is epidermal growth factor (EGF)-dependent [174], suggesting that additional hormones and growth factors may contribute to breast cancer biology and may mask or complicate interpretation of progestogen-dependent effects. Nevertheless, it is essential to delineate the actions of PR that are dependent on ER and those that are independent.

Studies in the DMBA-induced model of mammary carcinogenesis showed that tumor formation is inhibited by anti-progestins [167, 168], a phenomenon later confirmed in PR-knockout mice [175] and further proving the importance of PR. Removal of the progestin pellet following tumor growth causes abrupt tumor regression, even in the presence of estradiol [104] (**Figure 1.2**). It is possible that the effects of progestins and estrogen overlap, though studies such as the aforementioned strongly support the role of PR in promoting tumor growth. For example, progestins have been implicated in transforming otherwise dormant hormone responsive breast cancer cells into stem-like cancer cells. Estrogen might exert mitogenic activities in the latter cells, causing increased proliferation [103]. Alternatively, this proliferative response could be caused by progestin-induced VEGF [19]. It is therefore difficult to establish the precise role of estrogen with respect to PR and to ascertain whether or not estrogen acts synergistically with synthetic progestins. However, since postmenopausal women produce low levels of estrogen, and epidemiological evidence shows that combination estrogen/progestin HRT increases breast cancer risk significantly compared with estrogen alone [9, 12], models that combine estrogen and progestins are valuable for delineating progestin-dependent effects on breast cancer development.

Studying the effects of progestins is further complicated by their varying pharmacokinetics, as structure, half-life, and receptor affinity vary greatly between the different progestins (**see Table 1.3**), resulting in diverse activities. Benakanakere et al (2010) reported that synthetic progestins have different effects; norethindrone and MPA stimulate tumor growth, while norgesterol suppresses tumor development [176]. Progestins also have glucocorticoid and androgen receptor activity [177] and may exert off-target proliferative actions. Additionally, commonly used PR antagonists such as RU-486, are promiscuous and bind to GR [87], clouding their activities. Such issues can be

overcome by using models of GEM, such as PR knockout mice. Difficulties aside, the array of models currently available to researchers makes it possible to gain important insights into progestin-dependent breast cancer that will pave the way for future clinical studies aimed at elucidating mechanisms of disease and developing therapies to circumvent the deleterious effects of combination HRT.

1.6 Use of Dietary Compounds in Breast Cancer

Among the various factors associated with breast cancer, diet is one of the easiest to modify. Numerous epidemiological studies link the benefits of certain diets to decreased breast cancer risk. Of particular interest in this regard are the flavonoids, a class of naturally occurring organic phytochemicals that are found widely throughout the plant kingdom. Flavonoids possess valuable antioxidant and anti-inflammatory properties and when consumed, have many health benefits [178, 179]. They are becoming increasingly recognized as being anti-cancerous [180]; indeed a number of epidemiological studies suggest that flavonoids reduce breast cancer risk in women [181-183]. Since flavonoids are a basic component of the diet, humans are predisposed to absorb and utilize them. As a consequence they provide a readily available, safe class of agents that can be used easily to supplement chemopreventative and therapeutic treatment regimens [184].

Several dietary flavonoids have so far been reported to be protective in postmenopausal women. A study on Long Island, NY, of postmenopausal women consuming flavonoids, showed a reduced risk of breast cancer [183]. Furthermore, two independent studies of dietary soy intake in China [185] and Korea [186], found a reduced risk of hormone-responsive breast cancer in postmenopausal women. It is becoming increasingly apparent that certain dietary compounds exert their actions through a variety of mechanisms to arrest cancer. They prevent tumor cell proliferation and disrupt cell metabolism, inhibit angiogenesis and stimulate apoptosis, all classic hallmarks of cancer

[187]. There is mounting evidence that phytochemicals are not only anti-cancerous, but also have hormone-like properties, primarily acting through downstream effectors such as MAPK [187]. For example, the flavonoid genistein was reported to reduce cell proliferation, an effect that was overcome by excess estradiol, suggesting genistein competes with estradiol for the ER [188]. The ER antagonist properties of genistein were independently confirmed by Zava et al. [189]. Genistein is one of the most potent estrogens of the flavonoids, whereas apigenin exhibits weak estrogenic activity but is strongly progestational [190]. Besides being competitive antagonists, phytochemicals may interfere upstream of hormonal signal transduction by altering serum hormone binding proteins, effectively changing hormone delivery rate, metabolism and elimination [191].

The anticancer effects of phytochemicals have been confirmed in a variety of in vitro and in vivo systems [187]. Over 50% of all anticancer drugs currently in use are either directly derived from plants (>25%) or are chemically altered phytochemicals (~25%). Examples include vincristine, vinblastine, docetaxel, irinotecan and teniposide, all of which are plant-derived drugs used clinically to treat cancer [192]. Generally speaking, phytochemicals are safe to use for chemo-prevention and therapy since they are relatively non-toxic to normal tissue [193]. They arrest cancer development without noticeably affecting “normal” cells, though their mechanisms of action are probably varied and likely compound-specific; for example, via differential expression of signaling cascades and/or transcriptional regulation of tumor suppressor genes such as p53 [193]. A number of flavonoids disrupt the development of breast tumors by acting as hormone disruptors, inhibiting aromatase and effectively reducing estrogen biosynthesis and bioavailability [194-196].

While dietary phytochemicals offer promise as future anticancer therapies, their oral consumption leads to the formation of a number of metabolites that may be rapidly excreted, rendering them pharmacologically inactive [184]. It is therefore necessary to identify naturally occurring agents with anti-cancer properties that may be modified in ways that enable them to retain their therapeutic potential [197]. With this in mind we have studied phytochemicals that are antagonistic towards progestins and which may therefore be used to prevent and/or reverse hormone-responsive breast cancer, which represents approximately 70% of all breast cancer diagnoses. Earlier we described studies aimed at assessing the chemo-preventive and chemo-therapeutic potential of luteolin in hormone responsive tumors, as well as its effects against metastatic triple-negative basal-like breast cancer. In the next section I will provide a brief overview of the anti-cancer properties of luteolin.

1.6.1 Luteolin in Cancer Prevention and Therapy

Luteolin [2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxy-4H-1-benzopyran-4-one], is a yellow crystalline flavone found widely in over 300 different plant species [198]. Like other dietary flavonoids, luteolin exerts an array of anti-cancer effects and disrupts key processes essential for tumor development [187]. Luteolin induces apoptosis in lung and colon cancer cells [199, 200]; inhibits cell proliferation of immortalized hepatocytes and prostate cancer cells [201, 202]; decreases pancreatic and lung cancer associated angiogenesis [203-205]; and reduced epidermoid carcinoma and pancreatic cell metastatic potential [206, 207]. The flavonoid effectively inhibits the proliferation of several different types of cancer cell in a non-toxic manner [29, 184, 208]. Herein I will concentrate on three of the most important anti-cancer properties of luteolin; the inhibition of cell proliferation, angiogenesis and metastasis.

A hallmark of cancer is the loss of cell cycle control, resulting in unrestricted cell proliferation [131]. Studies of human gastric and prostate cancer, as well as studies in melanoma cells show that luteolin restricts the cell cycle and causes arrest in the G1 phase [209-211]. Cell cycle arrest occurs through luteolin-mediated direct inhibition of CDK2 and/or indirect upregulation of CDK2 inhibitors [210, 211]. Furthermore, luteolin is able to induce cell cycle arrest indirectly via p53, by suppressing DNA topoisomerases I and II [210], which are essential for DNA repair. Besides direct or indirect suppression of the cell cycle luteolin has also been shown to suppress growth factor-induced proliferation by inhibiting insulin-like growth factor (IGF)-induced cell proliferation through cyclin D1 suppression [202]. This is important because progestins cause a cyclin D1-dependent increase in hormone-responsive cell-intrinsic proliferation [97]. In vascular smooth muscle, luteolin inhibits platelet-derived growth factor (PDGF)-induced proliferation by preventing PDGF-phosphorylation, i.e. activation [212]; though the relevance of this to its anti-cancer activity is unclear. Luteolin has been reported to suppress prostate in both an androgen-dependent and androgen-independent manner [213, 214], the mechanism of action in this case likely being its anti-estrogenic activity [190]. Overall, these studies suggest that luteolin suppresses cell proliferation by means of specific signaling pathways.

Angiogenesis is an essential component of tumor maintenance, progression and metastasis and is dependent upon the production of VEGF. When this process is inhibited, neovascularization ceases and solid tumors cannot grow beyond 1-2 mm [22, 24]. Bagli et al. (2004) showed that luteolin inhibits VEGF-induced angiogenesis in both a murine xenograft model [215] and a rabbit corneal assay, which confirmed earlier findings in the cornea [216]. Furthermore luteolin inhibits VEGF synthesis in endothelial cells [217], providing a direct means of suppressing cancer by disrupting its

microenvironment. The production of VEGF is controlled by HIF-1 α , which would seem to make it a likely candidate for luteolin inhibition; however it has been reported that suppression of VEGF by luteolin is independent of HIF-1 α [205]. A more recent report shows that luteolin suppresses VEGF-induced phosphorylation of VEGFR-2, resulting in disruption of angiogenesis and consequent inhibition of prostate tumor growth [218]. Inhibition of PDGFR by luteolin also reduced VEGF production [204]. Luteolin has been shown to promote STAT3 degradation [219], potentially leading to down-regulation of STAT3 targeted genes such as cyclin D1, survivin, Bcl-xL, and VEGF [220]. Intriguingly luteolin was reported to inhibit hyaluronidase [221], which hydrolyzes hyaluronic acid, a CD44 substrate involved in neovascularization [222]. The flavonoid also suppresses NF- κ B, which in turn inhibits metalloproteases (MMPs; [223]), extracellular matrix proteins that are involved in VEGF binding [224]. In summary, the above reports show that luteolin could be used to reduce both angiogenesis and cell proliferation via a number of targets whose activities and levels are progestin-dependent: cyclin D1, BCLs, CD44, and NF- κ B to name but a few.

More than 90% of all cancer-related deaths occur following metastasis of the primary cancer to distant organs [131]. So far we know very little regarding the capacity of luteolin to inhibit metastasis. One report on prostate cancer cells shows that luteolin inhibits the invasion of PC3 cells by up-regulating E-cadherin via down-regulation of the AKT/mdm2 pathway [225]. Cancer stem-like cells, and in particular those that express CD44, possess metastatic potential [126, 129]. Luteolin may reduce metastatic potential by suppressing the CD44 ligand through inhibition of hyaluronidase [221]. Furthermore, it may indirectly suppress metastasis by inhibiting potential metastatic pathways. For example, luteolin may reduce cell invasion and metastasis by blocking the EGFR pathway [206]. Furthermore, luteolin has been shown to block NF- κ B [223, 226] which is

essential for proper MMP and Twist expression, both of which facilitate EMT and metastasis [227, 228].

The capacity of luteolin to interfere with various signaling pathways central to cancer development and progression, makes it a promising candidate as an anti-cancer drug. At present there is scant data supporting the use of luteolin as an anti-progestin, though indirect evidence does exist suggesting that the flavonoid has potential in this regard. Our laboratory is dedicated to identifying non-toxic, naturally-occurring alternatives to combat the harmful effects of progestins and to reduce the incidence of breast cancer in postmenopausal women undergoing combination HRT. Thus I have explored the effects of luteolin as a therapeutic and preventive compound against progestin-dependent breast cancer in detail. I have also explored the effects of luteolin on suppressing metastasis in triple-negative breast disease which is a disease with no therapeutic targets currently for systemic chemotherapy.

CHAPTER 2

LUTEOLIN SUPPRESSES DEVELOPMENT OF MEDROXYPROGESTERONE ACETATE-ACCELERATED 7,12-DIMETHYLBENZ(A)ANTHRACENE-INDUCED MAMMARY TUMORS IN SPRAGUE-DAWLEY RATS

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2.1 Abstract

Postmenopausal women undergoing hormone-replacement therapy containing both progestins and estrogens are at an increased risk of developing breast cancer compared with women taking estrogen alone. We recently demonstrated that medroxyprogesterone acetate, a progestin commonly used for hormone-replacement therapy, accelerates development of mammary carcinogenesis in 7,12-dimethylbenz(a)anthracene-treated Sprague-Dawley rats. Synthetic antiprogestins used to block the deleterious effects of progestins, are themselves associated with toxic side-effects. In order to circumvent this, we used the aforementioned model to identify less toxic natural compounds that might prevent the development of progestin-accelerated tumors. Luteolin, a naturally-occurring flavonoid commonly found in fruits and vegetables, has previously been shown to possess anti-cancer properties. In our studies, both low (1 mg/kg) and high (25 mg/kg) doses of luteolin significantly suppressed progestin-dependent increases in tumor incidence, while increasing tumor latency and reducing the occurrence of large (> 300 mm³) mammary tumors. However, an intermediate dose of luteolin (10 mg/kg), while suppressing the development of large tumors, did not affect either tumor incidence or latency. Immunohistochemical analysis of tumor tissues revealed that all concentrations of luteolin (1, 10, and 25 mg/kg) significantly reduced levels of VEGF within tumors. The suppressive effects of luteolin on tumor incidence and volume, together with its ability to reduce VEGF and blood vessels, persisted even after treatment was terminated. This suggests that luteolin possesses antiangiogenic properties which could mechanistically explain its capacity to control tumor progression. Thus luteolin may be a valuable, non-toxic, naturally-occurring anticancer compound which might potentially be used to combat progestin-accelerated mammary tumors.

2.2 Introduction

Breast cancer is the second most commonly diagnosed type of cancer, and the leading cause of cancer-related death in American women. In the United States, 200,000 mostly postmenopausal women are diagnosed with the disease every year [31]. A number of recent independent clinical trials and studies have shown that postmenopausal women undergoing combined estrogen and progestin hormone-replacement therapy (HRT) have an increased risk of developing metastatic breast cancer compared with women taking only estrogen [9-11, 229]. Combination HRT is commonly prescribed to women with an intact uterus to alleviate postmenopausal symptoms. The progestin component is added to minimize the risk of endometrial hyperplasia, which may precede endometrial cancer [230, 231]. Recent laboratory studies have shown that progestin stimulates proliferation of normal and neoplastic breast cancer cells [14, 101], correlating with clinical findings for combination HRT use.

Studies designed to elucidate the mechanism(s) underlying the increased incidence of breast cancer associated with combination HRT have shown that progestins induce a potent angiogenic factor, VEGF, in human breast cancer cells [17, 104, 124].

Furthermore, such studies have demonstrated that both natural and synthetic progestins increase the synthesis and secretion of VEGF in breast cancer cells that express mutant p53 tumor-suppressor protein, but not wild-type p53 [20, 124]. Locally produced VEGF acts in a paracrine manner to stimulate both endothelial and tumor epithelial cells; the latter cells are also stimulated by VEGF in an autocrine fashion [19]. In addition, progestins such as medroxyprogesterone acetate (MPA), a commonly prescribed component of HRT, have been implicated in reactivating breast cancer stem cell subpopulations in hormone-responsive cell lines [106, 124]. Progestins are also believed to act in concert with RANKL to increase cellular proliferation [100, 101], and increase

tumor vasculature [96, 124], thereby providing an enriched environment for tumor growth and metastasis.

Previous studies in our laboratory have shown that progestins drive 7,12-dimethylbenz(a)anthracene (DMBA)-induced hormone-dependent mammary tumors in Sprague Dawley rats [96, 171, 176]. We have exploited this finding to establish an *in vivo* model for testing progestin antagonists in an inclusive microenvironment. Although the exact mechanism behind progestin-accelerated tumor growth in the DMBA model is not fully understood, evidence suggests that progestin-induced VEGF production resulting in increased angiogenesis is likely responsible [96, 171, 176]. Previous studies have reported that antiprogestins (both synthetic, such as RU-486, and naturally occurring compounds, such as apigenin) block the incidence and growth of mammary tumors in the progestin-accelerated DMBA-induced model [96, 171, 232, 233]. These studies suggest that progestin-accelerated DMBA-induced mammary tumor incidence is largely influenced by the inherent ability of progestins to increase production of VEGF, resulting in decreased latency and increased tumor incidence, burden, and multiplicity. For this reason, this model provides an excellent means of studying hormone-dependent breast cancer and is particularly suitable for identifying naturally occurring, nontoxic antagonists of progestin-induced VEGF.

Luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one) (LU) is a low molecular weight, naturally occurring flavonoid commonly found in fruits and vegetables. A number of studies have shown that LU possesses a myriad of anticancer functions that are superior to other flavonoids, and that it suppresses tumor development in several types of human cancers [198, 234, 235]. Studies show that LU inhibits VEGF production and has antiprogestin capabilities [215, 218, 219, 236, 237], demonstrating its potential effectiveness against hormone-responsive cancers. Collectively, these

findings suggest that LU has important chemopreventive properties. Herein, we provide evidence supporting the ability of LU to substantially inhibit MPA-accelerated tumor latency, incidence and growth in the DMBA-induced mammary tumor model. We also show that LU possibly arrests the growth of mammary tumors by suppressing VEGF production and angiogenesis, vital components of breast tumor formation and development.

2.3 Materials and Methods

2.3.1 Animals

All surgical and experimental procedures were approved by the University of MissouriColumbia Institutional Animal Care and Use Committee (IACUC). Intact adult female SpragueDawley rats (45-55-day old) were purchased from Harlan Breeders (Indianapolis, IN) and maintained under 12-hour light/dark cycles with *ad libitum* access to food (LabDiet 5008; St. Louis, MO) and water in accordance with guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.3.2 Luteolin

LU (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one) was purchased from Indofine Chemical Company (Cat # L-101; Hillsborough, NJ) and dissolved in sterile filtered dimethyl sulfoxide (DMSO; Sigma Aldrich; Cat # D2650; St. Louis, MO). Solutions of LU were prepared weekly, aliquoted for daily use, and stored at -20°C until use.

2.3.3 Experimental Design

We modified the protocol of DMBA-induced mammary tumor formation described previously [96, 171] (summarized in **Figure 2.1**). Animals were given a 1 mL bolus of 10

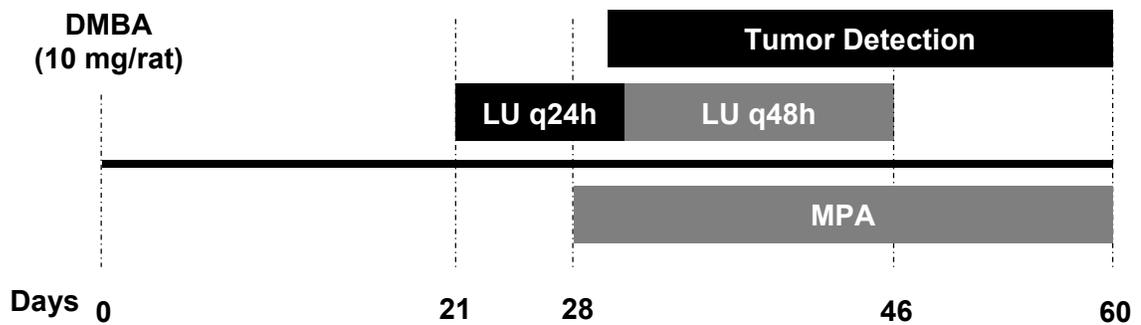


Figure 2.1. Luteolin treatment protocol. Sprague-Dawley rats were given 10 mg DMBA orally and subsequently implanted with a 25 mg 60-day release MPA (or placebo) pellet on Day 28 as described in Materials and Methods. Luteolin (LU; 1, 10, or 25 mg/kg) or vehicle (DMSO) was injected every 24 hours (q24h) for 10 days beginning on Day 21, and then every 48 hours (q48h) until Day 46. Animals were palpated for tumors every other day beginning on Day 29 and continuing until Day 59.

mg of DMBA (Sigma Aldrich Cat # D3254) dissolved in peanut oil by oral gavage (Day 0). Three weeks post-DMBA administration (Day 21), animals were divided into 5 treatment groups (n = 10–12 animals/group). Animals in the control group and those given only MPA were administered DMSO by intraperitoneal injection. Those animals given LU (1, 10, or 25 mg/kg) received injections of the flavonoid in DMSO every 24 hours for 10 days, followed by another 8 injections at 48-hour intervals. LU doses were selected based on previously reported *in vivo* studies [202, 225, 238]. Four weeks post-DMBA administration (Day 28), 25 mg 60-day release MPA or placebo pellets (Innovative Research of America; Cat # P-161; Sarasota, FL) were implanted subcutaneously on the dorsal part of the neck. Animals were weighed twice a week and, starting on Day 29, palpated every other day to detect tumor latency and incidence. On Day 59, all animals were sacrificed and tumor number and volume ($1/2 L \times W^2$) [239, 240] determined. Tumors and contralateral inguinal mammary gland tissue devoid of tumors were collected postmortem for analysis.

2.3.4 Histology and Immunohistochemical Analysis

Immunohistochemical staining of mammary and tumor tissue was performed following previously described procedures [96, 171]. The following polyclonal antibodies were used: anti-VEGF antibody 1:100 dilution [Santa Cruz Biotechnology Cat # SC-152; Dallas, TX]; and anti-Ki67 antigen antibody (1:400 dilution [Thermo Scientific Cat # RB1510-P; Waltham, MA]). Cell death immunohistochemistry was determined using a Roche (Basel, Switzerland) terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) detection kit. Histological samples were analyzed and quantified using Fovea Pro 3.0 (Reindeer Graphics) and Image J. Images were captured at 20 and 40X magnification and threshold intensity was adjusted for measurement in pixels. Tumors and representative contralateral inguinal mammary gland tissues were excised

from animals in each treatment group, fixed in formaldehyde, and embedded in paraffin for immunohistochemistry. One section from each individual tumor and mammary gland was placed on the corresponding slide for each of the immunohistochemical stains and 4 random fields captured from every section to minimize errors due to differences in cellularity. All the mammary tumors were collected and assessed for IHC biomarkers. The availability of tumor sections was dependent upon tumor occurrence (while 6-9 tumors developed in control, MPA and MPA + 10 mg/kg LU groups, only 2 and 3 animals developed tumors in the 1 and 25 mg/kg LU treated animal group, respectively). Regions of staining within tumors and areas of mammary hyperplasia in contralateral inguinal mammary gland tissue were recorded. For the mammary gland tissue analysis only 4 glands were used in each group. Fovea Pro 3.0 was used to quantitate the percent area of VEGF, Ki67 and TUNEL staining in tumor tissue using the color threshold feature in Image J. This facilitated precise discrimination between positive/negative cells and background. CD31, a blood vessel marker, was used to quantitate blood vessels in excised tumor tissue. Three CD31-labeled 10x sections were taken from each tumor to minimize intratumoral variation, as previously described [124]. The total number of vessels were counted in each section and then averaged per corresponding tumor. Data was then reported as means per treatment group with each group having an $n \geq 3$; except for the group given 25 mg/kg LU, which contained 1 tumor.

2.3.5 Statistical Analysis

Tumor latency was analyzed using the LIFETEST procedure in SAS software (9.4) to determine differences in time-to-event. Comparisons between groups were made using the Wilcoxon Log-rank Test in which the time-to-event represents time to appearance of the first tumor in each animal. Tumor-free animals were censored upon death or termination of the study (Day 59). All other tumor burdened animals, regardless of

survival, were uncensored. Tumor incidence and number of tumors per category (up to 300 mm³ or > 300 mm³) were compared using the general linear model (GENMOD) procedure in SAS software to determine the differences in least squared means among groups (logit link function and a binomial distribution). A logit link with a distribution binomial p cannot be equal to 0 [logit=ln (p/1-p)], thus the log of 0 is undefined. In the group treated with MPA + 25 mg/kg LU, there were 0 tumors formed in >300mm³ group, therefore a “1” was added to this group for statistical analysis (i.e. 1 tumor in 10 animals, instead of 0 in 10 animals). Immunohistochemical data was analyzed using an ANOVA followed by an all pairwise multiple comparison test (Student-Newman-Keuls Test) in Sigma Plot 12.5; except for the mammary tissue VEGF IHC analysis, in which MPA and MPA + LU 25 mg/kg was analyzed using t-test in Sigma Plot 12.5. In the instance where tumor tissue was n=1 (LU 25 mg/kg group), specifically for the tumor VEGF IHC analysis, the Student-Neman-Keuls multi-range test met the critical value between MPA and MPA + LU 25 mg/kg due to the large difference between treatment groups which resulted in statistical significance, this test assumes MPA + 25 mg/kg LU is a true representation of the mean (% area). Similar statistical significance was not reached with the MPA + 25 mg/kg LU groups for other markers analyzed by IHC. For all comparisons, P ≤ 0.05 was regarded as statistically significant.

2.4 Results

2.4.1 Luteolin Suppresses Development of Progestin-accelerated DMBA-induced Mammary Tumors

Using our well-established model of DMBA-induced mammary tumors [96, 171, 176], we examined the potential of LU to prevent MPA-driven tumor development. Three weeks after DMBA administration and 1 week prior to implantation of the MPA pellet (Day 21), various doses of LU were administered to determine its ability to impede MPA-

dependent tumor development by preventing the progression of neoplastic lesions to frank tumors. MPA reduced tumor latency in DMBA-treated rats compared with controls (DMBA-treated rats implanted with placebo pellets) (**Figure 2.2A**; $P < 0.05$).

Interestingly, the latency curve for the 10 mg/kg LU group was similar to that of the MPA group, with no significant difference between the two profiles. In contrast, in those animals given MPA + either 1 or 25 mg/kg LU, time-to-event data (latency) increased significantly (LIFETEST; $P < 0.05$, represented by *) compared with animals given MPA alone.

At cessation of LU treatment (Day 46), tumor incidence increased in animals treated with MPA alone and MPA + 10 mg/kg LU compared with controls and those administered 1 or 25 mg/kg LU + MPA ($P < 0.05$) (**Figure 2.2A**). Following cessation of LU treatment, tumor incidence in animals receiving the flavonoid at a dose of 1 or 25 mg/kg remained relatively low until the end of the experiment on Day 59. As a result, tumor incidence at Day 59 in groups given 1 or 25 mg/kg LU and controls was significantly reduced compared with that in animals treated with MPA alone (**Figure 2.2A**). Interestingly, administration of 10 mg/kg LU appeared to have little or no inhibitory effect on MPA-driven tumor incidence (**Figure 2.2A**).

Animal weights were not significantly affected by LU at even the highest dose used (25 mg/kg) throughout these studies (**Figure 2.2B**), indicating that the flavonoid had little or no toxicity. There was no significant difference in tumor multiplicity between groups and only 1 tumor developed per animal in this study. The majority of tumors formed in this animal model were ductal carcinomas with cribriform, papillary or a combination of cribriform and papillary patterns. Ductal carcinomas were also the predominant type of neoplasm detected in LU-treated rats and there was no observable trend for a particular classification of neoplasm as a response to the different treatments.

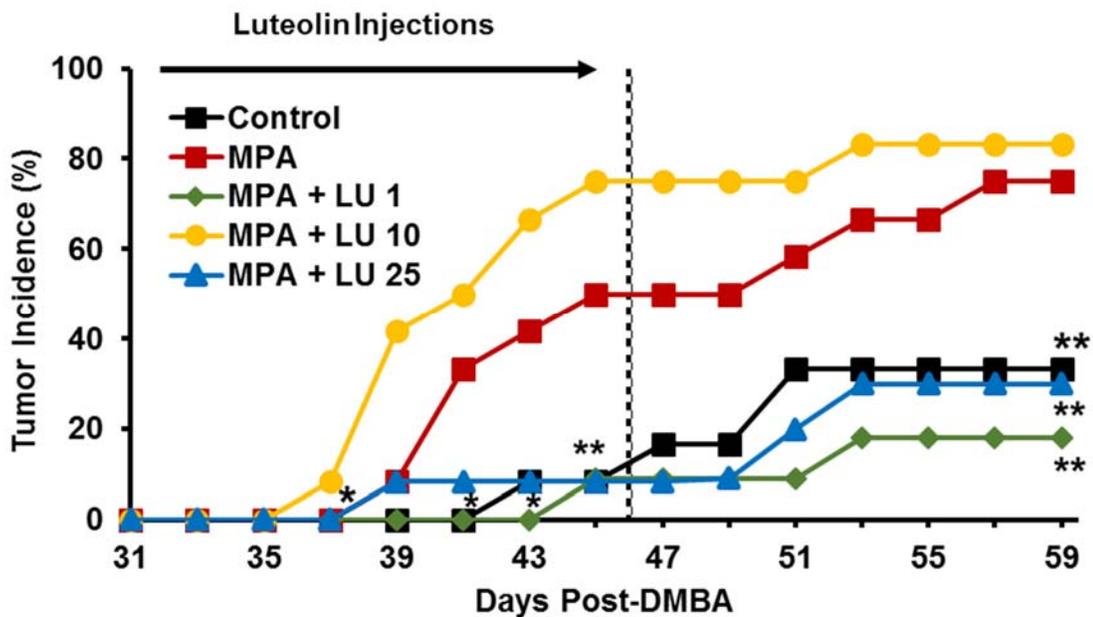


Figure 2.2A. Luteolin prevents development of DMBA-induced MPA accelerated tumors. Effects of LU on tumor latency and tumor incidence. *indicates $P < 0.05$ compared with tumor latency in the MPA-treated group; **indicates $P < 0.05$ compared with tumor incidence in the MPA-treated group at Day 45 and 59. Asterisks are placed above data points. Data represent the percent of animals with tumors in each treatment group at each time point ($n = 10-12$ animals/group). Control animals were given DMBA, implanted with a placebo pellet, and subjected to vehicle injections as per the protocol described in Figure 2.1. MPA animals were treated identically to control animals, except that they were implanted with an MPA pellet rather than a placebo pellet on Day 28.

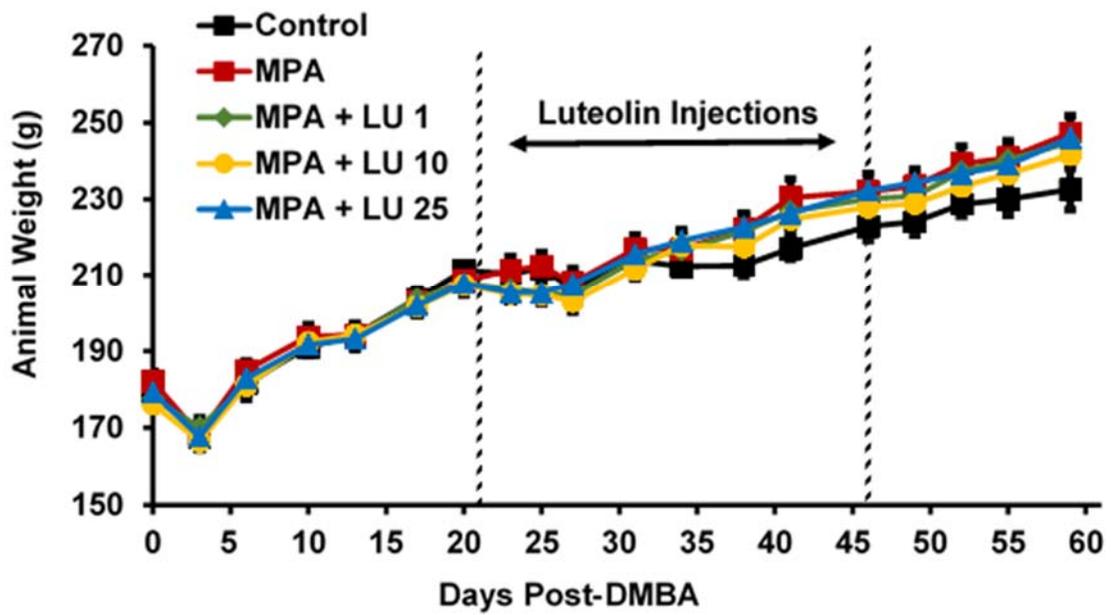


Figure 2.2B. Luteolin has no effect on Sprague-Dawley animal weight. Effects of LU on animal weights in the treatment groups throughout the study. Data represent the mean \pm SEM.

