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Roles of Resveratrol and Genistein in Invasion and Metastasis of Breast Cancer

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Roles of Resveratrol and Genistein in Invasion and Metastasis of Breast Cancer

A Thesis

Presented in

Partial Fulfillment of the

Requirements for the Degree of

Master of Arts

June, 2012

BY

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Figure key

 = Activation

 = Inhibition

LIST OF ABBREVIATIONS

AIF: Apoptosis inducing factor
ALK-2 (TGF- β R1): Transforming growth factor beta type I receptor
ALK5 (TGF- β): Transforming growth factor beta receptor
AP-1: Activator protein-1
APAF1: Apoptotic protease activating factor 1
ATF3: Activating transcription factor 3
ATM kinase: Ataxia telangiectasia mutated kinase
Bak: Bcl-2 homologous antagonist/killer
Bax: Bcl-2 associated X-protein
BCE: Bovine capillary endothelial cell
Bcl-2: B-cell lymphoma 2
Bcl-xL: B-cell lymphoma-extra large
bFGF: Basic fibroblast growth factor
Bid: BH3 interacting-domain death agonist
BME: Bovine microvascular endothelial cells
BPGF: Bone-derived growth factor
c-IAP: Baculoviral IAP repeat-containing protein
c-MYC: Cellular MYC
Cdk (Cdc): Cyclin-dependent kinase
CDKIs: Cyclin-dependent kinase inhibitors
Chk1(2): Serine/threonine-protein kinase
COX-2: Cyclooxygenase-2
CXCL12: C-X-C motif chemokine
CXCR4: C-X-C chemokine receptor type 4
DMBA: Dimethylbenz(a)anthracene
E-cadherin: Epithelial cadherin
EGF: Epidermal growth factor
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
Egr1: Early growth response protein 1
ELAM: Endothelial leukocyte adhesion molecule-1
EMT: Epithelial-mesenchymal transition
ER: Estrogen receptor
ER α : Estrogen receptor alpha
ER β : Estrogen receptor beta
ERE: Estrogen response element
ERK: Extracellular signal-regulated kinase
FAK: Focal adhesion kinase
FGF: Fibroblast growth factor
FoxM1: Forkhead box protein M1
G1 phase: Growth 1 phase (Post-mitotic phase)
G2 phase: Growth 2 phase (Pre-mitotic phase)
GSK3: Glycogen synthase kinase 3

H₂O₂: Hydrogen peroxide
HER-2 (c-erbB-2): Human epidermal growth factor receptor 2
HIF- α : Hypoxia-inducible factor alpha
HRG- β 1: Heregulin-beta1
HSP27: Heat shock protein 27
HUVECs: Human umbilical vein endothelial cells
IAP: Inhibitor of apoptosis proteins
ICAM-1: Intercellular adhesion molecule 1
IGF-1: Insulin-like growth factor 1
IGF-1R: Insulin-like growth factor 1 receptor
IGF: Insulin-like growth factor
IKK: Inhibitor of kappa B kinase
IL: Interleukins
JNK: c-Jun N-terminal kinase
LPA: Lipopophatidic acid
LPS: Lipopolysaccharide
M phase: Mitotic phase
MAP-KAPK2: Mitogen-activated protein kinase-activated protein kinase 2
MAPK: Mitogen-activated protein kinase
Mcl-1: induced myeloid leukemia cell differentiation protein
MEF: Mouse embryonic fibroblast
MEK (MEKK): Mitogen activated protein kinase kinase
MMP: Matrix metalloprotease
MT1-MMP: Membrane-type matrix metalloprotease
mTOR: mammalian target of rapamycin
Myt1: Myelin transcription factor 1
NF- κ B: Nuclear factor-kappa B
p21waf1: aka cyclin-dependent kinase inhibitor 1
p300/CBP: CREB-binding protein
p53: Tumor protein 53
PAE: Porcine aortic endothelial cell
PAI-1: Plasminogen activator inhibitor-1
PAR-2: Protease activated receptors
PDGF: Platelet-derived growth factor
PI3K: Phosphatidylinositol 3-kinase
PIP2: Phosphatidylinositol 4,5-bisphosphate
PIP3: Phosphatidylinositol (3,4,5)-triphosphate
PSA: Prostate-specific antigen
PTEN: Phosphatase and tensin homolog
PUMA: p53 upregulated modulator of apoptosis
Rb: Retinoblastoma protein
ROS: Reactive oxygen species
S phase: Synthesis phase
Smac: Second mitochondria-derived activator of caspase
SMAD1/3: Mothers against decapentaplegic homolog 1/3

SPARC: secreted protein acidic and rich in cysteine

TGF- α : Transforming growth factor alpha

TGF: Transforming growth factor

TKI: Tyrosine kinase inhibitor

TNF- α/β : Tumor necrosis factor alpha/beta

TNF: Tumor necrosis factor

TSP1: Thrombospondin 1

uPA: Urokinase plasminogen activator

VCAM: Vascular cell adhesion protein 1

VEGF: Vascular endothelial growth factor

XIAP: X-linked inhibitor of apoptosis protein

Summary

Breast cancer is a cellular disease characterized by the exploitation of several cellular and molecular mechanisms involved in cell proliferation, apoptosis, motility, and invasion. Effective treatment is available for non-invasive breast cancer at diagnosis, leading to a very high survival rate compared to the low survival rate for those where the breast cancer has spread. Thus, identifying effective therapies and preventative agents is imperative to successfully treat breast cancer. Since diet comprises a large component of the risk factors for breast cancer, it would be beneficial to examine dietary compounds that could potentially play a beneficial role in inhibiting cancer progression. For this thesis the two dietary compounds chosen for investigation are resveratrol and genistein. Both of these compounds can be naturally found within the diet. Resveratrol is present within the skin of red grapes and genistein is commonly found in soy products. Preliminary studies reveal that these agents have a cancer fighting potential at the level of invasion, proliferation, apoptosis, angiogenesis and motility, steps of the complex process of metastasis.

Since current therapies are ineffective in treating the most advanced form of breast cancer, examining dietary agents and their potential role in inhibition of breast cancer may provide valuable insight on the influence of diet in inhibiting cancer progression. Our research aims to investigate the use of two dietary agents (resveratrol and genistein) to effectively combat metastasis at multiple targets simultaneously. The hypothesis for this research is that the combination of two different dietary compounds (genistein and resveratrol) could lead to a greater success in treating breast cancer invasion and metastasis by targeting multiple steps in metastasis. This thesis aims at identifying where

these agents overlap in their mechanism of action and differ in their exploitation of pathways in metastasis. This finding could lead to a combinatory therapy to treat and prevent metastasized breast cancer via dietary agents.

From an extensive review of research literature in this thesis, resveratrol may play a significant role in preventing/treating breast cancer by decreasing the invasive phenotype of cancer cells, altering MMP expression, adhesion and migration of the cancer cells. In addition, resveratrol also decreases cell proliferation, apoptosis and effectively represses angiogenesis. These findings suggest that resveratrol exploits multiple signaling pathways involved in cancer progression to effectively combat cancer.

From an extensive review of research literature on genistein revealed that it effectively reduces metastasis through a wide variety of mechanisms. Genistein decreases invasion of cancer cells by inhibiting migration, cell adhesion, proliferation, and played a significant role in modulating the expression of angiogenesis facilitators and angiogenesis inhibitors.

This thesis identifies the similar and different targets in breast cancer progression that are utilized by both resveratrol and genistein to inhibit metastasis. These include: inhibition of MMPs for invasion, adhesion and migration of cancer cells, angiogenesis, cell cycle proteins and cell proliferation pathways employed by cancer cells, and an increase in caspase activity and apoptosis (Fig 14a-d).

Thus, the combined effects of genistein and resveratrol should lead to a more substantial increase by inhibiting tangential or multi-layered events in metastasis and would probably have a greater potential preventing and treating metastasized breast cancer at multiple levels.

This thesis also proposes future studies to explore the combinatory effects of resveratrol and genistein in inhibiting breast cancer progression at the cellular level.

1. INTRODUCTION

1.1 Disease facts

Breast cancer is a cellular disease characterized by uncontrolled cell proliferation and spread to various tissues in the body through a process known as metastasis. According to the American Cancer Society, treating early stage breast cancer that is diagnosed before it is able to metastasize has a high success rate. Unfortunately, on average, 1 in 8 women in their lifetime will actually develop the invasive form of breast cancer, which leads to metastasis. Invasiveness indicates the ability of breast cancer cells to leave the site of origin by breaking down the basement membrane (barrier that separates tissues), allowing for movement of cells through the tissue and entrance into the neighboring blood vessel. Migration and spread of the cancer cells then occurs to other surrounding tissues and organs, and eventually to several parts of the body via the process of metastasis. In 2010 alone, 28% of the new cancer diagnoses were attributed to this particular form of invasive breast cancer (1). The individuals diagnosed with this advanced form of breast cancer that has reached distant sites and metastasized at the time of diagnosis have a mere 5-year survival rate of 25% (1). In contrast, the 5-year survival rate for localized breast cancer that has not spread is 98%, which highlights the importance of early detection (1). Within the United States, among cancer related deaths, breast cancer is now the second highest mortality factor in women, ranking just below lung cancer (1). Therefore, it is important to improve our understanding of this disease through research and increase the effectiveness of available therapies in combating metastatic breast cancer.

1.2 Steps of metastasis

The high mortality rate that is attributed to breast cancer stems from its metastatic potential. Thus, it is important to understand the cellular and molecular processes that underlie metastatic progression in order to design effective therapeutic strategies against it. It is necessary to be aware of the different targets and pathways that can be inhibited within this process, especially since these steps may be potential sites for multiple target therapy use. Cancer is a cellular disease, and carcinogenesis initiated by one transformed or mutated cell, that gets damaged by external or internal stimuli, thereby altering the normal function of the cell, into an uncontrollable state of growth.

Following these initial transformation events, the process of metastasis ensues and this metastatic process occurs via:

1. Attachment of the cancer cells to the basement membrane, the tissue barrier between the cells and other tissues or neighboring blood vessels
2. Breakdown of the basement membrane through the release of degrading enzymes
3. Invasion of the cancer cells into the bloodstream by a process called intravasation
4. Migration of the cancer cells throughout the body and arrest in secondary and tertiary organs
5. Continuous growth and division through microcolonization, and
6. Obtaining a blood supply via angiogenesis

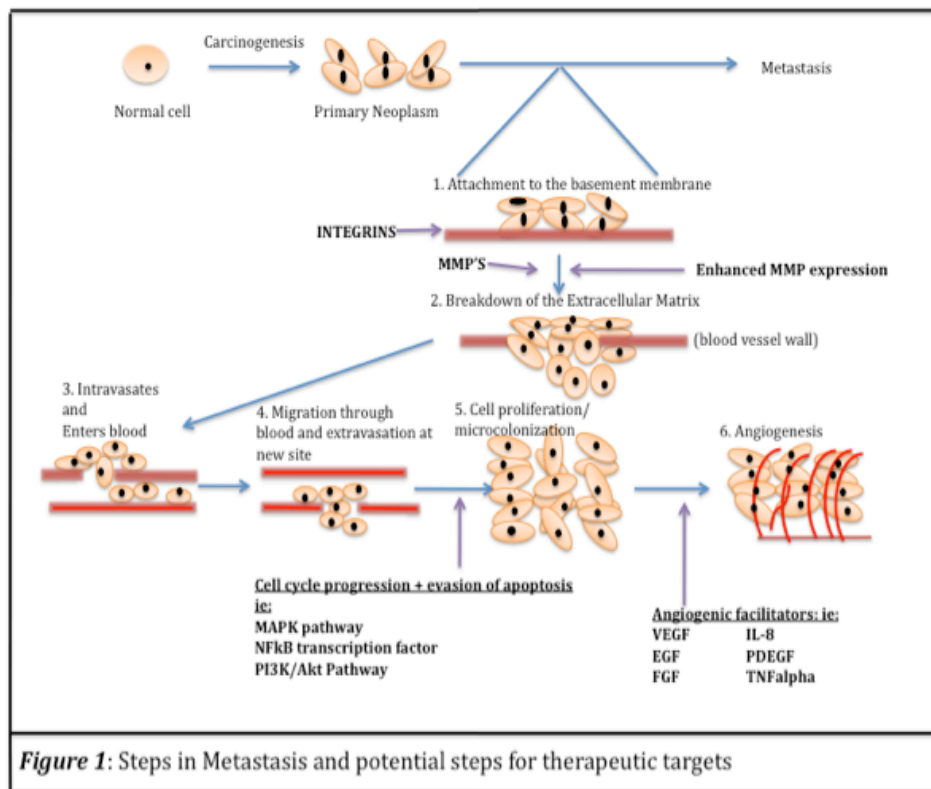
1.2.1 Attachment (step #1 in figure 1)

The process of migration from the primary tumor site begins when the cancer cells attach to the neighboring basement membrane (Fig 1). At this point, there are certain essential cellular functions that must occur for cell migration to take place. As shown in the figure above, attachment begins with a loss of adhesion between neighboring cells. This

reduction in adhesiveness occurs by the cancer cells losing several cell-to-cell binding proteins, such as e-cadherins (2). This reduces the connections and cohesiveness of cancer cells, allowing for detachment to happen, leading to an enhanced freedom for motility by these cells.

Next, increased expression of receptors present on the plasma membrane of cells is seen.

These receptor proteins, also known as integrins, are composed of both alpha and beta



subunits and have the ability to control cell-to-basement membrane adhesiveness (Fig 1) (2). Integrins can mediate attachment to fibronectins, laminins and

collagens found in the basement membrane. It is important to emphasize the basic structure of the integrins since specific alpha and beta subunits are more common in certain cancer states. The attachment to the basement membrane is mediated through the $\beta 1$ subunits of the integrins. For example, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins bind to certain types of collagen and laminin in the basement membrane, while integrins with the αV subunit attach to the fibronectins and vitronectins in the basement membrane. In breast cancer

certain integrins are up-regulated or down-regulated depending on their function (3). Integrins $\alpha 2$, $\alpha 5$, and $\beta 1$ have increased expression in breast carcinomas, which allows for enhanced invasiveness and metastatic potential. It has also been shown that $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins among others, are activated in metastasized breast cancer, and coincide with an increase in cell adhesion, migration and microcolonization (4). Other integrins are also linked to breast cancer aggressiveness and include; $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 6\beta 1$, $\alpha V\beta 1$ and $\alpha V\beta 5$. Upon binding and activation of integrins, invasion of the cancer cells can occur and increased cell motility effects can be seen by the promotional activation of downstream signaling molecules. An increased amount of integrins seen within the plasma membrane can be markers for breast cancer aggressiveness (2).