2.4.2 Luteolin Suppresses Progesterin-driven Mammary Tumor Growth

Tumor volumes were measured in the various treatment groups at the end of the experiment (**Figure 2.3**). Due to the biological variance in the volume of tumors within and among animal treatment groups, they were divided into two size groups, separating them into small and large tumors (small, up to 300 mm³; large > 300 mm³).

No statistical differences were observed among the various treatment groups with respect to the numbers of small tumors (< 300 mm³) occurring in experimental animals (**Figure 2.3A**, left panel), though more small tumors developed in the group given MPA + 10 mg/kg LU, reflecting a higher incidence of tumors in this group compared with other LU-treated groups (**Figure 2.2A**). However, the number of large (> 300 mm³) tumors arising in animals receiving only MPA, was significantly higher than in the control group (**Figure 2.3A**, right panel). Administration of 1 or 25 mg/kg LU significantly reduced the number of large tumors compared with the number observed in the MPA-treated group (**Figure 2.3A**, right panel), suggesting that LU interfered with MPA-driven tumor volume increases. Interestingly, although by Day 59 no difference was observed in tumor incidence between animals given MPA alone and those administered MPA + 10 mg/kg LU (**Figure 2.2A**), more of the tumors in the latter group were small (< 300 mm³). This finding suggests that a dose of 10 mg/kg LU, while not affecting tumor incidence, suppresses MPA-driven tumor growth and prevents the development of small tumors into larger ones. **Figure 2.3B** depicts images of representative tumor size seen in the treatment groups.

2.4.3 Luteolin Promotes Mammary Tumor Regression

Our initial results demonstrated that doses of 1 or 25 mg/kg LU most effectively suppressed progesterin-dependent increases in tumor incidence and growth. In the 1

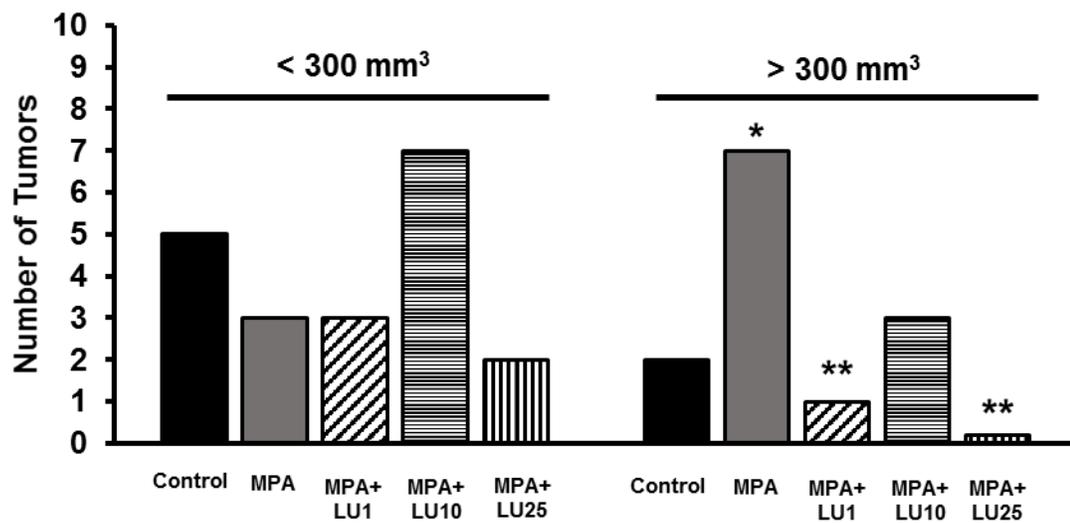


Figure 2.3A. Luteolin suppresses MPA-driven growth of DMBA-induced mammary tumors. Evaluation of tumor size in different groups at the end of the LU treatment protocol. Tumor volumes were calculated, and the total number of tumors < 300 mm³ and > 300 mm³ in each treatment group determined. *indicates P < 0.05 compared with the > 300 mm³ control group; **indicates P < 0.05 compared with the > 300 mm³ MPA-treated group. Please note that there were no tumors > 300 mm³ in the MPA + LU25 group.

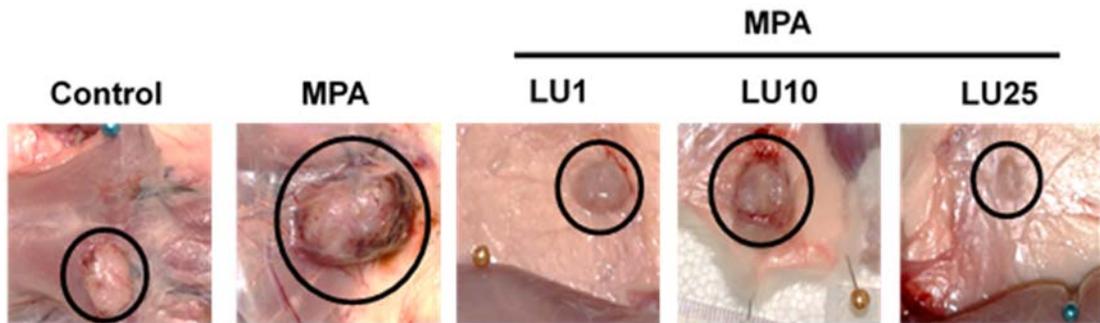


Figure 2.3B. Photographs of representative tumors from different treatment groups during necropsy. MPA treatment induced large vascularized tumors and LU suppressed MPA-driven growth.

mg/kg LU treatment group, a total of only four tumors were detected (in two of eleven animals), while just three tumors were observed in the group given 25 mg/kg LU (in two of ten animals) (**Figure 2.2A**).

In the 25 mg/kg group, only two of the three tumors were present during the last week of LU treatment. These tumors were initially palpated on days 39 and 51, while the third tumor was first palpated on Day 53. Tumors detected on Days 51 and 53 developed well after termination of LU treatment. The tumor detected on Day 53 contained a hypercellular stromal compartment surrounded by nests of cribriform, hyperplastic glandular tissue (**Figure 2.4**, left panel). The first tumor which arose on day 39 during LU treatment had decreased in size by the time it was excised and examined on Day 59 (**Figure 2.4**, right panel). This mass was composed of tightly-packed tubular structures with empty lumens and lined with a flattened epithelium. The cause of this change is not known, but has features suggestive of epithelial atrophy. The tumor detected on Day 51 was too small and not collectable at the end of the experiment. Consequently, the tumor that emerged on Day 53 (**Figure 2.4**, left panel) was used alone for all subsequent immunohistochemical analysis of tissues representing the 25 mg/kg LU treatment group.

2.4.4 Luteolin Reduces Expression of VEGF and CD-31, Markers of Angiogenesis, in Mammary Tumor Tissue

In previous studies, we showed that continuous production of VEGF by breast cancer cells is a vital component of MPA-dependent angiogenesis and subsequent tumor development [96, 124, 171]. In the current studies, we postulated that LU would reduce progestin-accelerated tumor growth by suppressing MPA-induced VEGF levels, thereby increasing tumor latency and reducing tumor number.

MPA + LU25

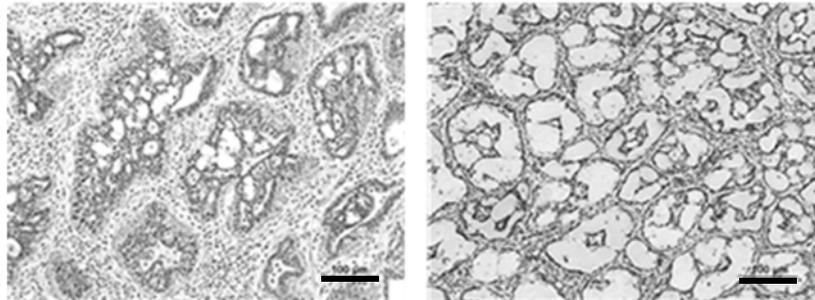


Figure 2.4. Luteolin induces tumor regression. H&E staining of MPA-driven LU (25 mg/kg) treated tumors. Left, tumor that emerged post-LU treatment (palpated at Day 53); this was the only tumor available for analysis from the 25 mg/kg LU group shown in Fig 5. Right, tumor that developed during LU treatment (palpated at Day 39), but then regressed. 10x images, bar represents 100 µm.

Assessment of the immunohistochemical data pertaining to the expression of specific markers showed that LU significantly reduced levels of tumor-associated VEGF in all treatment groups (1, 10, and 25 mg/kg) compared with MPA alone (**Figure 2.5A**). Non-tumor-associated levels of VEGF in mammary glands was significantly reduced by the highest dose of LU (25 mg/kg) compared with controls (Supplementary **Figure 2.1**). MPA alone had no significant effect on VEGF levels in either tumor or mammary gland tissues though there was a trend towards higher levels of VEGF within tumors derived from MPA treated animals (**Figure 2.5A** and *Supplementary Figure 2.1*). Tumors from MPA-treated animals exhibited a higher number of blood vessels compared with controls and all three doses of LU significantly suppressed tumor blood vessel formation (**Figure 2.5B**).

2.4.5 Luteolin Increases Expression of Ki67, a Marker of Proliferation, in Mammary Tumor Tissue

Levels of the proliferation marker Ki67 were significantly higher within end-point tumor tissues derived from animals given 10 mg/kg LU compared with either MPA alone, MPA + 1 mg/kg LU or controls (**Figure 2.6A**). Expression of Ki67 was reduced in tumors obtained from the group given 25 mg/kg LU compared with either MPA alone or controls, however, this effect was not statistically significant (**Figure 2.6A**). In contrast, compared with controls, levels of Ki67 were significantly higher in non-tumor mammary gland tissue collected from animals given the lowest dose of LU (1 mg/kg;) but not in the other treatment groups (data not shown).

TUNEL assays of tumor tissue demonstrated no significant differences in levels of apoptosis between groups treated with LU (**Figure 2.6B**). However, significantly less cell death occurred in non-tumor mammary gland tissue obtained from animals administered 25 mg/kg LU, compared with controls (data not shown).

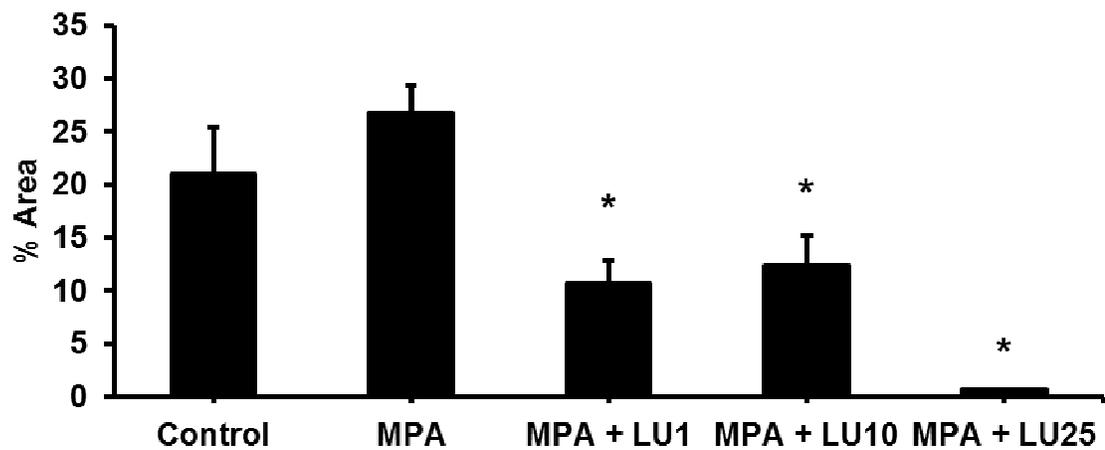
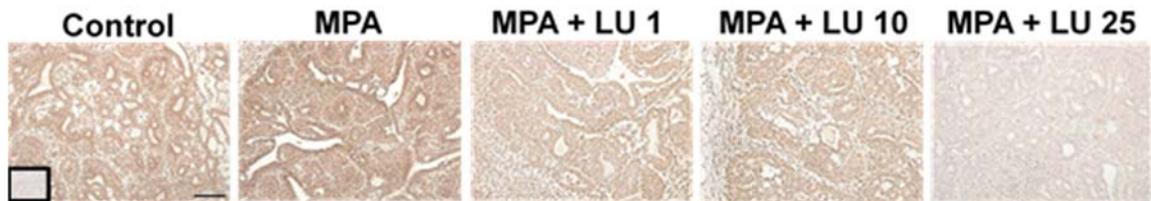


Figure 2.5A. Luteolin reduces expression of VEGF, a marker of angiogenesis, in mammary tumors. Tumors were removed 2 weeks after the last injection of LU (Day 59) and tissues subjected to immunohistochemistry. Immunohistochemical data from tumors was quantitated per group ($n \geq 3$; except for 25 mg/kg LU tumor tissue). Upper panel, representative immunohistochemical analyses of tumor, images were captured at 20x and scale bar represents 100 μm . Insert, negative control without primary antibody. VEGF quantification, mean \pm SEM shown in lower panel. VEGF staining in tumor tissues *indicates $P < 0.05$ compared with MPA-treated groups.

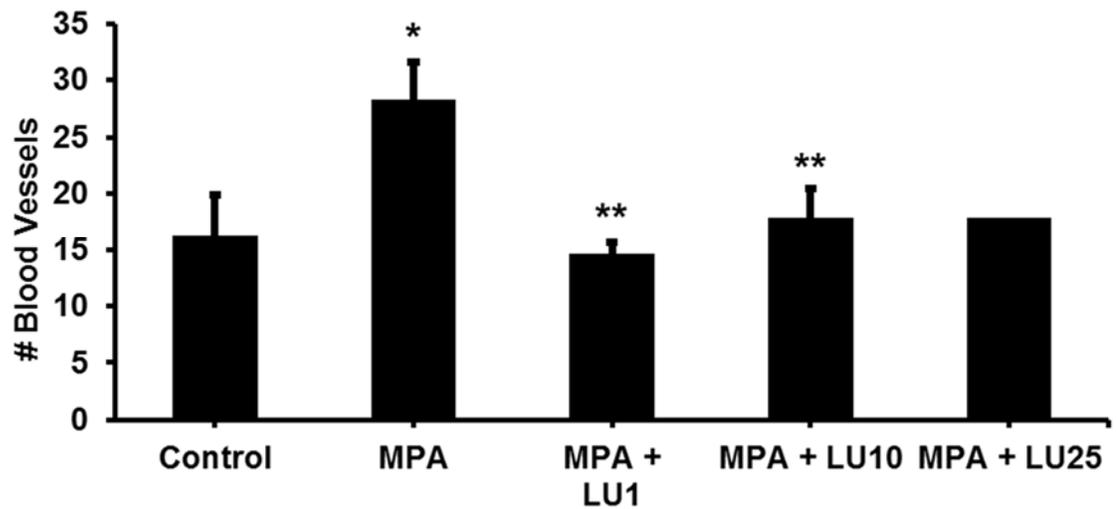
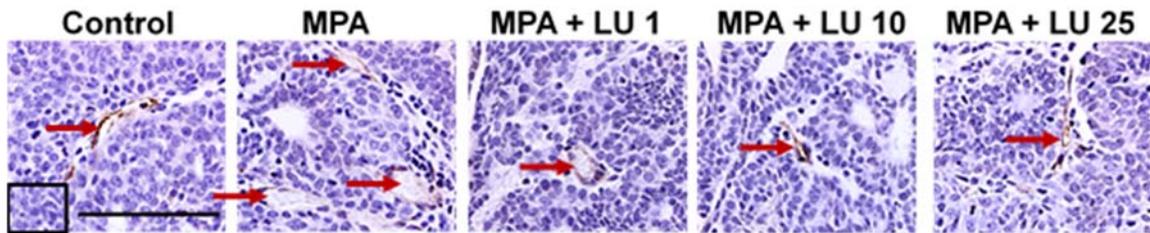


Figure 2.5B. Luteolin reduces expression of CD31, a marker of angiogenesis, in mammary tumors. Tumors were removed 2 weeks after the last injection of LU (Day 59) and tissues subjected to immunohistochemistry. Immunohistochemical data from tumors was quantitated per group ($n \geq 3$; except for 25 mg/kg LU tumor tissue). Upper panel, CD31 staining quantified by number of blood vessels present; representative pictures are at 20x and scale bar represents 100 μm . Insert, negative control without primary antibody. Lower panel, bar graph represents mean \pm SEM number of blood vessels captured at 10x per treatment group, as described in Methods. *indicates $P < 0.05$ compared to control group, while **indicates $P < 0.05$ compared to MPA-treated group.

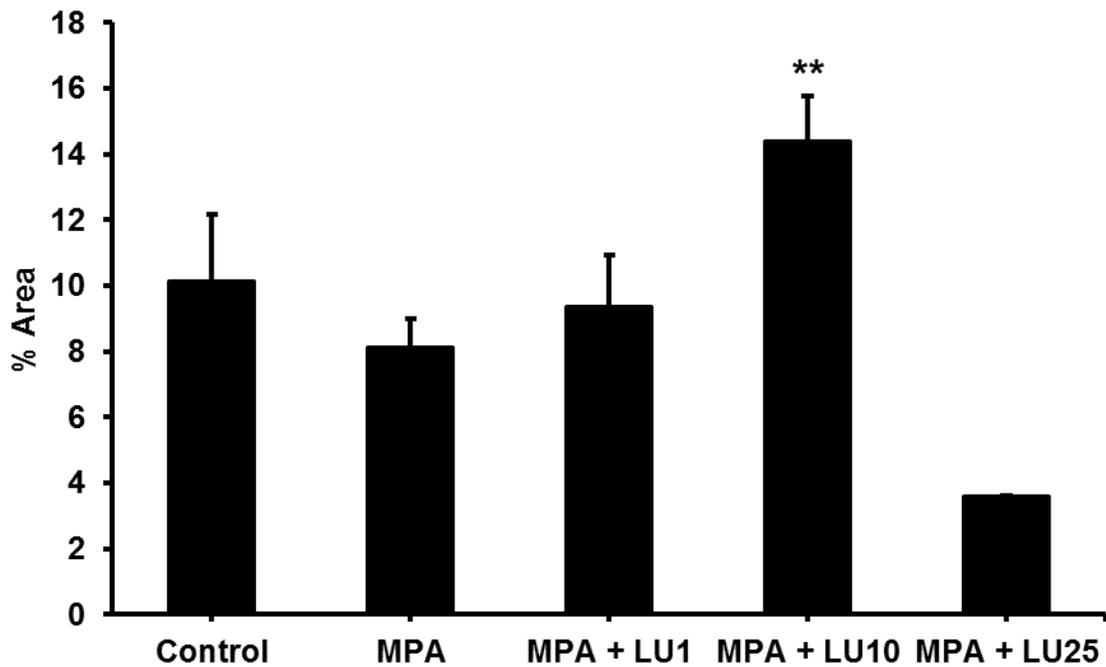


Figure 2.6A. Luteolin increases expression of Ki67, a marker of proliferation, in mammary tumors. Tumors were prepared as previously described in Methods. Immunohistochemistry data from tumors was quantitated per group ($n \geq 3$; except for 25 mg/kg LU group). Ki67 was quantified and reported as mean \pm SEM. Ki67 staining in tumor tissues **indicates $P < 0.05$ compared with control and MPA-treated groups.

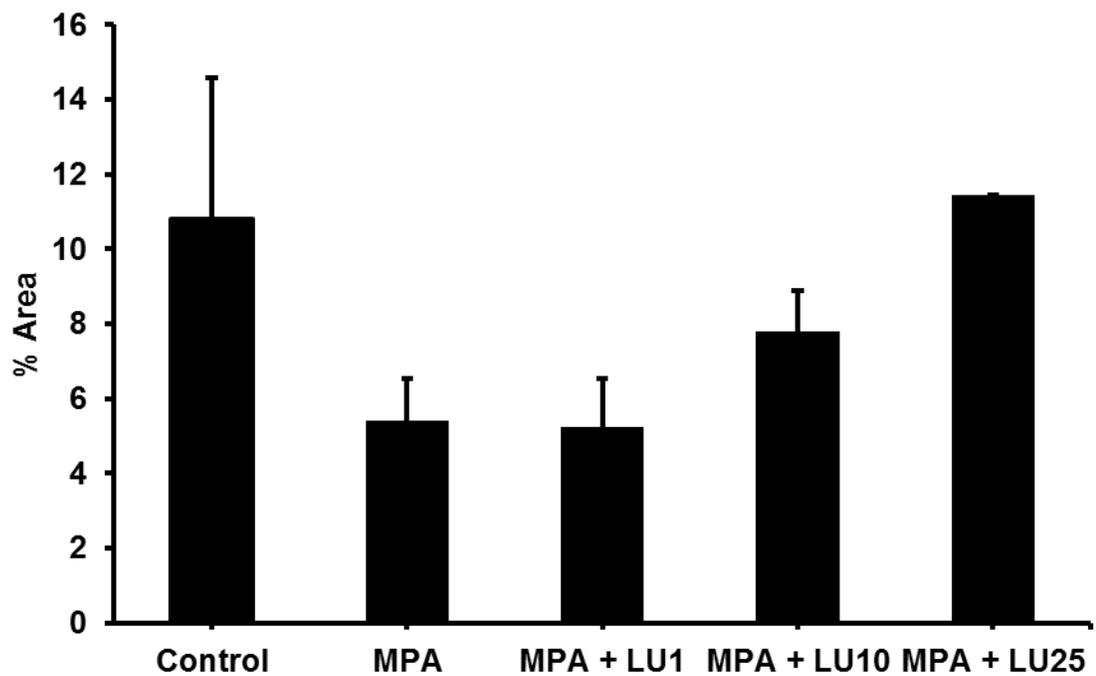


Figure 2.6B. Luteolin failed to induce apoptosis in DMBA mammary tissue. Tumors were prepared as previously described in Methods. Immunohistochemistry data from tumors was quantitated per group ($n \geq 3$; except for 25 mg/kg LU group). Tissue was prepared and stained for TUNEL; quantification was reported as mean \pm SEM.

Taken together, these data suggest that the reduced levels of cell death and increased proliferation observed at the end of the study in some animals given LU might occur in response to the lifting of selective inhibitory pressure on Day 46 when LU injections were terminated.

Immunohistochemical analysis of tissue derived from mammary tumors and contralateral non-tumor mammary glands showed that in the latter, LU was unable to prevent and/or reverse the formation of hyperplastic lesions arising in response to MPA (*Supplementary Figure 2.1*). These data suggest that LU exerts its effects and prevents MPA-induced tumor development in breast tissue at a stage subsequent to the formation of precancerous lesions.

2.5 Discussion

The consumption of combination HRT, which contains both estrogen and progestin, puts millions of postmenopausal women at higher risk of developing breast cancer compared with those taking estrogen alone [9-11, 229]. It is therefore imperative that we develop new and effective safe synthetic and/or naturally occurring compounds with antiprogestin activity that can be taken in conjunction with combination HRT regimens to eliminate the progestin-dependent increase in breast cancer risk. With this in mind, we conducted studies to investigate the ability of the flavonoid LU to act as a preventive compound in MPA-driven breast cancer, given that LU has been shown to have anticarcinogenic properties in other test systems [234, 235]. Using an established progestin-dependent DMBA mammary tumor model previously developed in our laboratory [96, 171, 176], we determined that LU suppresses the development of progestin-driven mammary tumors. Surprisingly, when LU was used as a chemopreventive agent, a biphasic response was observed, whereby both low and high doses (1 and 25 mg/kg), but not an intermediate

dose (10 mg/kg), of the flavonoid decreased MPA-induced mammary tumor incidence and increased mammary tumor latency. These observations resulted in a nonmonotonic U-shaped dose-response curve [241]. It should be noted that treatment with 10 mg/kg LU, in the absence of MPA, did not cause induction of tumors or toxicity to animals (*Supplementary Figure 2.2*), indicating that the flavonoid does not have any inherent tumor-stimulating properties. Importantly, no adverse effects were observed with any of the doses of LU used in these studies.

Having determined that LU is an effective means by which to block the development of progestin-dependent tumors, we sought to elucidate the mechanisms responsible by examining VEGF, blood vessel density and Ki67 expression, as well as conducting TUNEL assays in sections from mammary tumors and contralateral inguinal mammary glands. Because LU treatment stopped two weeks prior to the termination of the study, these results represent the lasting effect of LU on the targeted tissues.

Tumor incidence was low in animals given 1 and 25 mg/kg LU, an observation which may be explained by a sustained loss of VEGF within tumor tissue resulting in an inability of preneoplastic lesions to form frank tumors, as previously observed with apigenin treatment [171]. The loss of VEGF, is correlated with reduced blood vessel density in regressing tumors, which may result in the suppression of tumor nourishment. Although LU caused a sustained downregulation of tumor-associated VEGF that was independent of dosage, it had no effect on VEGF levels in non-tumor mammary gland tissue. The ability of LU to significantly reduce both VEGF production and tumor volume in all three dosage groups (1, 10, and 25 mg/kg) is most likely explained by the flavonoid acting as both an antiprogestin and possibly an antiestrogen [190, 198, 237, 242, 243]. This proposed mechanism could explain why tumor volumes in the 10 mg/kg group were reduced even though the incidence of tumors in the same group was high. Since LU did

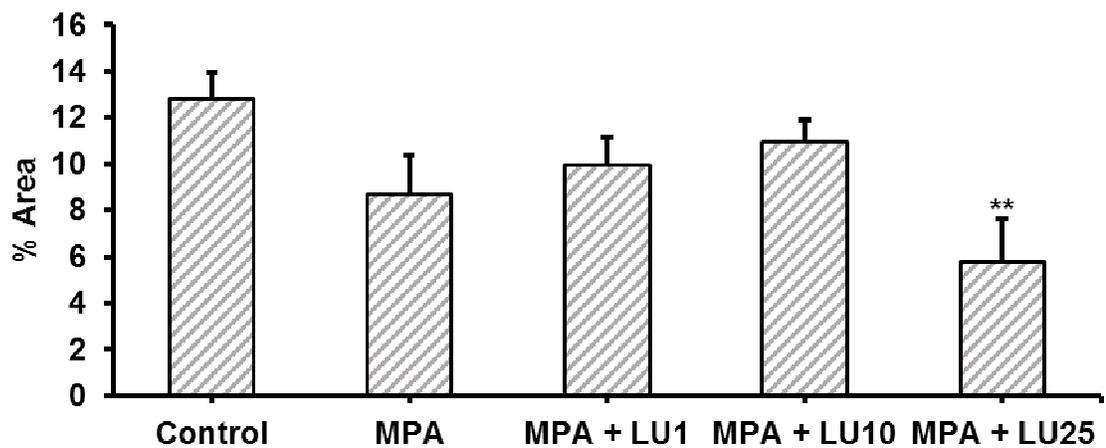
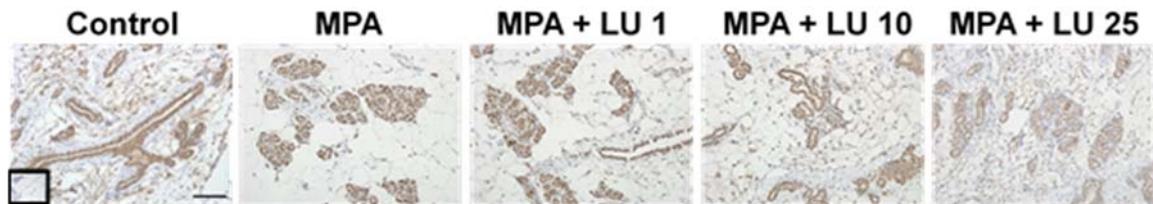
not influence progesterone receptor levels (data not shown), its inhibitory effect may be due to its ability to attenuate the post-ligand binding signal transduction pathway normally known to promote VEGF production in tumor cells. For example, hypoxia inducible factor alpha (HIF-1 α) plays an important role in progesterone receptor-mediated VEGF induction [244]. Thus, inhibition or downregulation of HIF-1 α may suppress VEGF activity. It is also possible that LU may modify the activity of progesterone receptor post-transcriptionally, as shown by others [245]. These possibilities remain to be explored.

Ki67 and TUNEL measurements provided little insight into the mechanism through which LU prevents tumor formation in the MPA-driven DMBA-induced mammary tumor model. While neither Ki67 nor TUNEL signals changed markedly in response to doses of 1 and 25 mg/kg LU, Ki67 expression was significantly increased in tumor tissue obtained from animals given a dose of 10 mg/kg LU, suggesting that in this group, circulating levels of LU caused tumor cell proliferation, which in turn resulted in LU losing its ability to control the formation of MPA-driven tumors. Such effects of flavonoids have been reported previously [171, 233]. It is likely that our inability to gain meaningful data for ki67 and TUNEL assays is due to the time lag between cessation of LU treatment and tumor collection. During this period when the suppressive pressure of LU was removed, tumor cell proliferation most likely increased, while apoptosis decreased. In future studies we will address this time-lag by collecting tumors at the time LU treatment is terminated. Suppression of VEGF expression and consequent disruption of angiogenesis by LU might explain why these tumors remained smaller compared with those under the influence of MPA alone, even though tumor incidence was equivalent.

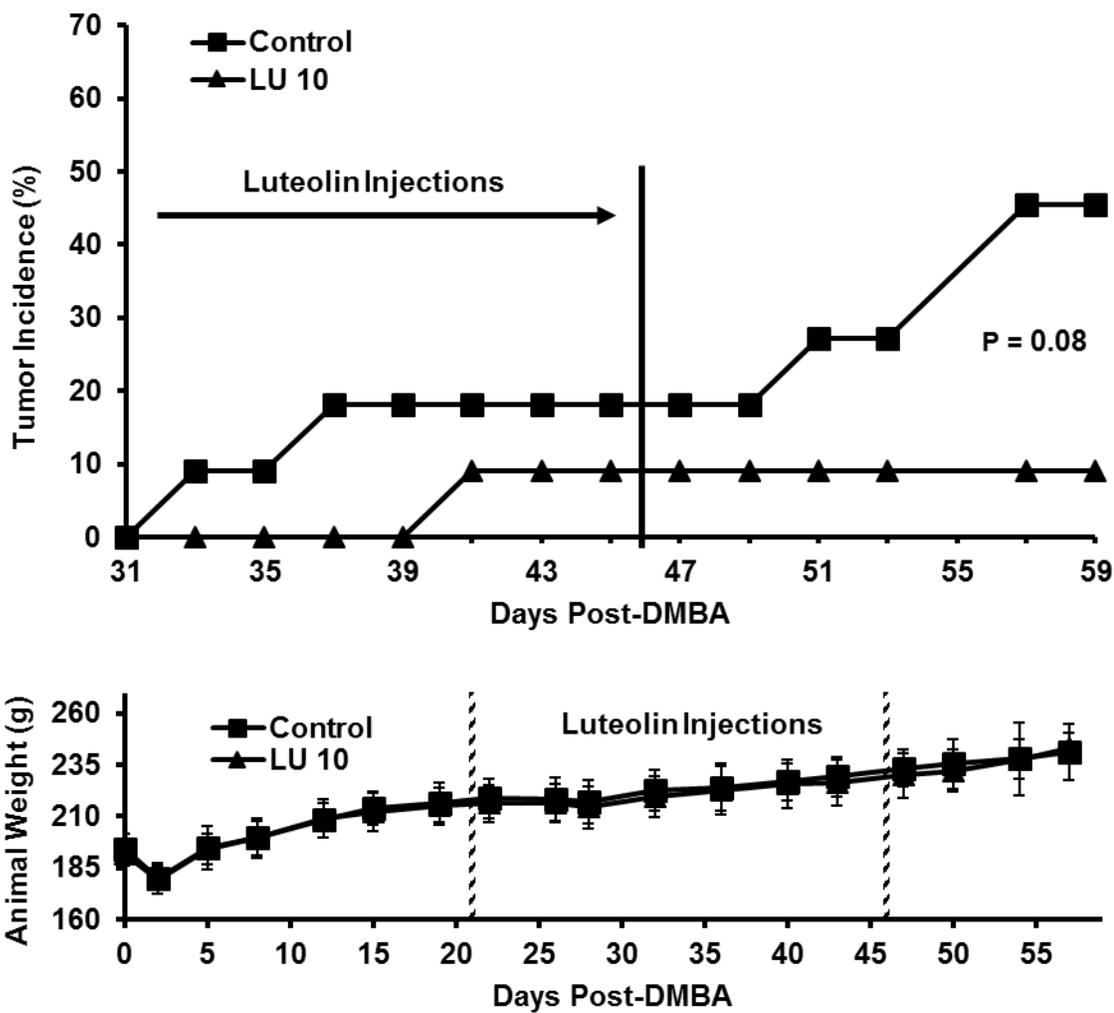
It is well known that progestins increase VEGF in hormone-responsive tumor cells [16, 96, 124, 171]. Recent evidence suggests that progestins not only provide a

microenvironment conducive to growth and metastasis, but that they also enrich the tumor cell population [117, 246]. It is therefore imperative that we improve the available therapeutic antiprogestin options. The minimal increase in tumor-associated VEGF observed in the MPA group in this study likely occurred as a result of the length of time involved in the experiment. Due to a large number of variously sized (both large and small) tumors present in the first place, maximum tumor growth may have already occurred prior to the end of the experiment, influencing final VEGF levels. Alternatively, seasonal variations may have caused fluctuations in levels of VEGF [169]. Nevertheless, it is interesting to note that LU brought about a persistent and significant reduction in VEGF production within mammary tumors, and that suppression of VEGF was independent of dosage. To our knowledge, this is the first report of any lasting effect of LU (i.e., persistent inhibition of VEGF) even when cessation of LU administration occurred at a point well before the end of the experiment. These observations suggest that LU may cause epigenetic changes in the VEGF gene, though this remains to be determined. Importantly, these findings indicate that LU has the ability to prevent tumors from establishing a microenvironment conducive to growth. Our observations also suggest that LU may suppress cancer stem cells since tumor incidence remained low even when LU supplementation was discontinued. Thus in vitro studies to examine the effects of LU on the self-renewal properties of stem-like cells are warranted. Considering that treatment with 25 mg/kg LU was nontoxic, resulted in the lowest total number of tumors (3 tumors in the 25 mg/kg group vs. 4 tumors in the 1 mg/kg group), and largely counteracted the effects of MPA on DMBA-induced mammary cancer by reducing both tumor volume and incidence, we propose that the flavonoid should be further evaluated as a naturally occurring chemopreventive compound. LU possesses important antitumor properties that may well be extremely advantageous to women who are either undergoing combination HRT or who have already been exposed to this type of therapy.