In summary, the cancers cells, now displaying decreased cell-to-cell adhesiveness, and have gained an increased ability to attach themselves to the basement membrane through these integrin receptors (2). This attachment between integrin and basement membrane proteins leads to the regulation and release of chemotactic molecules that aid in motility and proteases that aid in basement membrane degradation (5).

1.2.2 Breakdown of the basement membrane (step #2 in figure 1)

The cancer cells, now attached to the basement membrane, promote the release of matrix metalloproteases (MMPs) from tumor cells (2). MMPs are proteolytic enzymes, that when activated lead to the degradation of the basement membrane, thereby breaking open the barrier between the tissues (Fig 1) (2). MMPs are part of the urokinase plasminogen activator (uPA) system (6). This system works by allowing the MMPs to transition from inactive to their active form. Upon this change in expression, the enzymes degrade the basement membrane. (3).

Specific MMPs play a role in the breakdown of different areas of the basement membrane. They are classified into four main groups known as collagenases, gelatinases, stromelysins and membrane-type MMPs (7). Collagenases degrade different forms of collagen by using MMP-1, -2, and -8, while gelatinases degrade denatured collagen referred to as gelatin through MMP-2 and -9. Stromelysins degrade several basement membrane proteins including proteoglycans, laminin, fibronectin and include MMP-3, -11, -10 and -7 (8). The final group of membrane-type MMPs have a transmembrane domain and consists of MT1-MMP which degrade several basement membrane substrates (8).

Individuals with breast cancer have been shown to have enhanced expression of MMP-2 and MMP-9, which may lead to further activation of other MMPs within the tissue (8). These proteases promote tumor progression, and lead to metastasis through invasion, subsequently activating growth factors and promoting angiogenesis (8). Hence, the breakdown of the basement membrane by specific MMPs, such as MMP-9 and MMP-2 would be a therapeutic step that can be targeted for the inhibition of invasion. (Fig 1)

1.2.3 Invasion (step #3 in figure 1)

Once the basement membrane is compromised the cancer cells are motile and enter the blood vessel through a process called intravasation (Fig 1). The motility acquired occurs by actin polymerization, new actin filaments produced at the leading side of the cell allow for elongation of the cell to occur (9). Three main cytoskeleton fibers are present within the cell leading to its normal structure and they include microfilaments, microtubules, and intermediate filaments. In order for the for cell motility to occur, actin fibers reorganize and polymerize into the leading edge allowing the cell to move forward at the leading end of the cell (9). The actin structures reorganize during movement and the

different cellular structures, filopodia, lamellipodia, and focal adhesions, are extended from the surface of the cell allowing for attachment to the substrate and movement to ensue. The rear of the cell must then be able to retract forward through the myosin II and actin interactions that allow for the final movement seen in cell crawling (10). Once in the blood stream, these cancerous cells may encounter immunological factors of the infected individual, and some die off. Those cells that are best suited for metastasis survive the journey through the blood. This passage may be facilitated by the binding of these cells to coagulation factors such as tissue factor, fibrinogen, fibrin, and thrombin (11).

1.2.4 Migration and invasion (step #4 in figure 1)

Once the cancer cells reach the target site they exit the blood vessels at the capillary level, by a process called extravasation (Fig 1). The cancer cells then establish a suitable microenvironment for their growth and survival (11). Breast cancer cells have shown preferential distribution of cancer to certain sites like the liver, bone and lung since these are the first pass organs in the vascular system. (11). This preferential dissemination has been attributed to the production of chemoattractants, which are growth factors released by target sites that provide a conducive environment for the cancer cells to survive and grow (11).

1.2.5 Microcolonization (step #5 in figure 1)

Growth ensues once the cells find their secondary and tertiary sites, creating increased cell division in these tissues. Cancer cells acquire an increase in proliferative ability by activating certain cell pathways, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factors (TGF), insulin-like growth factor (IGF), tumor necrosis factor (TNF), and interleukins (IL)

(12). When growth factor expression is enhanced, for a number of different or abnormal reasons, as seen in cancer, an increase in proliferation and evasion of apoptosis is seen

(12). Inhibiting growth signal pathways would be a beneficial target for dietary agents, allowing the proliferative process to be halted and inducing apoptosis in the cancer cells.

There are several other key molecular targets for therapy to focus on including nuclear factor-kappa B (NF-kB). This particular transcription factor can be activated by an assortment of carcinogens as well as cytokines and inflammatory mediators among others (12). NF-kB can induce transcription of over 200 genes including those that aid in the metastatic process. For example, cyclin D1, a cell proliferation gene, can be expressed through NF-kB and subsequent activator protein-1 (AP-1) activation as well as adhesion molecules, and Bcl-2 which is anti-apoptotic (12). Other proteins may also be up regulated during the course of cancer. This may include the cell survival kinase Akt, which promotes cell survival through NF-kB activation as well as promoting anti apoptotic proteins.

Several other pathways may also be compromised including tumor suppressor gene and oncogene pathways (12). Some examples of these altered regulators of the cell cycle include p53, beta-catenin, and the Jak-stat pathway. The mitogen-activated protein kinase (MAPK) pathway, is thought to further advance cell growth and initiate growth factor activation important in metastasis (12). Cell adhesion molecules such as ICAM-1, VCAM, and ELAM have also shown enhanced activation during metastasis.

Increased activation of these regulators and promoters during breast cancer makes these proteins and genes essential targets in the microcolonization step in metastasis. Within this proliferative and cell survival step, migratory factors are noted for their help in movement of cells to secondary and tertiary sites. This movement is done mainly through

chemoattractants and chemokines, which direct the cell to particular sites of the body and allow for cell progression to occur. For example, the chemokine IL-8, upon binding with its receptor, is able to promote proliferation and angiogenesis of the cancer cells (12). But, cancer cells are only able to proliferate to a certain point before they must rely on this outside blood supply and nutrients to continue their growth and metastasis.

1.2.6 Angiogenesis (step #6 in figure 1)

The blood supply necessary for tumor growth occurs through angiogenesis. This process takes place when cancer cells release angiogenic factors that enable new growth of pre-existing blood vessels (13). Breast cancer cells release signaling molecules known as chemoattractants and angiogenic factors that are up regulated in cancer such as: vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), interleukin 8, platelet-derived endothelial growth factor (PDEGF), transforming growth factor alpha, and tumor necrosis factor alpha which direct the endothelial cells toward the cancer site or a specific area needing a blood supply (13). Receptors are present on the endothelial cells that facilitate binding between the chemoattractants and their receptors on the endothelial cells which leads to the induction of a signaling cascade and ultimately activated gene expression of the endothelial cells. Endothelial cell activation gives rise to multiple changes in gene expression inducing endothelial cell proliferation, releasing and activating matrix metalloproteases (MMPs), and endothelial cell migration to the cancer cells which reorganizes the dividing cells in the cytoskeleton via angiogenesis (13).

1.3 Compensatory vs. essential cell migration mechanisms

There are certain compensatory mechanisms that the cancer cells can trigger which allow them to overcome obstacles that may potentially block metastatic spread. The initial compensatory process cancer cells are able to overcome is the loss of e-cadherins, which is important in cell-cell adhesion. The loss of this adhesiveness may force single cell migration of the cancer cells to occur through the basement membrane. Before migration can occur, integrin-mediated attachment of the cells to the basement membrane must take place, as shown in figure 1 step number 1. These cancer cells have the ability to compensate for this integrin attachment through initiating different cell-matrix interactions, and/or utilizing additional integrins that may be in or around that same area, as well as promoting cytoskeletal shape changes of the actual cell itself (14). Normally, after integrin attachment occurs in step 1, release of MMPs ensues, causing proteolytic degradation of the basement membrane. If certain MMPs are inhibited through treatments or other cellular mechanisms interfering with their expression, cancer cells may potentially work to enlist the help of other available MMPs in the surrounding tissue. The cancer cells may also acquire the ability to completely evade the use of degradation enzymes by changing cellular cytoskeletal shape, that may allow for the cells to squeeze through the membrane in areas of the least resistance (14). Lastly, activation of chemotactic factors and cytokines, which help guide the cancer cells through the body, may also potentially be compensated for. This is accomplished by the cells when they retain specific cytoskeleton organization that leads to the maintenance of cell polarity and migratory movement via cytoskeletal proteins, regardless of the presence or absence of chemotactic factors (14). Hence, certain parts of the cell migration process may have the ability to be compensated

for by some cancer cells, but these mechanisms are still very important, as the most utilized routes of cancer cells for metastasis, and should not be overlooked in treating the disease.

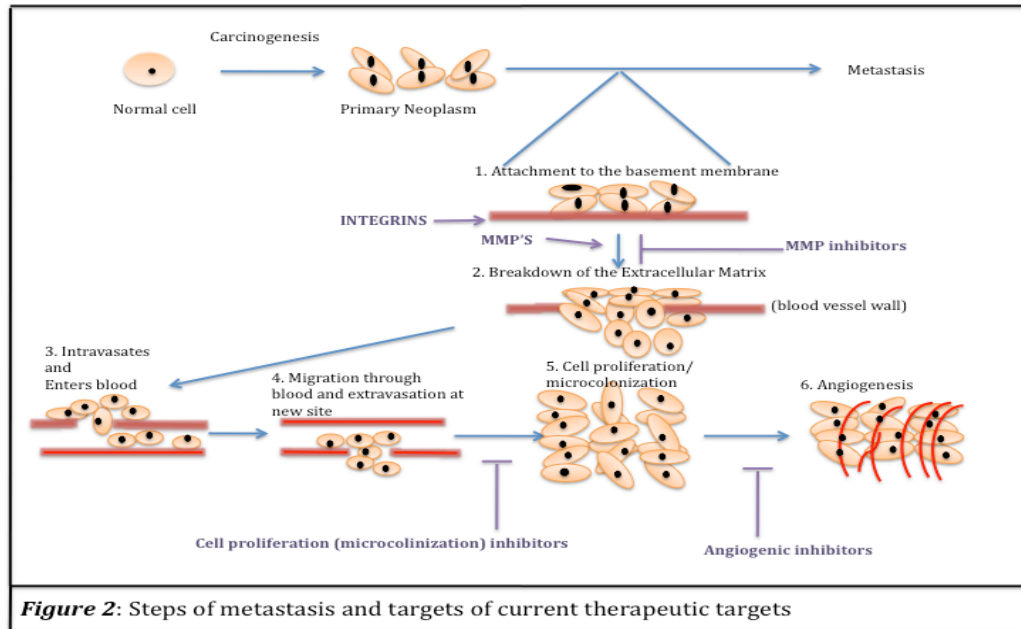
On the other hand, there are essential components of cell migration that cannot be compensated for by cancer cell machinery. Since the cells are capable of having enhanced movement, it is necessary that they have some sort of physical contact with the basement membrane in order to migrate through, and leave the original tissue (14). Once they have pushed or squeezed through the membrane, the cells need to be able to maintain polarity, which allows the cell to sense its surrounding environment. Once it is able to sense its surrounding environment, it proceeds to move in the direction of movement through actin filaments reorganization in the cell that promotes the formation of cellular protrusions. These cytoskeleton reorganization events occur through actin polymerization and depolymerization by the cell, utilizing the actin-myosin filaments. Consequently, cell motility may be impaired through the inhibition of actin polymerization by drugs including cytochalasin D, latrunculin and others that target myosin-II-activation of the actin filaments (14). A form of therapy that could target these essential functions would focus on the cytoskeleton and cell motility structures. It would also be useful to target cytokinesis as well as chemotaxis, the ability of the cells to move based on chemicals in their environment.

A beneficial form of therapy that can be examined would include targets within several specific steps of cancer cell migration, as discussed above, and inhibiting these targets might potentially have a broad range of inhibition than specific targeted therapies. Successful therapies for invasion and metastasis would need to focus on inhibiting both compensatory and essential, mechanisms in migration. Currently, there are only a few different therapy options available for treating those with the advanced form of breast

cancer. such as inhibitors of cell proliferation and angiogenesis, however, there are no therapies that target essential steps involved in cell migration.

A therapy that would include both metastasis and cell motility in its mode of action needs to be researched into as tumor cells have the ability to employ multiple pathways within metastasis. The use of these multiple routes allows the cancer cells to become resistant to radiotherapy, drug/hormone therapy and chemotherapy treatment, which is currently available for unmetastasized breast cancer patients.

1.4 Current therapies: benefits and inefficacies:



There are currently three main types of therapy available for those with metastasized breast

cancer including the use of matrix metalloprotease (MMP) inhibitors, angiogenic inhibitors, and inhibitors of cell microcolonization. Each form of therapy is able to target a specific step in the metastatic process.

1.4.1 MMP inhibitors

MMP inhibitors can target general MMP expression (Fig 2). Targeting proteases, allows for the inhibition of both attachment and break down of the basement membrane,

inhibiting invasion (Fig 2). MMP expression increases in malignancies, treatment to combat this increase is through the use of mitogen activated protein kinase (MAPK) inhibitors, which inhibit general MMP expression, leading to a decrease in cancer invasiveness. (15). Certain inhibitors in use include PD 166286, and SB 203560 (15). Antisense oligonucleotides are also utilized to target specific MMPs, slowing the progression of cancer (15). Potential therapeutic agents designed for metastasized breast cancer include the use of peptidomimetic MMP inhibitors, nonpeptidic MMP inhibitors, tetracycline derivatives and bisphosphonates (15).

1.4.2 Angiogenic inhibitors

Angiogenesis, as mentioned earlier, is the growth of new blood vessels from the endothelial cells lining the existing blood vessels (16). Typically angiogenesis during adulthood is activated during female reproduction, tissue and wound repair. In which case angiogenesis is tightly regulated to ensure a proper blood supply to the site of injury or in shedding of the endometrial lining monthly during the menstrual cycle. This fine regulation is controlled by the concentration of both activator and inhibitor molecules of angiogenesis (17). Typically when a tumor initiates angiogenesis it produces a greater amount of activators, while decreasing the amount of angiogenic inhibitors, allowing for endothelial cells to sprout and grow towards the tumor site (12). The tumor gains a blood supply alongside oxygen and nutrients, allowing it to continuously grow and metastasize. For this reason, inhibiting blood vessel growth is a key target for chemotherapeutic agents in patients with tumor growth, much like metastasized breast cancer individuals.

Angiogenic inhibitors combat activating expression produced by breast cancer cells. The inhibitors have a dual role, targeting angiogenesis both directly and indirectly (18).

The direct angiogenic inhibitors do not target the cancer cells directly, instead they stop the progression of cell growth, migration and induce apoptosis in the vascular endothelial cells (18). Current commercial drugs that target this process include vitaxin and angiostatin. Indirect angiogenic inhibitors, on the other hand, such as herceptin, prevent signaling proteins in cancer cells from activating angiogenesis, and/or blocking receptor expression (final step in figure 2). High therapeutic doses are used to ensure that an inhibitory effect on the metastatic cascade. Finally, synthetic inhibitors may be applied as a therapy and they work by blocking the signaling of the stimulators elicited through the cancer cells. Angiostatin, endostatin, thrombospondin, avastin are all examples of natural and synthetic inhibitors (13). Hence, agents that could block angiogenic signaling would be beneficial in slowing the progression of breast cancer. If this step is not inhibited, a blood supply is established, growth ensues, and the cancer cells look to expand their horizons yet again in a continuous metastatic process (Fig 2).