Further studies are justified to elucidate fully the effects of LU *in vivo* and to gain a better understanding of its potential for human use. Future animal studies should also investigate oral consumption of LU, in order to determine its preventive properties when ingested as a dietary supplement. Such studies would serve as a means of assessing its potential use in humans.



Supplementary Figure 2.1. Luteolin reduces mammary-associated VEGF, a marker of angiogenesis. Contralateral mammary glands (devoid of tumor) were removed 2 weeks after the last injection of LU (Day 59) and the tissues subjected to immunohistochemistry. Top, Representative immunohistochemical analyses of mammary gland tissue. All images were captured at 20x, and scale bar represents 100 μ m. Insert, negative control without primary antibody. Bottom, Hatched bars represent contralateral tumor-associated mammary gland tissue from each treatment group ($n \geq 3$; except for 25 mg/kg LU tumor tissue). Data represent the mean \pm SEM. VEGF staining. ** $P < 0.05$ compared with the control group in mammary gland tissues.



Supplementary Figure 2.2. Luteolin prevents development of DMBA-induced MPA accelerated tumors. Effects of LU on tumor latency and tumor incidence. Data represent the percent of animals with tumors in each treatment group at each time point (n = 11–12 animals/group). Control animals were given DMBA and subjected to vehicle and 10 mg/kg LU injections (Day 21–46) as per previously described protocol. Upper, LU suppressed DMBA-induced mammary tumorigenesis in Sprague-Dawley, P = 0.08 (GENMOD). Bottom, LU had no effect on animal weight.

CHAPTER 3

LUTEOLIN INHIBITS PROGESTIN-DEPENDENT VEGF INDUCTION, STEM CELL-LIKE CHARACTERISTICS, AND TUMOR PROGRESSION OF HUMAN BREAST CANCER CELLS

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3.1 Abstract

Clinical trials and epidemiological evidence show that combined estrogen (E) and progestin (P) hormone replacement therapy (HRT) increases the risk of breast cancer in postmenopausal women. Tumor progression is dependent on angiogenesis, which provides nutrients vital to the developing cancer. We showed, both in vitro and in vivo, that both natural and synthetic P, including the widely used progestin medroxyprogesterone acetate (MPA) increase production of a potent angiogenic factor, vascular endothelial growth factor (VEGF) in human breast cancer cells. This effect is blocked by the anti-progestin RU-486, suggesting the involvement of progesterone receptors in the process. Evidence from our laboratory using in vivo breast cancer models suggests that P accelerates the development of tumors from latent tumorigenic cells, leading to the development of palpable tumors, a process that may be attributed to increased production of VEGF. RU-486 blocks P-dependent VEGF production and thereby reduces tumor growth; however, the anti-progestin has severe side-effects and therefore cannot be used in the long-term. Recently, we have been studying less toxic naturally-occurring compounds for their ability to antagonize P-dependent VEGF induction and block tumor progression. In this study, we tested the effects of luteolin, a flavonoid commonly found in fruits and vegetables, on proliferation of BT-474 and T47-D breast cancer cells and their P-dependent production of VEGF. Luteolin treatment (25-100 μ M) for 24-48 h reduced in vitro tumor cell viability. Interestingly, treatment with a lower concentration of luteolin (10 μ M) blocked the production of P-dependent VEGF, indicating that VEGF suppression precedes luteolin-mediated loss of cell viability. Furthermore, luteolin (20 mg/kg, i.p.) suppressed growth of MPA-dependent T47-D human xenograft tumors in nude mice. Immunohistochemical analysis showed that luteolin reduced VEGF in tumor xenografts, strongly suggesting that it disrupts

tumor progression by blocking P-dependent VEGF production and consequent angiogenesis and preventing tumor cell proliferation. Furthermore, luteolin blocked the MPA-induced acquisition of stem cell like properties by breast cancer cells; CD44 expression, ALDH activity and mammosphere formation were all reduced by the flavonoid. We contend therefore that luteolin is a compound with valuable therapeutic properties. Its ability to reduce levels of VEGF, coupled with its capacity to interfere with the acquisition of stem-cell like properties by breast cancer cells, make luteolin a compound with significant clinical potential in the battle against P-dependent human breast cancer.

3.2 Introduction

Breast cancer is the most commonly diagnosed cancer and second leading cause of cancer-related death in women. In 2015, an estimated 232,000 new cases of breast cancer will be diagnosed, with approximately 40,000 cancer-related deaths [34]. A subset of these new cases and cancer-related deaths are linked to menopausal hormone replacement therapy (MHT). Postmenopausal women routinely undergo hormone replacement therapy (HRT) containing estrogen or, an estrogen and progestin combination, to ameliorate the life-altering symptoms of menopause. Estrogen is typically sufficient to overcome these symptoms but a progestin component is necessary to counteract the increase risk of endometrial cancer [7, 230, 231]. In 2002, the Women's Health Initiative found correlations between an increased risk of breast cancer and postmenopausal women taking combined estrogen plus progestin HRT compared to the placebo or estrogen alone arms [11]. Providentially, the increased breast cancer risk is transient and is greatly reduced after cessation of the progestin component [59], correlating to a 7% decline in breast cancer incidence after the results of the WHI trial were announced. Consequently because of these studies and others the use of MHT has become increasingly controversial. Nevertheless, an estimated 1.6 million U.S. women are currently taking combined estrogen plus progestin HRT in order to alleviate the symptoms of menopause [13].

Recently our lab and others have shown that progestins drive hormone-responsive breast cancers through the increase in production of vascular endothelial growth factor (VEGF), thereby, increasing neovascularization, proliferation, and metastasis. In the BT-474 xenograft model progestin is shown to stimulate lymph node metastasis, implicating progestin as a possible accelerant [124]. Due to the rapid onset of HRT driven tumors and the similarities of metastatic cancer cells to stem-like cells, it is suggested that the

previously progestin-starved latent tumor cells or cancer stem-like cells are revitalized with the exogenous progestin, thereby causing increased proliferation and ultimately leading towards a more aggressive phenotype. Supporting this supposition, progestins have been shown to enrich the stem cell-like cancer cell population in vitro by dedifferentiating progenitor cells back to a stem cell-like origin [103]. Taken together, progestins promote breast cancer not only through the potent mitogenic factor VEGF, by stimulating tumor and endothelial cell proliferation, but also enriching the stem cell-like population enabling tumors to grow and metastasize at an alarming rate.

Luteolin is a low toxic, prolific flavonoid found in over 300 plant species, many of which are readily available in our diets. Recently, luteolin has been shown to inhibit a variety of cancers both in vitro and in vivo [198, 234, 235] with little to minimal toxicity. Our lab has previously shown that luteolin prevents and delays medroxyprogesterone acetate (MPA)-dependent tumor development in the 7, 12-dimethylbenz(A)anthracene (DMBA)-induced tumor model and possess long-lasting anti-cancerous effects [247]. In the present study, we propose luteolin as a possible therapeutic to treat progestin-dependent breast cancer using the T47-D in vivo xenograft model [104] and provide a possible in vitro mechanism for which luteolin may prevent tumor stem cells from proliferating and subsequently metastasizing.

3.3 Materials and Methods

3.3.1 Reagents

Luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one) was purchased from Tocris (Cat # 2874; Minneapolis, MN) and dissolved in sterile filtered dimethyl sulfoxide (DMSO; Sigma Aldrich; Cat # D2650; St. Louis, MO). Solutions of LU were prepared weekly, aliquoted for daily use, and stored at -20°C until use.

Medroxyprogesterone acetate (MPA), progesterone, norethindrone, norgestrel, and RU-

486 were purchased from Sigma-Aldrich. Pierce bicinchoninic acid (BCA) protein reagents were obtained from Fisher Scientific (Pittsburg, PA). 17- β estradiol (E2; 1.7 mg), MPA (10 mg), and placebo sixty-day release pellets were obtained from Innovative Research of America (Cat # E-121, P-161; Sarasota, FL).

3.3.2 Cell Lines and Culture

Hormone responsive BT-474 and T47-D breast cancer cell lines were obtained from American Type Culture Collection (ATCC) and maintained and grown at 37°C in phenol red-free DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich) in a humidified atmosphere of 5% CO₂. 0.05% trypsin-EDTA (Invitrogen) was used for cell harvesting. For all in vitro experiments cells were allowed to reach 50-60% confluence, after which they were washed in phosphate-buffered saline (PBS) and maintained in DMEM/F12 supplemented with 5% dextran-coated charcoal (DCC) for 24 hours prior to treatment. Subsequently, cells were washed and further incubated in fresh 5% DCC-DMEM/F12. Incubations were carried out in the presence of MPA (10 nM) either alone or with RU-486 (1 μ M) or LU, as well as with the two aforementioned inhibitors alone. Unless otherwise stated, cells were treated with RU-486 and LU for 30 minutes prior to the addition of progestins. Control cells were treated with DMSO.

3.3.3 Cell Viability Assay

Viable cells were quantitated using the previously described sulforhodamine B (SRB) assay [248], a cell protein dye-binding assay. In brief, 1.0 x 10⁴ cells in 100 μ l 5% DEMEM/F12 medium were seeded into each well of a 96-well plate and incubated at 37°C overnight in 5% CO₂. Cells were treated with either the indicated levels of LU, or DMSO (controls) for periods up to 48 h. Subsequently adherent cells were fixed in situ for 1 h at 4°C using 50% cold trichloroacetic acid (TCA), after which they were washed with ice-cold water and allowed to dry. Fixed cells were stained at room temperature in

50 μ l of 4% SRB for 8 min. Excess dye was removed by washing with cold 1% acetic acid and allowed to dry. Protein-bound stain was solubilized in 10 mM Tris and absorbance at 520 nm measured using a SpectRA MAX 190 microplate reader (Sunnyvale, CA). Treatments were performed in sextuplet.

3.3.4 Apoptosis Assay

The extent of LU-induced apoptosis in breast cancer cells was evaluated by FACS analysis with Annexin V-FITC and PI binding assay as described previously [112]. T47-D cells were grown to 50-60% confluence in 10% DMEM/F12, at which point the media was switched to 5% DCC-DMEM/F12. After 24 h cells were treated with LU \pm MPA for an additional 16 h. Treated cells were harvested using 0.5% trypsin and stained for FACS analysis per manufacturer's protocol.

3.3.5 VEGF ELISA

Quantikine human VEGF ELISA kit (Cat # DVE00) was purchased from R&D Systems, Inc. (Minneapolis, MN). T47-D and BT-474 hormone responsive breast cancer cells were treated with vehicle (DMSO), MPA (10 nM), and either LU (at the indicated doses) or RU-486 \pm MPA for 18 hours at 37°C. Supernatant from each group was collected and VEGF concentrations measured by ELISA kit according to manufacturer's protocol. Experiments were performed in triplicate, and each sample was analyzed in duplicate on a microplate reader. Inter- and intra-assay coefficients of variance given by the manufacturer for cell culture supernatant assay are 5% to 8.5% and 3.5% to 6.5%, respectively.

3.3.6 Bicinchoninic Acid Protein Assay

Cells were harvested and pellets resuspended in 300 μ L lysis buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, and 1% Nonidet P-40). Protein concentration was determined by measuring absorbance at 562 nm on a microplate reader and using bovine serum

albumin (Thermo Fisher Scientific, Cat # 23209; Waltham, MA) as standard.

Experiments were performed in triplicate, and samples were analyzed in duplicate.

3.3.7 Western Blot

Total cellular protein extracts were prepared as described previously [249]. Protein aliquots (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. Protein bands were detected and quantified by blotting with PR (1:200 dilution; Santa Cruz Biotechnology; Cat # SC810), ERα (1:150 dilution; Santa Cruz Biotechnology; Cat # 8005), and A-actin (Sigma-Aldrich; Cat # SAB2500963) using an anti-mouse or anti-goat secondary antibody (1:2000 dilution; Santa Cruz Biotechnology). Visualization was achieved using an Amersham ECL Prime western blotting detection reagent (GE Healthcare, Cat # RPN2232; Little Chalfont, UK), following the manufacturers protocol.

3.3.8 RNA Preparation and Primers Used for RT-PCR

RNA from progestin-treated cells was purified and RT-PCR conducted as described previously [249]. The primers used were

VEGF

F 5' - CTG CTG TCT TGG GTG CAT TGG

R 5' - CAC CGC CTC GGC TTG TCA CAT

PR

F 5' - AGC CCT AAG CCA GAG ATT

R 5' - TAG GAT CTC CAT CCT AGA CC

GAPDH

F 5' - ATG AGA AGT ATG ACA GCC

R 5' - TGA GTC CTT CCA CGA TAC C

3.3.9 Fluorescence-activated Cell Sorting

T47-D breast cancer cells were treated for 16 hours as indicated and harvested using accutase (BD Biosciences, San Jose, CA) in place of 0.5% trypsin. 1 X 10⁶ cells were suspended in 100 µL staining buffer and placed in conical microcentrifuge tubes. 20 µL PE mouse anti-human CD24 (BD Biosciences, Cat # 555428) and APC mouse anti-

human CD44 (BD Biosciences Cat #559942) were added to each sample, along with the necessary FACS dye controls and samples were incubated on ice for 45 minutes. Cells were washed twice in staining buffer and resuspended in 500 μ L staining buffer.

Aldehyde dehydrogenase activity, a non-immunological identifier of human stem/progenitor cells, was assessed using STEMCELL Technologies ALDEFLUOR kit (Cat #01700; Vancouver, BC), per manufacturers protocol. All samples were processed within 15 minutes of the final wash. Cells were visualized using a Beckman Coulter CyAn ADP FACS machine running Summit 5.2 software and results analyzed as previously described [55].

3.3.10 Mammosphere Assay

T47-D cells were grown in 10% FBS DMEM/F12 medium. Cells were cultured in 5% FBS-DCC DMEM/F12 medium for 24 hours, and then treated for 48 h with (1) 10 nM MPA, (2) 25 μ M LU, (3) both 10 nM MPA and 25 μ M LU. Cells from each group were subsequently seeded into six-well plates (5,000 cells/well) in Complete MammoCult medium (StemCell Technologies Inc., Vancouver, BC) and treatment continued for a further 7 days. Culture medium (1 ml) containing the indicated agents, was added on days 2, 4 and 6 to ensure drug availability. Light microscopy (10X) pictures of mammosphere formation in response to the different drug treatments were captured after 7 days using an EVOS light microscope. T47-D cell mammospheres were counted by size exclusion using a 60 μ m standard.

3.3.11 Progestin-dependent Growth of Human Breast Xenograft Tumors.

Female athymic nu/nu nude mice, 5 to 6 weeks old (18–22 g), were purchased from Harlan Sprague-Dawley, Inc. Mice were housed in a laminar air-flow cabinet under specific pathogen-free conditions. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) in accordance with current federal regulations and standards. Nude mice were inoculated with E2 pellets 48

hours before implantation of T47-D cells. Cells were harvested by trypsinization and washed twice with DMEM/F12 medium. Cell pellets were then suspended in DMEM/F12 medium and injected subcutaneously (s.c.; 1×10^7 cells per 150 μ L) into the side flanks of each mouse. Tumor volume was measured every 3 days using a digital caliper and calculated using the formula $L \times W \times H \times \pi/6$, as previously described [104]. Tumors began to regress once they reached 60 to 100 mm³ in size, usually after 6–10 days. On day 10, mice were inoculated with a 10 mg 60-day release MPA pellet. On days 60 and 61 intraperitoneal (i.p.) injections of LU (20 mg/kg/day) (loading dose) were given. LU was then administered every other day until Day 80.

3.3.12 Histology and Immunohistochemical Analysis

Immunohistochemical staining of tumor tissue was performed following previously described procedures [104, 232]. The following polyclonal antibodies were used: anti-VEGF antibody 1:100 dilution [Santa Cruz Biotechnology Cat # SC-152; Dallas, TX]; CD31 antibody and PR antigen antibody (1:50 dilution [DAKO Cat # M0823 and A0098; Carpinteria, CA]). Cell death immunohistochemistry was determined using a Roche (Cat # 11684817910, Basel, Switzerland) terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) detection kit. Histological samples were analyzed and quantified using Fovea Pro 3.0 (Reindeer Graphics) and Image J. Images were captured at 20X magnification and threshold intensity was adjusted for measurement in pixels. Tumors were excised from animals in each treatment group, fixed in formaldehyde, and embedded in paraffin for immunohistochemistry. One section from each individual tumor was placed on the corresponding slide for each of the immunohistochemical stains and 4-5 random fields were captured from every section to minimize errors due to differences in cellularity. Mammary tumor sections were collected from both flanks of each mouse and at least 3 tumors per group were collected for analysis. Regions of staining within tumors were recorded. Fovea Pro 3.0 was used to quantitate the percent area of VEGF,

while PR and TUNEL staining was calculated using the color threshold and counting feature in Image J, respectively. This facilitated precise discrimination between positive/negative cells and background. CD31 stained blood vessels were counted using Adobe Photoshop CS 5.5 (San Jose, CA).

3.3.13 Statistical Analysis

Statistical significance was tested using one-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparison test to determine the difference in mean between groups. A two-way repeated measures ANOVA was used for animal weights. If normality failed, the data was tested using a nonparametric one-way ANOVA on ranks (Kruskal-Wallis), followed by the Newman-Keuls nonparametric multiple comparisons test. Data were reported as means \pm SEM. For all comparisons, $P \leq 0.05$ was regarded as statistically significant and analysis was accomplished in SigmaPlot 12.5 software.

3.4 Results

3.4.1 Luteolin Inhibits Viability of Human Breast Cancer Cells

The effect of LU on cell viability was assessed in two ER and PR positive breast cancer cell lines (BT-474 and T47-D cells), measuring cell protein content by sulphorhodamine B assay, as described previously [112]. Breast cancer cells were exposed for 24 and 48 h to varying concentrations of LU (0–100 μ M) that have been used in previous studies [198, 234, 235]. LU markedly reduced the viability of T47-D and BT-474 cells in both a dose- and time-dependent manner ($P < 0.05$; **Figure 3.1A** and **Figure 3.1B**). Following 24h exposure, LU exhibited an IC_{50} value of approximately 50 μ M against tumor cells, with minimal or no effect observed at 10 μ M during the same treatment period. Thus unless otherwise stated, in subsequent experiments T47-D cells were treated with 10 μ M LU for 16-18 h in order to determine initial biological effects of the nutraceutical (e.g. suppression of VEGF) without inducing cell death.

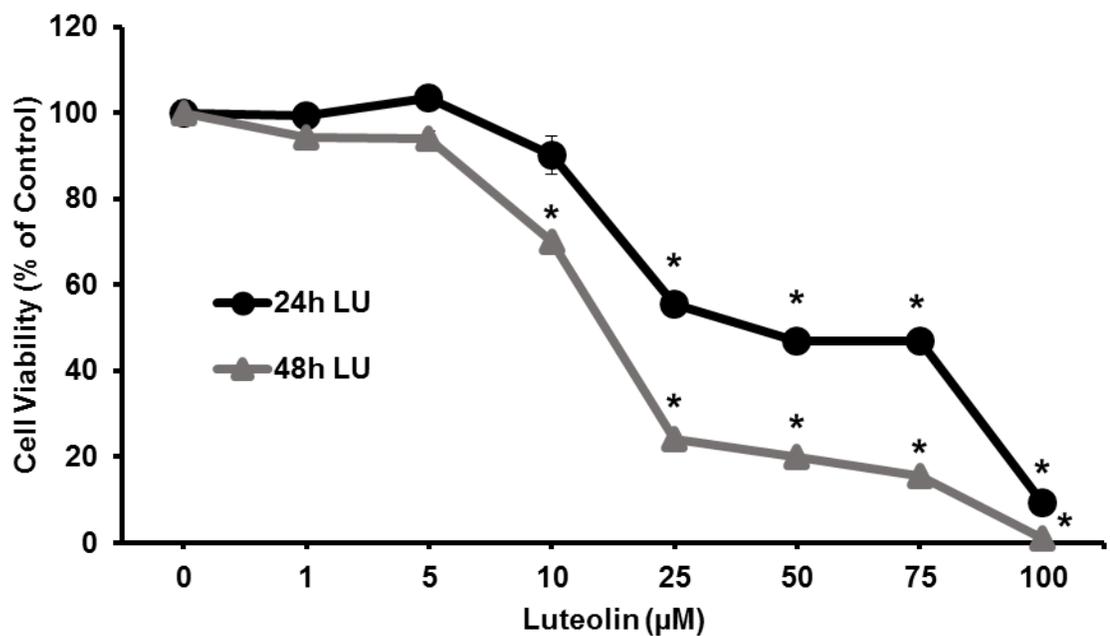


Figure 3.1A. Luteolin inhibits T47-D human breast cancer cell viability. T47-D breast cancer cells were seeded overnight in a 96-well plate (1.0×10^4 cells/well). Cells were then washed and treated with the indicated concentration of LU for either 24 or 48 hrs. Cell viability was determined as described in Methods. *Significantly different from control (ANOVA, $P < 0.05$).

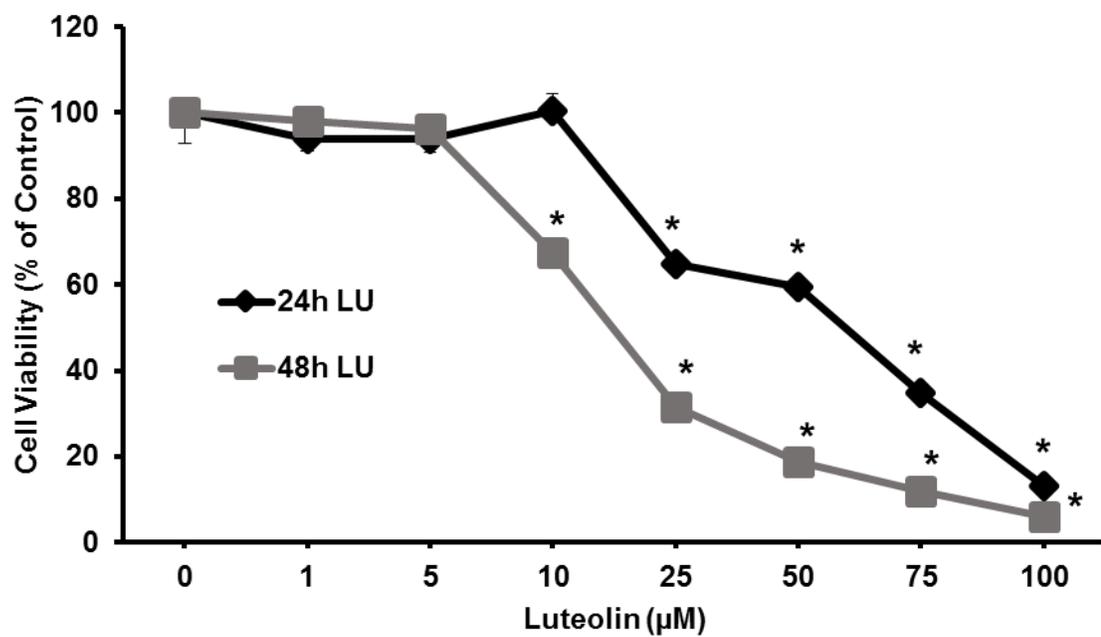


Figure 3.1B. Luteolin inhibits BT-474 human breast cancer cell viability. BT-474 breast cancer cells were seeded overnight in a 96-well plate (1.0×10^4 cells/well). Cells were then washed and treated with the indicated concentration of LU for either 24 or 48 hrs. Cell viability was determined as described in Methods. *Significantly different from control (ANOVA, $P < 0.05$).

3.4.2 Luteolin Inhibits Progesterin-induced VEGF from Breast Cancer Cells

We previously established that both natural and synthetic progestins induce synthesis and secretion of the potent angiogenic factor VEGF from both T47-D and BT-474 human breast cancer cells [4, 16, 17, 19, 20]. Using the synthetic anti-progesterin RU-486 we further showed that VEGF induction was PR-dependent [109, 250]. We have extended our studies to examine the ability of LU, a naturally-occurring plant flavonoid, to block progesterin-induced secretion of VEGF protein from breast cancer cells. We contend that if LU blocks VEGF production by tumor cells, it could be used as a natural, non-toxic alternative therapeutic option to prevent angiogenesis and tumor cell survival [23, 24, 131]. Initially T47-D cells were treated for 16 h with MPA, both with and without LU or RU-486. MPA-dependent release of VEGF into the culture medium was measured by ELISA. Levels of MPA-dependent VEGF secreted by T47-D cells were significantly reduced by 10 μ M LU; however, 2 μ M LU had no effect (**Figure 3.2A**; ANOVA $P < 0.05$). Similarly, 10 μ M LU lowered levels of VEGF secreted in response to both progesterone and a number of commonly used synthetic progestins (**Figure 3.2B**). Importantly, LU itself did not induce VEGF, therefore behaving in a similar fashion to RU-486.

In order to ascertain whether the LU exerted similar effects in other breast cancer cell types we measured its ability to block MPA-induced VEGF production in BT-474 cells. As shown in **Figure 3.2C**, 10 μ M LU also reduced levels of VEGF secreted in response to MPA by BT-474 cells. At higher concentrations (25 μ M) LU blocked even basal levels of VEGF production.

3.4.3 Luteolin Suppresses Progesterin-induced VEGF at a Transcriptional Level

We used T47-D cells to determine whether LU also suppressed P-induced VEGF at the RNA level. Cells were treated for 6 h with MPA \pm either 25 μ M LU or 1 μ M RU-486, after

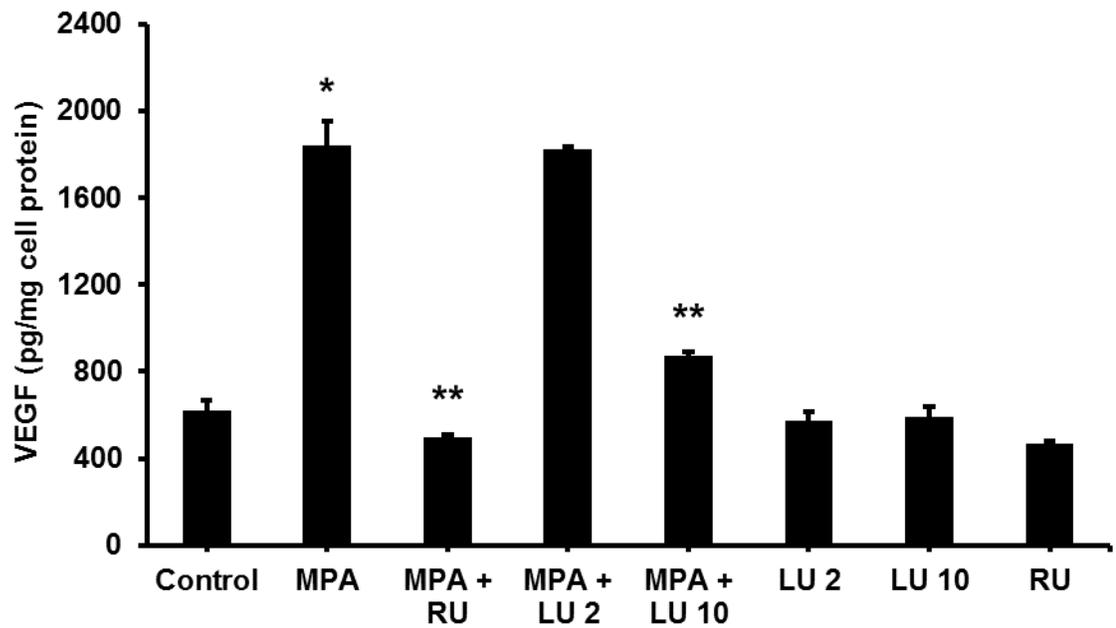


Figure 3.2A. Luteolin inhibits MPA-induced VEGF protein secretion in human T47-D breast cancer cells. T47-D cells were treated at 37°C for 18 h with 10 nM MPA ± 1 µM RU or RU alone and 2 or 10 µM LU and with 10 µM LU alone. Administration of LU and RU was 30 min prior to MPA. Levels of VEGF in supernatants were measured by ELISA as described in Methods. Results represent means ± SEM (n=3, in duplicate). *P < 0.05, compared with control. **Significantly different from progestin alone (ANOVA, P < 0.05). MPA, medroxyprogesterone acetate; RU, RU-686 (mifepristone).