1.4.3 Microcolonization inhibitors

Invasive cells are able to acquire and use several signaling pathways, leading to continual progression of the cell cycle and evasion of apoptosis. Some signaling pathways important for metastatic progression include the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, the mitogen activated protein-kinase (MAPK), and Nuclear factor kappa Beta (NF- κ B) transcription factor (19). Although they are not the only ones recognized in advanced breast cancer metastases, they play a vital role in cancer progression. Current use of drugs such as gefitinib, may act as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI), inhibiting the activation and growth of epidermal growth factor expressing cells (19). Growth factors are a key target among others, including the down-

regulation of the MAPK pathway, leading to a decline proliferation and an increase in apoptosis.

1.4.4 Metastasis

All three therapies discussed above work by targeting important steps in the formation of breast metastases. Unfortunately, tumors formed during metastasized breast cancer are able to oppose the killing effects of drugs and procure a resistance to treatment (20). This resistance can be seen with increased breast cancer therapy use, leading to a greater resistance to form that can be applied to a large spectrum of drugs rather than an individual/specific drug.

These therapies act by eliciting apoptosis in cancer cells, but the cancer can evade this process by utilizing pathways that hinder cell death, allowing DNA damaged cells to resist destruction by numerous therapies (20). This can be seen in metastasized breast cancer, when the tumor shrinks with therapy use, but upon treatment completion, the remaining tumor cells replenish the cancer population. The use of multiple drugs at the most effective level is a good approach, but there are several problems that still arise in regards to toxicity, effectiveness and long-term usage with current treatments. Other forms of multiple target therapy must be investigated as such large portions of metastasized breast cancer patients have a low survival rate.

1.5 Gaps in research: multiple target therapy

In order to stop or slow the invasion process, therapeutic agents that have potential to combat cancers cell's ability to exploit multiple pathways at multiple steps need to be explored. It would be beneficial to look at a main environmental factor that already has an impact on cancer occurrence, the diet. In particular, it would be useful to investigate the

breast cancer fighting potential of both resveratrol and genistein, which are utilized in the diet. The research throughout this paper will focus on these two dietary compounds for their potential as multi-targeted therapeutic agents on invasion and metastasis. Current therapy options include use of inhibitors against angiogenesis, cell proliferation and MMPs; with each focusing on one single molecular target within metastasis. Thus, each current therapy works on a single target, focusing on only one step in the metastatic cascade. Implementing this type of therapeutic strategy enables the cancer cell to compensate by employing other signaling pathways/mechanisms for its growth and survival, thereby leading to the ineffectiveness of these therapeutic agents. Combining therapies together (such as genistein and resveratrol) to have a multiple targeted approach at inhibiting several steps in the metastatic process simultaneously is important and would be effective against metastasis. This would be achieved by targeting several pathways and mechanisms within the metastatic process, creating a better chance for treatment to inhibit the cancer cells. *Our research aims to investigate the use of dietary agents, resveratrol and genistein in effectively combating metastasis at multiple targets simultaneously.* The combinational effects of these two dietary agents could potentially lead to an increased multiplicity of inhibition of the several layers included in metastasis. In particular, the following cellular processes will be focused: MMPs, angiogenesis, cell proliferation, and cell motility. Overall, this thesis attempts to explore if these dietary agents can give an overarching coverage for metastasized breast cancer therapy through the individual's dietary intake or physiological concentrations of genistein and resveratrol, leading to the best possible outcome for the patient and their survival.

1.6 Advantages of using dietary agents

One of the advantages of using these dietary compounds are that they can be utilized at physiological concentrations, which is important for the patient and normal processes occurring in the body. These agents when combined may lead to an overarching multiplicity effect on the levels of metastasis.

Since these compounds are already consumed in the normal diet, they present an advantage over ingestion of other chemicals that might present toxicity effects. And, also, since these dietary compounds are utilized on a daily basis, they are widely and globally available and rather inexpensive. This is of particular importance due to the higher toxicity potential of certain therapies that becomes a problem with continuous use. Hence, these dietary compounds have the potential to be utilized for undesignated amounts of time and present none of the toxic effects and unwanted side effects that are normally seen in chemotherapy and radiotherapy.

The nature of these dietary compounds suggest that they could potentially act via a multi-targeted action, as discussed previously; multiple target therapies can inhibit more than one-step of the metastatic process at a time. For example, resveratrol has been shown to inhibit certain MMPs compromised in the degradation of the extracellular matrix, as well as acting as an angiogenic inhibitor, inhibiting multiple micro-colonization pathways, and halting cell motility (21). Hence, resveratrol targets multiple metastatic steps at one time, leading to a greater inhibition of breast cancer progression than a single target therapy. Treatment with more than one dietary agent, allows the two compounds to act on various steps in metastasis, leading to a new and possibly beneficial therapy option that works through the diet and may be utilized for prevention and treatment of metastasized breast cancer.

1.7 Hypothesis

Dietary compounds can utilize several pathways to cause cell cycle arrest, induce apoptosis in the cancer cells, act to inhibit angiogenesis, prevent the breakdown of the basement membrane, and increase cell motility, and thus would have tremendous potential in treating breast cancer. **The hypothesis of this thesis is that the two different dietary agents (genistein and resveratrol) could have the potential ability to inhibit metastasis at multiple steps and therefore could be more effective in treating cancer metastasis.** The approach taken for this thesis was to examine current literature and identify potential areas of overlap and differences in their mechanism of action on exploitation of pathways in metastasis. This could lead to a better-fit combinatory therapy to treat and prevent metastasized breast cancer via the diet. The effects of these two dietary agents will be examined on parameters employed by the cancer cells for angiogenesis, invasion and migration.

1.8 Focus of review

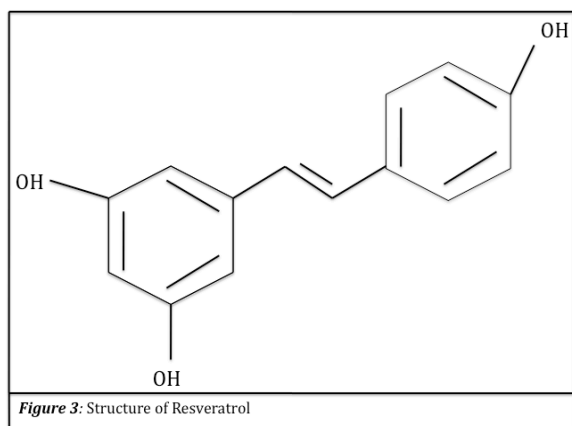
To view the role of genistein and resveratrol in invasion and metastasis the following factors must be examined and will be the focus of my investigation. As a starting point, the inhibition of matrix-metalloprotease expression, degrading enzymes, and integrins present in the basement membrane (step #1 and 2 in figure 1) was examined. The next step explored was the role of both compounds in down-regulating pathways on cell proliferation and microcolonization (step #5 in figure 1). Down-regulating these pathways could potentially also down-regulate the spread of cancer throughout the body. Inhibition of angiogenic growth factors and regulators (step #6 in figure 1), and finally, the effect of resveratrol and genistein on actin polymerization and myosin II activity that occurs during

cell migration (step #2, 3 and 4 in figure 1) was explored. Above all the other process, this final mechanism might be a critical factor in metastasis, as it involves an essential step that the cell cannot bypass, and hence may have an usefulness that could potentially extent beyond breast cancer alone.

The other main focus, that encompasses all of the above criteria, is to see if these compounds can exploit multiple targets/pathways in reducing metastasis at one time, and if combining these agents would lead to multiple levels being inhibited. Overall, this would lead to a higher multiplicity of inhibition as discussed earlier. The type of therapy to look for throughout this thesis is one that keeps all of these mechanisms and issues mentioned thus far into account. In order to examine these dietary compounds and their effects on invasive breast cancer, individual research on resveratrol and genistein must be explored in depth at each target of metastasis, giving the cellular and molecular components that are at work in helping with cancer.

2. Review of literature

2.1 Resveratrol



Resveratrol, a plant-derived polyphenol phytoestrogen (Fig 3), has been found within the skin of red grapes and other food products/plants including raspberries, blueberries, red wine, peanuts and certain types of pines (22). It has also been produced,

to a lesser extent, as an antimicrobial agent by certain plants that have been under pathogenic attack (22).

Resveratrol was widely tested and studied and showed several health benefits (heart, neurological, liver, lungs, etc.) and has displayed a pertinent role in several age-related illnesses including diabetes, arthritis, neurodegeneration and pulmonary disease (23). Resveratrol's effects on aging-relating conditions appears to be through the activation of certain sirtuin NAD⁺-dependent proteins (24). Sirtuin NAD⁺-dependent proteins are known protein deacetylases that regulate transcription of genes relevant for aging related processes, inhibiting apoptosis and cell cycle progression (24).

Resveratrol's effects on cardiovascular health has been demonstrated in a phenomenon called the "French paradox" (25). It was noticed that the French population had a lower incidence of cardiovascular disease even though they consumed a high-fat diet. Studies have shown that this could be due to the increased intake of red wines high in resveratrol, leading to cardio protective qualities including anti-platelet aggregation, and vasorelaxation (26). Resveratrol has also been shown to have beneficial cancer fighting properties such as cell cycle inhibition, inhibition of DNA synthesis through reducing DNA polymerase and ribonucleotide reductase activity (22, 27, 28), pro-apoptotic actions, cell proliferation inhibition, as well as inhibition of angiogenesis, inflammation, adhesion-related invasion and progression of metastasis (24).

Resveratrol was investigated in a number of different studies to explore its role in the invasion of breast cancer cells by influencing MMPs, signaling pathways, and angiogenesis which led to a decreased invasive potential and metastatic spread in breast cancer phenotypes. In animal models it has been noted that resveratrol is quickly

metabolized by the liver, while in humans who consumed 25mg of resveratrol, only 70% was absorbed into the blood through the action of carrier molecules. The absorbed resveratrol displayed a half-life of approximately 10 hours, reaching maximal plasma levels near 2 μ M (29). These next few subsections examined the beneficial role of resveratrol in inhibiting the growth and promotion of metastasized breast cancer via inhibition of MMPs, cell microcolonization pathways, angiogenesis and cell motility.

2.1.1 Resveratrol and MMP inhibition

Several studies have looked at the possible MMP inhibition by resveratrol in breast cancer cells. The findings of extensive breast cancer research suggest an up-regulation of the gelatinases, MMP-2 and MMP-9 in these cells. These particular MMPs, as discussed earlier, are important in degrading collagen IV in the basement membrane. When over-expression of MMP-2 and MMP-9 occurred, as displayed in several cancer states, a greater invasive potential of the cancer cells was observed.

Resveratrol's effects on MMPs were studied in multiple cancers, and have shown agreement thus far on the beneficial role of resveratrol on inhibiting the invasive stage of cancer. Studies included breast cancer trials, where estrogen receptor positive (ER+) individuals had an increased likelihood of over-producing human epidermal growth factor receptor 2 (HER-2), a known transmembrane tyrosine kinase (30). When up regulated, HER-2 led to greater MMP-9 expression, and increased invasive potential of tumors through its ability to activate multiple signaling pathways in the cell (30). This growth factor receptor, among others, including HER-2, -3, and -4, were activated by heregulin- β 1 (HRG- β 1), a growth factor that is expressed in around 1/3 of metastasized breast cancer patients (30).

Tang and colleagues utilized MCF-7 cell lines, which are estrogen receptor positive human breast adenocarcinoma cells, and examined resveratrol's effects on MMP-9 expression through modulation of HER-2 (30). The cell lines received low concentrations of resveratrol (2, 5 and 10 μ M), which were slightly higher than physiological concentrations (100 pM to under 2 μ M). Resveratrol treatment displayed a decrease in HRG- β 1 induced MMP-9 expression (23, 27). The decreased expression of MMP-9 was mediated through inhibition of ERK1/2 activation within the MAPK cascade, which reduced MMP-9 expression and mediated breast cancer cell invasion (30). The MAPK pathway, a cell proliferative signaling cascade, was shown to lead to the activation or inactivation of several transcription factors related to various metastatic processes. In breast cancer, any defect or mutation in the MAPK pathway has been shown to lead to increased invasion, among other processes. In the absence of resveratrol on the MCF-7 cell lines, the kinase pathway was activated and promoted expression of the growth factor, HRG- β 1, which led to subsequent HER-2 mediated MMP-9 expression (similar in most cancer states) (30). Upon resveratrol treatment, HER-2 activation was not induced, inhibiting downstream signaling, including repressing MMP-9 expression, which inhibited invasion.

HRG- β 1, being down regulated upon resveratrol use, also led to decreased activation of Akt of the PI3K/Akt pathway (23, 27). In the context of metastasis, Akt activation normally promoted cell proliferation by enabling cell survival (as discussed in greater detail in the subsequent sections). Hence, resveratrol treatment on breast cancer cells exhibited a subdued invasive phenotype, through inhibited MMP-9 expression. Further *in vitro* studies performed on MCF-7 cell lines agreed with these findings even though this cell line was non-aggressive it did show targeting of MMPs. Demonstrating that

after cancer cells were administered varying concentrations of resveratrol (10, 25, and 50 μ M), they displayed a dose-dependent decrease in MMP-9 expression (31).

Similarly, Banerjee et al, through an *in vivo* animal study on breast cancer, further evaluated resveratrol's effects on NF- κ B, cyclooxygenase-2 (COX-2) and subsequent MMP-9 expression (31). Female Sprague dawley rats containing 7,12-Dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors were used for this study. DMBA is a carcinogen that causes mutations to occur leading to tumor progression. The results showed that rats fed 100 μ g/rat resveratrol over a minimal period of 120 days displayed decreased expression of MMP-9 suggesting that resveratrol was effective in reducing tumor incidence, development, and growth in Sprague Dawley female rats (28).

It is important to understand the mechanism through which resveratrol down-regulated MMP expression. One such factor, NF- κ B, a transcription factor, played an important role in promoting breast cancer metastasis by activating the transcription of several genes, including inducing expression of MMP-9 and Cyclooxygenase 2 (COX-2). COX-2 is an enzyme expressed normally during inflammation, but was up regulated in breast cancer and allowed progression of tumor metastases. NF- κ B activation induced transcription of the MMP-9 gene and subsequent MMP-9 expression, which was upregulated in disease/cancer states (32). Treatment with resveratrol has demonstrated down-regulation of NF- κ B, which has led to inhibition of MMP-9 activation, and consequently decreased its expression and invasive potential (33).

MMP-2 and MMP-9 (gelatinases) are the two main MMPs up regulated in breast carcinomas, and target the denaturation of collagen in the basement membrane in order to invade into the neighboring tissues by entering the blood stream. It is also important to

mention the effects of resveratrol on MMP-2. Resveratrol showed inhibition of MMP-2 expression, reducing breakdown of the basement membrane through this protease as well.

Resveratrol effects on MMP-2 expression have been documented in multiple studies discussed in the following section. In estrogen receptor negative breast cancer cells a specific hormone, insulin-like growth factor 1 (IGF-1), was needed for the metastatic spread of cancer cells (34). In certain cancers, IGF-1, when activated, displayed enhanced protease expression, and increased invasiveness of cancer cells (34-36). IGF-1 activated and induced both MMP-2 and MMP-9 expression, the two MMPs up regulated in metastasis of breast carcinomas, as discussed (31). It was also documented that IGF-1 expression in prostate carcinoma cells, stimulated MMP-2 and MMP-9 expression through different signaling routes (31).

Tang and colleagues investigated IGF-1's role on MMP-2 expression in breast cancer progression. Human breast cancer cell lines, MDA-MB 435, treated with resveratrol showed a dose-dependent decrease in IGF-1 induced MMP-2 expression, maximal inhibition occurred at 20 μ M via down-regulation of the PI3K/Akt pathway (34), thereby reducing the invasive potential of these breast cancer cells.

MMP-2 expression was also explored in an animal *in vivo* model. Nude mice were transfected with heptoma cancer cells (liver cancer) (37). 50 and 100 μ g/kg of resveratrol were administered over 3 times per week after implantation of the tumor cells in the mice (34). The results suggested that resveratrol caused a dose-dependent decrease of MMP-2 expression, similar to the results experienced in MDA-MB-435 cell lines. It was hypothesized that the mechanism by which resveratrol promoted these effects, occurred

through modulation of NF- κ B expression and hence, down-regulated MMP-2 in the heptoma cells of the nude mice (32).

MMP-2 inhibition was further demonstrated by Gagliano and his team when they investigated resveratrol's effects on glioblastoma cells (38). Glioblastoma cell lines, highly metastatic brain tumors coupled with a very poor prognosis, were tested (T60, T63 and GBM) and administered resveratrol concentrations between 1 and 50 μ M (35). The data was analyzed on MMP-2 expression and secreted protein acidic and rich in cysteine (SPARC) expression. SPARC, a glycoprotein was evaluated because it had the capability of inducing expression of MMP-2 and MMP-9 and mediating cell-matrix interactions in the basement membrane (35).

MMP-2 mRNA levels decreased in a dose-dependent manner after 72 hours of resveratrol administration (between 1 and 50 μ M concentrations) (35). The SPARC gene protein expression levels decreased as well following 72-hour treatment with resveratrol (35). The down-regulation of MMP-2 expression that occurred was attributed to the inhibition of NF- κ B activation (33). Thus, an extensive literature review, supported resveratrol's influential role on inhibiting the breakdown of the basement membrane and inhibiting the invasion of cancer cells through MMP-2 and MMP-9.

Literature reports throughout this section revealed resveratrol's inhibitory properties regarding over activation and initiation of proteases, glycoproteins, transcription factors like NF- κ B, and signaling pathways that help to modulate proteolytic breakdown of the basement membrane (24-33). The down regulation of MMPs (MMP-9 and MMP-2) was essential in causing a reduction of the invasive phenotype of cancer cells. The cellular mechanisms involved in invasion included up regulation or down regulation of

several signal transduction pathways compromised within metastasis. Some of these have been mentioned above, but will be covered in depth in the following cell microcolonization section.

2.1.2 Resveratrol and cell microcolonization inhibition

Prior research demonstrated that various cell proliferative and survival signaling pathways were important components of microcolonization, and were altered by resveratrol. These cellular behaviors are important for the establishment of micro metastases. This section thoroughly examined those pathways that resveratrol influences, beginning with cell proliferation, cell cycle proteins, and finally apoptosis.

2.1.2.1 Resveratrol and cell proliferation

Resveratrol inhibited cancer cell proliferation through regulation of various transcription factors including NF-kB, AP-1, and modulated estrogen receptors (ER) expression. These transcription factors have been either shown to be affected by, as well as cause effects on, various factors and stimuli, such as oncogenes, tumor suppressor genes, tumor necrosis factor (TNF), p53, NO synthase, MMP-9, adhesion molecules, COX-2, c-MYC, MAPK, reactive oxygen species (ROS), and the PI3K/Akt/mTOR pathway.

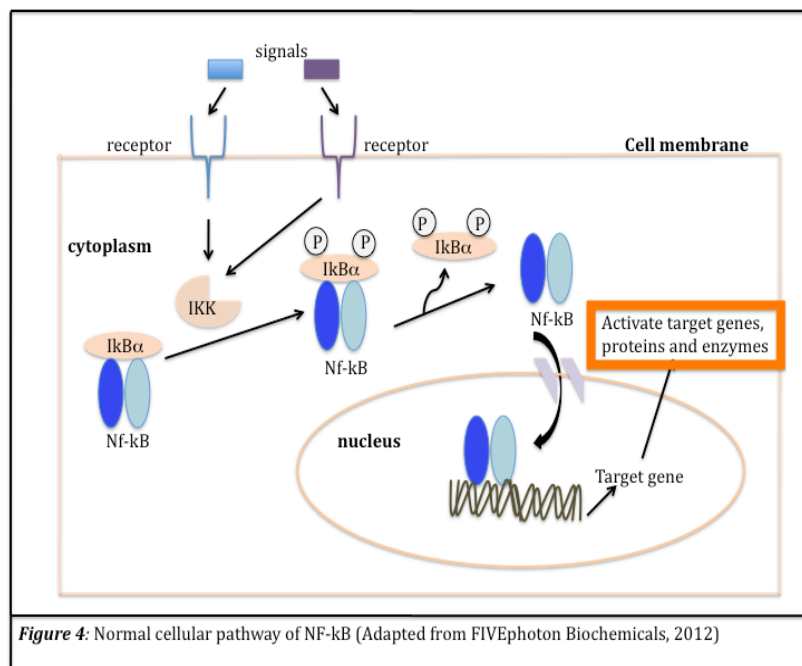
Research has explored resveratrol's role on these pathways and mechanisms in cancer. Studies displayed some of resveratrol's actions occurring through the ER, due to its similar structure to estrogen. This similarity to estrogen structure causes resveratrol to bind to the estrogen receptor (ER), and inhibit cellular transcription causing a decreased activation of gene products that aided in growth (25).

When estrogen was present in the system, resveratrol displayed an increased binding to the ER in prostate cancer cell lines (LNCaP androgen sensitive cell lines) which

allowed the compound to act as both an ER agonist (activated the receptor like estrogen would) at lower concentrations of 10 and 50 μM and antagonist (inhibited activation of the receptor) with increased concentrations of greater than 50 μM (39). When resveratrol displayed agonistic properties it still allowed proliferation to occur, but when it acted as antagonist, proliferation was inhibited. Resveratrol acted as an ER agonist/antagonist in ER positive breast cancer cell lines additionally (40-43). The concentrations that caused both the agonistic and antagonistic effects varied between the different breast cancer lines. The agonistic effects for a variety of breast cancer cell lines (T47D, MCF-7) were exhibited at resveratrol concentrations of 10-25 μM , while antiestrogenic effects occurred at concentrations greater than 25 μM resveratrol (42). ER-negative cell lines (MCF-10, MDA-MB-231 and MDA-MB-435) also displayed dose-dependent anti-proliferative effects upon resveratrol treatment (40-44). Thus, resveratrol exerted biphasic effects on the cell cycle (for proliferation) in a time and dose-dependent manner, based on the form of receptor present (androgen or estrogen) (39). By mimicking estrogen, resveratrol reduced breast cancer growth by inhibiting activation of the necessary transcription factors that led to proliferation in metastasized cancers (25).

Another important transcription factor resveratrol regulated was NF- κB . NF- κB is involved in regulation of several cellular processes (22, 45). In its normal state, NF- κB was activated through a variety of signals that included oncogenes (i.e. Ras and Bcr-Abl), growth factors, and kinases (i.e. Akt, p38), mitogens, cytokines, UV, ionizing radiation, bacterial toxins, TNF, ROS, lipopolysaccharides (LPS), H₂O₂, okadaic acid and ceramide (Fig 4) (22, 46). When the stimuli were bound to their receptors in the cell membrane, extracellular signals caused activation of the enzyme inhibitor of kappa B kinase (IKK). NF-

kB, which was present in the cytosol, was inactive due to the formed complex with the



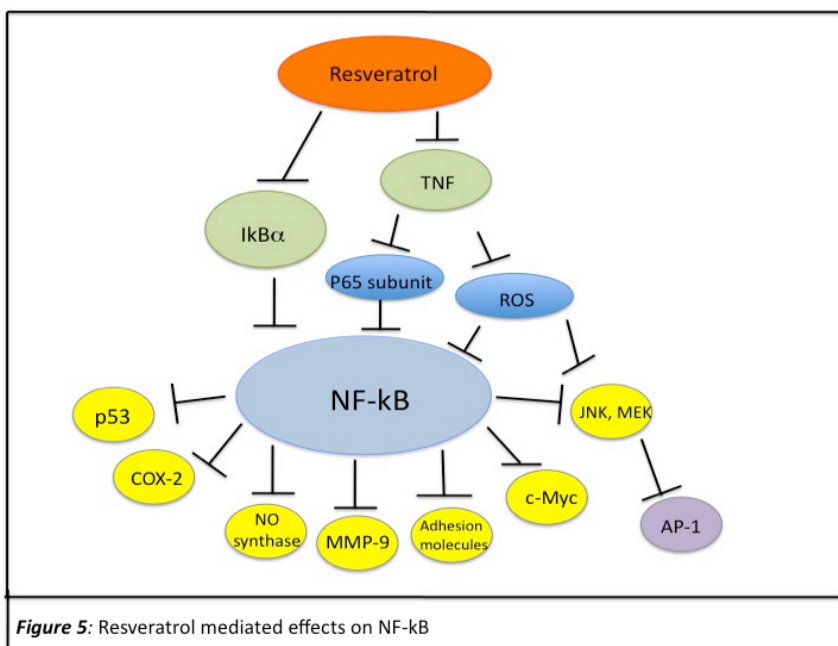
inhibitor of kappa B alpha (IκBα) protein. When IKK was activated, phosphorylation of IκBα occurred and was subsequently degraded, which allowed NF-κB to enter the nucleus of the cell. Within the nucleus, NF-κB initiated

transcription and translation of certain genes that altered several cellular functions, which included proliferation of the cell (Fig 4) (22, 47).

NF-κB was constitutently activated in several cancers, which led to enhanced gene expression of proteins and enzymes implicated in tumor proliferation, and metastasis (22, 32). Research on resveratrol use in multiple cancers, has displayed a reduction in NF-κB activation, which has led to decreased proliferation of cancer cells through the modulation of genes (or promoters) that NF-κB normally activated (IL-6, Bcl-2, Bcl-xL, XIAP, c-IAP, VEGF, and MMP-9) (Fig 5) (47). The stimuli (as mentioned previously) that caused increased NF-κB expression and were present in metastasized forms of cancer included; oncogenes such as Bcr-Abl, Ras, growth factors, and kinases (Akt, p38, MAPK), were implicated by resveratrol consumption (22, 28).

Resveratrol acted through multiple routes that decreased NF-κB expression in cancer. Resveratrol targeted the promotional stage of cancer which occurred through

activated TNF, phorbol ester and okadaic acid (46). TNF was a cytokine and inflammatory mediator, which induced cell proliferation through activating NF-kB (46, 48). This occurred because TNF produced reactive oxygen species (ROS) and caused lipid peroxidation, which allowed protein kinases associated within the MAPK pathway to be activated, which promoted expression of transcription factors like NF-kB and downstream AP-1 (facilitator



of tumorigenesis) (46). It was demonstrated in TNF deficient mice models, that TNF was necessary for tumor growth (46, 49) (Fig 5). Resveratrol demonstrated inhibition of TNF activated NF-kB expression in an *in vitro*

experiment performed by Manna and colleagues (46). Resveratrol concentrations from 1 to 25 μM were used on U-937 myeloid cell lines, Jurkat lymphoid cell lines, HeLa epithelial cell lines and H4 glioma cell lines (46). The mechanism through which resveratrol decreased NF-kB activation was contrary to what had been seen in other phytochemicals, resveratrol failed to block IκBα phosphorylation, so it was not degraded in the NF-kB pathway (46, 50, 51). Resveratrol prevented the phosphorylation and binding of the p65 NF-kB subunit, which decreased transcription and subsequent translation that was normally induced via TNF activation (46, 51).

Resveratrol also demonstrated inhibition of the transcription factor AP-1 through TNF. AP-1 was inhibited by reduced kinase activity associated with the MAPK pathway, which included JNK and MEK, which also reduced ROS production. The TNF-mediated events were inhibited by 90% upon 5 μ M use of resveratrol, it was inhibited maximally 4 hours and on after use (which is comparable to the 25 μ M administered to the skin of rats during *in vivo* studies, and equivalent to physiological concentrations obtained from the diet) (46). Hence, resveratrol demonstrated its importance in metastasis through its repression of TNF-mediated NF-kB gene expression, which was crucial for activation of several genes, proteins and enzymes in cancer (Fig 5) (31, 46, 52, 53).

Another essential pathway that was compromised in metastatic cancers was the PI3K/Akt pathway, which included the tumor suppressor protein, PTEN. Under normal conditions this pathway was activated via growth factors and other stimuli that induced MAPK pathway activation. Activated PI3K phosphorylated membrane-bound PIP2, which gets converted into PIP3. This step (conversion of PIP2 to PIP3) is regulated by a tumor suppressor protein that functions to keep the concentrations of PIP2 and PIP3 balanced for the cell. In cancer, increased concentrations of PIP3 were seen which activated the protein kinase Akt, causing minimal cell cycle arrest and consequently, proliferation (54). This pathway also activated an array of downstream cell proliferative proteins such as glycogen synthase kinase 3 (GSK3) and mammalian target of rapamycin (mTOR).