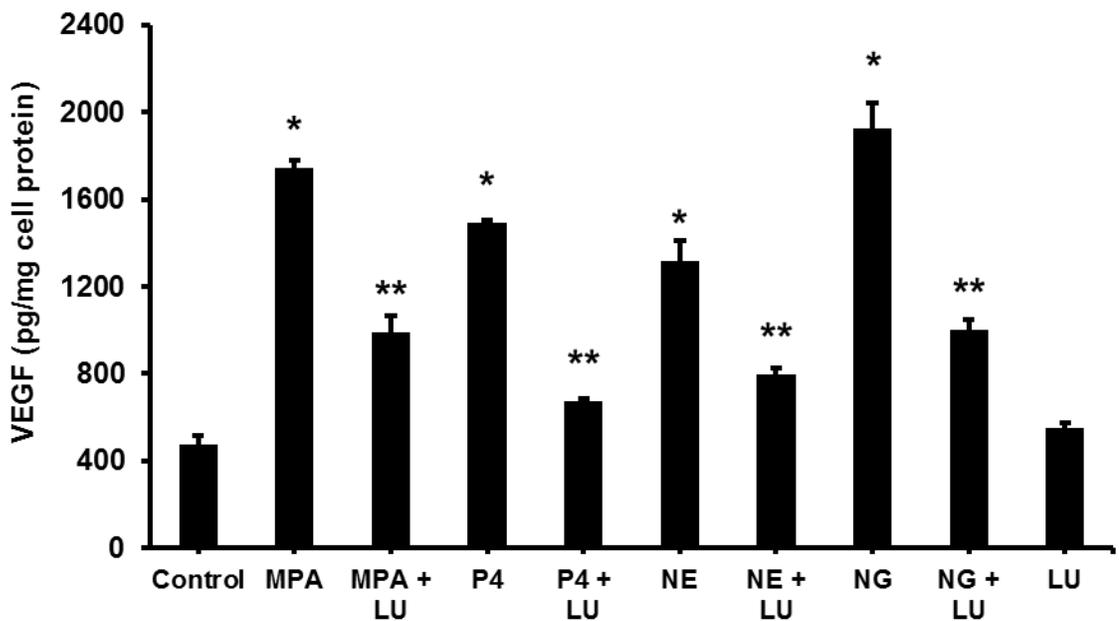


Figure 3.2B. Luteolin suppresses VEGF secretion induced by natural and synthetic progestins in T47-D cells. T47-D cells were treated at 37°C for 18 h with 10 nM MPA, progesterone (P4), norethindrone (NE), or norgestrel (NG) ± 10 µM LU. Administration of LU was 30 min prior to the progestin. Results represent means ± SEM (n=3, in duplicate). *P < 0.05, compared with control. **Significantly different from progestin alone (ANOVA, P < 0.05). MPA, medroxyprogesterone acetate.

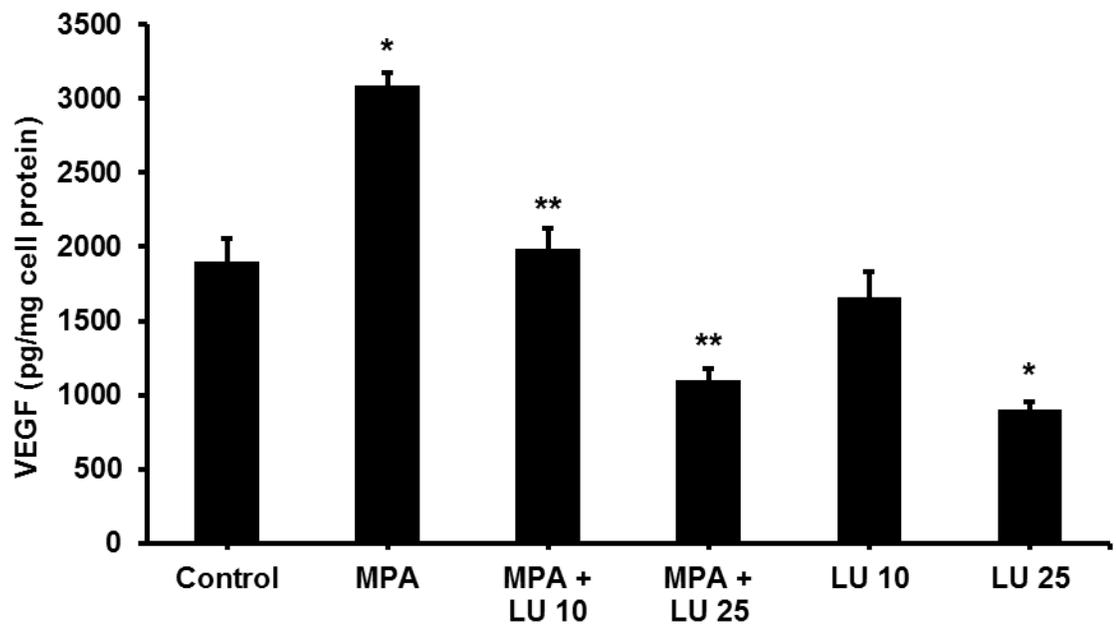


Figure 3.2C. Luteolin inhibits progestin-induced secretion of VEGF from human BT-474 breast cancer cells. BT-474 cells were treated with 10 nM MPA \pm 10 or 25 μ M Luteolin (LU) at 37°C for 18 h, as described in Methods. Results represent means \pm SEM (n=3, in duplicate). *P < 0.05, compared with control. **Significantly different from progestin alone (ANOVA, P < 0.05). MPA, medroxyprogesterone acetate.

which RNA was prepared. As shown in **Figure 3.3**, LU and RU-486 at the concentrations tested both suppressed MPA-induced VEGF. The anti-hormones themselves did not induce VEGF mRNA.

3.4.4 Luteolin Induces Apoptosis in Breast Cancer Cells

In another study we sought to determine whether LU induced breast cancer cell apoptosis. T47-D cells were treated with either 25 or 50 μM LU for 16 h. Using FACS analysis we then analyzed for expression of Annexin V, a marker of apoptosis. As shown in **Figure 3.4**, LU induced apoptosis in breast cancer cells whether or not 50 μM MPA was present, indicating that the progestin is unable to protect breast cancer cells from LU-induced cell death.

3.4.5 Luteolin Inhibits MPA-induced Tumor Growth in Nude Mice

Having shown that LU is effective in vitro, we studied its therapeutic effect in a progestin-dependent T47-D xenograft model previously developed in our laboratory [104]. The experimental protocol is shown in **Figure 3.5A**. E2 is present in all groups since without it tumors do not grow in vivo. Intraperitoneal administration of 20 mg/kg LU or vehicle commenced once tumor-bearing mice reached an average tumor volume of 60 mm^3 , at approximately day 60. As seen in **Figure 3.5B**, LU blocked progestin-induced T47-D tumor progression that reached control levels by day 76 ($P < 0.05$). At day 79, mean tumor volume was $90 \pm 15 \text{ mm}^3$ in the MPA (vehicle treated) group, $38 \pm 6 \text{ mm}^3$ in animals injected with LU, and $31 \pm 7 \text{ mm}^3$ in the control group (with E2 pellets alone). No LU-related toxicity was observed in any of the experimental animals, as determined by animal weight (average body weight was slightly higher in MPA group compared to controls) (**Figure 3.5C**). Furthermore animal behavior, i.e. eating, grooming, and mobility, was no different in LU treated mice, again suggesting that the flavonoid had little to no toxicity.

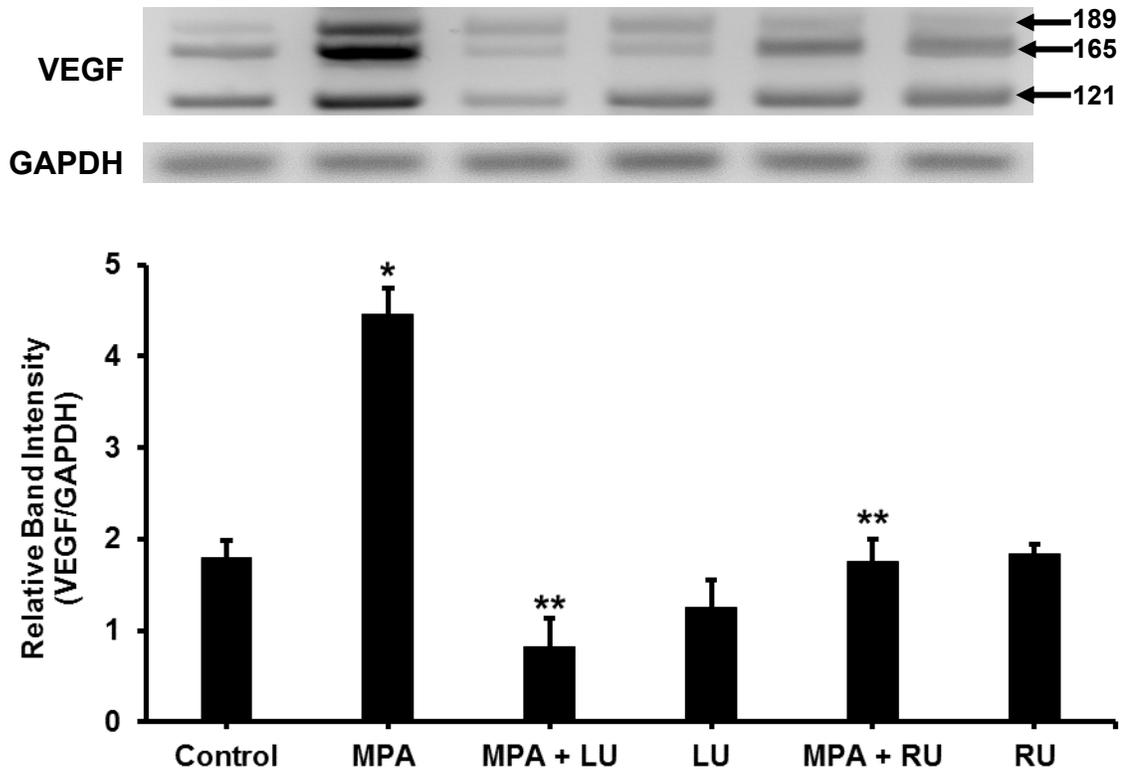


Figure 3.3. Luteolin inhibits MPA-induced VEGF mRNA expression in T47-D breast cancer cells. Cells were treated for 6 h with MPA (10 nM) ± LU (25 μM) or RU (1 μM), after which RNA was isolated and RT-PCR for VEGF isoforms performed as described in Methods. Upper panel is a representative Figure of PCR amplified VEGF bands, showing VEGF 189, 165, and 121 and the GAPDH band used for normalization. Bottom panel shows mean values from three independent experiments. Results are expressed as mean band intensities (VEGF/GAPDH) ± SEM (n=3). *p<0.001, significantly different from control. **p<0.001, significantly different from MPA (ANOVA).

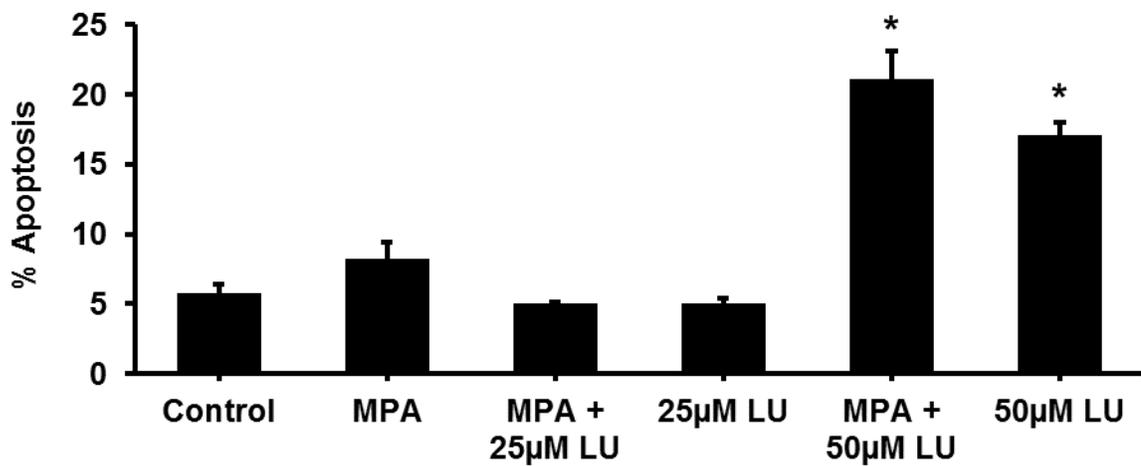


Figure 3.4. Luteolin induces apoptosis in breast cancer cells. T47-D human breast cancer cells were treated with MPA (10 nM) \pm LU (25 or 50 μ M), as described in Methods. Cells were stained with Annexin V (AV) and propidium iodide (PI) and analyzed by FACS. 50 μ M LU effectively induced apoptosis irrespective of whether or not cells were exposed to MPA, while 25 μ M LU had no effect. Bars represent AV + AV/PI means \pm SEM (n=3). *p<0.001 compared with control (ANOVA).

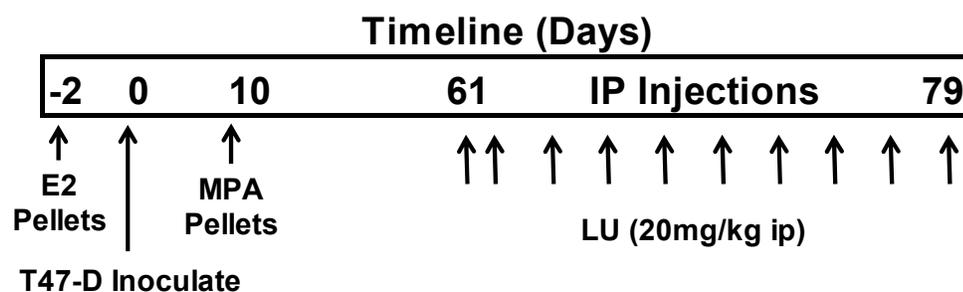


Figure 3.5A. Protocol for MPA-accelerated human breast cancer xenograft growth and treatment model. T47-D cells (1×10^6) were injected subcutaneously into both flanks of nude mice. An estradiol (E2) pellet was implanted two days prior to injection of tumor cells. MPA pellets were implanted on day 10 and when tumors reached about 60 mm^3 , treatment with LU (20 mg/kg) or placebo began (arrow, day 61). LU was injected i.p. daily for two days (loading dose), followed by injections every other day until day 79.

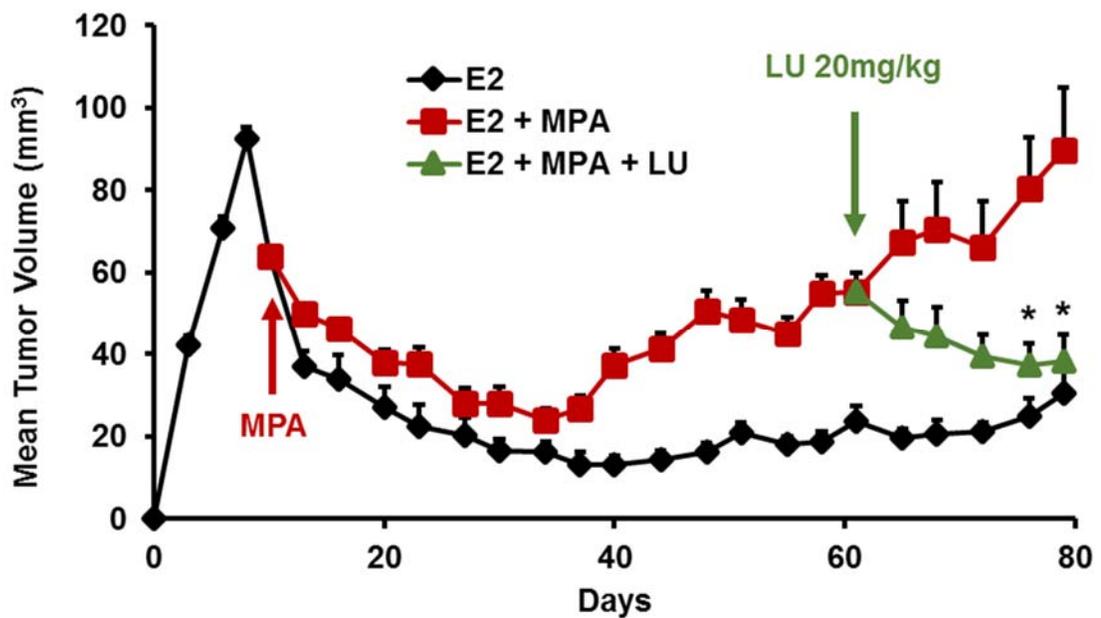


Figure 3.5B. Luteolin suppresses growth of MPA-accelerated human breast cancer cells in a xenograft model. T47-D xenograft tumor growth curve in vivo. MPA pellets were implanted on day 10 (arrow) and when tumors reached about 60 mm³, treatment with LU (20 mg/kg) or placebo began (arrow, day 61). LU was injected i.p. daily for two days (loading dose), followed by injections every other day until day 79. Each data point represents mean tumor volume \pm SEM (E2 group, n=4 tumors; E2 + MPA group, n=6 tumors; E2 + MPA + LU group, n=8 tumors). *Significant difference compared to MPA group ($p < 0.05$, ANOVA).

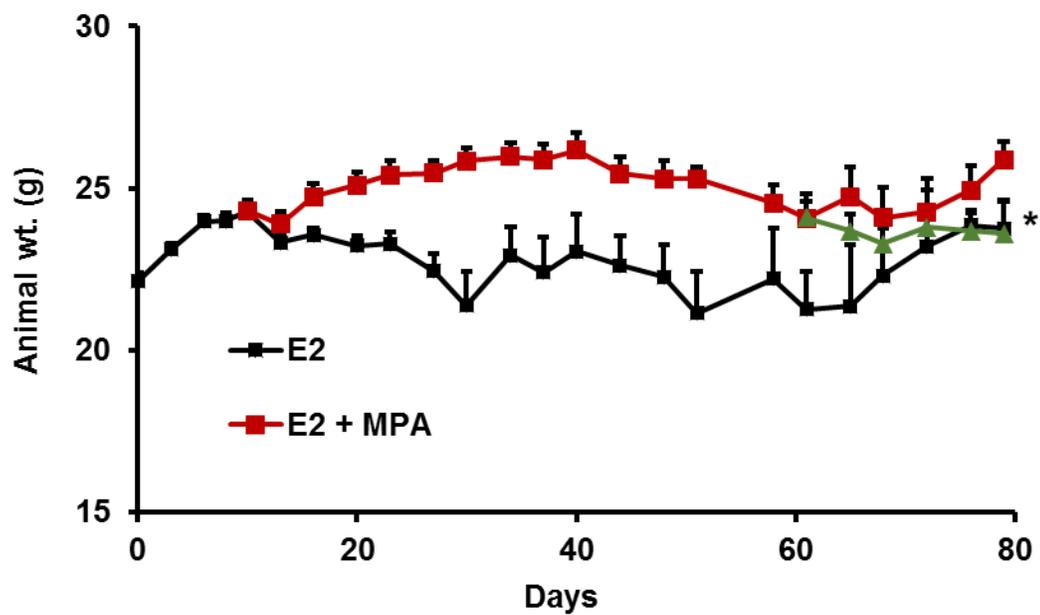


Figure 3.5C. Luteolin has little to no effect on nude mice weight. Treatment with LU (20 mg/kg) or placebo began (arrow, day 61). LU was injected i.p. daily for two days (loading dose), followed by injections every other day until day 79. Animal weights were measured throughout the experiment. By day 79 there was a slight, though significant weight gain in animals treated with MPA. * $p < 0.05$, ANOVA.

3.4.6 Immunohistochemical Analysis

Apoptosis and Proliferation markers: The reduction in tumor volume associated with LU might be explained by either increased apoptosis or lower levels of proliferation. With this in mind we used TUNEL and Ki67 assays to measure these parameters in tumors excised on day 79. In both cases, LU elicited no significant changes in either parameter (data not shown). This suggests that neither mechanism is involved in the LU-induced decline in tumor growth or that we are studying tumor decay too far in advance to observe such changes.

Luteolin reduces expression of VEGF and CD-31, markers of angiogenesis: We previously showed that progestins increase tumor burden by inducing VEGF, thereby promoting tumor growth [104, 124]. In the present study we used our xenograft model to examine the ability of LU to block VEGF induction and thus reduce angiogenesis, since in vitro studies suggest this may be possible. MPA significantly increased intratumoral VEGF production compared with tumors derived from placebo treated animals (**Figure 3.6A**, left panels). Compared with animals given MPA alone, i.p. injections of LU (20 mg/kg) caused a significant reduction in VEGF production (**Figure 3.6A**; ANOVA $P < 0.05$). Tumors from MPA-treated animals exhibited a higher number of blood vessels compared with controls (ANOVA $P < 0.001$) and 20 mg/kg LU significantly suppressed MPA-driven tumor blood vessel formation (ANOVA $P = 0.003$, though the inhibitory effect did not reach control levels (**Figure 3.6B**, ANOVA $P = 0.024$).

Progesterone Receptor (PR): Immunohistochemical analysis of tumor tissue demonstrated an almost complete loss of PR in animals given MPA alone (**Figure 3.6C**; ANOVA $P < 0.05$), concurring with previous reports [232, 251]. LU did not prevent the MPA-induced loss of PR in these tumor tissues (**Figure 3.6C**), suggesting that it does not block PR activation, but rather, acts at a point beyond the PR activation step, or

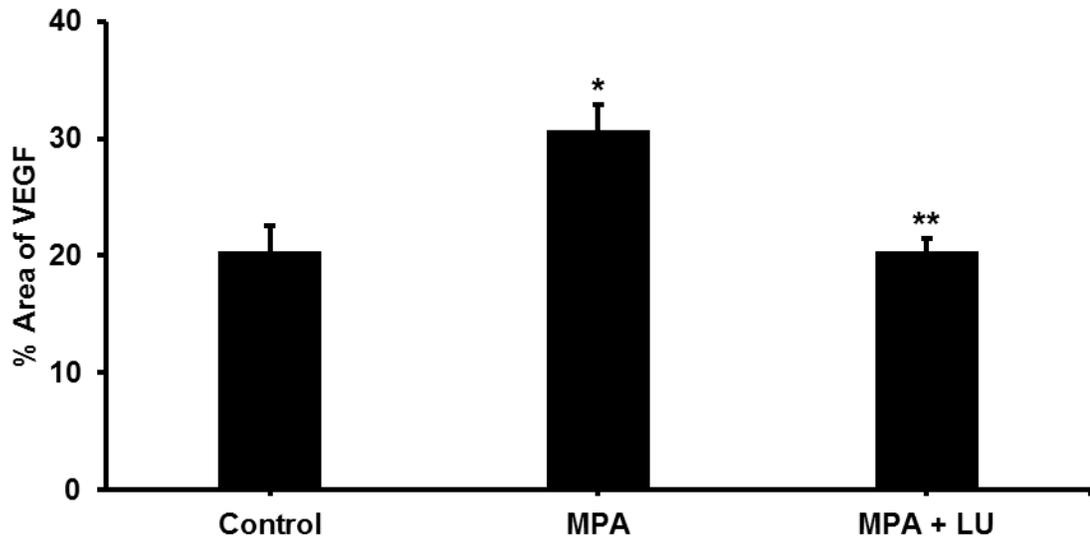
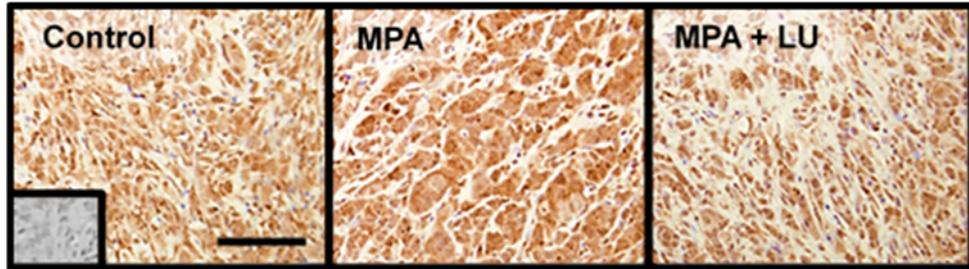


Figure 3.6A. Immunohistochemical analysis of T47-D derived xenograft tumors. Luteolin (LU) suppresses MPA-driven VEGF production in T47-D xenografts. Top: images represent VEGF (brown) staining from one tumor per group, 100 μ M scale. Insert, negative control without primary antibody. Bottom: quantification of VEGF data with bars representing mean \pm SEM (E2, n=3 tumors; E2 + MPA, n=6 tumors; E2 + MPA + LU, n=8 tumors) and analyzed as described in Methods. *Significantly different than control, P = 0.007; **significantly different than MPA, P < 0.001; ANOVA.

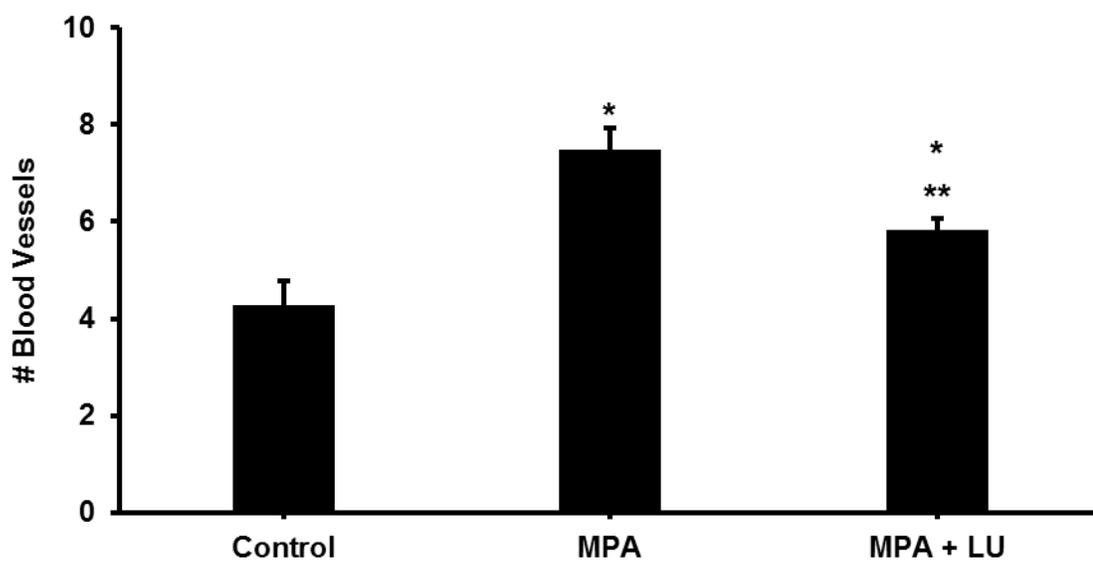
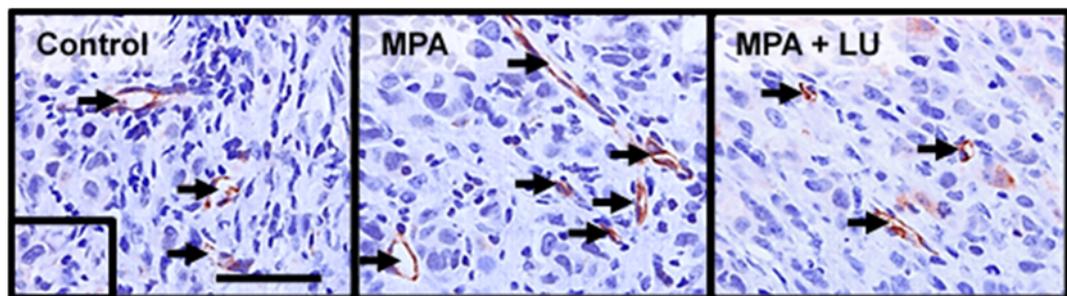


Figure 3.6B. Luteolin suppresses MPA-driven blood vessel growth in T47-D xenografts. Top: CD31 immunohistochemical endothelial stain (reddish-brown), representative pictures show blood vessels from sections of a 20x field at captured resolution, bar 50 μ m. Insert, negative control without primary antibody. Five captures at 20x were taken per tumor in each group. Tumor captures were averaged for each individual tumor, and the data represents means \pm SEM. The control group contained 3 tumors from 1 animal while the groups given MPA alone and MPA + LU had 7-8 tumors from at least 4 animals. *indicates significantly different than control (MPA: $P < 0.001$; MPA + LU: $P = 0.024$). **indicates significantly different than control and MPA alone ($P = 0.003$; ANOVA followed by Student-Newman-Keuls multi-range comparison test).

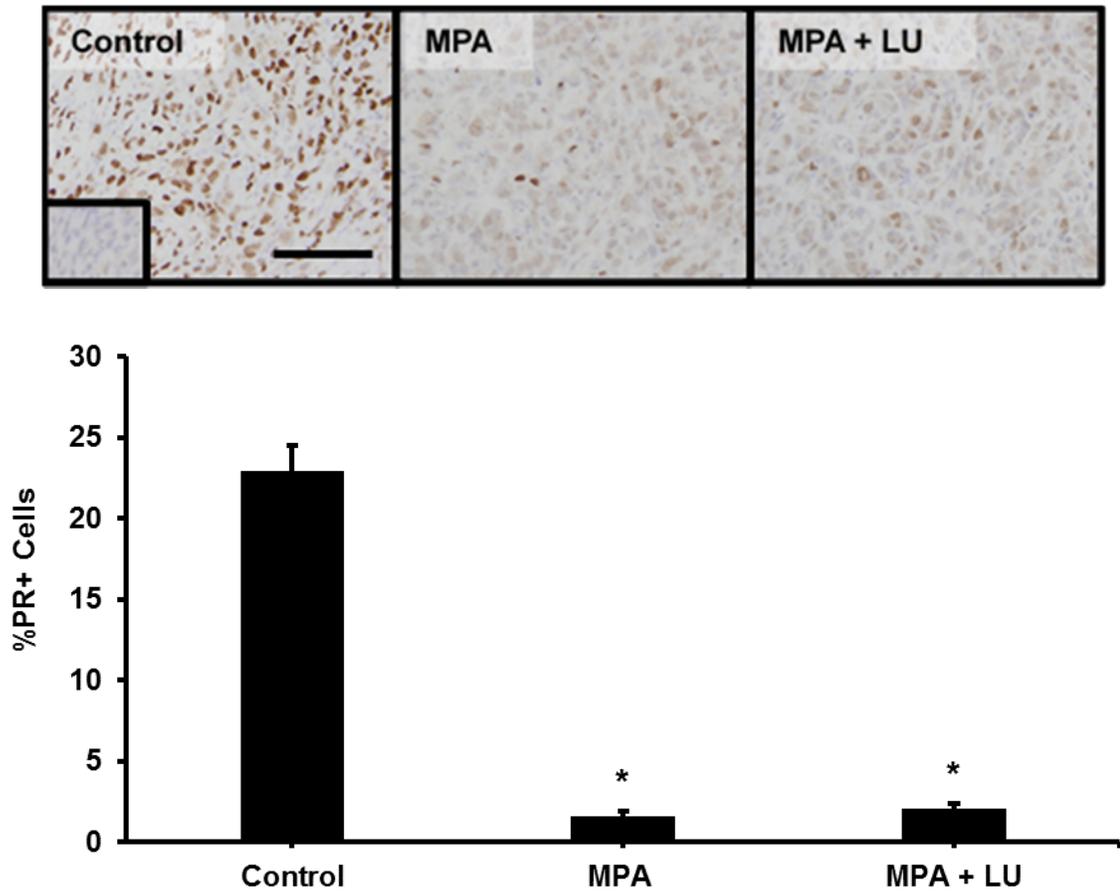


Figure 3.6C. Luteolin fails to abrogate MPA-driven PR loss in T47-D xenografts. Top: images represent progesterone receptor (PR) expression, insert represents negative control without primary antibody. Bottom: quantification of data. Statistical analysis was carried out using ANOVA, followed by a Kruskal Wallis on Ranks (* $p < 0.05$).

exerts other post-transcriptional effects on VEGF RNA or protein. The inability of LU to prevent PR activation was verified in vitro by Western blot analysis. MPA was again shown to lower PR, whether LU was present or not (data not shown). By virtue of these findings, one may infer that loss of tumor growth in this model is due to loss of VEGF, which leads to a suppression of angiogenesis and reduced tumor cell survival.

3.4.7 Luteolin Inhibits MPA-induced Expression of Stem Cell-like Markers in Breast Cancer Cells

CD44 expression: As previously shown [104, 124], MPA rescues in vivo tumor cell growth, a phenomenon that is likely linked to the ability of the progestin to enrich the stem-cell-like properties in a small sub-portion of tumor cells [52, 103]. In this study we used in vitro FACS analysis to show that MPA induced a large and highly reproducible CD44⁺ shift in T47-D breast cancer cells. CD44⁺ is a well-recognized marker of breast cancer stem cells and this finding suggests that MPA induces an increase in stemness or progenitor-like cells as previously shown [52, 53]. The MPA-induced CD44⁺ shift was significantly reduced by exposure to both 25 μ M LU and to the anti-progestin RU-486 (**Figure 3.7A**). Furthermore LU countered the MPA response dose-dependently, since 10 μ M LU given in the presence of MPA did not significantly attenuate the effect, but 10 μ M LU on its own appeared to slightly, though significantly, bring about an increase in CD44 expression compared with control (**Figure 3.7A**). This suggests that at a concentration of 10 μ M, LU has a slight agonist activity with respect to CD44 induction, an effect that is completely overcome at 25 μ M.