The PI3K/Akt/mTOR pathway is overactive in cancer, usually from a faulty or mutated PTEN protein. When PTEN was absent, the cell cycle progressed continuously and failed to respond to any external regulatory signals (34). Hence, mutations in this pathway have been observed in many cancers, including breast cancer. For example, in estrogen

receptor negative breast cancer cells, IGF-1, a growth factor that was necessary for the metastatic cells to spread and induced cell migratory events, acted through the PI3K/Akt pathway (34). IGF-1, when bound to its receptor, IGF-1R, caused activation of the PI3K/Akt pathway, which allowed the cell cycle to progress from G1 to the S phase, causing DNA synthesis (55). An *in-vitro* study on resveratrol's effects on the IGF-1 stimulated proliferation of MDA-MB 435 ER negative breast cancer cells was investigated by Tang and colleagues (34). Resveratrol concentrations (10 and 20 μ M) were administered to the IGF-1 (10 ng/mL) cell lines. It was demonstrated that in the presence of IGF-1 alone, MDA-MB 435 cells had increased Akt activation and proliferation that occurred through an ER independent mechanism (34). When resveratrol was added, the IGF-1 mediated activation of Akt was effectively inhibited by 70% at 20 μ M of resveratrol (34). The observed results displayed resveratrol's ability to down-regulate the PI-3K/Akt signaling pathway effectively, which caused decreased cell cycle progression in ER negative cell lines. But, resveratrol also displayed these effects on estrogen receptor positive breast cancer cell lines.

In MCF-7 breast cancer cells, PI3K activity was analyzed in the presence of resveratrol (10 to 150 μ M), and shown to have a biphasic effect on ER positive cells (54). Resveratrol demonstrated greater PI3K activity at lower concentrations, and diminished activity at increasing concentration levels (54). Upon resveratrol being administered, ER α regulated genes were also inhibited in a non-biphasic pattern, which showed that resveratrol still inhibited cell proliferation of MCF-7 cells through the PI3K pathway.

Activated PI3K/Akt pathway has also led to the activation of the serine/threonine protein kinase mammalian target of rapamycin (mTOR), which was part of the PI3K

protein family and regulated cell proliferation (56). In U251 glioma cells, resveratrol displayed inhibited PI3K/Akt/mTOR pathway activation after 100 μ M use over 24 hours (56). This was also examined in other forms of cancers, including gastric, breast, pancreatic, thyroid and ovarian cancer cells, where Akt1 or Akt2 (or both) were up regulated and over expressed, and resveratrol use reduced their activation and inhibited proliferation (57-59).

Therefore, resveratrol's extensive influence over this pathway has been documented, and has potential to treat metastasized forms of cancer, including breast cancer.

Resveratrol has demonstrated preliminary effects on other proliferative pathways, which included the Wnt/ β -catenin pathway. Studies on colon cancer cells examined the effect of resveratrol on IGF-1's influence on activation of the Wnt/ β -catenin pathway, which increased colon cancer cell production in cancer (55). Resveratrol demonstrated decreased proliferation at 100-150 μ M concentrations, which was done through inhibited IGF-1R, which caused a reduction in the phosphorylation and subsequent activation of β -catenin, decreasing transcription in the Wnt pathway. At lower concentrations (50 μ M) there was no significant effect on the pathway, and proliferation remained similar to normal HT-29 cell lines (55).

These results indicate that resveratrol repressed pathways and mechanisms that promoted proliferation its specific targets include transcription factors and extensive pathways (ER, NF-kB, PI3K/Akt, mTOR and PTEN), which play an important role in cellular growth. Resveratrol supplementation could be a useful therapeutic tool, as it targeted many key regulators in the growth process.

2.1.2.2 Resveratrol and cell cycle proteins

Another important part of the microcolonization process was the cell cycle, which is controlled via several cell cycle regulators. These include various cdks, cyclins, oncogenes, and c-MYC. The modulators of the cell cycle are regulatory cyclin subunits (cyclin A, B, Ds or E) that interact with cyclin-dependent kinases (Cdk 1, 2, 4 or 6) that allow the cell to progress through G1, G2, M and S phases of the cell cycle. Cell cycle regulation can also occur through inhibitor proteins, which include p21WAF1, and p27KIP1 (22, 60, 61). Activators of the cell cycle such as cyclin D1, were over expressed in certain cancers allowing for continuous progression through the cell cycle. Notably, cyclin D1 revealed maximal cell cycle inhibition by resveratrol at 100 μ M concentrations in U251 glioma cells after a treatment period of 24 hours (56). The cdk-cyclin complex has been shown to bind to and activate the retinoblastoma protein (Rb), allowing for the transcription of genes that facilitate the progression of the cell from G1 to S phase (22).

Resveratrol also demonstrated repression of the cell cycle by modulating the cdk-cyclin activity in MCF-7 breast cancer cell line (62). Proliferation was inhibited at the G1/S and G2/M checkpoints following resveratrol use in prostate cancer cells (39). Resveratrol's mechanism of action appears to be regulating the activation of p53. p53 is a tumor suppressor protein, referred to as “the guardian of the genome” which, when activated, mediates p21 expression which inhibits cdk-cyclins that subsequently cause cell cycle arrest (25, 39). p53 is typically activated under stressful conditions by an array of protein kinases, which include the MAPK pathway (JNK, ERK, p38, MAPK) that lead to the expression of growth inhibiting genes including p21WAF1, p300/CBP, APAF1, and Bak (22, 63).

Resveratrol's effects on p53 expression was also investigated by Zhou and his team who worked with pancreatic cancer cell lines capan-1, capan-2, colo357, miapaca-2, and bxp-3 (22, 64). Various concentrations of resveratrol were used this study (20, 50, 100 and 200 μ M), and upon treatment, p53 was up regulated by increased ATM phosphorylation, causing inhibition of cancer cell proliferation (22, 64, 65). Resveratrol also demonstrated MAPK activation, maximally seen at 200 μ M concentrations, which led to downstream up-regulation of p21 (through p53), causing cell cycle arrest (64).

This Ras-MAPK dependent activation of p53-induced cell cycle arrest that was discussed above was also observed in both papillary and follicular thyroid carcinoma (22, 66). When activated by resveratrol, p53 induced p21 activation, which inhibited cdk-cyclins that caused G2 arrest. It was noted that p53-independent p21 activation and cell cycle arrest also occurred upon resveratrol use, thus resveratrol worked through both p53 dependent and independent pathways (65, 67).

Resveratrol also induced S phase arrest; by targeting those cancers that over expressed cellular MYC (c-MYC) (22, 68). Resveratrol targeted and inhibited expression of Cdk1, which downregulated survivin, a protein necessary for survival of over expressed c-MYC tissues, and this led to cell cycle arrest (22, 68, 69).

Resveratrol regulated c-MYC-inhibition can cause cell cycle arrest as observed in *in vitro* studies as well as *in vivo* studies in mouse lymphoma and hepatoblastoma carcinomas (22, 68). A study on medulloblastoma cell lines (UW228-2 and UW228-3) that expressed c-MYC displayed these similar effects upon resveratrol treatment. S phase was arrested and observed in over 50% of the cells after 100 μ M concentrations of resveratrol were utilized for a period of 24 hours, while 0% of the cells exhibited movement through the G2/M phase

at that time point (69). Hence, resveratrol demonstrated reduced transcription of c-MYC in cancer cells that led to cell cycle arrest. The research presented in this section, demonstrated how several cell cycle regulators were compromised in different carcinomas, and the effects of resveratrol on exerting anti-carcinogenic effects.

2.1.2.3 Resveratrol and apoptosis

Resveratrol inhibited proliferation and cell cycle proteins, as documented from the research above, but it also promoted apoptosis of cancer cells as discussed within this section. Apoptosis, or cellular death, is evaded by cancer cells, through exploitation of several signaling mechanisms (Fig 6). Several studies have shown that apoptosis, a complex process, was induced with resveratrol supplementation.

Resveratrol activated both caspase 2 and casapase 8 in the apoptotic caspase cascade, which initiated the programmed cell death process. When caspase 2 was activated, it caused a conformational change in pro-apoptotic proteins like Bax and Bak and Bid (70), altering the permeability of the mitochondrial membrane, leading to the release of mitochondrial proteins such as cytochrome c in the cytoplasm, that form the apoptosome. The apoptosome structure consists of cytochrome c, APAF-1 and caspase 9 proteins (22, 71). Subsequently, this leads to activation of caspase 9, followed by activation of caspase 3, 6 and 7. Caspase 3 activation executes apoptosis (72, 73). The second mitochondria-derived activator of caspase (SMAC), a protein, that when present, binds to inhibitor of apoptosis proteins (IAP) and causes its deactivation, resulting in cytochrome c-dependent apoptosis (71). Anti-apoptotic proteins, such as Bcl2 inhibit cytochrome c release, while pro apoptotic proteins such as Bax and Bid (previously mentioned), relocate into the mitochondria and induce cell death (74). Activation of the mitochondria is also believed to

initiate activation of caspase-independent death effectors, apoptosis inducing factor (AIF) and endonuclease G, which aid in cellular death (70).

Apoptosis can also be mediated by caspase 8, which signals through membrane-bound death receptors, and is not dependent on cytochrome c release (75, 76). This mitochondria-independent route, where activated caspase-2 also triggers caspase 8 activation, followed by caspase 3 activation leading to apoptosis. In a diseased or cancer state, overexpression of antiapoptotic proteins like Bcl-2 and Bcl-xL were seen, and subsequently cytochrome c release was inhibited (73). Inhibition of both proapoptotic proteins and reduced cytochrome c release led to drug resistant forms of several carcinomas (73). Resveratrol induced apoptosis in cancer via both mitochondrial pathways and to a lesser extent, non-mitochondrial pathways (Fig 6) (62, 70, 76).

Apoptosis is a key component for therapies to target in cancer. Resveratrol brought about this apoptotic effect in prostate cancer cell lines DU-145 (10-100 $\mu\text{g/mL}$), as well as leukemia cell lines (50 μM) through caspase-9 activation (77, 78).

Both the intrinsic (mitochondrial) route and extrinsic (mitochondrial-independent) route of apoptosis were effective in causing cellular death in cancer cells, yet research shows that the pathways and proteins that activate these two routes are separate or there is cross-talk between the two. Benitez and colleagues investigated resveratrol's apoptotic action on prostate cancer lines, and observed that prostate cancer cell lines over expressed estrogen receptors, and lacked expression of androgen receptors in highly metastasized forms of the disease. Hence, they utilized PZ-HPV-7 non-tumorigenic cell lines, LNCaP androgen sensitive cell lines and PC-3 androgen insensitive cell lines (39). Concentrations of over 100 μM (including 150 μM) resveratrol induced apoptosis (39). The mechanism of

apoptotic action was due to the altered Bax/Bcl-2 ratio. Resveratrol caused increased Bax accumulation, and enhanced caspase 9 activation with subsequent caspase 3 induced apoptosis in both LNCaP and PC-3 cell lines (39). Of the prostate cancer cell lines tested, the LNCaP (androgen sensitive) cell line was most sensitive to the effect of resveratrol. Thus, the results suggested that resveratrol caused apoptosis to occur via the intrinsic (mitochondrial) route, which included activation of caspase 3 and caspase 9 (39); and to a lesser extent, through the extrinsic pathway, which included caspase-8 that was also activated slightly in the PC-3 (androgen insensitive) cell line, and could be involved in prostate cancer cell apoptosis.

This same mitochondrial intrinsic apoptotic pathway, activated by resveratrol in the prostate cancer cell lines, was also seen in other cell lines. This was observed when resveratrol supplementation of 100 μ M and above, increased apoptosis in a time-dependent manner in Y79 retinoblastoma cells (79). Activated apoptosis occurred via several mechanisms in different cancers, as noted previously. Similar to the prostate cancer cell lines above that were androgen sensitive, estrogen sensitive MCF-7 breast cancer cells also displayed a marked increase in apoptosis due to resveratrol administration. The MCF-7 cells induced apoptosis through a non-caspase dependent route (39, 62). At less than 50 μ M concentrations of resveratrol, MCF-7 cells activated cell cycle regulators, causing increased expression of p27, p53 and p21, which led to induction of apoptosis without direct caspase activation (62). This suggested that the presence or absence of estrogen, or androgens in the case of prostate cells, could have an effect on the type of apoptotic mechanism resveratrol utilized. It was proposed that those cells expressing steroid hormone receptors could be capable of inducing apoptosis with resveratrol as well (62).

However, MDA-MB-231 cells that were treated with resveratrol at higher concentrations, 200 μ M, failed to enhance tumor suppressor proteins p27, p53 and p21, and the apoptotic cascade was not initiated (62). Thus, supporting that cell death could be promoted via different mechanisms (62, 80).

Further *in vivo* studies on resveratrol's apoptotic potential were evaluated. Mohan and colleagues observed the role of resveratrol on inducing apoptosis in mouse embryonic fibroblasts (MEF) cell lines containing a Bax knock-out, Bak knock-out, double knock-out and wild type (70). The MEF Bak and Bax deficient cells were utilized from the mouse model and treated with 50 μ M concentrations of resveratrol, which still showed a reduction in apoptosis through this route, this suggested that resveratrol activated apoptosis through a mitochondria independent route as well in these cells, activating caspase 2 or 8, or both (70).

Human colon adenocarcinoma (HCT 116) cell lines were also investigated following resveratrol treatment (25, 50, 75 and 100 μ M) over 24 to 72 hours (70). Resveratrol induced apoptosis at or higher than 50 μ M through caspase activation upstream of the mitochondria, and the mitochondrial route appeared to play a major role in cellular death (70). The death signals, induced by caspase-8 and caspase-2 in the colon cancer cell lines, were mediated by a conformational change that occurred in both Bax and Bak, that subsequently activated caspase 9 and 3 (70, 81, 82). However, the mechanism through which resveratrol induced caspase-2 activation, upstream of the mitochondria, was not well known. Studies suggested that ceramide production in MDA-MB-231 breast cancer cells could be a possible trigger for activated caspase 2, yet the reason for it in colon cancer cells remained unclear at this point (83). Following resveratrol administration to the colon

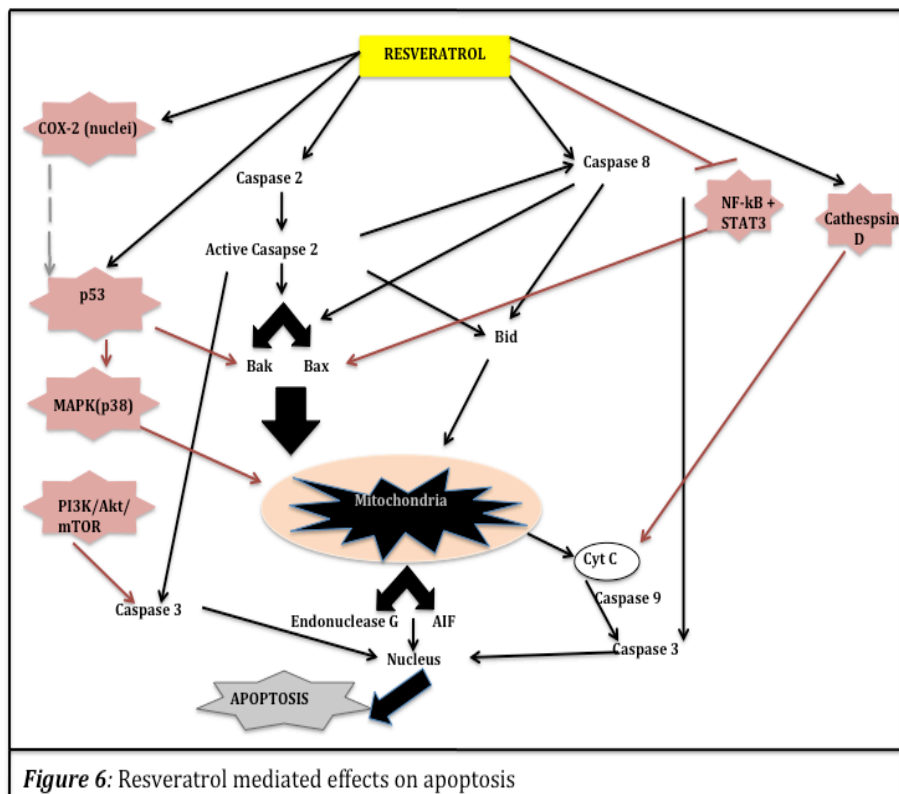
cancer cells, cytochrome c, AIF, and endonuclease G were released, which triggered the apoptotic events seen in these cancer cells.

Thus, these results support the notion that resveratrol is an inducer of apoptosis in several different cancers. As documented, resveratrol supplementation promoted this action through first, the main route of mitochondrial dependent apoptosis utilizing caspase 2, -8 and Bax or Bak accumulation which produced cytochrome c, endonuclease G and AIF being released from the mitochondria, and initiated apoptosis. But, resveratrol also acted through an alternative mitochondrial independent route that relied on caspase 2 and caspase 8 activation that led to downstream caspase promotion and apoptosis (70). Hence, resveratrol would be considered a useful therapeutic tool for cancer to target apoptosis via two distinct routes.

Multiple *in vitro* studies have investigated the apoptotic action of resveratrol use, including those investigated below. For example, MCF-7 and MDA-MB-231, which displayed apoptotic effects at 10 μ M concentrations and above, had maximal effects seen after 24 hours (84). Additionally, studies with pancreatic cancer cell lines showed similar results upon resveratrol treatment at 20, 50, 100 and 200 μ M (22, 64). Capan-2 and colo357 colon cancer cells also induced apoptosis through 200 μ M of resveratrol treatment, in a time-dependent manner (64). Other colon cancer cell lines, HT-29 and SW480, demonstrated apoptotic action at 100-150 μ M (maximally seen at 100 μ M) resveratrol concentrations over a period of 24 to 72 hours (55). Resveratrol's apoptotic effects were also observed in Drug-resistant human multiple myeloma cell lines (U266 and RPMI 8226) which revealed 50 to 100 μ M and above of resveratrol use, induced apoptosis (85).

However, lower plasma levels of resveratrol, from 20 nM to 2 μ M (physiological concentrations) were the most effective in inducing apoptosis in *in vivo* studies (31, 86, 87).

Resveratrol has been shown to alter several proteins that aid in the release of death promoting factors and initiate the caspase cascade. The following section investigates several proteins that resveratrol has an effect on and are involved in activation of different areas within the apoptotic cascade. The role of resveratrol will be demonstrated first on COX-2 accumulation, followed by up regulated p53 and STAT3, which includes MAPK-induced apoptosis. Discussion will then occur on resveratrol's influential role that has been displayed within the PI3K/Akt/mTOR pathway, on NF-kB inhibition, and finally how it inhibited cathepsin D. These various proteins and pathways were regulated by resveratrol



and displayed initiation of apoptosis in multiple cancers (Fig 6).

It is clear from the extensive research on resveratrol, that this compound has played a significant role on targeting several important proteins like

receptor tyrosine kinases, protein kinases, cyclooxygenase and p53 (88). Tang and colleagues explored the role of the enzyme cyclooxygenase-2 (COX-2) and its apoptotic

abilities within breast cancer cells. COX-2 is produced through the inflammatory response and allows tumor growth to occur, as well as invasion and evasion of apoptosis in normal expression (84). COX-2 produces prostaglandins, which is part of a lipid group that is known to aid in the metastatic process of several carcinomas (22, 89-91). Abnormal or increased expression of COX-2 was observed in cancerous tissues, which led to malignant phenotypes. It was observed that COX-2, upon conversion to arachidonic acid, produced ROS, which created oxidative stress and damaged the DNA, which helped to create this phenotype (22, 89, 92). COX-2 also monitored p53 expression, which, as mentioned, is a tumor suppressor protein that regulates cell cycle progression, and is involved in apoptosis. p53, a central transcription factor, activates other genes that are involved in regulating normal cellular functions.

Treatment with resveratrol ($>10 \mu\text{M}$) down-regulated the expression of COX-2. Resveratrol regulated the phosphatase, MKP5, which is an inflammatory mediator that inactivates MAPKs (93, 94), this was previously observed in prostate cancer cell lines where MKP5 production was increased (95). In particular p38 and JNK protein kinases were inhibited, causing decreased NF- κB and COX-2 expression (22, 93, 94).

COX-2 activated apoptosis in not only prostate cancer cells, but human breast cancer cells, where resveratrol acted as an anti-inflammatory agent (84). Upon investigation of MCF-7 and MDA-MB-231 cell lines, COX-2 expression was induced by resveratrol, which caused COX-2 to accumulate in the nuclei of the MCF-7 cells at $10 \mu\text{M}$ of resveratrol and above, and maximal effects were seen after 24 hours (84).

The above-mentioned effects of resveratrol were brought about through increased DNA binding activity of the transcription factor AP-1. Activated AP-1 was necessary for

resveratrol mediated COX-2 expression. The accumulated COX-2 in the nuclei caused increased Ser15-phosphorylated p53 inside the cells as well, which led to p300 activation (a transcriptional co activator) and initiated downstream apoptosis.

The results suggested that COX-2 expression could have a dual role, causing enhanced cancer progression under normal (untreated) conditions, and actually influenced p38 mediated apoptosis of MCF-7 breast cancer cells possibly through a ceramide signaling route (referred to as the tumor suppressor lipid), after being treated with 10 μ M (and above) of resveratrol (84). This pathway though not unclear, presents the possibility that the actions of resveratrol are upstream to the mitochondrial events in the cascade (84). Thus inflammatory mechanisms and inflammatory related pathways were important in resveratrol's cancer fighting process.

The next important protein that induced apoptosis was p53 (Fig 6). COX-2 production, as mentioned, activated and caused Ser15-phosphorylated p53 to increase inside the cells, and induced p300 activation. p300 had antitumor effects because it is a transcription co activator, which alters the binding and activation ability of several transcription factors (NF-kB, p53, Egr1) which play a role in cell death (96). Ser15-phosphorylated p53 also displayed increased ERK1/2 activity in the MAPK pathway through resveratrol use and caused apoptosis in the cells via this route (84). The interaction that occurred between resveratrol and COX-2/p53/p300, which led to apoptosis, was observed to be dependent on ERK 1/2 activity (84). This showed that p53 acted through the MAPK pathway as well as p300, to progress apoptosis through multiple mechanisms.

Another way resveratrol displayed its beneficial effects on p53, was through pro-apoptotic factors. Apoptosis was initiated when an enhanced expression of pro-apoptotic factors (Bax, Bak, PUMA, Noxa, and Bim) was seen, while a simultaneous decrease in anti-apoptotic factors (Bcl2, Bcl-XL, and Mcl-1) also occurred (97). Zhou and his team observed resveratrol mediated p53 effects on colon cancer cell lines (capan-2, colo357, miapaca-2, and bxp-3) upon 20, 50, 100 and 200 μ M treatment with resveratrol (22, 64). Apoptosis was detected in a time and dose-dependent manner (maximal effects after 200 μ M concentrations) upon resveratrol administration, which caused increased caspase 3 activity (64).

Resveratrol also enhanced p53 accumulation within the cells, in order for resveratrol to do this, it was necessary that the cell lines had a functional p53 pathway (64). Thus, mutated p53 pathways observed in colon cancer cells (miapaca-2, bxp-3, and capan-1) were unable to be stimulated by resveratrol treatment and could not induce apoptosis (64). Though mutant p53 pathways blocked resveratrol induced apoptotic effects through this mechanism, a multitude of cancers were still able to utilize the p53 protein, thus these cancers still yielded to these cellular death events (44, 55, 64, 98-100).

Another important pathway involved in multiple protein interactions, which can lead to apoptosis, is the MAPK pathway (figure 6). For example, resveratrol inhibited p38 and JNK protein kinases within this signal transduction route, causing decreased NF- κ B expression as well as COX-2 expression (22, 93, 94), which indirectly allowed other pathways to decrease cell survival. The MAPK pathway also demonstrated an essential role in mediating COX-2 expression after 10 μ M of resveratrol was administered, which produced the increased Ser15-phosphorylated p53 (84). Hence, this showed that COX-2

accumulation in the nuclei from resveratrol treatment was MAPK pathway dependent. In colon cancer cells (Capan-2 and colo357), treatment with 200 μ M of resveratrol caused downstream effects, phosphorylating ERK (one of the MAPKs), which initiated PUMA activation. This protein caused apoptosis in the cells, and was further up regulated by resveratrol treatment (64). Further roles of resveratrol impacting the MAPK dependent apoptosis pathway need to be documented and evaluated by additional research at this time.

Another major pathway involved in resveratrol-mediated apoptosis was the PI3K/Akt/mTOR pathway. In relation to this pathway, IGF-1's influence was explored. IGF-1 appears to be pertinent in several cancers, and elevated expression levels during obesity usually considered relevant to several obesity linked cancers. Vanamala and his research team observed resveratrol's apoptotic effects in the presence of IGF-1 (55). Their research on colon cancer cells (HT-29 and SW480) displayed that varying concentrations of resveratrol (50 to 150 μ M over 24, 48, and 72 hours) were shown to exert apoptotic effects (maximally at 100-150 μ M concentrations) through suppression of IGF-1R, which caused reduced Akt activation, and also increased p53 expression (55). Lower concentrations of resveratrol (50 μ M) on colon cancer cells, displayed no significant effect on the apoptotic pathways, and cell survival remained relatively unchanged (55). Thus, resveratrol showed therapeutic potential on those cancers that were promoted by IGF-1, as was seen in certain forms of breast cancer.

Research also presented findings on resveratrol-mediated effects on the PI3K/Akt pathway, when IGF-1's influence was absent. Akt, discussed previously, was a group of serine/threonine protein kinases that mediated cell proliferation and apoptosis via a

variety of routes that included regulation of Bad, caspase-9, glycogen synthase kinase 3 (GSK3), Forkhead transcription factors and NF- κ B (56, 101). Resveratrol treatment induced a time and dose-dependent increase in apoptosis in such cell lines as gliomas (U251) that were partially regulated by the PI3K/Akt/mTOR route (102). Jiang and colleagues further examined the glioma cells after resveratrol treatment and also observed increased caspase 3 expression, maximally seen at 100 μ M concentrations over a period of 24 hours (56). Resveratrol inhibited the pro survival ability of the PI3K/Akt pathway, which cleaved pro-caspase 3 to activated caspase 3, increasing its expression up to 27.7 fold (102). Hence, resveratrol's increased ability to down regulate this pathway was important in initiating apoptosis.

A further regulatory component of the apoptotic cascade is the transcription factor, NF- κ B (figure 6). As discussed in prior sections, NF- κ B activation led to transcription of cell proliferative genes, but NF- κ B also promoted transcription of cell survival genes, and thus plays an influential role in apoptosis as well. Importantly, resveratrol suppressed NF- κ B activation in multiple myeloma cell lines, which led to increased apoptosis (85). Resveratrol advanced apoptosis through inhibiting both, NF- κ B expression, and other transcription factors like STAT3 expression. Resveratrol mediated NF- κ B and STAT3 expression reduced transcription of antiapoptotic gene products (Bcl-xL, IL-6, Bcl-2, cyclin D1) (85, 103). Bhardwaj et al (2007) explored resveratrol's effects on NF- κ B as well as STAT3, and how they influenced antiapoptotic gene expression (85). Drug-resistant human multiple myeloma cell lines (U266 and RPMI 8226) after being treated with 50 μ M concentrations of resveratrol and above, displayed an increase in apoptosis (85). Again, it was observed that resveratrol down-regulated the constitutively activated NF- κ B and

STAT3 with this concentration of resveratrol (85). Resveratrol reduced the expression of NF- κ B, and also induced caspase 2 expression, independent of NF- κ B. Hence, apoptosis occurred after resveratrol treatment through both of these routes (70).

Resveratrol-induced inhibition of I κ B α kinase and p65 sub-unit mediated NF- κ B activation (85). Due to this down-regulation, Bax increased and caused subsequent cytochrome c release, which led to activated caspase-3 (an initiator in the apoptotic death cascade), and inhibited several of the antiapoptotic products that included cyclin D1, Bcl-2, and Bcl-xL (85). Overall increased apoptosis was observed in the cells lines that were administered with resveratrol.

Normally, the intrinsic pathway displays increased activation of proapoptotic proteins, like Bax and Bak, both members of the Bcl-2 family. It was hypothesized that they were released from upstream signals of caspase 2, which produced cytochrome c release (figure 6) (70). It was observed in certain conditions, such as epithelial breast cancer, proteins like cathepsin D, an aspartic endoprotease (a tumor marker of breast cancer aggressiveness) were overexpressed (104). In ER-positive cells, meaning the estrogen receptors are over-expressed in the tissues, estrogen and growth factors (IGF1 and EGF) regulate lysosomal cathepsin D production (22, 104). When cathepsin D matured, it could lead to inhibited release of cytochrome c and reduced caspase 3 and 9 activation, and decreased apoptosis to occur (22, 104). It was observed that resveratrol exerted its apoptotic effects partly through inhibiting the lysosomal pathway of cathepsin D, which was a novel target for cancer cells. Resveratrol displayed that effect in ER positive, but not ER negative, breast cancer cells in a biphasic manner. Resveratrol inhibited cathepsin D expression and IGF-II at higher concentrations of 10^{-4} M, and enhanced production

cathepsin D at lower concentrations of 10^{-6} M resveratrol (22, 105). Thus resveratrol demonstrated the vast role it has in the induction of apoptosis in several cancers.

It was apparent from the extensive amount of research, that resveratrol regulated cell microcolonization in cancer cells via multiple routes that involved inhibition of cell proliferation, cell cycle proteins, and induction of apoptosis. Resveratrol had the ability to exert these effects through a variety of mechanisms including the similar estrogenic structure it displays, its down regulative ability of transcription factors such as NF- κ B, AP-1 and STAT3, increased production of antiapoptotic gene products (Bcl-xL, IL-6, Bcl-2, cyclin D1) and finally by inhibition of proapoptotic factors (Bax, Bak). Resveratrol's effects were observed at higher concentrations through inhibited cathepsin D production. Apoptotic effects were displayed through enhanced cytochrome c release, caspase 3, 8, 7, and 9 release, which worked in initiating the final cell death signals. Resveratrol was also shown to increase activated p53, and influenced apoptotic mediators. Resveratrol's influential role has been displayed on several kinases, which included regulator of the extensive MAPK pathway (ERK1/2, Jun).