Aldehyde dehydrogenase (ALDH) activity: Increased ALDH1a levels are recognized as an established stem cell marker in a variety of cancers, including breast cancer [55]. With this in mind we sought to determine whether MPA induced an increase in the ALDH signal, and if so, whether LU would counter such a response. As shown in **Figure 3.7B**,

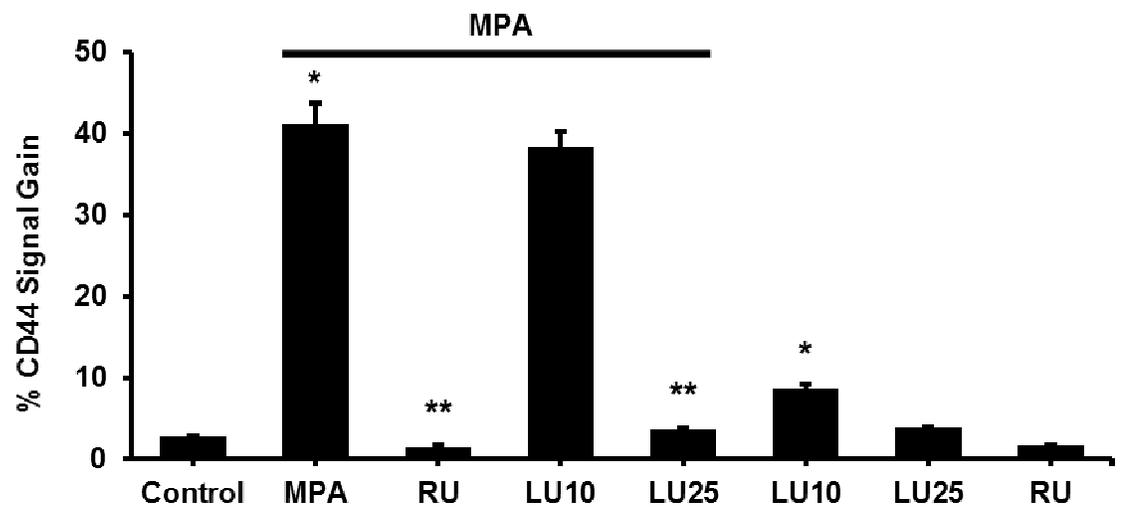
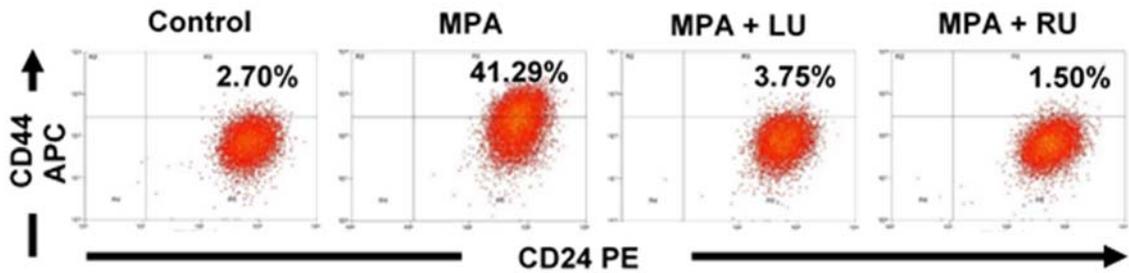


Figure 3.7A. Luteolin suppresses MPA-induced stem-cell-like properties of breast cancer cells. T47-D cells were incubated for 24 h with 10 nM MPA, 10- or 25 μ M LU, and/or 1 μ M RU-486. Cells were labeled with CD44-APC and CD24-PE and analyzed by FACS using Summit, upper panel. Bottom, quantitation of data from 3 different experiments conducted as described. 25 μ M LU effectively blocked induction of CD44 in response to MPA, while this concentration of LU alone had little effect. Bars represent means \pm SEM (n=3). ANOVA p<0.05 *statistically significant CD44+ signal gain compared with controls, **statistically significant CD44+ loss compared with MPA-induced CD44+ gain.

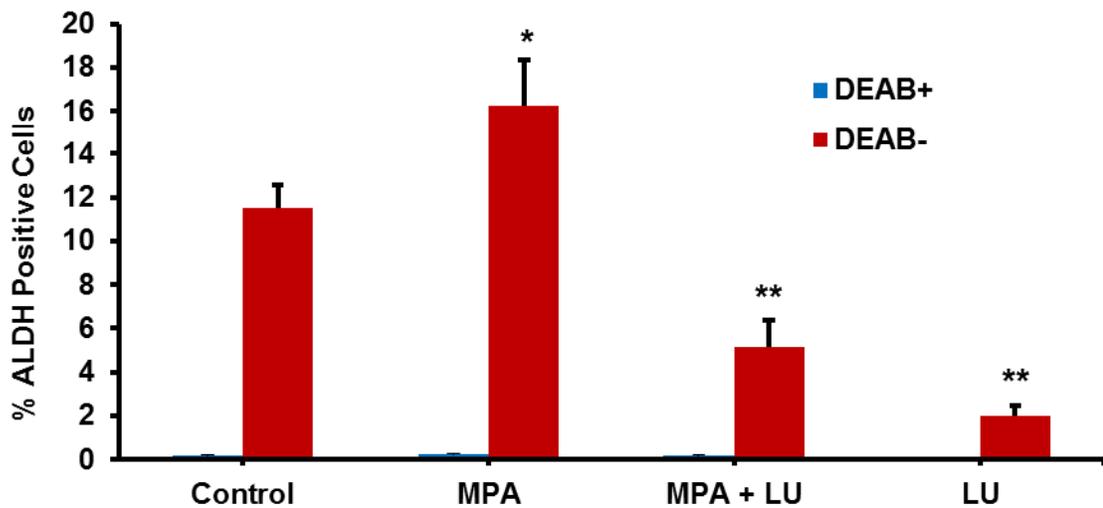
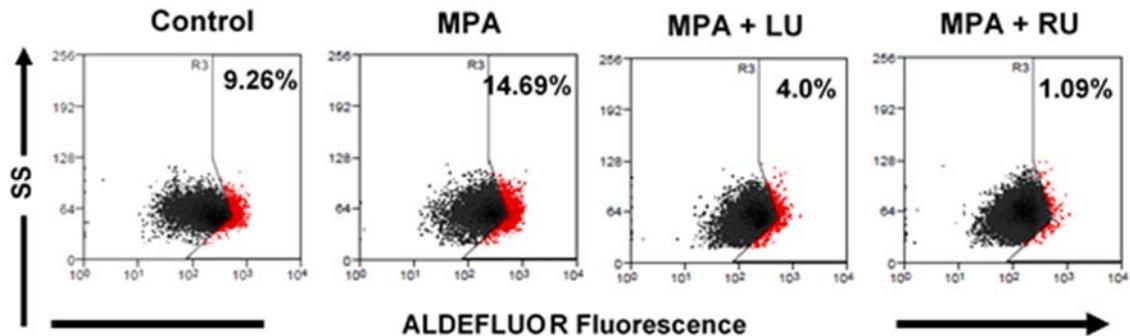


Figure 3.7B. Luteolin inhibits MPA-induced ALDH induction. T47-D cells were grown overnight in DMEM/F12 with 5% dextran-coated charcoal (DCC)-stripped FBS. Media was replaced and cells treated with MPA (10 nM) \pm 25 μ M LU. After 24 h incubation cells were harvested with Accutase (StemPro) and ALDH activity measured by FACS analysis, as described by the manufacturer (Stemcell Technologies ALDEFLUOR assay). The FACS plot shown in the top panel represents ALDH bright cells (red) shifting right with MPA compared with controls, an effect negated by LU. The ALDH bright gate was set using negative controls DEAB+. Viable cells were gated using PI; once the gates were set they were carried throughout the analysis. Bottom panel represents FACS analysis (Summit 5.2) summation of 4 different experiments, mean \pm SEM (n=4). Compared with controls, the ALDH signal was effectively reduced in response to 25 μ M LU, both in the presence and absence of MPA. *Statistically significant increase in ALDH positive cells compared with controls. **Statistically significant ALDH loss compared to controls and MPA-induced gain (ANOVA followed by SNK multiple comparisons procedure, $p < 0.05$).

MPA induced a significant increase in ALDH bright activity, an effect that was dramatically reduced by LU (ANOVA $P < 0.05$). LU treatment alone significantly reduced even basal ALDH1a activity (**Figure 3.7B**), suggesting that the flavonoid not only diminishes high ALDH activity induced by MPA, but also has an inherent ability to reduce the stem-cell like properties of breast cancer cells.

Mammosphere formation: Based on our previous findings we conducted studies to determine whether LU inhibited the formation of mammospheres. It is recognized that only stem cells are capable of seeding mammospheres in this anchorage independent 3D environment [114, 252, 253]. MPA alone caused a small but significant increase in the number of mammospheres formed by T47-D cells, an effect that was blunted when cells were simultaneously exposed to LU (**Figure 3.7C**; ANOVA $P < 0.05$). It is important to note that LU alone did not increase the number of mammospheres. Also notable was the observation that MPA not only increased the number of mammospheres, but also amplified their size. This suggests that the progestin has a proliferative effect, a notion supported by evidence that MPA increases the progenitor cell population [52, 103]. LU treated mammospheres appeared more dispersed than those in either the MPA alone or control groups (**Figure 3.7C**).

3.5 Discussion

A number of recent clinical trials and studies show that using an HRT regimen consisting of a combination of progestin and estrogen to alleviate the symptoms of menopause leads to a significant increase in the risk of breast cancer [9-12, 56, 59, 229].

Investigators have attempted to understand the role of progestins in this process.

Studies from our laboratory showed that induction of the potentially angiogenic VEGF in both T47-D and BT-474 cells, is one possible mechanism which might explain elevated cases of breast cancer arising in response to combination HRT [16, 17, 19]. Other mechanisms such as progestin-dependent increases in tumor cell proliferation, and

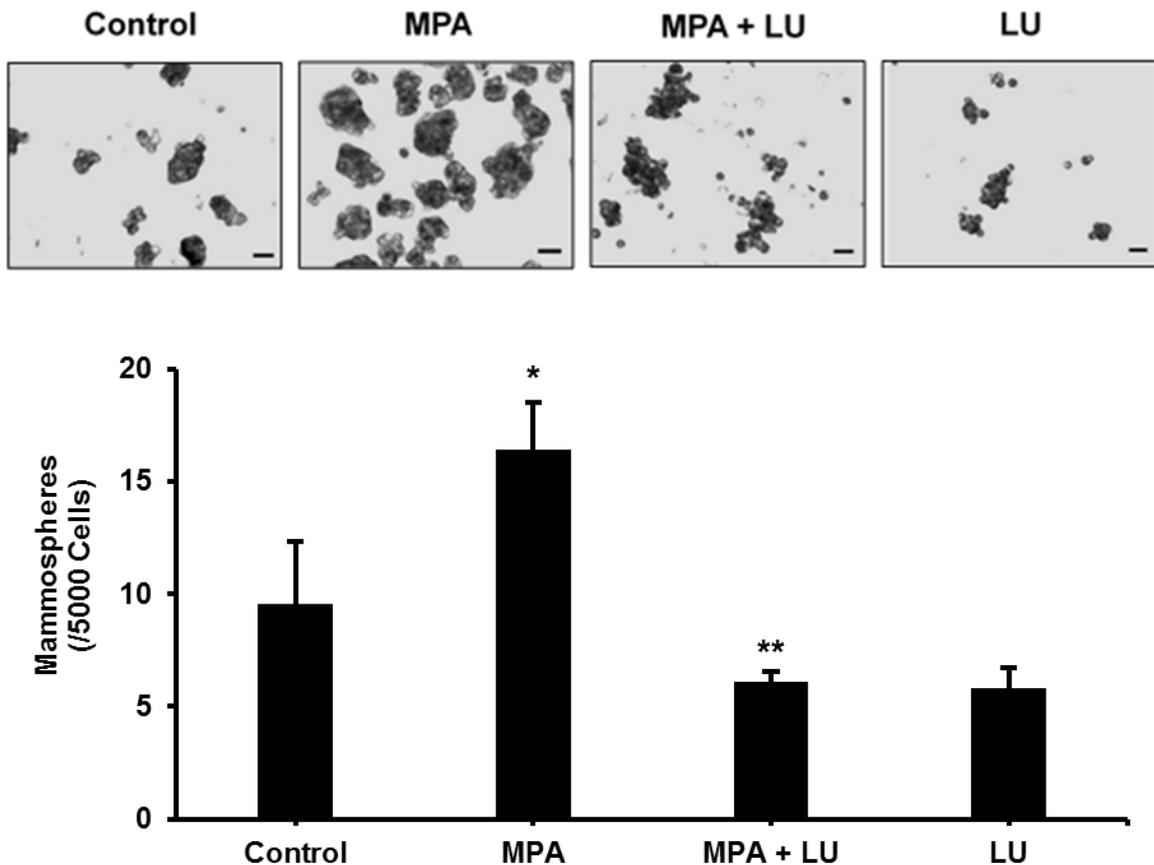


Figure 3.7C. Luteolin suppresses self-renewal and proliferation of T47-D breast cancer stem like cells. T47-D cells were grown in 10% FBS DMEM/F12 medium and then incubated for a further 24 h in 5% FBS-DCC DMEM/F12 medium. Cells were then treated for 48 h with vehicle, 10 nM MPA, 10 nM MPA plus 25 μ M LU or 25 μ M LU alone. After 48 h treatment, 5000 cells from each group were seeded into six-well plates and treated for 7 days in Complete MammoCult medium (StemCell Technologies Inc., Vancouver, Canada). Cells were re-treated with 1 ml culture medium on days 2, 4, and 6. Top: representative light microscopic images (10X magnification) of T47-D mammospheres formed in Complete MammoCult medium after 7 days; scale represents 60 μ m. Bottom: bars represent mean number of mammospheres per group (n=3). Number of mammospheres \geq 60 μ m was quantitated from 6-9 images per well, 3 wells per group. *Significant differences compared with control group (P< 0.05); **Significant differences compared with MPA group (P< 0.05). ANOVA on Ranks followed by Dunn's Method of multiple comparisons.

generation, within tumor cells, of stem-cell like properties, have also been suggested [19, 103, 112, 117, 118]. Using induction of VEGF as a biomarker by which to determine the role of both natural and synthetic compounds in tumor cell development, we examined the therapeutic ability of the naturally-occurring flavonoid LU to suppress the growth and progression of progestin-dependent human breast cancer cells in vitro and in vivo.

Low concentrations of LU, up to about 10 μM , had no effect on cell viability of either T47-D or BT-474 cells, a finding in accordance with those of other investigators [198, 234, 235]. However, at doses exceeding 10 μM LU caused significant loss of cell viability following treatment of cells for 24 - 48 h (IC₅₀ values of approximately 50 μM at 24 h). Loss of cell viability occurred irrespective of whether or not MPA was present (data not shown). Although treatment of cells for 16h with 50 μM LU caused an increase in tumor cell apoptosis, it was interesting to note that a dose of 25 μM LU failed to induce apoptosis during the time frame tested, even though there was a loss of cell viability at this concentration (**Figure 3.1A** and **Figure 3.4**). It is therefore likely that lower concentrations of LU cause cell cycle arrest, a phenomenon previously reported in prostate cancer cells [254], while induction of apoptosis occurs at higher concentrations. Progestin-induced VEGF production was blocked in both T47-D and BT-474 cells by LU in a similar fashion to inhibition of VEGF by apigenin, another naturally-occurring flavonoid tested in our laboratory [249]. It appears however that LU might be more effective pharmacologically against tumors than apigenin [235]. Since 10 μM LU was ineffective in killing tumor cells during the 16-20 h treatment period, we selected this concentration to monitor whether VEGF suppression represented an early event that preceded LU-induced apoptosis. 10 μM LU suppressed VEGF production in a manner equal to 1 μM RU-486, an anti-progestin that is commonly used to demonstrate PR-

mediated effects, including suppression of progestin-induced VEGF [27, 87]. LU blocked production of VEGF by both natural and several synthetic progestins including MPA, Norgestrel and Norethindrone, all of which are common components of HRT in the USA and Europe. Furthermore, LU suppressed increases in P-induced VEGF mRNA following 6h of tumor cell treatment. Interestingly, LU had no effect on PR at either the mRNA or protein level (data not shown), suggesting that the flavonoid may interfere with the PRE on the VEGF promoter [17, 108], or act at a downstream step by modifying co-activators needed for PR-dependent gene transcription [67-69]. The novel ability of LU to curb P-induced VEGF production in human breast cancer cells could also be due to suppression of the phosphoinositide-3'-kinase (PI3-kinase) pathway [215], or inhibition of the SP-1 transcription factor [109], both of which are known to control P-induced VEGF induction in human breast cancer cells [250]. These possibilities remain to be tested. In order to further examine the efficacy of LU as a potential chemotherapeutic agent against progestin-driven breast cancer we studied its effects in a T47-D human breast cancer xenograft model, previously developed by Liang et al [104]. In this model progestins have been shown to increase VEGF production, which in turn exerts paracrine and autocrine mitogenic effects on the cancer cells themselves, as well as the vasculature [19]. Using the T47-D xenograft model we found that LU immediately suppresses tumor growth and, in fact, causes tumor regression, observations which we propose are likely due to inhibition by LU of MPA-induced VEGF production and subsequent angiogenesis. LU did not prevent PR loss in vivo, a finding in accord with results obtained in vitro which is indicative of PR activation [245, 251]. However, it has recently been shown that progestins may cause dedifferentiation of cancer cells into cancer stem-like or progenitor cells [103, 117], providing another possible mechanism by which LU diminishes tumor growth in the T47-D xenograft model. In other words, by decreasing the population of cancer stem-cell like or progenitor cells, LU may effectively

reduce the tumor burden while maintaining a similar proliferative index compared with non-treated tumors.

A number of established assays were used to determine whether LU possesses properties that would prevent tumor cells from acquiring stem-cell-like properties.

Acquisition of stem cell properties by tumor cells is associated with a CD44^{high}/CD24^{low} phenotype [53]. As shown previously, MPA caused an increase in CD44 levels in T47-D cells, though cells still maintained their levels of CD24 and did not acquire the CD24^{low} phenotype [15]. Nevertheless, cells that have increased expression of CD44 have been shown to be more aggressive in terms of growth and motility [53, 103, 128], and could be responsible for increased tumor growth seen in our established model [104, 124]. Both RU-486 and 25 μ M LU prevented MPA-induced increases in CD44 after 16 h. It was interesting to note that a low-dose of LU appeared to increase CD44 production slightly compared with controls. Previous reports suggest that LU has low ER activity [237, 242], which may explain this observation. It has been reported that CD44^{hi}/CD24^{low} cells show the most cancer-stem cell-like characteristics [53]. However, this report was based on data from patient-derived metastatic lesions which, unlike the homogeneous T47-D human breast cancer cells used in this study, are phenotypically heterogeneous in nature. The homogeneity of our T47-D cells likely explains why we don't see a distinct CD24^{low} subpopulation. We also carried out additional studies to determine whether MPA influences two other determinates of stem-cell properties, mammosphere formation and ALDH⁺⁺ enzyme activity [55, 255], and furthermore, ascertained whether or not LU affected them. MPA caused a significant increase in mammosphere formation and size, an effect likely due to an expansion of progenitor cells. Likewise, MPA brought about a small but significant increase in ALDH activity. LU inhibited the formation of mammospheres in response to MPA and also reduced their size. In addition ALDH activity was abolished by the flavonoid. Interestingly, LU alone

significantly reduced basal ALDH activity. Taken together these data suggest that LU reduces the number of cancer stem cell-like cells and/or progenitor cells in progestin-responsive breast cancer and may thereby be able to reverse tumor growth [19, 103, 117].

In summary, our studies provide evidence that LU inhibits P-dependent induction of VEGF mRNA and protein and reduces the amount of VEGF secreted by PR-expressing T47-D and BT-474 breast cancer cells. The latter cells express high levels of Her-2-neu, which correlates with aggressive types of cancer and has also been shown to metastasize in response to progestins [124]. Consequently, LU has the potential to disrupt angiogenesis and thereby prevent the development of progestin-driven tumors, a concept that was further confirmed in our in vivo studies in which LU potently inhibited MPA-driven VEGF production, leading to tumor regression. While VEGF is a potent mitogenic factor that is important to the establishment of tumors and vital to their growth and metastasis, its loss may not completely explain the regression in tumor burden, therefore blood vessel density was determined. CD31, a blood vessel marker, analysis provided evidence to suggest that LU mitigates tumor growth through suppression of P-driven vascularization; i.e. LU established an inauspicious tumor microenvironment. Finally, we provide evidence to show that in addition to being anti-angiogenic, LU also reduced the MPA-driven cancer stem-cell-like and/or progenitor cell subpopulation, strongly suggesting that it exerts its anti-tumor effects in a variety of ways. The MPA-induced production of CD44, a cell surface glycoprotein that is associated with extracellular VEGF [256, 257], was suppressed by LU. ALDH activity, which is a recognized characteristic of stem cells, was also reduced by the flavonoid, as was the mammosphere forming capacity of progestin-treated cells. Considering that both VEGF and CD44 are controlled by PR-mediated events, and that both LU and the antiprogestin RU-486 effectively block MPA-induced effects, it is highly likely that an association exists

between these two proteins. Based on our overall novel findings, we contend that LU demonstrates significant potential as a new and novel agent which might be used in a multifaceted manner to combat particularly aggressive and hard to treat types of breast cancer. If administered in conjunction with currently used HRT regimens LU could protect against the formation of tumors that would otherwise arise as a result of MPA consumption. It is therefore essential that we investigate more thoroughly the mechanisms by which LU moderates the effects of progestins in order to fully exploit its therapeutic potential.

CHAPTER 4

LUTEOLIN INHIBITS LUNG METASTASIS OF HUMAN TRIPLE-NEGATIVE BREAST CANCER CELLS

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4.1 Abstract

Metastasis accounts for more than 90% of all cancer-related deaths (Weigelt 2005). Triple-negative breast cancers, most of which are basal-like, are extremely aggressive and have a high-rate of recurrence (Perou 2000). Women with triple-negative breast cancers who present with early metastatic lesions have limited treatment options and therefore have a poor prognosis. Most treatment protocols involve high-dose chemotherapeutic strategies. With this in mind we examined the ability of luteolin (LU), a naturally occurring, non-toxic plant compound that has previously been shown to inhibit the growth of several types of tumor cells, to suppress the growth, mobility and metastasis of triple negative cell lines and to disrupt their ability to form metastatic colonies in the lung. The triple-negative basal-like breast cancer cell line MDA-MB-231 (4175) LM2 was chosen since it represents an aggressive subpopulation of clinically relevant cells with a molecular signature for preferential metastasis to the lungs (Minn 2005). Herein we report that LU displayed a novel ability to suppress metastasis of the aforementioned cells to lung. Animals injected with MDA-MB-231 (4175) LM2 cells alone developed 67.6 ± 27.1 colonies, while in those receiving both MDA-MB-231 (4175) LM2 cells and 40 mg/kg LU, only 22.8 ± 3.6 colonies developed ($P = 0.035$). The effect of LU was confirmed using another triple negative cell line, MDA-MB-435, which also metastasize to the lungs but less aggressively than the aforementioned variant. The corresponding values were 14.1 ± 1.6 colonies vs 5.3 ± 0.5 colonies in the presence of 20 mg/kg LU; $P < 0.05$). LU (0-50 μM) significantly reduced cell viability, dose-dependently increased apoptosis ($P = <0.001$), and reduced tumor cell migration suggesting inhibition by LU of several steps involved in the metastatic process. Furthermore, at relatively low levels (10 μM), LU significantly inhibited VEGF secretion from tumor cells ($P = <0.001$), suggesting that the flavonoid has additional anti-metastatic properties, suppressing a potent angiogenic and cell survival factor. Antibody

suppression of the VEGF receptor, KDR, produced a similar decrease in migration potential in the presence or absence of LU, suggesting that LU may partially act through the KDR receptor to inhibit in tumor cell migration, survival and angiogenesis. These studies demonstrate that LU has the ability to disrupt metastatic breast cancer through a variety of mechanisms. Its use as a potential therapeutic option in women suffering from breast cancer warrants further investigation.

4.2 Introduction

Triple-negative breast cancers comprise 15-20% of all tumors of the breast and are so named because they fail to express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2/neu) [46, 258]. Compared with hormone-responsive breast cancers, i.e. tumors that are positive for ER, PR, and/or HER2, the triple-negative phenotype is associated with a significantly poorer clinical outcome [46-48]. Hormone receptor positive tumors can be treated therapeutically, using antagonists to disrupt normal hormone/receptor binding. Unfortunately however, those suffering from triple-negative breast cancer have limited treatment options and such patients must rely on high-doses of highly toxic chemotherapeutic drugs. Triple-negative tumors represent a cancer phenotype that is very hard to treat, and cancers of this type that escape adjuvant treatment and surgery generally emerge as particularly aggressive tumors. That being said, recent reports suggest that membrane-bound PR may play a role in triple-negative breast cancer stem cell-like maintenance, providing a possible chemotherapeutic target [259].

Perou et al. reported that women with basal-like (specifically, triple-negative) breast cancers have an increased risk of tumor recurrence compared to women with other cancer subtypes [43]. Triple-negative breast cancers frequently metastasize to the viscera, i.e. lung, brain, liver and to a lesser extent bone [260-263]. Not surprisingly the rate of cancer recurrence 3-5 years post-therapy is greater in women suffering from triple-negative breast cancers than in their non-triple negative counterparts [262, 263]. The increased virulence of basal-like breast cancers, which represent ~80% of all triple-negative tumors [264] may be attributed to increased VEGF secretion. It is well-established that increased VEGF promotes tumor growth and metastasis [23, 24, 131, 265], resulting in higher morbidity [266-269]. VEGF-stimulated migratory and proliferative pathways provide capable adjunctive chemotherapeutic options. Previous

reports have established that vascular endothelial growth factor (VEGF), specifically VEGF₁₆₅, increases migration and invasion potential in T47-D human breast cancer cells [136, 270]. More recently our lab has shown that VEGF induces mitogen effects in MDA-MB-231 breast cancer cells mediated through the interaction of VEGFA and KDR [19]. These reports suggest that indirectly blocking VEGF-induced migration and proliferation may decrease the metastatic potential of Stage IV breast cancer. Ongoing studies in our laboratory are aimed at identifying naturally-occurring compounds with low toxicity that can be used chemotherapeutically to treat breast cancer. Angiogenesis is fundamentally important for tumor development, hence our focus centers on agents with anti-angiogenic properties which will arrest and possibly reverse tumor progression and metastasis. We previously reported on the ability of luteolin (LU), a non-toxic, natural plant flavonoid with the full chemical name 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone, to block progestin-driven VEGF expression and suppress in vivo tumor progression in two different progestin-associated animal models [247]. There are currently no clinically accepted standardized chemotherapeutic options to combat metastatic triple-negative cancers, and current treatment regimens increase survivability by a modest 13 months [271]. With this in mind we tested LU for its ability to disrupt the growth of triple-negative metastatic breast cancer cells, both in vivo and in vitro, and to prevent metastasis. Our findings are encouraging, showing that LU effectively suppresses the viability of triple-negative breast cancer cells and blocks tumor metastasis to the lungs.

4.3 Materials and Methods

4.3.1 Reagents

Luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one) was purchased from Tocris (Cat # 2874; Minneapolis, MN) and dissolved in sterile filtered dimethyl sulfoxide (DMSO; Sigma Aldrich; Cat # D2650; St. Louis, MO). Solutions of

luteolin (LU) were prepared weekly, aliquoted for daily use, and stored at -20°C until use. Pierce bicinchoninic acid (BCA) protein reagents were obtained from Fisher Scientific (Pittsburg, PA). hVEGF₁₆₅ (AF293NA) and hKDR (AF357) antibodies were purchased from R&D Systems (Minneapolis, MN).

4.3.2 Cell Lines and Culture

Triple negative human breast cancer MDA-MB-435 cells were maintained at 37°C in phenol red-free DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) in a humidified atmosphere of 5% CO₂ [272]. The MDA-MB-231 (4175) LM2 breast cancer cell line is a metastatic subpopulation isolated from MDA-MB-231 cells [260]. MDA-MB-231 (4175) LM2 cells were maintained in DMEM supplemented with 10% FBS at 37°C. 0.05% trypsin-EDTA (Invitrogen) was used for cell harvesting. For all in vitro experiments cells were allowed to reach 50-60% confluence, after which they were washed in phosphate-buffered saline (PBS) and maintained in DMEM/F12 or DMEM supplemented with 5% FBS for 24 hours prior to treatment. Incubations were carried out in the presence of LU at the various concentrations described below. Control cells were treated with DMSO.

4.3.3 Cell Viability Assay

Viable cells were quantitated using the previously described sulforhodamine B (SRB) assay [248], a cell protein dye-binding assay. In brief, 1.0-1.5 x 10⁴ cells in 100 µl 5% DMEM/F12 or DMEM medium were seeded on a 96-well plate and incubated at 37°C overnight in 5% CO₂. Cells were subsequently treated for 48 h with the described concentrations of LU. Adherent cells were fixed in situ for 1 h at 4°C using 50% cold trichloroacetic acid (TCA), after which they were washed with ice-cold water and allowed to dry. Fixed cells were stained at room temperature in 50 µl of 4% SRB for 8 min. Excess dye was removed by washing with cold 1% acetic acid and allowed to dry. Protein-bound stain was solubilized in 10 mM Tris and absorbance measured at 520 nm

using a SpecTRA MAX 190 microplate reader (Sunnyvale, CA). Treatments were performed in sextuplet.

4.3.4 Migration Assay

As metastasis proceeds, migration is essential at multiple steps of the process. Viable MDA-MB-435 or MDA-MB-231 (4175) LM2 cells were counted and plated (4×10^4 cells/well) on an Oris Pro Cell Migration Assay 96-well tissue culture treated plate (Platypus Technologies, Cat # PROCMA1) and 3 h later treated with LU and/or hVEGF₁₆₅ and hKDR antibodies for 48 h. Images were taken at 0, 24, and 48h intervals on an EVOS XL Cell Imaging System utilizing a 4x objective. Migration was analyzed by determining the difference in area of the detection zone between pre-migration (0 h) and subsequent time points, using the lasso and measurement tools in Adobe Photoshop CS 5.5 (San Jose, CA) on an inductive pen-enabled Wacom touchscreen. Inter-well coefficients of variability given by the manufacturer for the detection zone created by the biocompatible gel were $\leq 12\%$.

4.3.5 Apoptosis Assay

LU-induced apoptosis of breast cancer cells was measured by fluorescence activated cell sorting (FACS) analysis using the Annexin V-FITC and PI binding assay (BioVision, Cat # K101-100; San Francisco, CA). MDA-MB-231 (4175) LM2 cells were grown to 50-60% confluence, washed twice, and treated with varying concentrations of LU in 5% FBS/DMEM for an additional 48 h. Cells were harvested using 0.5% trypsin and stained for FACS analysis per manufacturer's protocol.

4.3.6 VEGF ELISA

Quantikine human VEGF ELISA kit (Cat # DVE00) was purchased from R&D Systems, Inc. (Minneapolis, MN). MDA-MB-435 and MDA-MB-231 (4175) LM2 hormone-independent breast cancer cells were treated with vehicle (DMSO) and LU at the indicated doses and incubated for 16 hours at 37°C. Supernatant from each group was

collected and VEGF concentrations measured by ELISA according to manufacturer's protocol. Experiments were performed in quadruplicate, and each sample was analyzed in duplicate on a microplate reader. Inter- and intra-assay coefficients of variance given by the manufacturer for cell culture supernatant assay were 5% to 8.5% and 3.5% to 6.5%, respectively.