But, these were not the only roles of resveratrol regarding its cancer therapeutic potential. Resveratrol demonstrated increased accumulation of COX-2 in the cancer cell nuclei, which activated a variety of other factors that aided in repressing cancer growth and metastasis. In certain cancers, such as ER negative breast cancer, IGF1 mediated the overproduction of PI3K/Akt, and resveratrol again demonstrated inhibition of the PI3K/Akt/mTOR survival pathway. Resveratrol was observed to exert its effects most efficiently at a consistent range from 5 to at least 100 μ M concentrations for many *in vitro*

studies (31, 55, 106), but lower plasmas levels of 20 nM to 2 μ M were documented in *in vivo* studies (31, 86, 87).

Thus, resveratrol has played an essential role in repressing the cell microcolonization pathways that were important in progressing cancer metastasis and this compound should be considered in furthering research on cancer prevention and treatments.

2.1.3 Resveratrol and inhibition of angiogenesis

Resveratrol has been shown to regulate cancer metastasis via cell microcolonization and MMP expression, but also to decrease angiogenesis. Angiogenesis is a necessary process for tumors in their metastatic progression, in order to acquire nutrients through the blood supply. Current research has reported resveratrol as a potential novel angiogenic inhibitor, especially when consumed normally through the diet (22, 107).

VEGF, an essential growth factor in the angiogenic pathway, works to promote proliferation, movement and tubular growth of endothelial cells, aid in proteolytic break down of the basement membrane (fig 7) (107). Under normal conditions angiogenesis is induced during times of wound healing, which provide a blood supply to the infected area. While during cancer states, the normal balance between angiogenic facilitators and inhibitors are disrupted. Metastasized cancer cells only obtain a limited amount of oxygen from their environment before they enter a hypoxic state and die off; in order to counter this problem the tumor cells up regulate hypoxia inducible factor-1 α (HIF-1 α) (96, 108). HIF-1 α causes activation of several angiogenic factors, including over expression of growth factors such as VEGF. HIF-1 α binds to p53, and p53 produces the opposite effect of HIF-1 α and increases angiogenic inhibitors such as thrombospondin-1 (TSP1) (108-110). VEGF, on

the other hand, further promotes cancer progression by activating the PI3K pathway and subsequently Akt, which continuously encourages endothelial cell blood vessel growth, and provides an endless source of oxygen and nutrients to the growing metastases (22, 111, 112).

In cancer states, up-regulation of growth factors through multiple routes (one example being HIF-1 α discussed above), allows increased amounts of VEGF to be released from the

tumor. Subsequently VEGF binds to the VEGF receptor (VEGFR) on endothelial cells, inducing activation of multiple signaling pathways, which leads to downstream effects that promotes angiogenesis (113).

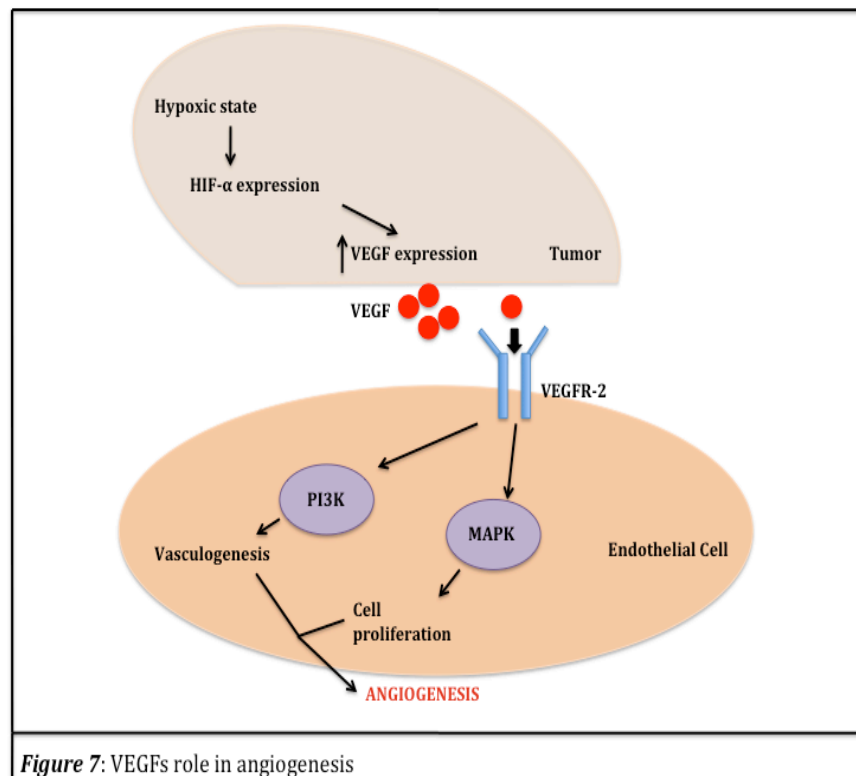


Figure 7: VEGFs role in angiogenesis

One such pathway that is activated is the MAPK pathway (22, 114). ERK1/2 within this pathway is phosphorylated following receptor binding of the growth factor, which promotes the signaling cascade events in this pathway and induce proliferation and survival of the endothelial cells, as well as plasminogen activation (115). Overall, these promotional events allow angiogenesis occurrence and aid in cancer progression. Findings

have demonstrated how resveratrol is beneficial in reducing VEGF-dependent angiogenesis through signaling pathway (22, 116).

Resveratrol's effect on angiogenesis has been the target of multiple studies. Research performed with resveratrol *in vitro* has shown that it inhibited angiogenesis via multiple routes, as discussed below. In myeloma cell lines (YUZA6, M14 and A375) co cultured with a monolayer of vascular endothelial cells, resveratrol treatment inhibited angiogenesis at a concentration of 50 μ M and above over 48 hours (108). Down-regulation of angiogenesis occurred via suppressed HIF-1 α expression by resveratrol. HIF-1 α , as mentioned previously, was strongly correlated with VEGF levels, thus inhibited expression of HIF-1 α causes a reduction in VEGF as well (108).

It was hypothesized that resveratrol mediated angiogenesis production in cell lines through up-regulation of p53, which targets down-stream signalers including secreted matrix protein, thrombospondin 1 (TSP1). When TSP1 is activated, it functions as an angiogenic inhibitor (108), showing that resveratrol can function through the p53 pathway promoting TSP1 expression and subsequent inhibition of angiogenesis. Those cancers with a mutant p53 pathway did not have enhanced amounts of TSP1, and consequently did not reduce angiogenesis.

Other studies have displayed direct inhibition on the secretion of both VEGF and basic fibroblast growth factor (bFGF) when treated with 25 μ M of resveratrol in multiple myeloma cell lines (107). Resveratrol concentrations of 100 μ M and above for a period of 48 hours also exhibited a reduction in VEGF activity in MDA-MB-231 breast cell lines (114). This decrease in VEGF activity by resveratrol in these *in vitro* studies could possibly be through a decreased phosphorylation of MAPK, in particular, ERK1/2 in the MAPK

pathway. Thus, the down-regulation of this pathway was shown to inhibit endothelial cell growth and reduce plasminogen activation leading to decreased angiogenesis to the tumor site (117-119). Resveratrol also further mediated this decrease in VEGF secretion through ER β in breast cancer cells (114).

Other cell lines that were studied pointed to a decrease in essential growth factors and MAPK signaling after resveratrol treatment. For example, in bovine capillary endothelial (BCE) cell lines, resveratrol was shown to inhibit FGF-2 induced phosphorylation of ERK1/2 in the MAPK pathway at concentrations of 10 and 20 μ M, which led to suppression of angiogenesis in these cell lines (113). Porcine aortic endothelial cell lines, which consistently express VEGFR-2 (PAE/VEGFR-2), have also demonstrated a reduction of angiogenesis upon resveratrol treatment. In these cell lines, 1 μ M of resveratrol inhibited VEGF production and subsequent migration of endothelial cells and neovascularization (113). This demonstrated how resveratrol inhibited growth factors and MAPK expression that were important for angiogenic progression (113). This was particularly important because the MAPK signaling pathway, as mentioned, was a key route for endothelial cell proliferation that was exhibited in angiogenesis (113). Therefore, inhibition of both the FGF-2 and VEGF receptors by resveratrol produced a large reduction in endothelial cell growth pertinent to angiogenesis.

Resveratrol's anti-angiogenic ability was additionally observed in gliomas, where enhanced vessel formation was linked with tumor aggressiveness (117, 120, 121). VEGF levels were reduced in RT-2 glioma cells with 10, 25, and 100 μ M concentrations of resveratrol over a 24-hour period, showing increased suppression with increasing concentrations. As opposed to previous findings where VEGF suppression was related to a

decrease in MAPK activity, glioma cell inhibition of VEGF was thought to be due to decreased micro vessel density and decreased proliferation (121). In particular, resveratrol treatment revealed VEGF to have a decreased binding ability to human umbilical vein endothelial cells (HUVECs), important for producing micro sized tubes significant for angiogenesis (121). But, the mechanism for VEGF suppression was not similar to the breast cancer cell lines that have been previously reported. Through the work of Tseng et al (2004), the MAPK pathway was not down regulated (as seen with breast cancer cells), and no significant change in expression was observed, meaning it might not be the only signaling pathway inhibiting angiogenesis in glioma specific cancers, and other pathways such as PI3K/Akt may be working at that point to increase VEGF expression (121). Typically higher concentrations of resveratrol ranging from 50 to 100 μ M produced the down-regulation of MAPK expression, leading to VEGF suppression in several forms of cancer which included both breast and pancreatic forms (118, 119). Therefore, this data suggested that angiogenesis could be inhibited through suppression of VEGF working through down-regulation of MAPK pathway in certain cancers, as well as down regulation of other possible pathways relating to angiogenesis that have not been as thoroughly investigated.

In vivo studies have also explored resveratrol's influence on angiogenesis. Studies that examined the mouse cornea related to tumor production have shown reduced angiogenesis via resveratrol inhibiting both VEGF and bFGF secretion (107, 113, 121). VEGF is an important target for anti-angiogenic therapies, and the mechanism through which resveratrol suppressed VEGF induced neovascularization could be through different routes, as explored previously.

In studies relating to breast cancer cells, the estrogen response element (ERE) has been found in the VEGF gene, meaning estrogen (and other sex steroids) would initiate transcription of VEGF through the ERE (114, 122). This is an important factor when considering the role of angiogenesis in breast cancer patients. Nude mice when injected with MDA-MB-231 breast cancer cells and treated with resveratrol inhibited angiogenesis *in vivo* at concentrations of 25 mg/kg per day for a time period of 3 weeks, which was comparable to pharmacological levels (114). VEGF displayed a decrease in expression under these conditions in mice, and it was hypothesized that the mode of inhibition could have been through decreased activation of the MAPK pathway, reducing VEGF mediated effects on endothelial cells (114).

Brakenhielm and colleagues further examined resveratrol induced VEGF inhibition via the MAPK pathway *in vivo* as well. This was done through testing male and female C57B16/J mice implanted with murine fibrosarcomas. Oral intake of resveratrol took place, with mice consuming 5.7 µg/ml (equivalent to 25 µM) resveratrol (113). This oral administration revealed that resveratrol had the ability to hinder angiogenesis in murine fibrosarcoma tumor cells in mice. But, at the same time, reduced angiogenesis occurrence also correlated with an inhibition of wound healing under these circumstances.

Other studies have shown that angiogenesis can be inhibited when resveratrol was administered orally, such as through the consumption of red wine or grapes in humans. Similarly, mice implanted with FGF-2 and VEGF corneas, which consumed a total of 48µg/kg resveratrol (equivalent to 3 glasses of wine/day for humans), showed greater inhibition of neovascularization in the cornea due to VEGF and FGF-2 suppression (113). Similar to these findings, it was seen that resveratrol inhibited FGF-2 and VEGF activation

and subsequent phosphorylation of ERK1/2 in the MAPK pathway (113). Thus, FGF-2 and VEGF were again shown to be reduced due to resveratrol treatment, which coincidentally decreased vessel density (113).

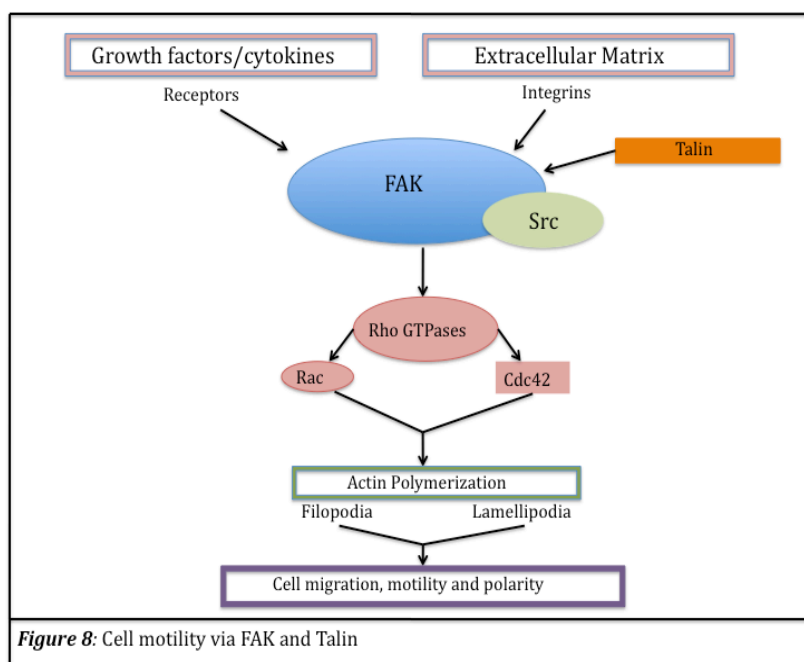
To further validate resveratrol's anti-angiogenic effects, RT-2 glioma cells were injected into a rat model, and resveratrol (40 mg/kg/day) was administered over 4 weeks (121). This amount of resveratrol consumption was equivalent to around 25 μ M in peak plasma levels. Resveratrol was shown to increase survival time and decrease the tumor growth rate, concurrent with inhibiting angiogenesis. The reduction in angiogenesis was thought to be due to resveratrol decreasing micro vessel density in the gliomas (121). This decline in density was associated with decreased angiogenesis as well, and thus may be attributed to resveratrol working at higher concentrations on reducing angiogenesis *in vivo*.