4.3.7 Bicinchoninic Acid Protein Assay

MDA-MB-231 (4175) LM2 cells were washed 2x then harvested, pelleted, and re-suspended in 300 μ L lysis buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, and 1% Nonidet P-40). Protein concentration was determined using a microplate reader to measure absorbance at 562 nm with a bovine serum albumin standard (Thermo Fisher Scientific, Cat # 23209; Waltham, MA). Experiments were performed in triplicate, and samples were analyzed in duplicate.

4.3.8 RNA Preparation and Primers Used for RT-PCR

MDA-MB-231 (4175) LM2 cells were grown to 60% confluence and treated with 10, 25, and 50 μ M LU for 6 h, after which total mRNA was collected using the phenol based extraction method (RNAzol RT: Molecular Research Center; Cincinnati, OH), per manufacturer's recommendations. Total mRNA was converted to cDNA and amplified

VEGF

F 5' - CTG CTG TCT TGG GTG CAT TGG
R 5' - CAC CGC CTC GGC TTG TCA CAT

GAPDH

F 5' - ATG AGA AGT ATG ACA GCC
R 5' - TGA GTC CTT CCA CGA TAC C

using the Invitrogen SuperScript III One-Step RT-PCR System (Cat # 12574-026) in combination with the following primers:

RNA was run on 1.5% agarose gel for 1.5 h at 100v and quantified using a Bio-Rad Laboratories imager and software.

4.3.9 Breast Cancer Lung Metastasis Xenograft Model.

Female athymic nu/nu nude mice, 5 to 6 weeks old (18–22 g), were purchased from Harlan Sprague-Dawley, Inc. Mice were housed in a laminar air-flow cabinet under specific pathogen-free conditions. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) in accordance with current federal regulations and standards. Cells were harvested by trypsinization and washed twice with DMEM/F12 or DMEM medium. Cell pellets were then suspended in medium and injected intravenously (i.v.) into the tail vein. Numbers of MDA-MB-435 and MDA-MB-231 (4175) LM2 cell lines injected were 2.2×10^6 and 2.0×10^5 cells per 150 μL , respectively (**Figure 4.1**). Intraperitoneal (i.p.) injections of LU began on the fifth day post-inoculation and occurred every other day until the end of the study, though the MDA-MB-231 (4175) LM2 xenograft model received a daily loading dose of LU for the first week. Mice inoculated with MDA-MB-435 cells were given i.p. injections of 10 or 20 mg/kg LU; those inoculated with MDA-MB-231 (4175) LM2 cells received a higher LU dose of 40 mg/kg due to the more aggressive nature of the MDA-MB-231 (4175) LM2 breast cancer cell line. Animal weights were monitored throughout the study for signs of drug intolerance and lungs were harvested periodically to monitor tumor progression. The final collections occurred on Days 51 and 42 for mice inoculated with MDA-MB-435 and MDA-MB-231 (4175) LM2 cells, respectively. Lungs were collected and fixed in Bouin's Fixative Solution (Ricca Chemical Company, Cat # 1120-32), per manufacturer's recommendations. Superficial lung lesions were counted using a stereoscopic dissection microscope and results from two independent researchers were averaged, and statistically analyzed using Sigma Plot.

4.3.10 Statistical Analysis

Statistical significance was tested using one-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparison test to determine the difference in mean between groups. If normality failed, the data was tested using a nonparametric one-way

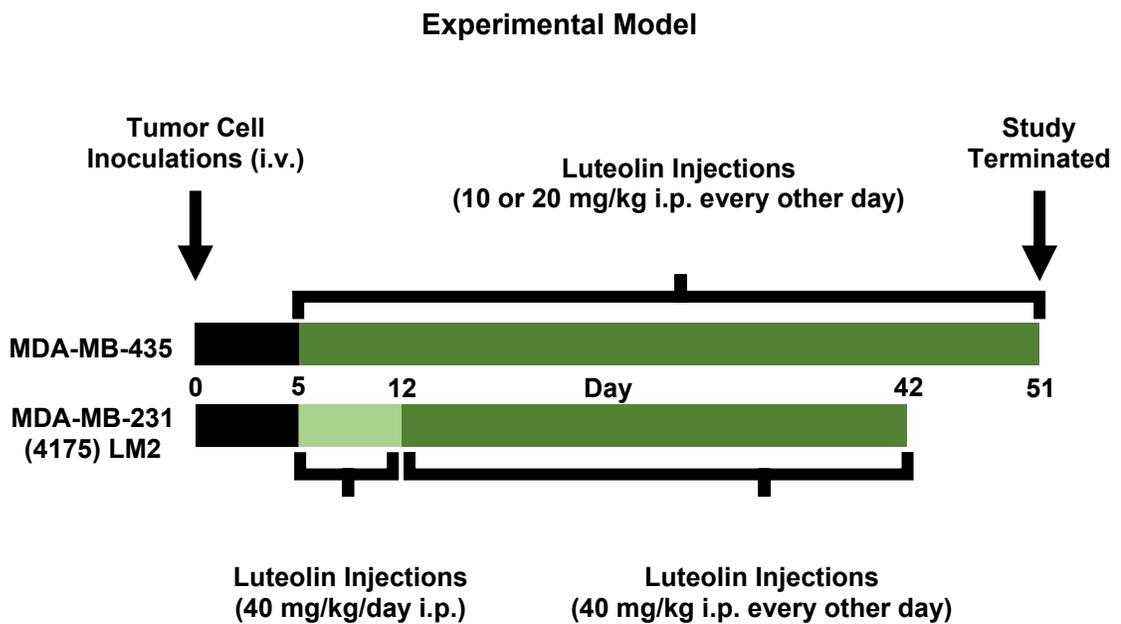


Figure 4.1. Luteolin treatment protocol and experimental design. Female athymic nu/nu mice were inoculated intravenously (i.v.) via the tail vein with triple-negative cells on Day 0, as described in Material and Methods. Luteolin (LU; 10, 20, or 40 mg/kg) or vehicle (DMSO) was intraperitoneal (i.p.) injected every 48 hours until study termination (Day 42 or 51), with the exception of MDA-MB-231 (4175) LM2 receiving a 1 week everyday loading dose. Animals were sacrificed and lungs were excised for analysis.

ANOVA on ranks (Kruskal-Wallis), followed by the Newman-Keuls nonparametric multiple comparisons test or Dunn's Method in the presence of uneven n. Student t-test was used to report the difference between two group means; when normality failed the Mann Whitney Rank Sum Test was used. Animal weight comparisons were analyzed using a Two-way Repeated Measure (RM) ANOVA. Data were reported as means \pm SEM. For all comparisons, $P \leq 0.05$ was regarded as statistically significant and analysis was accomplished using SigmaPlot 12.5 software.

4.4 Results

4.4.1 Luteolin Inhibits Lung Metastasis in Mouse Models

In order to determine the effectiveness of LU as an anti-metastatic compound that might be used to combat breast cancer we utilized a xenograft model that mimics secondary site colonization. In this model MDA-MB-435 and MDA-MB-231 (4175) LM2 tumor cells (2.2×10^6 and 2.0×10^5 respectively) were introduced through the tail vein and allowed to circulate to distant organs. Metastatic breast cancer cells were given five days to circulate to the luminal side of capillaries, after which intraperitoneal (i.p.) injections of luteolin were given according to the protocol (**Figure 4.1**). At the end of the experiment lungs with metastatic colonies were excised and fixed in Bouin's Fixative. In the MDA-MB-435 xenograft model, an average of 13 superficial lung colonies formed in the control group. Colonies were markedly reduced by LU in a dose-dependent manner (**Figure 4.2A**). Injections of 20 mg/kg LU significantly suppressed MDA-MB-435 lung colony formation (5.3 ± 0.5) compared with controls (14.1 ± 1.6) (ANOVA on Ranks, $P < 0.05$). The lower dose of 10 mg/kg LU also reduced the number of metastatic colonies (8.4 ± 0.9), though this did not reach significance. It is important to note that there was no significant difference in animal weights between sham controls and animals given LU (**Figure 4.2B**).

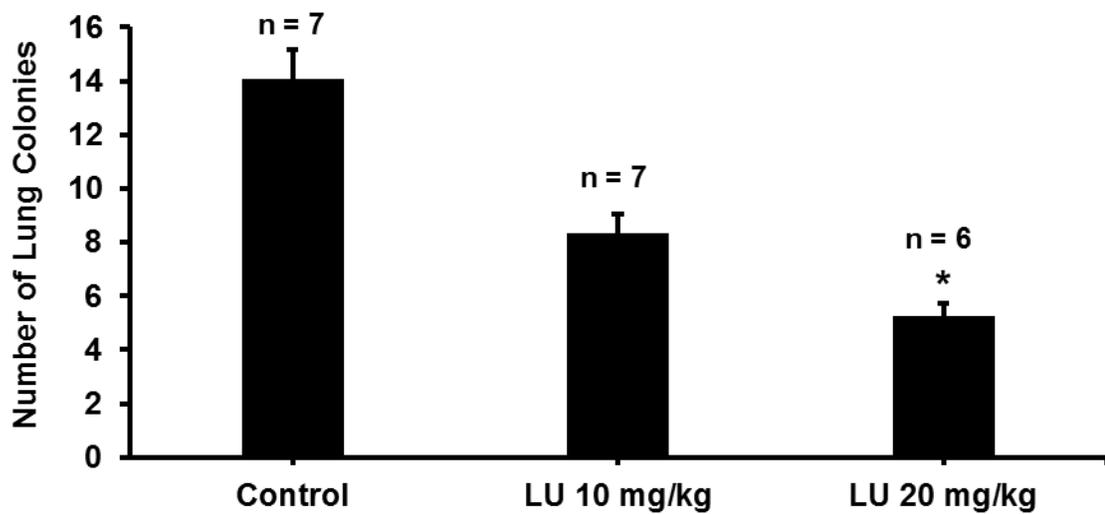


Figure 4.2A. Luteolin suppresses lung metastasis in nude mice. MDA-MB-435 triple-negative breast cancer cells (2.2×10^6) were inoculated via tail vein and treatment with LU (10 or 20 mg/kg) or placebo began 5 days post inoculations. LU was injected i.p. every other day until termination of study. Bar graph represents mean number of lung colonies \pm SEM, n = number of animals. *Significantly different compared to control group ($P < 0.05$, ANOVA on Ranks followed by Dunn's Method).

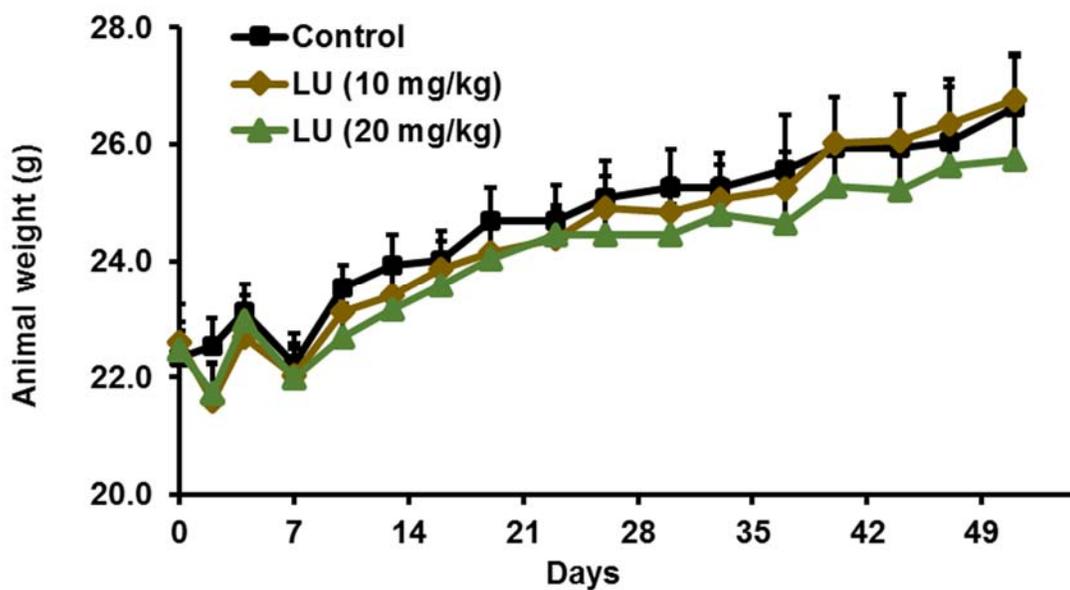


Figure 4.2B. Luteolin treatment did not affect animal weight. LU (10 or 20 mg/kg) or placebo was injected i.p. every other day starting at Day 5. Animal weights were measured every 2-3 days throughout the experiment. There was no significant change between animal weights throughout the study. 2-way Repeated Measures ANOVA.

Having shown that LU reduced metastasis in the MDA-MB-435 model we sought to determine whether this inhibitory effect was cell line specific. Using an alternative triple-negative breast cancer MDA-MB-231 variant (LM2-4175), that grows aggressively and has a molecular signature specific to lung metastasis [260] we injected cells via tail vein to establish metastatic lung colonies. Due to the highly aggressive nature of the MDA-MB-231 (4175) LM2 cell line we opted for a ten-fold reduction in the injection fraction (based on Minn 2005) and increased LU to levels approaching the maximum reported in the literature [213]. Injections of LU began with a loading dose of 40 mg/kg/day for 7 days followed by 40 mg/kg every other day until termination of the study on Day 42. Injection of MDA-MB-231 (4175) LM2 cells increased the average number of lung colonies 5-fold compared with MDA-MB-435 cells (67.6 ± 27.1 colonies vs 13.8 ± 1.6 colonies respectively), a finding that was highly significant (Mann-Whitney Rank Sum Test, $P = <0.001$). Administration of LU reduced significantly the number of lung colonies formed by the highly tumorigenic MDA-MB-231 (4175) LM2 cells (22.8 ± 3.6 colonies) (**Figure 4.2C**; Mann-Whitney Rank Sum Test, $P = 0.035$). Mice in the MDA-MB-231 (4175) LM2 group tolerated LU just as well as those given MDA-MB-435 cells; animal weights (**Figure 4.2D**) and behavioral patterns were not significantly different from controls.

4.4.2 Luteolin Inhibits Tumor Cell Migration.

Migration is an essential characteristic of metastasis and inhibiting the migration of tumor cells reduces their metastatic potential. Using MDA-MB-435 and MDA-MB-231 (4175) LM2 cells we examined the effects of LU on cell migration. Cells were grown to 60% confluence, trypsinized, harvested and used to seed a 96-well plate containing a bead of biocompatible gel (4×10^4 cells/well). Within an hour the gel dissolves, leaving a circular zone of detection, and 2 h later, once cells had properly adhered to the plates they were treated with LU.

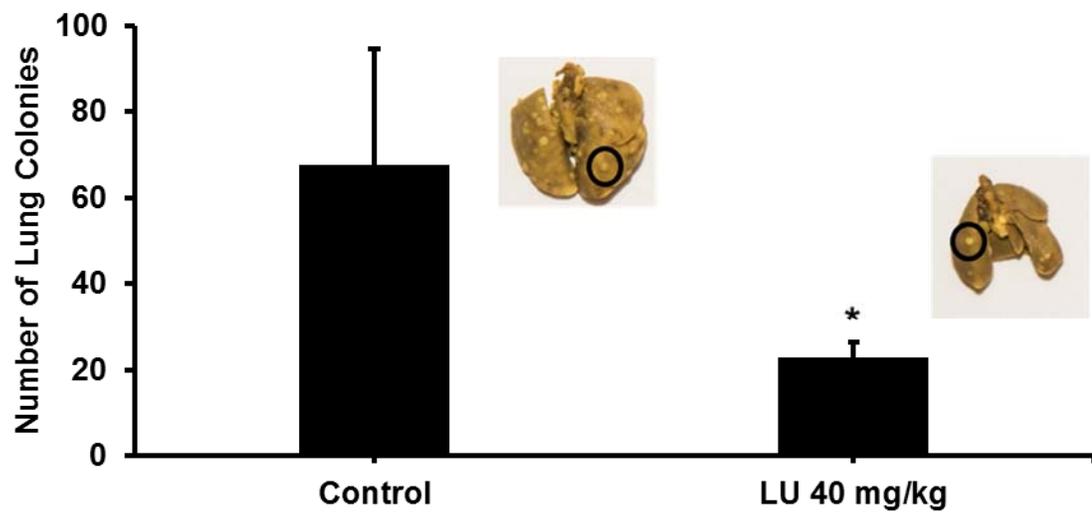


Figure 4.2C. Luteolin inhibits MDA-MB-231 (4175) LM2 lung colony formation in nude mice. MDA-MB-231 (4175) LM2 triple-negative breast cancer cells (2.0×10^5) were inoculated on Day 0, and subsequently treated with LU (40 mg/kg), as previously described. Inserts are representative pictures from each group, colonies appear as off-white specs on the lungs (circled). Bar graph represents mean number of lung colonies \pm SEM (placebo, n=7 mice; LU 40 mg/kg, n=6 mice). *Significantly different compared to control group (P = 0.035, Mann-Whitney Rank Sum Test).

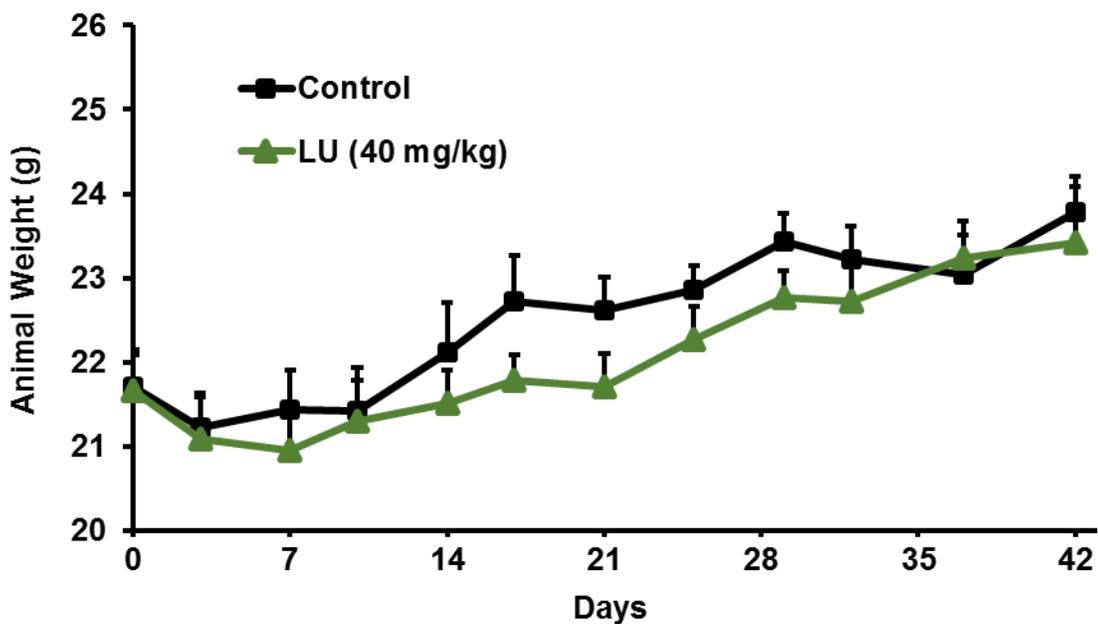


Figure 4.2D. Luteolin has little to no effect on animal weight. LU (40 mg/kg) or placebo was injected i.p. every day starting at Day 5 for 1 week, followed by every other day injections until study termination. Animal weights were measured every 3-4 days throughout the experiment. There was no significant change between the placebo and luteolin animal weight curves. 2-way Repeated Measures ANOVA.

MDA-MB-435 breast cancer cells were treated with LU for 24 h and 48 h. Even with very low doses of LU, a marked reduction in migration capacity was observed after both periods of treatment, as shown in the representative panel in **Figure 4.3A**. After 24 h 5 μ M LU significantly suppressed migration of MDA-MB-435 breast cancer cells compared with controls (40.5 ± 4.7 vs 13.2 ± 6.8 % closure; $P = 0.003$). Migration was further suppressed in a dose- and time-dependent manner at higher concentrations (**Figure 4.3A** graph, $P = <0.001$). After 48 h migration was also significantly reduced by LU, starting at a LU concentration of 10 μ M (54.2 ± 5.8 vs 15.9 ± 5.5 % closure; $P = <0.001$). A concentration of 50 μ M LU completely abolished the migratory potential of MDA-MB-435 breast cancer cells after 24 h and 48 h (**Figure 4.3A**).

MDA-MB-231 (4175) LM2 cells (4×10^4 cells/well) were similarly plated and treated with concentrations of LU ranging from 1 to 50 μ M. The difference in migration potential between MDA-MB-435 and MDA-MB-231 (4175) LM2 cells was surprisingly large; 40.5 ± 4.7 vs 73.4 ± 1.7 % closure after 24 h and 54.2 ± 5.8 vs 99.8 ± 0.1 % closure after 48 h, respectively (data not shown; Mann-Whitney $P = <0.001$). Nevertheless, LU significantly reduced the migration capacity of MDA-MB-231 (4175) LM2 cells after 24 h of exposure. A concentration of 5 μ M LU was sufficient to reduce migration significantly ($P = <0.001$) and inhibition of migration potential increased in a dose- and time-dependent manner (**Figure 4.3B**, $P = \leq 0.001$). Exposure of MDA-MB-231 (4175) LM2 cells to LU for 48 h had a similar effect as that seen in MDA-MB-435 cells; in both cases cells overcame the effects of 5 μ M LU to inhibit migration. However, with increasing concentrations of LU, from 10 μ M to 50 μ M, a dose-dependent reduction in MDA-MB-231 (4175) LM2 cell migration occurred (**Figure 4.3B**) (ANOVA on Ranks, $P < 0.05$). Interestingly, there was no significant difference in % closure after 24 and 48 h exposure to 50 μ M LU (42.6 ± 2.25 vs 46.5 ± 1.8 % closure; Student t-test, $P = 0.189$), suggesting

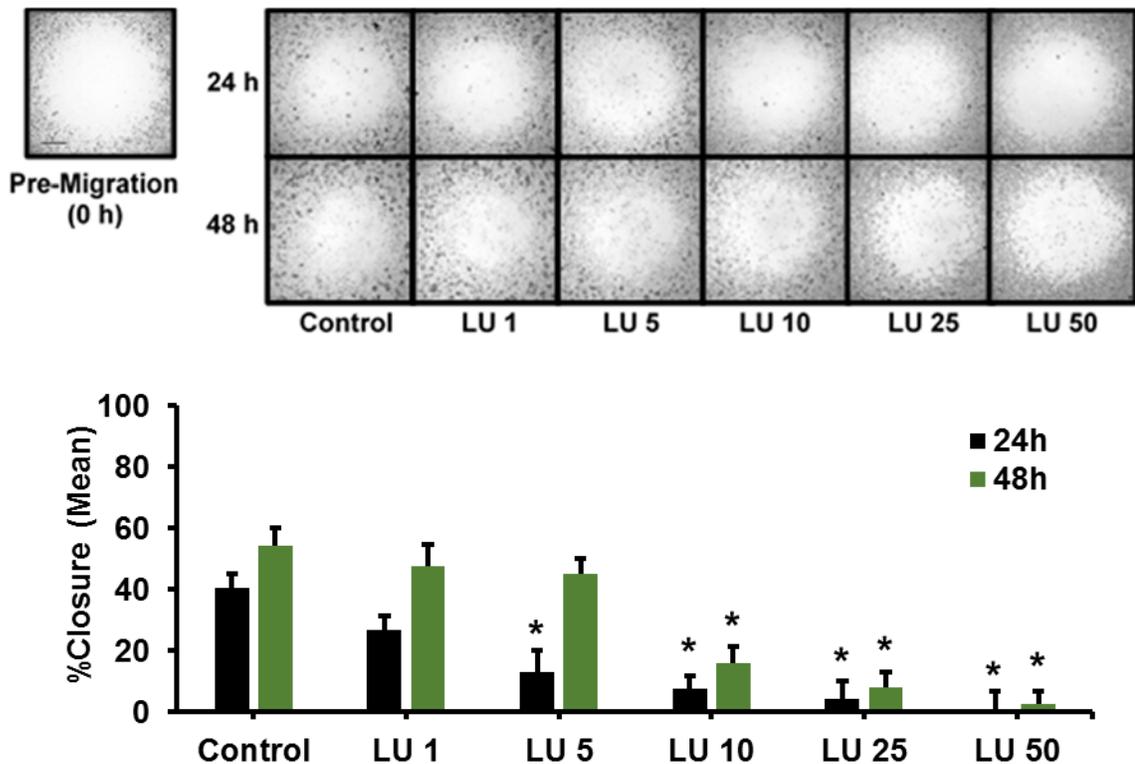


Figure 4.3A. Luteolin abolishes migration of MDA-MB-435 cells in vitro. Triple-negative breast cancer cells were plated (4×10^4) in a 96-well migration assay plate and allowed to adhere. Cells were treated with DMSO (controls) or varying concentrations of LU (1-50 μ M) and pictures were captured upon treatment (0 h) and periodically over the subsequent days (24 and 48 h). Representative images (top) capture migration over two days, and show LU-mediated migration inhibition. Bottom, bar graph represent means \pm SEM (n=8). *indicates significant difference from control ($P = \leq 0.003$, ANOVA).

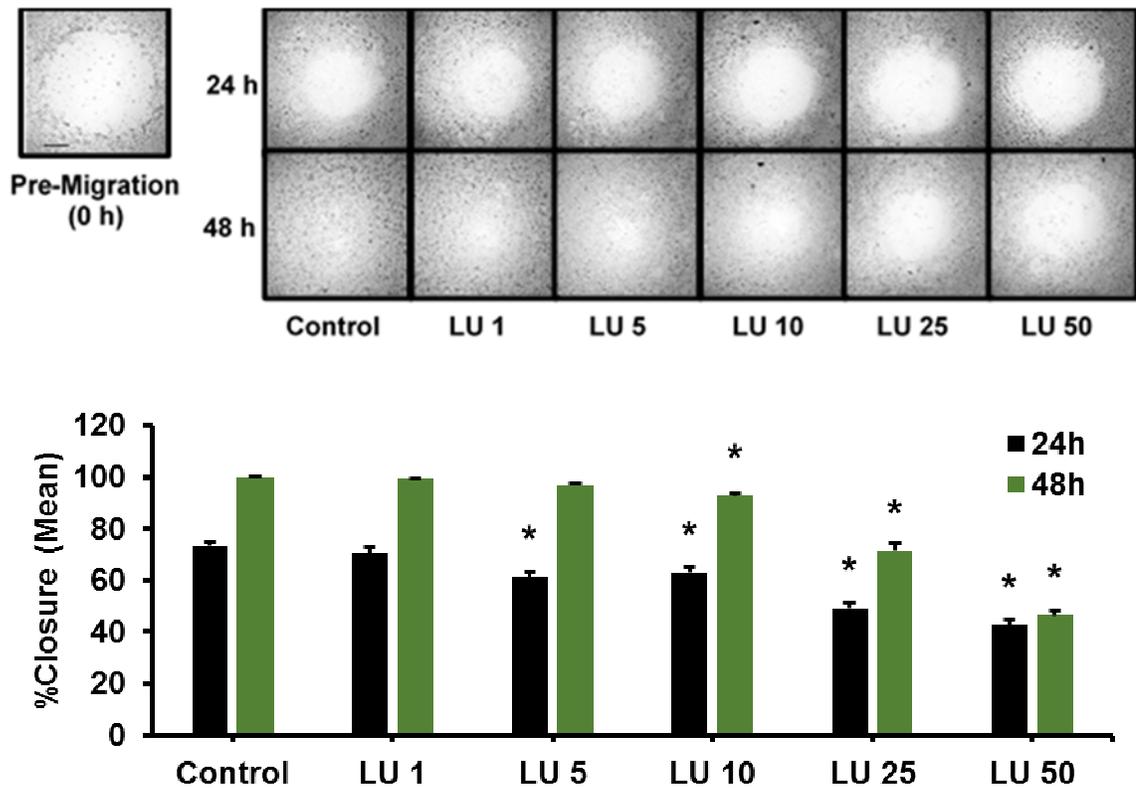


Figure 4.3B. Luteolin suppresses MDA-MB-231 (4175) LM2 cell migration in a time-dependent manner. MDA-MB-231 (4175) LM2 breast cancer cells were plated (4×10^4) in a 96-well migration assay plate and allowed to adhere. Cells were treated with DMSO (controls) or varying concentrations of LU (1-50 μ M) and pictures were captured upon treatment (0 h), 24 and 48 h. Representative images (top) capture LU-mediated migration inhibition at 24 and 48h. Bottom, bar graph represent treatment group means \pm SEM (n=8). *indicates significant difference from control (24 h, $P < 0.001$; ANOVA; 48 h, $P < 0.05$ ANOVA on Ranks).

that at this concentration, the flavonoid completely halts the migratory potential of MDA-MB-231 (4175) LM2 cells.

4.4.3 Luteolin Attenuates VEGF Production in MDA-MB-231 (4175) LM2 Breast Cancer Cells

It is well established that the potent angiogenic factor VEGF₁₆₅ increases migration and invasion potential of T47-D human breast cancer cells [270] and that VEGF has mitogenic effects in MDA-MB-231 breast cancer cells that are mediated through the interaction of VEGFA and its receptor, KDR [19]. Since MDA-MB-231 (4175) LM2 cells aggressively metastasize to lungs in xenograft models, and VEGF production is associated with increased propensity for metastasis and cell migration, we sought to determine whether LU suppresses VEGF production in these cells. MDA-MB-231 (4175) LM2 cells were grown to 60% confluence, washed twice and treated with 10, 50, or 100 μ M LU or DMSO for 16 h. Medium was collected and VEGF levels measured using a Quantikine human VEGF ELISA kit, which predominantly binds VEGF₁₆₅ (normalized to total protein concentrations, as described in methods). VEGF levels secreted by MDA-MB-231 (4175) LM2 cells were significantly reduced by 10 μ M LU ($P = <0.001$) (**Figure 4.4A**). Interestingly, although VEGF production was significantly reduced by 50 μ M LU, a slight, but repeatable increase in VEGF arose compared with 10 μ M LU treatment (20.8 ± 1.1 vs 22.2 ± 1.0 ng/mL VEGF, $P = 0.253$). This “resistance” to the lower concentrations of LU was completely overcome by 100 μ M LU, which significantly reduced the production of VEGF compared with either 10 or 50 μ M LU (ANOVA, $P = <0.001$).

4.4.4 Luteolin Fails to Abolish Transcriptional Regulation of VEGF

The ability of LU to suppress transcriptional regulation of VEGF in MDA-MB-231 (4175) LM2 cells was examined using RT-PCR. MDA-MB-231 (4175) LM2 cells were treated with 10, 25 and 50 μ M LU for 6 h, after which total mRNA was collected using RNazol

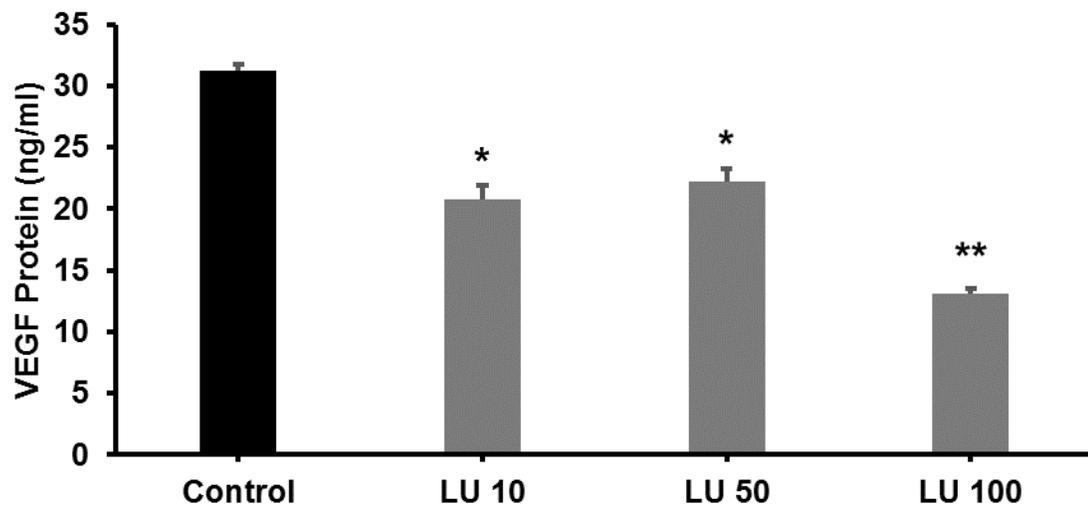


Figure 4.4A. Luteolin inhibits VEGF protein secretion from human MDA-MB-231 (4175) LM2 breast cancer cells. Triple-negative breast cancer cells were treated at 37°C for 18 h with 10, 50, or 100 μ M LU. Levels of VEGF in supernatants were measured by ELISA as described in Methods. Results represent means \pm SEM (n=4, in duplicate). *Significantly different compared to the control (P < 0.001). **compared to 50 μ M LU (ANOVA, P < 0.001).

RT. Total mRNA was reverse transcribed into cDNA and amplified using VEGF and GAPDH primers (see methods). Relative band intensity was determined using a Bio-Rad imager and software. LU had no effect on total VEGF mRNA at the three concentrations used (ANOVA, $P = 0.149$; **Figure 4.4B**), suggesting that the ability of LU to suppress VEGF secretion is mediated through post-transcriptional regulation.

4.4.5 Blocking VEGF Receptor but Not VEGFA Inhibits Migration of MDA-MB-231 (4175) LM2 cells in the Presence or Absence of Luteolin.

We previously alluded to VEGF increasing the migration and invasion potential of breast cancer cells [270]. Since 10 μM LU suppresses VEGF secretion (**Figure 4.4A**) and migration (**Figure 4.3B**), we conducted studies to determine whether squelching VEGF₁₆₅ would decrease MDA-MB-231 (4175) LM2 migration. MDA-MB-231 (4175) LM2 cells were plated on an Oris Pro 96-well migration plate (4×10^4 cells/well) and treated with corresponding amounts of LU (10 μM) and/or hVEGF₁₆₅-ab (5.0 $\mu\text{g}/\text{mL}$) or KDR-ab (0.5 $\mu\text{g}/\text{mL}$). Percent closure of the detection zone was analyzed as previously described in methods.

MDA-MB-231 (4175) LM2 cell migration was not inhibited by the hVEGF₁₆₅-ab; however, as previously shown, migration was significantly reduced by 10 μM LU after both the 24 h and 48 h exposure intervals, see **Figure 4.5A** graph (ANOVA on Ranks; $P < 0.05$). Intriguingly, a combination of 10 μM LU and 5.0 $\mu\text{g}/\text{mL}$ hVEGF₁₆₅-ab elicited a more robust effect than either agent alone, significantly reducing MDA-MB-231 (4175) LM2 migration compared with 10 μM LU alone (ANOVA on Ranks; $P < 0.05$). This suggests that LU-induced loss of VEGFA (or another component in the transduction pathway) may sensitize MDA-MB-231 (4175) LM2 cells to further suppression by LU in the presence of VEGF165 antibody.

Considering that VEGF₁₆₅ actions are often potentiated through vascular endothelial growth factor receptor-2 (KDR) and that KDR is stimulated by other VEGF isoforms, we

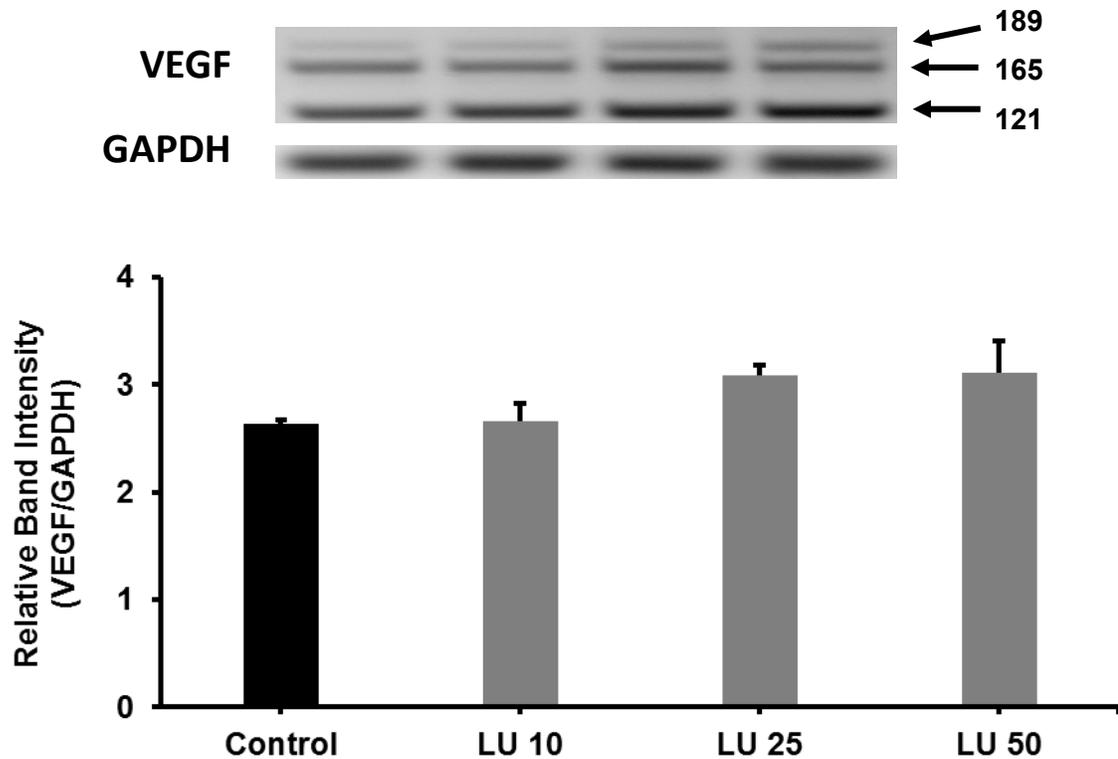


Figure 4.4B. Luteolin fails to abrogate VEGF mRNA production in human triple-negative breast cancer cells. Cells were treated for 6 h \pm LU (10, 25, 50 μ M), after which RNA was isolated and RT-PCR for VEGF isoforms performed as described in Methods. Upper panel is a representative Figure of PCR amplified VEGF bands, showing VEGF 189, 165, and 121 and the GAPDH band used for normalization. Bottom panel shows mean values from three independent experiments. Results are expressed as mean band intensities (VEGF/GAPDH) \pm SEM (n=3). No significance was reached (P = 0.149, ANOVA).

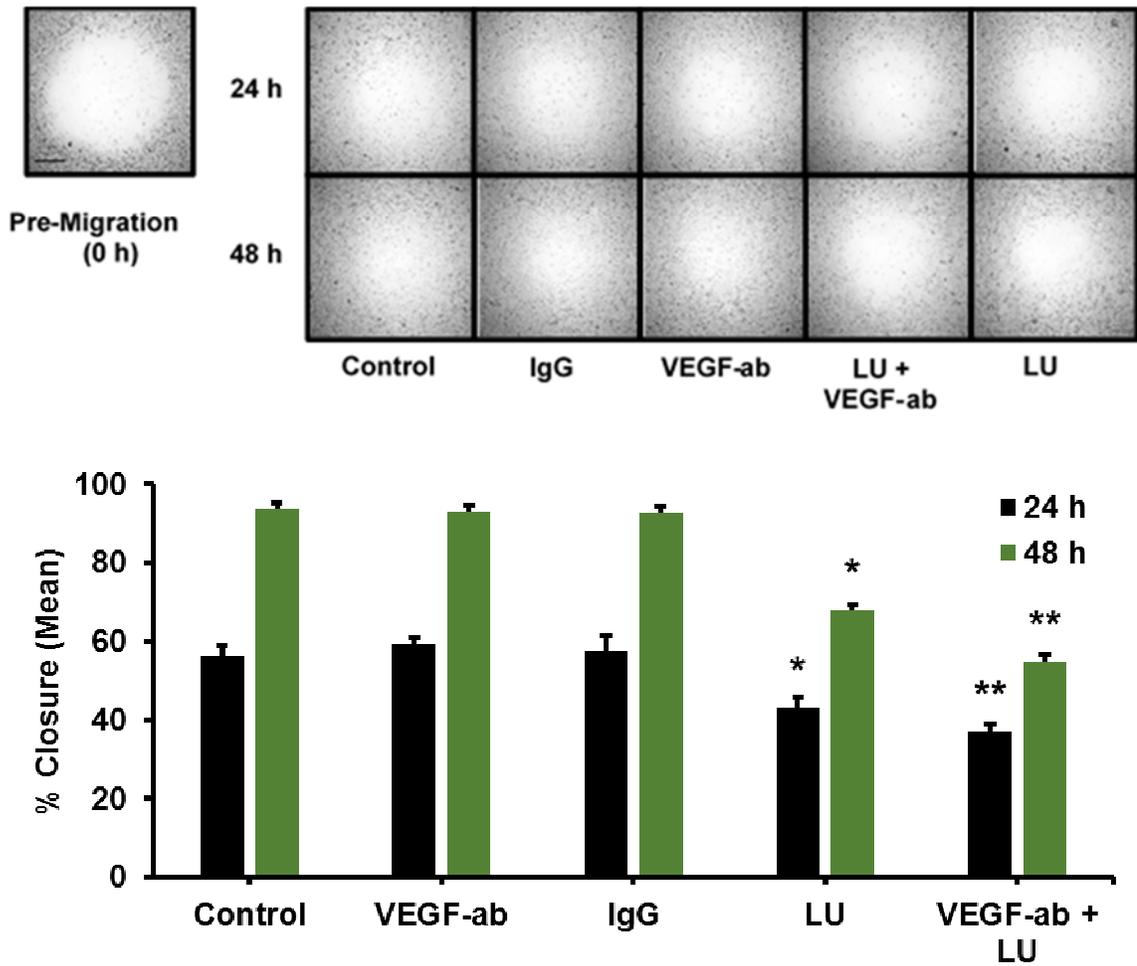


Figure 4.5A. Luteolin and VEGFA-ab synergistically suppress migration of human MDA-MB-231 (4175) breast cancer cells. Triple-negative breast cancer cells were plated (4×10^4) in a 96-well migration assay plate and allowed to adhere. Cells were treated with DMSO (controls) or $10 \mu\text{M} \pm$ hVEGF-ab ($5.0 \mu\text{g/mL}$). Pictures were captured at three intervals for analysis: upon treatment (0 h), 24 and 48 h. Images (top) represent LU migration inhibition in the presence or absence of hVEGFA antibody. Bottom bar graph shows mean values from each treatment group \pm SEM (n=8). *indicates significant difference from control ($P < 0.05$). **Significant difference compared to LU (24 and 48 h, $P < 0.05$ ANOVA on Ranks).

used a human KDR-ab to determine how the receptor is involved in the migration of MDA-MB-231 (4175) LM2 cells. The migratory potential of MDA-MB-231 (4175) LM2 cells was inhibited by blocking hKDR using either a KDR specific antibody or 10 μ M LU for 24h (ANOVA on Ranks, $P = <0.05$) (**Figure 4.5B**). Interestingly, after 48h the effect of hKDR-ab was less than that of 10 μ M LU, since the flavonoid suppressed migration significantly more than did the KDR-ab alone ($P = <0.05$). A combination of LU and KDR-ab had no effect over and above that of LU alone. Taken together these results suggest that LU may act on KDR or its downstream effectors to inhibit MDA-MB-231 (4175) LM2 breast cancer cell migration.

4.4.6 Luteolin Attenuates Viability of Triple-negative Breast Cancer Cells.

The effect of LU on cell viability was assessed in both MDA-MB-435 and MDA-MB-231 (4175) LM2 hormone-nonresponsive breast cancer cell lines as described in methods. Breast cancer cells were exposed for 24 and 48 h to varying concentrations of LU (0-100 μ M). The viability of MDA-MB-435 cells was markedly reduced by LU in both a dose- and time-dependent manner (ANOVA on Ranks, $P = <0.05$; **Figure 4.6A**). Following 24h exposure, the IC_{50} value for LU was between 50 and 75 μ M for MDA-MB-435 cells, with minimal or no effect observed at 10 μ M. MDA-MB-231 (4175) LM2 breast cancer cells were more resistant to LU; after 48h the IC_{50} was approximately 50 μ M. LU had minimal effect after 24 h until concentrations exceeded 10 μ M (ANOVA on Ranks, $P = <0.05$; **Figure 4.6B**). Our findings suggest that 24 h exposure to 10 μ M LU had little to no effect on the proliferation of either MDA-MB-435 or MDA-MB-231 (4175) LM2 cells. Moreover, 10 μ M LU had little effect on MDA-MB-231 (4175) LM2 proliferation, even after 48 h of exposure. However, at concentrations of LU greater than 10 μ M, the viability of both MDA-MB-435 and MDA-MB-231 (4175) LM2 cells was disrupted in a dose- and time-dependent manner (ANOVA on Ranks, $P = <0.05$; **Figure 4.6A and 4.6B**).

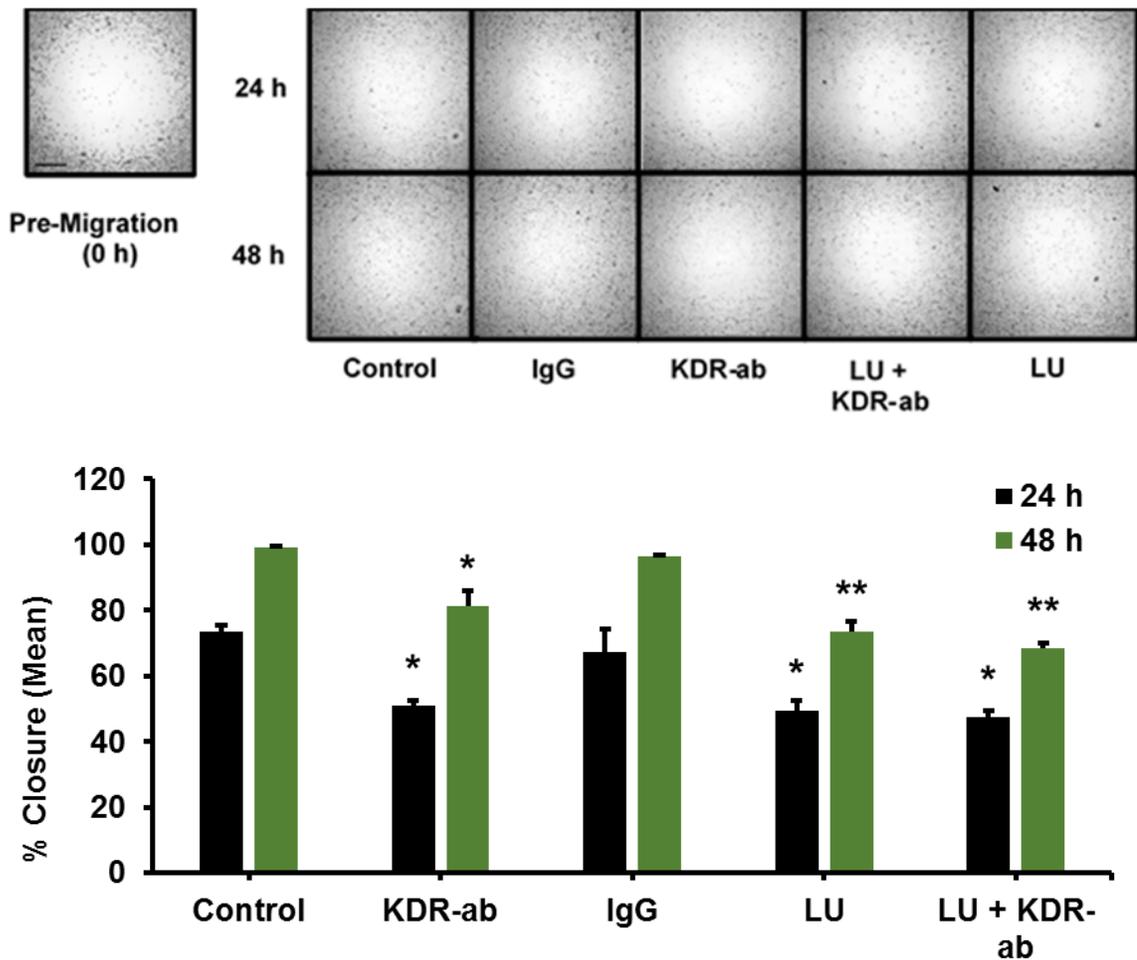


Figure 4.5B. Luteolin in the presence or absence of KDR-ab equivocally suppresses MDA-MB-231 (4175) cell migration. Triple-negative breast cancer cells were plated (4×10^4) in a 96-well migration assay plate and allowed to adhere. Cells were treated with DMSO (controls) or $10 \mu\text{M}$ \pm KDR-ab ($0.50 \mu\text{g/mL}$). Pictures were captured upon treatment (0 h), 24 and 48 h. Images (top) represent LU migration inhibition in the presence or absence of KDR antibody. Bottom bar graph shows mean values from each treatment group \pm SEM (n=6). *indicates significant difference from control ($P < 0.05$). **compared to LU (24 and 48 h, $P < 0.05$ ANOVA on Ranks).

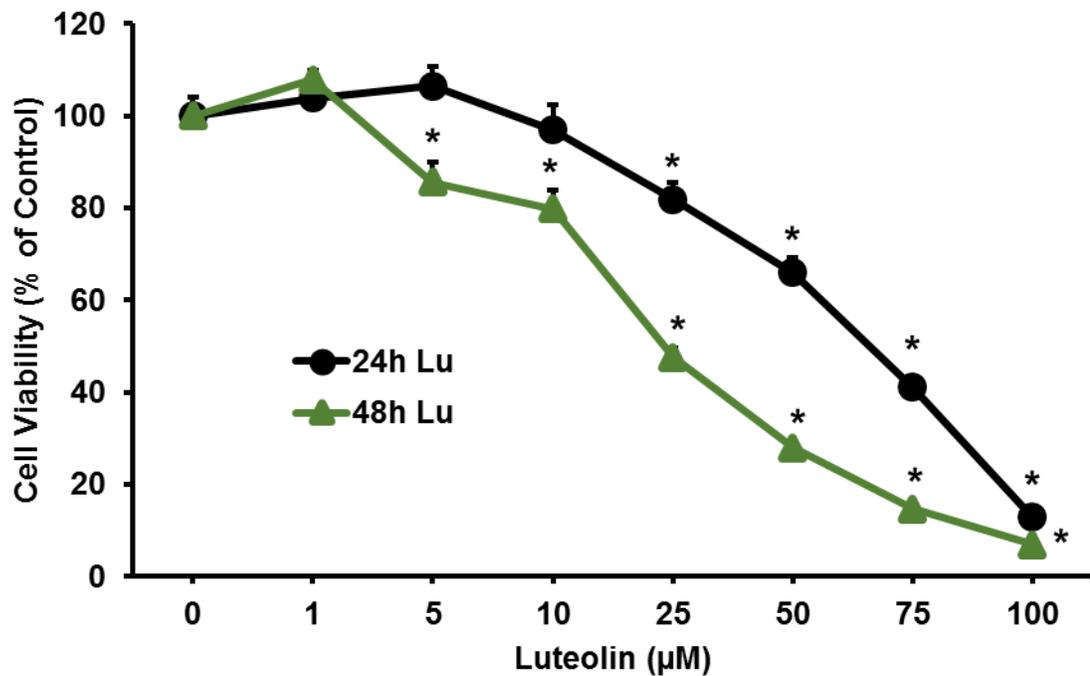


Figure 4.6A. Luteolin inhibits triple-negative MDA-MB-435 human breast cancer cell viability. MDA-MB0435 triple-negative breast cancer cells were seeded overnight in a 96-well plate (1.5×10^4 cells/well). Cells were then washed and treated with the indicated concentration of LU for either 24 or 48 hrs. Cell viability was determined as described in Methods. *Significantly different from control (ANOVA, $P < 0.05$).

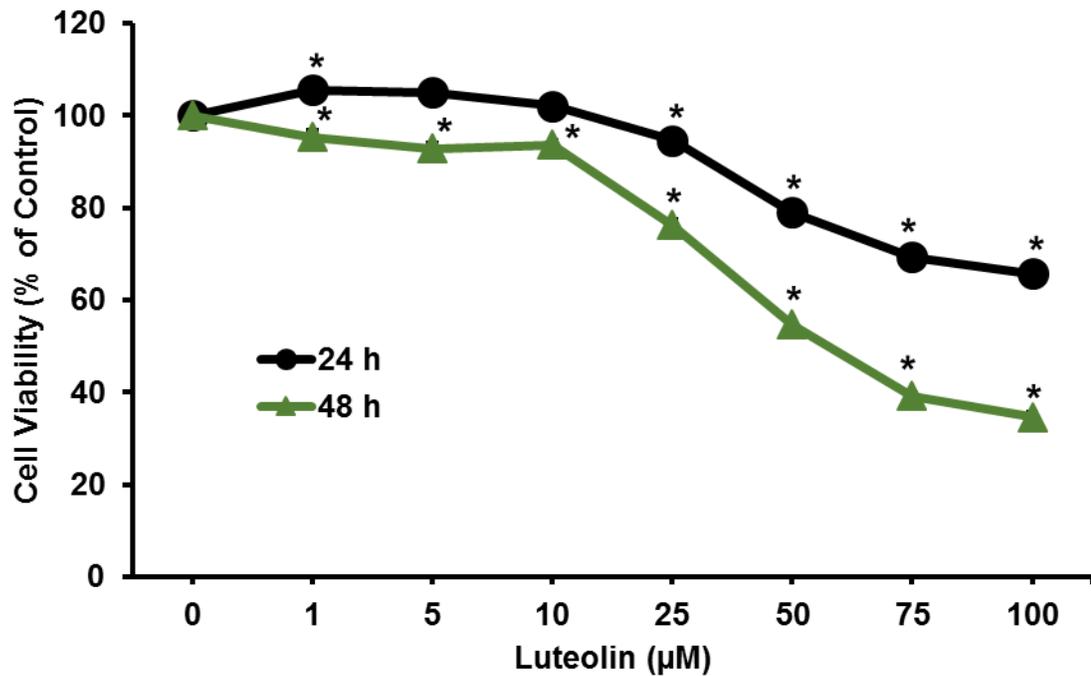


Figure 4.6B. Luteolin inhibits triple-negative MDA-MB-231 (4175) LM2 human breast cancer cell viability. Triple-negative breast cancer cells were seeded overnight in a 96-well plate (1.0×10^4 cells/well). Cells were then washed and treated with the indicated concentration of LU for either 24 or 48 hrs. Cell viability was determined as described in Methods. *Significantly different from control (ANOVA, $P < 0.05$).

4.4.7 Luteolin Induces Apoptosis in MDA-MB-231 (4175) LM2 Breast Cancer Cells

In a separate study we carried out experiments to determine whether LU induces apoptosis of clinically relevant MDA-MB-231 (4175) LM2 breast cancer cells. MDA-MB-231 (4175) LM2 cells were grown to 50-60% confluence and washed twice prior to treatment with medium containing different concentrations of LU (0, 10, 25, 50 μ M). After 48h cells from each treatment group were collected and assessed for apoptosis by FACS analysis, measuring Annexin V and PI labeling. As shown in **Figure 4.7**, LU induced apoptosis in MDA-MB-231 (4175) LM2 breast cancer cells, starting at 10 μ M in 24h, indicating that loss in cell viability is likely due to apoptosis.

These data suggest that low concentrations of LU (10 μ M) lower the migratory potential of triple-negative breast cancer cells, likely through inhibiting KDR signaling. Moreover, at increasingly higher concentrations LU reduces breast cancer cell viability and eventually induces apoptosis.

4.5 Discussion

Breast cancer is a potentially deadly disease that afflicts millions of women worldwide. Hormone-responsive breast cancer is a form of the disease that often affects post-menopausal women undergoing combination HRT. There is increasing evidence to show that the progestin component of HRT increases the production of VEGF, a vital component of new blood vessel formation (angiogenesis). At present there is a dearth of effective treatments for breast cancers that are hormone-responsive and for those that are not hormone-dependent, such as triple-negative tumors. Many existing treatments are highly toxic and resistant tumors often arise despite the use of aggressive chemotherapy regimens. With this in mind we embarked upon a study of naturally-occurring compounds that might be used to block tumor progression and metastasis. Such compounds have the benefit of being either non-toxic, or of low toxicity, and therefore confer significant potential advantages over currently used drugs.

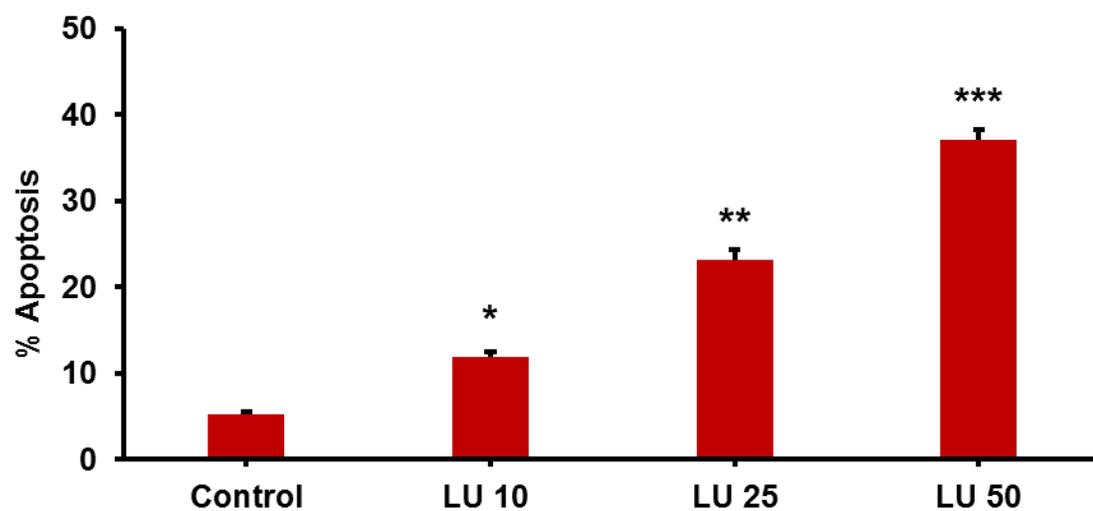


Figure 4.7. Luteolin induces apoptosis in MDA-MB-231 (4175) LM2 human breast cancer cells. Triple-negative breast cancer cells were treated with DMSO (control) or LU (10, 25, or 50 μ M) for 24 h, as described in Methods. Cells were stained with Annexin V (AV) and propidium iodide (PI) and analyzed by FACS. LU effectively induced apoptosis in a dose-dependent manner. Bars represent means (AV + AV/PI) \pm SEM (n=4). Asterisks indicate significantly different compared to *control, **control and 10 μ M LU, *** control, 10 μ M LU, and 25 μ M LU (P < 0.001, ANOVA).

The main thrust of our studies has been to determine the effectiveness of specific compounds with the potential to block angiogenesis. Tumor growth is dependent upon the formation of new blood vessels, which provides nourishment for the developing cancer. Metastasis also relies on neovascularization for the dissemination of tumor cells to distant sites. We previously reported on the ability of the flavonoid, LU, to block progestin-driven tumor formation and progression in two different progestin-associated animal [247]. In the studies described herein we assessed the capacity of LU to reduce the metastatic potential of triple-negative breast cancer. There are no recognized chemotherapeutic targets for triple-negative cancers, therefore patients suffering from this type of disease have poor prognosis [46-48, 105].

In order to determine whether LU blocks metastasis we employed well-established xenograft models of lung metastasis. Nude mice were inoculated i.v. with two different triple-negative breast cancer cell lines; MDA-MB-435 [272] and MDA-MB-231 (4175) LM2 [260]. Both cells are known to metastasize to lung, though the latter cell line is particularly aggressive [260] and gives rise to significantly more tumors of the lung per animal, a testament to its virulence. LU significantly inhibited tumorigenicity of both MDA-MB-435 and MDA-231 (4175) LM2 triple-negative breast cancer cells with little to no toxicity (**Figure 4.2**). To our knowledge, this is the first time it has been reported that LU attenuates the ability of triple-negative breast cancer cells to metastasize to the lungs, though it has been suggested in other models that inhibition of metastasis by LU may be potentiated through suppression of cellular migration and neovascularization [215], indispensable steps in tumor metastasis [265]. With this in mind we examined the capacity of LU to inhibit migration of triple-negative breast cancer cells and found that relatively low concentrations of the flavonoid (10 μ M LU at 24 and 48 h) significantly reduced migration. This suggests that at low concentrations, LU lessens expansion of the colony at the site of metastasis. Higher concentrations of LU (50 μ M) actually

abolished migration in a manner that was both time-dependent and dependent upon the particular cell line (**Figure 4.3**). LU inhibited migration of MDA-MB-435 cells relatively quickly, while in contrast it took approximately 24 h of exposure to suppress migration in the more aggressive MDA-MB-231 (4175) LM2 cells. The delayed response seen with MDA-MB-231 (4175) LM2 cells suggests that the ability of LU to interfere with migration is most likely an indirect effect, perhaps following LU-mediated suppression of VEGF secretion from tumor cells. Locally produced VEGF is known to act via VEGF receptors to promote tumor cell survival and migration [273-275].

Since VEGF plays such an important role in tumor cell migration, survival and proliferation [24, 268], we conducted studies using an hVEGF ELISA kit, to ascertain whether production of the growth factor by MDA-MB-231 (4175) LM2 cells was inhibited by LU. The MDA-MB-231 (4175) LM2 cell line was selected because these cells are particularly aggressive in their colonization of lung tissue following injection into nude mice. Very low concentrations of LU reduced basal levels of VEGF produced by tumor cells. Secreted VEGF was reduced by >30%, with further suppression occurring in response to 100 μ M LU (**Figure 4.4A**), though when cells were exposed to 50 μ M LU, a small, though reproducible increase in VEGF secretion was observed compared to low LU concentrations, suggesting that LU at these levels may stress the cells and stimulate slightly elevated VEGF production. It is interesting to note that LU did not influence levels of VEGF mRNA in tumor cells (**Figure 4.4B**), suggesting that it most likely influences VEGF production post-transcriptionally.

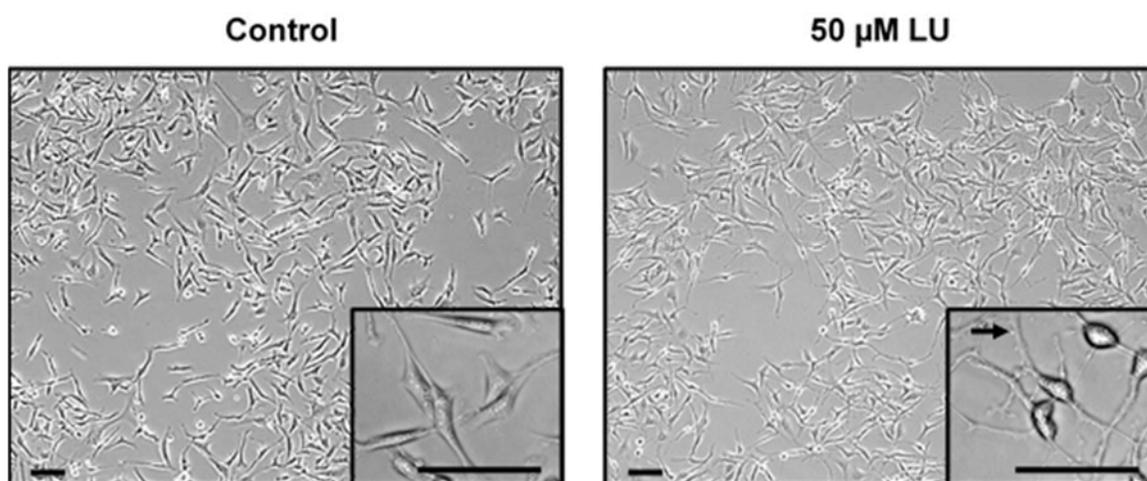
It has been reported that VEGF₁₆₅, the most abundant form of VEGF, promotes breast cancer cell migration [270]. Consequently, it is reasonable to speculate that suppression of VEGF bioavailability by LU is a plausible mechanism by which the flavonoid may modulate cell migration. To test this possibility we neutralized soluble VEGF with a VEGF₁₆₅-ab in MDA-MB-231 (4175) LM2 cells and determined differences in migration

potential. Unexpectedly MDA-MB-231 (4175) LM2 breast cancer cell migration was not affected by neutralization of serum VEGF levels (**Figure 4.5A**). LU (10 μ M) reduced migration, while surprisingly, a combination of LU and the VEGF₁₆₅-ab enhanced inhibition of the migratory potential of MDA-MB-231 (4175) LM2 cells, suggesting that the flavonoid sensitizes these cells so that their migration is inhibited via an alternative pathway, i.e. other than reduction of VEGF in the media. For example, the anti-migratory effects of LU may be hampered by an inability to block other isoforms of VEGF (e.g. VEGF₁₂₁ or VEGF₁₈₉), which continue to activate VEGFR2 in the absence of VEGF₁₆₅. However, considering the aforementioned observations concerning the effects on migration of sequestering VEGF₁₆₅ in the presence of LU, it is likely that LU acts downstream of VEGF receptor-2 (KDR) or on the receptor itself. To support this concept, there is evidence that LU suppresses VEGF-induced phosphorylation of VEGFR-2, resulting in inhibition of prostate tumor growth [218]. Accordingly, we used a KDR specific antibody to neutralize the receptor, both in the presence and absence of LU, in an effort to determine the effects on MDA-MB-231 (4175) LM2 migration. After 24 h exposure to the KDR-ab there was marked inhibition of migration in the triple-negative MDA-MB-231 (4175) LM2 cells. Inhibition of migration was no different than when cells were exposed to 10 μ M LU alone, or in combination with the KDR-ab, though the ability of KDR-ab to inhibit migration as effectively as LU decreased upon continued KDR exposure (48 h; **Figure 4.5B**). Collectively these data suggest that LU at least partially disrupts triple-negative cell migration through KDR-mediated signal propagation. Previous reports indicate that VEGF is potently mitogenic for breast cancer cells and that its actions are mediated through the interaction of VEGFA and its receptor [19]. Furthermore, it has been reported that 10 μ M LU is able to block VEGF-induced angiogenesis and proliferation through phosphatidylinositol 3'-kinase (PI3K) inhibition [215]. As previously described, low concentrations of LU inhibited the production of

VEGF by MDA-MB-231 (4175) LM2 cells (**Figure 4.4A**), which may also reduce cell survival. Initially we used a sulforhodamine B assay to determine the effect of LU on cell viability and found that relatively modest concentrations of LU, between 10 and 25 μM , effectively inhibited cell viability in a dose-, time-, and cell-dependent manner (**Figure 4.6**). We subsequently carried out FACS analysis to ascertain LU effects on apoptosis and to differentiate the cause in reduced cell viability. We found that concentrations of LU (10 μM) that seemingly did not affect MDA-MB-231 (4174) LM2 breast cancer cells, did in fact result in apoptosis (**Figure 4.7**), findings that are in accordance with previous reports in triple-negative breast cancer cells [218, 276]. The discrepancies between these two assays at low LU concentrations is likely due to the morphological changes seen in the triple-negative cells (*Supplementary Figure 4.1*), sensitizing them to mechanical manipulation involved in FACS analysis preparation. The precise role of VEGFA in these processes, and to what extent its attenuation by LU has on mediating triple-negative cell migration, proliferation and apoptosis remains unclear.

Besides our current studies, there is little direct evidence regarding the ability of LU to inhibit breast cancer metastasis, though there are reports demonstrating that LU potently inhibits in vivo metastasis in both prostate and colorectal cancer cells. Zhou and colleagues found that LU (5 mg/kg 3x/wk. i.p.) inhibited human PC3 prostate cancer cells from spontaneously metastasizing to lungs, through upregulation of E-cadherin [225]. LU was also shown to suppress Raf and phosphatidylinositol 3-kinase (PI3K), thereby diminishing mouse CT-26 colorectal cancer cell metastasis [277], a finding in agreement with Bagli et al. [215] and Pratheeshkumar et al. [218] who reported that low concentrations of LU (10-20 μM) acted as both as a potent PI3K inhibitor and an inhibitor of metalloproteinase MMP9. Considering that removal of VEGF₁₆₅ likely attenuates VEGF-induced PI3K phosphorylation, LU may self-promote its inhibitory effects. To this

end, additional evidence implicates down-regulation of PI3K phosphorylation and MMP9 as being responsible for the anti-invasive capacity of LU, perhaps explaining its ability to reduce the invasive potential of various cancer cell lines in vitro [278-282]. Since the VEGF-induced PI3K pathway mediates cell survival, migration and proliferation, we propose that our findings that LU inhibits migration support the notion that it acts via multiple mechanisms likely potentiated through blockade of PI3K phosphorylation and/or MMP-mediated VEGF signaling. While its precise mechanisms of action remain to be determined, we contend that these studies support the further investigation of LU as an anti-metastatic agent which might be used to combat Stage IV triple-negative breast cancer.



Supplementary Figure 4.1. Luteolin treatment instigates morphological changes in triple-negative breast cancer cells. Representative pictures of MDA-MB-231 (4175) LM2 triple-negative breast cancer cells after 24 h exposure to either control (DMSO) or 50 μM LU. Large field image was taken at 10x on an EVOS XL plate imager and inserts are at 40x, bar scale 100 μm. LU causes cytoplasmic blebs (arrow), possibly due to apoptosis.

CHAPTER 5: SUMMARY AND PERSPECTIVE STUDIES

5.1. SUMMARY

Breast cancer is one of the leading causes of cancer-related death in women throughout the world. In seventy percent of all breast cancer cases, both estrogen receptor (ER) and progesterone receptor (PR) are expressed by tumor tissue, which is problematic for post-menopausal women who seek hormone replacement therapy (HRT) to alleviate the symptoms of menopause. There is overwhelming evidence that combined HRT, which contains both estrogen and progestin, increases the risk of breast cancer [9-12, 56, 229]. This is chiefly due to the progestin component binding to PR and thereby inducing effects that may become pathologic [14, 245]. Common anti-progestins such as RU-486 counteract progestin-induced effects, but their clinical use is limited because they themselves have severe side effects. As a consequence the use of HRT has declined, though an estimated 1.6 million women still undergo combined HRT in the United States [13]. Despite a concerted effort to combat breast cancer, there has been little progress in the development of effective therapies to prevent the disease.

Our understanding of the precise role played by progestins in breast cancer progression is still at a preliminary stage. That being said, studies suggest that progestins promote proliferation, stem-cell enrichment, and metastasis [19, 103, 117, 123]. Findings from our laboratory and others show that progestins accelerate tumor progression by increasing levels of the potent angiogenic vascular endothelial growth factor (VEGF) [21, 104, 124]. The development of new blood vessels (angiogenesis) is essential for tumor survival, growth and metastasis [23, 24, 265] and is strongly associated with increased mortality [25]. Studies in our laboratory have focused on a number of different phytochemicals as potential chemo-preventive and/or chemo-therapeutic compounds since such compounds are naturally available and are usually non-toxic. Recently, we discovered that luteolin (LU), a naturally occurring flavonoid with well-recognized anti-

inflammatory and antioxidant properties, as well as being a free radical scavenger [198, 234, 235, 283], inhibits progestin-induced VEGF in breast cancer cells. In these studies we investigate the biological properties of LU and determine its capacity as an effective, naturally-occurring compound which might be used to safely prevent the progression of metastatic breast cancer.

LU is a flavonoid that occurs widely throughout the plant kingdom, with over 300 different plant species known to produce the compound [198]. Due to its wide availability and negligible toxicity, LU shows promise as an adjunctive and preventive therapeutic agent which could be used to combat breast cancer [234, 235]. In this study we focused on the ability of LU to suppress both progestin-driven effects and progestin-independent metastasis, the latter being studied because many tumors that are initially hormone-responsive lose nuclear steroid receptors in advanced disease, and many tumors that are negative for nuclear steroid receptors retain their membrane counterparts [259].

Initially we sought to determine whether LU reduces progestin-driven tumor growth in the well-characterized 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat model of mammary carcinogenesis. Previous studies from our laboratory show that medroxyprogesterone acetate (MPA), a commonly used component of HRT, accelerates tumor growth in this model [96, 171, 176]. During our initial study we initiated LU administration using three different concentrations of the flavonoid (1, 10 and 25 mg/kg given by i.p. injection) just before implantation of MPA pellets. Both low and high doses significantly reduced MPA-driven tumor incidence while also increasing tumor latency, i.e. tumors appeared significantly faster in response to MPA alone than in animals receiving both MPA and either 1 or 25 mg/kg LU. Administration of 10 mg/kg LU resulted in observations seemingly at odds with the other two groups, and tumor incidence was no different in animals given 10 mg/kg LU + MPA than in those receiving

MPA alone. To better understand this biphasic dose-response we assessed endpoint (Day 60) tumor growth by measuring tumor volume. Both low and high dose LU significantly reduced the number of large tumors. In the MPA \pm 10 mg/kg LU study, 70% of tumors were deemed large in response to MPA alone, whereas 70% were small when LU was also administered, i.e. the ratio of large to small tumors was reversed.

Considering that most tumors in the 10 mg/kg LU group had developed prior to cessation of LU administration and that 70% of these tumors were small compared with those in the MPA alone group, it would seem that this dose of LU is able to suppress tumor expansion but not MPA-accelerated tumor initiation. This was later confirmed immunohistochemically, which demonstrated both a significant loss of VEGF, and a corresponding reduction in intratumoral blood vessels, regardless of LU dosage (1, 10, or 25 mg/kg). Since these observations were made two-weeks after LU treatment ended, this suggests that the flavonoid may have long-lasting and/or epigenetic effects. In contrast, animals given MPA + 10 mg/kg LU showed a marked increase in tumor proliferation and no change in apoptosis two weeks after injections of LU ceased. However, the inability of this dose of LU to inhibit MPA-accelerated tumor progression might be explained by a MPA-driven 'catch-up' effect, i.e. release of the LU 'brake'. Importantly we examined the effects of the 10 mg/kg LU dose in the absence of MPA and observed tumor incidence to be lower than in controls, proving that this dose of LU does not in itself accelerate DMBA tumorigenesis.

Having demonstrated that LU is a potent chemopreventive agent with long-lasting effects, subsequent studies were performed to determine whether it might also have potential as a therapeutic agent for use against human breast cancer. Previous studies from our laboratory show that progestins stimulate VEGF production in PR containing mutant p53 human breast cancer cells and it has further been postulated that PR-

induced VEGF stimulates cancer cell proliferation in both an autocrine and a paracrine manner [19, 20]. This could explain the elevated risk of breast cancer in postmenopausal women undergoing combined HRT. In the current studies we used T47-D and BT-474 human breast cancer cells which contain both ER and PR; BT-474 cells also express human epidermal growth factor receptor-2 (HER2). In both cell lines, doses of LU greater than 10 μ M reduced cell viability in a dose- and time-dependent manner, with high doses initiating apoptosis. Using ELISA kits we measured levels of VEGF secreted by T47-D and BT-474 cells in response to progestin stimulation, both with and without anti-progestins or LU. Low level LU (10 μ M) significantly reduced progestin-driven VEGF production in T47-D breast cancer cells, regardless of the progestin used; likewise the same dose significantly reduced VEGF production to basal levels in BT-474 cells. The ability of LU to block MPA-induced VEGF protein secretion was also seen at the transcriptional level. However, LU failed to abolish PR loss, which is a necessary step in PR signal propagation [245]. Taken together these data suggest that LU blocks VEGF production somewhere between PR-activation and the VEGF-promoter.

In subsequent studies our goal was to ascertain whether or not the effectiveness of LU in vitro translated to the in vivo situation. This was accomplished using the progestin-stimulated nude mouse model established by our lab [104]. In this model, human breast cancer cells, in the absence of Matrigel, are injected into the mammary tissue of nude mice supplemented with E2 pellets. Initial tumor progression is observed followed by a regression phase within 8-10 days. Implantation of progestin pellets rescues tumor growth and drives tumor progression; removal of the progestin pellet or administration of an anti-progestin instigates tumor regression. In the current study, nude mice were inoculated with T47-D human breast cancer cells. LU (20 mg/kg) was injected i.p. after

establishment of MPA-driven tumor growth, resulting in a marked suppression of xenograft tumor growth. Immunohistochemical analysis of excised tumors showed that LU significantly reduced the expression of MPA-induced VEGF production. Compared with mice given MPA alone, the number of intratumoral blood vessels was also lower in animals receiving LU, corroborating our previous findings in the DMBA model. Similarly, LU failed to prevent MPA-stimulated loss of PR. In both the DMBA-induced rat model of mammary tumorigenesis and the human breast cancer cell xenograft nude mouse model, LU effectively reduced MPA-induced VEGF production, resulting in a corresponding decrease in intratumoral vasculature.

It is well-established that progestins stimulate the production of VEGF in hormone-responsive breast cancer cells and that the growth factor exerts stimulatory effects on both endothelial and breast cancer cells alike [17, 19, 21, 100]. Recent evidence suggests that progestins not only provide a microenvironment conducive to growth and metastasis, but that they also enrich the stem-like cancer cell subpopulation [103, 106, 117]. Considering how quickly LU inhibits MPA-driven tumor growth in the aforementioned nude mouse studies, it is possible that it affects progestin-driven breast cancer cell enrichment. A number of different assays designed to determine 'stemness' were used to examine such a possibility. CD44⁺ is a well-established marker of breast stem-cells [53, 256, 264]. More recently, high levels of aldehyde dehydrogenase (ALDH) have been associated with 'stemness' [55, 264]. In our studies MPA increased CD44 protein levels dramatically; this effect was completely blocked by the anti-progestin RU-486. Similarly, moderate levels of LU (25 μ M) completely blocked MPA induction of CD44. Importantly, LU at this level did not itself increase CD44. In a subsequent study we determined the effect of LU on ALDH activity which was slightly, though significantly increased in response to MPA. LU suppressed ALDH activity

beyond control levels, whether or not MPA was present. Taken together these studies suggest that the flavonoid effectively inhibits the development of progenitor and/or stem cell-like breast cancer cells. This possibility was tested using a mammosphere assay for investigating 'stemness' in a 3-D matrix. This type of assay capitalizes on the unique properties of stem cells, i.e. anchorage-independent proliferation and self-renewal, to provide a stem cell testing bed. In accord with previous reports [116] we found that MPA increased mammosphere formation, an effect that was blocked significantly by extremely low doses of LU. Collectively these observations suggest that LU blocks the enrichment of cancer stem cell-like cells. The multi-faceted actions of LU make it an extremely attractive therapeutic agent with significant potential against progestin-dependent breast cancer.

Metastatic cancer leads to more cancer-related deaths than any other form of the disease. Preventing metastasis, or at least slowing its progress, will improve an individual's quality of life and even extend life in high risk patients. Stage IV breast cancer, commonly referred to as metastatic breast cancer, and is currently considered incurable. Figures from the American Cancer Society (ACS) show that the relative survival rate is 22% for the first five-years. This is an alarmingly low rate of survival compared with earlier invasive stages of the disease in which cancer has not migrated beyond the lymph nodes. Stage II and Stage III breast cancers have a respective 90% and 72% five-year relative survival rate [34].

Breast cancer metastasizes chiefly to the bone, brain, lungs, and lymph nodes [105]. Due to variability in microenvironments between sites, it is believed that metastasizing cancer cells may have predestined niches. In 2005, Massague and colleagues elegantly showed that metastatic subpopulations of the triple-negative breast cancer cell line MDA-MB-231 had molecular niches. They separated metastatic cells in both lung and

bone from the parental line and enriched the cells to create highly virulent metastatic subpopulations that expressed different molecular signatures with preferential seeding sites [260]. Intriguingly, the molecular signature of the metastatic subpopulation isolated from lung was associated significantly with an increase in clinical breast cancer metastasis, but not bone metastasis. Considering the clinical relevance, their programmed nature and aggressive metastatic potential, these cells provide a novel in vivo model by which to study the treatment and prevention of breast cancer and its metastasis to lung.

Progestins have been shown to induce mammary tumor metastasis to lymph nodes and lungs in a variety of in vivo models [123, 124]. Although logistics may often preclude the use of these models, they do provide a direct measure of progestin-induced metastasis. We opted to use the well-characterized xenograft mouse model in which human breast cancer cells are introduced via tail vein injection, to investigate the ability of LU to suppress lung metastasis. Two different triple-negative breast cancer cell lines (MDA-MB-435 and MDA-MB-231 (4175) LM2 cells) were used. MDA-MB-231 (4175) LM2 cells express the clinically relevant signature of lung metastasis [260]. Triple-negative breast cancer cells were injected into the circulatory system via tail vein and allowed to settle in lung capillaries prior to i.p. injection of LU. Animals injected with MDA-MB-435 cells were administered either 10 or 20 mg/kg LU; those injected with MDA-MB-231 (4175) LM2 cells were given 40 mg/kg. Post-necropsy analysis of lung tissue showed that LU significantly suppressed the metastasis of both triple-negative breast cancer cell lines to lung. There was little or no evidence of toxicity to experimental animals, as determined by animal weights and behavior.

Several in vitro assays were employed to elucidate possible mechanism(s) of action behind the novel ability of LU to inhibit metastasis. Using migration assay plates we

found that LU inhibits the migration of both MDA-MD-435 and MDA-MB-231 (4175) LM2 triple-negative breast cancer cells in a time- and dose-dependent manner. Migration of the latter was not completely blocked by 50 μ M LU until after about 24 h, suggesting that inhibition of migration by LU is an indirect effect. VEGF, as previously outlined, is associated with increased morbidity and metastasis [266-269]. Since triple-negative breast cancer cells produce copious amounts of VEGF we tested whether LU could reduce basal VEGF production in such cells, and thereby reduce metastatic potential. Low levels of LU significantly reduced VEGF secretion in MDA-MD-231 (4175) LM2 cells, though this was not reflected at the level of transcription. A higher level of LU (50 μ M) appeared to actually increase levels of VEGF protein and transcript in these cells, though this did not achieve significance but this resistance was overcome at 100 μ M LU. Bearing in mind these observations we conducted studies to determine the importance of VEGF in cell migration, using antibodies (ab) against VEGF and its receptor, KDR. To our surprise, squelching VEGF with a VEGF-ab did not affect migration when LU was not present; however, a synergistic effect on cell migration was observed when VEGF-ab was combined with LU. Furthermore, when KDR was neutralized using a KDR-ab, migration was blocked significantly and no difference was found between KDR and LU alone or LU + KDR-ab at 24 h. Collectively these data suggest that LU may disrupt triple-negative cell migration through KDR-mediated signal propagation, the mechanism of action likely being downstream of KDR.

Previous reports indicate that VEGF acts as a potent mitogen in breast cancer cells, and that mitogenic effects are mediated through KDR [19]. As we saw previously, low concentrations of LU inhibited the production of VEGF by MDA-MB-231 (4175) LM2 cells, which may also reduce cell survival. We initially used a SRB assay to determine the effect of LU on cell viability and found that relatively modest concentrations of the

flavonoid, between 10 and 25 μM , effectively inhibited the viability of human breast cancer cells in a dose-, time-, and cell-dependent manner. These studies were followed with an Annexin V apoptosis assay in order to confirm that loss of cell viability measured by SRB was due to cell death. After 24 h incubation, apoptosis increased dose-dependently as cells were exposed to increasing levels of LU (10, 25 and 50 μM), even though the SRB assay detected no effect on cell viability at 10 μM LU. This discrepancy may be due at least in part to sensitization by LU of breast cancer cells to apoptosis and the increased agitation inherent in FACS preparation. As previously described, exposure of cells for 24 h to 50 μM LU did not increase the observed number of dead (i.e. floating) cells, but did change the morphology of MDA-MB-231 (4175) LM2 cells to cells that looked apoptotic in culture, which largely explains the discrepancy between assays.

In summary, LU effectively blocks the effects of progestins in the MPA-accelerated DMBA-induced rat model, and provides sustained protection against MPA-induced VEGF production and vascularization. In hormone-responsive human breast cancer cells LU inhibits progestin-induced VEGF production in vitro, as well as MPA-induced tumors grown in nude mice. Furthermore, progestin-stimulated stem cell-like enrichment is completely abolished by relatively low levels of LU. Lastly, studies in nude mice show that LU suppresses the metastasis of hormone-independent breast cancer cells to lung, a phenomenon that is most likely due in part to its ability to mediate the VEGF-KDR signal pathway. In all cases, LU reduces cell viability and increases apoptosis in both hormone-dependent and hormone-independent human breast cancer cells. These studies provide compelling evidence for the use of LU as an effective, non-toxic means to combat breast cancer.

5.1. PERSPECTIVE STUDIES

The preceding studies describe luteolin as a potent therapeutic and chemopreventive compound in combating human breast cancer, however, many questions remain unanswered. Further investigation is warranted to determine the exact mechanism(s) by which luteolin inhibits progesterin-dependent growth in both the DMBA-induced and human xenograft mammary cancer models. The mechanism by which luteolin blocks triple-negative cell migration likely involves inhibition of tyrosine-kinase pathway [206]. This remains to be proven.

Luteolin may introduce epigenetic changes in DMBA-induced mammary carcinogenesis in Sprague-Dawley rats providing 'long-lasting' protective effects against MPA-driven growth and VEGF production. Recent reports suggest that dietary compounds, such as flavonoids, may cause in utero epigenetic changes which result in reduced breast cancer risk [284]. Considering this, it would be insightful to administer luteolin in prepubescent rats to determine if luteolin causes 'long-lasting' epigenetic changes that may prevent MPA-driven DMBA-induced mammary cancer. It would also be of interest to examine tissue samples from tumors and mammary glands throughout the study to determine differences between direct and sustained actions of luteolin.

We showed that luteolin potentially blocked progesterin-induced VEGF, and that luteolin failed to block P-induced PR loss. This does not necessarily exclude luteolin from binding to PR as the likely mechanism of action, therefore a co-immunoprecipitation would help determine this outcome. Prospectively luteolin inhibits PI3K [215] or possibly SP-1 transcription factor, both of which are necessary for P-induced VEGF production [109]. A positive result of luteolin blocking either PI3K or SP-1 would suggest that luteolin acts indirectly to inhibit VEGF production and may have other downstream effectors. Due to the fact that VEGF induction is dependent not only on PR but also on

HIF-1 α [244], it will be useful to understand whether the HIF-1 α pathway is also a target for Luteolin mediated loss of VEGF production. Mechanistically, it will also be useful to determine whether luteolin blocks interaction of the mentioned transcription factors to the promoter region of VEGF using ChIP and transient transfection assays. Furthermore, it would be of interest to test the ability of luteolin to demonstrate anti-angiogenic properties using in vitro assays. This would help to differentiate whether the luteolin-induced loss of blood vessels seen in both the DMBA and nude mouse models is by inhibition of neovascularization directly or inhibition of preexisting vasculature and/or its function.

Metastasis is responsible for the vast majority of cancer-related death. Luteolin proved highly beneficial in mitigating aggressive human triple-negative breast cancer cells from metastasizing to the lungs in the xenograft nude mouse model. In this study we also showed that the ability of luteolin to inhibit migration is at least partly under the influence of KDR. Due to the synergistic affect with the combined treatments, VEGFA-ab and luteolin, further studies are necessary to elucidate if the ability of luteolin to suppress VEGF secretion is related to the decrease in migratory potential.

This study examined several possible mechanisms through which luteolin inhibits breast cancer growth and metastasis. In the DMBA model we saw a paradoxical dose-dependent curve with luteolin. Considering this, modification of route of ingestion of luteolin may prove more beneficial, especially if given orally. Inevitably human trials will be necessary to examine the pharmacokinetics of luteolin and its ability to combat breast cancer in humans.

APPENDIX

PUBLISHED MANUSCRIPTS

APPENDIX I:

**CHOLESTEROL BIOSYNTHESIS INHIBITORS AS POTENT NOVEL ANTI-CANCER
AGENTS: SUPPRESSION OF HORMONE-DEPENDENT BREAST CANCER BY THE
OXIDOSQUALENE CYCLASE INHIBITOR RO 48-8071**

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ABSTRACT

In most human breast cancers, tumor cell proliferation is estrogen dependent. Although hormone-responsive tumors initially respond to anti-estrogen therapies, most of them eventually develop resistance. Our goal was to identify alternative targets that might be regulated to control breast cancer progression. Sulforhodamine B assay was used to measure the viability of cultured human breast cancer cell lines exposed to various inhibitors. Protein expression in whole-cell extracts was determined by Western blotting. BT-474 tumor xenografts in nude mice were used for in vivo studies of tumor progression. RO 48-8071 ([4'-[6-(Allylmethylamino)hexyloxy]-4-bromo-2'-fluorobenzophenone fumarate]; RO), a small-molecule inhibitor of oxidosqualene cyclase (OSC, a key enzyme in cholesterol biosynthesis), potently reduced breast cancer cell viability. In vitro exposure of estrogen receptor (ER)-positive human breast cancer cells to pharmacological levels of RO or a dose close to the IC₅₀ for OSC (nM) reduced cell viability. Administration of RO to mice with BT-474 tumor xenografts prevented tumor growth, with no apparent toxicity. RO degraded ER α while concomitantly inducing the anti-proliferative protein ER β . Two other cholesterol-lowering drugs, Fluvastatin and Simvastatin, were less effective in reducing breast cancer cell viability and were found not to induce ER β . ER β inhibition or knockdown prevented RO-dependent loss of cell viability. Importantly, RO had no effect on the viability of normal human mammary cells. RO is a potent inhibitor of hormone-dependent human breast cancer cell proliferation. The anti-tumor properties of RO appear to be in part due to an off-target effect that increases the ratio of ER β /ER α in breast cancer cells.

APPENDIX II:

**EFFECTS OF DIETARY APIGENIN ON TUMOR LATENCY, INCIDENCE AND
MULTIPLICITY IN A MEDROXYPROGESTERONE ACETATE-ACCELERATED
7,12-DIMETHYLBENZ(A)ANTHRACENE-INDUCED BREAST CANCER MODEL**

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Study Publication: Nutrition and Cancer 2013; 65(8), 1184-1191.

ABSTRACT

Medroxyprogesterone acetate (MPA) is a synthetic progestin commonly administered to postmenopausal women for hormone replacement therapy and has been associated with increased risk of breast cancer. MPA has been shown to accelerate the development of mammary tumors in a 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast cancer animal model. Previously, we have shown that intraperitoneally administered apigenin effectively treated and prevented the progression of MPA-accelerated breast cancer in DMBA-induced and xenograft mammary cancer models. Here we used the DMBA model to examine the chemopreventive effect of dietary apigenin against MPA-accelerated tumors with 3 different levels of apigenin (0.02%, 0.1%, and 0.5% w/w) incorporated into a phytoestrogen-free diet. Results showed that 0.1% dietary apigenin reduced MPA-dependent tumor incidence; however, the same dietary level increased tumor multiplicity in animals that developed tumors. Neither 0.02% nor 0.5% dietary apigenin reduced MPA-dependent tumor incidence or latency, and tumor multiplicity increased significantly in response to 0.5% apigenin. These results contrast with previous chemopreventive effects observed when apigenin was administered intraperitoneally, suggesting that route of administration may influence its action. Consequently, until further research clarifies the effect of dietary apigenin on progestin-accelerated mammary tumors, caution should be exercised when considering the flavonoid as a dietary supplement for preventing hormone-dependent breast cancer.

APPENDIX III:

**FUNCTIONAL CFTR IN CRYPT EPITHELIUM OF
ORGANOTYPIC ENTEROID CULTURES FROM MURINE SMALL INTESTINE**

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C1492-C1503.**

ABSTRACT

Physiological studies of intact crypt epithelium have been limited by problems of accessibility *in vivo* and dedifferentiation in standard primary culture. Investigations of murine intestinal stem cells have recently yielded a primary intestinal culture in three-dimensional gel suspension that recapitulates crypt structure and epithelial differentiation (Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, Van Es JH, Abo A, Kujala P, Peters PJ, Clevers H. *Nature* 459: 262–265, 2009). We investigated the utility of murine intestinal crypt cultures (termed “enteroids”) for physiological studies of crypt epithelium by focusing on the transport activity of the cystic fibrosis transmembrane conductance regulator Cftr. Enteroids had multiple crypts with well-differentiated goblet and Paneth cells that degranulated on exposure to the muscarinic agonist carbachol. Modified growth medium provided a crypt proliferation rate, as measured by 5-ethynyl-2'-deoxyuridine labeling, which was similar to proliferation *in vivo*. Immunoblots demonstrated equivalent Cftr expression in comparisons of freshly isolated crypts with primary and passage 1 enteroids. Apparent enteroid differences in mRNA expression of other transporters were primarily associated with villous epithelial contamination of freshly isolated crypts. Microelectrode analysis revealed cAMP-stimulated membrane depolarization in enteroid epithelium from wild-type (WT) but not Cftr knockout (KO) mice. Morphological and microfluorimetric studies, respectively, demonstrated Cftr-dependent cell shrinkage and lower intracellular pH in WT enteroid epithelium in contrast to Cftr KO epithelium or WT epithelium treated with Cftr inhibitor 172. We conclude that crypt epithelium of murine enteroids exhibit Cftr expression and activity that recapitulates crypt epithelium *in vivo*. Enteroids provide a primary culture model that is suitable for physiological studies of regenerating crypt epithelium.

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VITA

Matthew Thomas Cook was born in Warrensburg, Missouri on November 23, 1980. He grew up on a small farm and had the spirit of the wind. For many years he discussed his ambitions to become a scientist, to all that would listen. Throughout his young adult life, he worked as an electrician's apprentice and wired new and old houses alike. While still in high school attendance his trade-job afforded him much success and allowed him to purchase his first new car. In college, Matthew immersed himself in work as a nurse assistant, ward secretary, and hospice at St. John's Mercy Hospital. Undecided about continuing his education, he graduated from his bachelors with marginal success and went to work in St. Louis as an Analyst II for Test America. In 2007, he realized that his childhood dreams to become a professor, and follow in the footsteps of his father Dr. Thomas Sommerkamp, had nearly slipped away. Matthew decided to give up his job, risk everything, and attend university.

In 2008 Matthew started his Masters studies in Biology at University of Central Missouri. In less than a semester he was recruited into the graduate assistantship program by the department and found an advisor, Dr. Scott Lankford, who would inspire him throughout his studies. The continued education and ability to teach classes kindled an unforeseen passion in Matthew and he finally felt the path was lit. Matthew began to work with renewed vigor and took the time to gain as much experience as possible. He became the departments student representative in faculty council, took courses in the mornings, taught as an adjunct instructor in both the biology and chemistry departments, as well as, taught his normal assistantship assignments throughout the days. He did this all while maintaining his research project on the intraovarian regulation of steroidogenesis in rainbow trout *Oncorhynchus mykiss*, at night. He graduated with a Masters in Biology

in 2010 with exceptional grades, and was awarded an outstanding graduate thesis award by the college.

In January 2011 Matthew was accepted into the Biomedical Sciences PhD program at the University of Missouri-Columbia, at which point he began his PhD rotations and education. At first, he struggled to find a research project he would be passionate about, until by happenstance he met with Dr. Salman Hyder. Within days of this newly developed third rotation he realized breast cancer research was for him. After Matthew joined Dr. Salman Hyder's laboratory he continued to produce both successful research and education. His advisor encouraged him to not only seek out research opportunities but to help the community. Matthew became a community leader and joined the Graduate Student Association, where he served as Treasurer, and subsequently, as President. His persistence and ambitions ultimately resulted in successful completion of his doctoral studies with near perfect marks and an excellent research project. His studies investigated the therapeutic and chemopreventive potential of luteolin against growth and metastasis of breast cancer. Matthew will be forever indebted to the great many people throughout his life who have helped him achieve this.