Through several studies angiogenesis was inhibited *in vivo* at concentrations of resveratrol ranging from 2.5 to 100 mg/kg for a period ranging from 15 days to 28 days (24, 113, 114, 121, 123, 124). Resveratrol also displayed anti-angiogenic properties *in vitro* in a range of amounts from 10 to 100 μ M with maximal effects seen after 24 to 48 hours, with many of those anti-angiogenic effects seen in a time and dose-dependent manner (24, 107, 108, 113, 114, 121). Resveratrol, therefore, could be beneficial as an angiogenic inhibitor. Structurally similar molecules to resveratrol could also have the potential to be used as angiogenic inhibitors, as they could induce similar effects on the angiogenic pathway, but that would be something to further explore in the future. From here, studies to investigate further how angiogenesis is inhibited through resveratrol could continue by using different breast cancer cell lines, animal models, and clinical trials.

2.1.4 Resveratrol and inhibition of cell migration and motility

Resveratrol thus far has been shown to be an angiogenic inhibitor, MMP inhibitor and influence different cellular pathways within metastasis. It is also essential to mention the effects that resveratrol could have on the actin cytoskeleton, especially when considering the mechanisms of cell migration that are essential in nature.

For directed cell migration to occur in any form of breast cancer, cell movement occurs through actin polymerization and production of actin-based protrusions including



lamellipodia and filopodia.

The filopodia-based protrusions are needed for sensing the cellular environment, as opposed to lamellipodia-based protrusions, which are necessary for actual cellular movement (10). Filopodia

could become lamellipodia via signals from growth factor receptors (Fig 8).

During the migratory process, integrins, attachment proteins on the plasma membrane of the cells create complexes referred to as focal adhesions, by which cells attach to the basement membrane. Focal adhesion kinase (FAK) and Src are recruited to the membrane by integrins and growth factor receptors, causing activation of the focal adhesions (10). This whole process can initiate breast cancer cell migration, invasion and also cell proliferation (10). During cell migration, the Rho family of GTPases, which include

Rac and Cdc42, play an essential role in the remodeling of the actin cytoskeleton that occurs during metastasis (10). In particular, Cdc42 contributes to filopodia production while Rac functions in lamellipodia formation (Fig 8) (10, 125).

As mentioned previously, the structure of resveratrol is similar to that of estrogen and therefore mimics estrogen by binding to the two different kinds of estrogen receptors, ER α and ER β (10). This suggests that resveratrol could produce estrogenic (ER agonist) and anti-estrogenic (ER antagonist) effects. This also could mean that certain forms of breast cancer, depending on the estrogen receptors present or absent, could be affected by resveratrol. In particular, metastasized breast cancer tends to lose the ER α form. This was why MDA-MB-231 breast cancer cell lines were utilized in multiple cell line studies, since they are ER α negative and ER β positive (10).

Estrogen and EGF, when added to the breast cancer cell lines, showed an increase of cell migration coupled with an increase in lamellipodia, which could enhance cellular movement (10, 126). Resveratrol supplementation in MDA-MB-231 cell lines had the opposite effect, and decreased cell migration in estrogen and EGF treated cells at 50 μ M concentrations of resveratrol, over a period of 8 hours. This could be occurring by restructuring of the filopodia, and decreasing recruitment of FAK and focal adhesions in the basement membrane (10, 126). This also points to an important component of cell migration, which is cell polarity that aids in the forward movement and migration of cells. With the increased presence of filopodia in the resveratrol-treated cells, their appeared to be no polarity and thus cell migration was also suppressed through this mechanism (10, 126). Thus resveratrol reduced cell motility in ER α negative and ER β positive breast cancer tissues that also expressed the EGF receptor (EGFR) (126).

MDA-MB-231 cells treated with 50 μ M showed inhibited cell motility through a decreased production of lamellipodia after 8 hours (10). This inhibition of cell motility complexes at higher concentrations of resveratrol were thought to be due to a more sustained and larger response through Cdc42, which did not promote the conversion of filopodia to lamellipodia, the motile actin structure (10). But, the production of unpolarized filopodia through higher concentrations of resveratrol could have worked independently of Cdc42, and other GTPases could have had a larger effect on the decreased mobility of these cells as well (10).

Resveratrol supplementation has also displayed the importance of cytoskeleton remodeling in the presence of IGF-1, which could be upregulated in several cancer states. In particular, FAK and IGF-1R worked together to induce survival and continual proliferation via activation of downstream signalers (55). Talin, a regulatory integrin protein, was thought to further influence the activity of FAK which could lead to actin-based enhanced motility cytoskeleton changes, as well as mediating cell survival pathways (55). The interaction of talin and FAK, produce changes in downstream effectors, as was seen in several carcinomas (55, 127, 128). It was found in colon cancer cell lines (HT-29) that resveratrol suppressed talin and phosphorylated FAK in IGF-1 present cells at a concentration of 150 μ M (55). This same finding was demonstrated in the absence of IGF-1 as well, but IGF-1R acted synergistically with FAK, which led to multiple downstream effects in IGF-1 upregulated cancer states. Resveratrol also displayed enhanced cell detachment, and decreased protein interactions in the extracellular matrix due to inhibition of the cytoskeleton talin-FAK pathway (55).

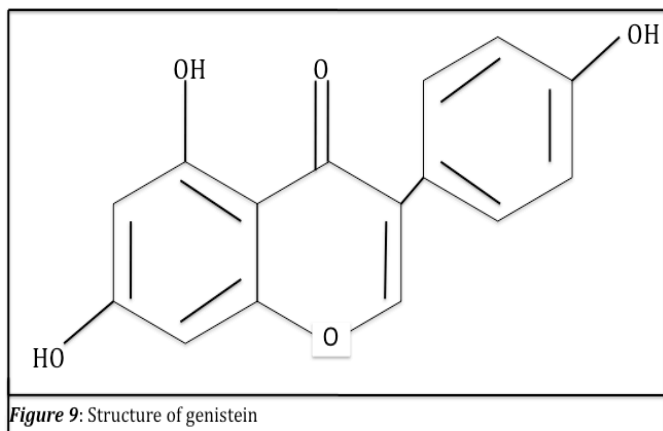
Thus resveratrol acted on the cytoskeleton structures of cells, which are important in motility and activating downstream signaling pathways that could promote metastasis as well.

2.1.5 Conclusions on resveratrol

With the extensive research that has been discussed above, it was obvious that resveratrol could play an influential role on invasive breast cancer. Not only did these studies give insight into the significant role of resveratrol, but also showed that more research is necessary regarding this specific phytochemical and its breast cancer fighting potential. Resveratrol had the potential to inhibit multiple targets in the metastatic cascade, which included MMP inhibition, modulating cell microcolonization events, suppressing angiogenesis and down-regulating cell motility structures that helped cancer cells to continually metastasize. In summary, the many molecular targets of resveratrol included, NF- κ B, MMP-2 and -9, VEGF, FGF, MAPK, PI-3K/Akt/mTOR, p53, TNF, AP-1, cMYC, COX-2, HRG- β 1, HER-2, multiple caspases, IGF-1, cathepsin D, STAT3, HIF-1 α , talin-FAK (17).

Thus, with research displaying resveratrol modulating many different pathways, proteins, and complexes, it would have great potential as a form of therapy for metastasized breast cancer. Resveratrol supplementation is already in clinical phase I trials utilizing COX-2 as a biomarker, on patients diagnosed with resectable colorectal cancer. As the safety of resveratrol on human trials, which has been positive thus far, continues to be investigated, other analogs of resveratrol should be explored and as they could have great promise in helping to treat cancer (22, 129).

2.2 Genistein



Another phytoestrogen (dietary estrogen), genistein, that has shown great promise in treating or preventing breast cancer. Further, several studies have demonstrated that it prevents breast cancer invasion and metastasis.

Genistein, an isoflavone, is a biologically active phenolic compound, and is found in several plants, most notably in soybeans and soy products (130). Dietary intake of genistein has been shown to aid in the prevention of cardiovascular disease by decreasing cholesterol and triglyceride levels (131).

Genistein ingestion has been linked to a reduction in bone loss by activating the process of bone formation, thus aiding in osteoporosis, a common disease condition in postmenopausal women (132). Genistein's mechanism of action, like resveratrol, has been proposed to be due to its similar structure to 17- β -estradiol, which allows it to bind to both ER α and ER β (with much greater affinity) and promotes their activation and estrogen-like effects (130, 133). Stimulation of the estrogen receptor by genistein has led to alleviation of several postmenopausal symptoms (134). Genistein has also been shown to reduce heart disease, atherosclerosis, and Type 2 diabetes (130, 131), and contains antioxidant, estrogenic and cancer fighting properties. This isoflavone appears to have the greatest impact on both breast and prostate cancer, as discussed throughout the following parts of this section (130, 131, 135, 136).

Population based studies have investigated the lifestyle and dietary factors that could have led to a lower incidence of breast cancer in specific populations. In fact, a lower incidence of breast cancer has been documented in Eastern Asian countries where the diet utilizes a higher proportion of soy (137-144). Japanese and Chinese women who lived in Asia had a 5-fold decrease in breast cancer occurrence compared to women also of Japanese and Chinese descent in North America and Western countries (137). High soy consumers of Southeast Asia had genistein blood concentrations 100 to 1,000 times higher than people in western populations who ingested a low soy diet (138). Typically, within higher soy-consuming individuals, genistein consumption was around 0.3 to 1.0 mg per kilogram per day (145-152). Genistein, upon ingestion (which is usually in the micro molar range), is broken down to a mostly conjugated form, and 10% free form (which was in nanomolar concentrations) (153, 154). After immigration of Asian women into the USA and Western Europe, and adoption of the Western diet and lifestyle factors, the risk of breast cancer was comparable to Caucasian women after only two generations (140, 155). Hence, an environmental factor, such a diet based on high soy consumption, could be attributed to this lower incidence of breast cancer in these Southeast Asian populations.

Southeast Asian male populations have also seen an associated decline in cancer, but in their case it was a reduction in prostate cancer incidence. Again, genistein consumption was much greater in these individuals and with this increase in dietary intake of soy; there was also a ten-fold decrease in metastatic prostate cancer occurrence (130, 138-140). Again, like breast cancer, it was seen that one generation after immigration to western cultures, that metastatic prostate cancer risk increased (140). Similar evidence has also been found that linked increasing genistein ingestion with a lower incidence of colon

cancer (144, 156). Thus, through environmental factors like the diet, genistein consumption could potentially contribute to the decreased incidences of breast, prostate and colon cancer in these populations with high soy-consuming diets (16, 130).

An array of *in vitro*, *in vivo* and clinical trials have been performed on genistein ranging from micro molar to nanomolar concentrations, in order to determine its effects on cancer at multiple stages. Genistein has been found to have a large impact on cancer metastasis and has been shown to inhibit several steps of this cascade. Importantly, genistein has been shown to be useful at inhibiting the activation of MMPs and integrins that aid in invasion, as well as inhibiting cell cycle regulators, inducing apoptosis, repressing angiogenesis and decreasing cellular motility (130, 157). The pathways and mechanisms involved in each of these processes will be discussed throughout the following sections.

2.2.1 Genistein and MMP inhibition

Cellular invasion, as mentioned previously, enlists activated MMPs in order to degrade the basement membrane (158). Activation of MMPs normally aid in endothelial cell proliferation and division, but has also been shown to increase tumor growth and metastasis by promoting growth factor release which leads to disorganized growth and division (159). Thus dietary agents like genistein, may show promise in targeting specific MMPs, such as the gelatinases (MMP-2 and MMP-9) and could provide assistance in targeting this process *in vivo* in advanced forms of breast cancer.

Genistein's ability to aid in down-regulation of specific MMPs has been seen in multiple cancer related studies. Concentrations of 10 nM (similar to physiological levels) and above have shown inhibition of metastatic prostate cancer cell lines *in vitro* (160).

Similar effects have also been documented in PC3 (hormone-dependent) and LNCaP (hormone-independent) prostate cancer cell lines. In this case, a reduction of MMP-2 activity was seen in both cell lines with concentrations starting at 5 µg/mL and showing enhanced inhibition with greater concentrations (50 µg/mL) and time (161). Higher concentrations of genistein varying from 30 to 50 µM have also demonstrated a reduction of MMP-2, as well as MMP-7 (a matrilysin) in the primary breast cancer cell line HCC13995 (162). More invasive breast cancer phenotypes (MCF-7 and MDA-MB-231) have confirmed these findings, that genistein administration inhibits invasion through suppression of MMP-2, MMP-7 and MMP-9 (163).

Inhibition of invasion following basement membrane degradation is one of the key features targeted in the progression of metastasized cancer forms. In human glioblastoma cells (U87MG) MMP-2 activation was observed and decreased with genistein administration in a dose and time dependent manner, ranging from 2 to 20 µM concentrations (164). This decrease in MMP-2 expression, at least in relation to glioblastomas, was thought to be partially due to the reduced protein levels of membrane type-1 MMP (MT1-MMP), present on the basement membrane, which aided in MMP-2 activation (164). A particular cellular component of the basement membrane, laminin 5γ2, also assists with membrane breakdown during metastasis, and was hindered as a result of genistein treatment (164).

MMP-9, the other key gelatinase was also down regulated upon genistein treatment in cancer cell lines. MMP-9, upon genistein treatment, was inhibited *in vitro* in both MDA-MB-231 and MCF-7 breast cancer cell lines, glioblastomas and pancreatic cancer cell lines (162-165). A pharmacological dosage of 50 µM of genistein concentration has displayed a

reduction of MMP-9 activation in PC3 prostate cancer cell lines as well (166). PC3 prostate cancer cells have also demonstrated decreased MMP-2 and MMP-9 expression with a concentration of 50 μ M of genistein (167, 168). In MDA-MB-435 cells, up-regulation of breast cancer cells was enhanced with c-erbB-2 expression. C-erbB-2, also known as human epidermal growth factor receptor 2 (HER2), is a protein that aids in the pathogenesis and progression of several forms of cancer (169). MDA-MB-435 cancer cell lines transfected with c-erbB-2, showed enhanced MMP-2 and MMP-9 secretion (169). These overexpressing c-erbB-2 cells, when treated with genistein, showed a reduction in c-erbB-2 expression and a subsequent decrease in MMP-2 and MMP-9 expression (169).

It is also of importance to note that clinical trials on genistein have begun, and in particular, phase II trials have documented decreased MMP activity in prostate cancer patients (170). Patients were administered 2 mg of genistein per kilogram of body weight (with a blood concentration of free genistein around 140 nM) compared to control individuals. Following genistein therapy, radical prostatectomies were performed, and prostate cells were viewed for prostate cancer invasive potential. It was seen through these trials that MMP-2 activity shrank to 24% the levels compared to the untreated group (control), which supported the role that genistein played in inhibiting the invasion process of cancer (170).

Thus, decreased MMP-2, MMP-9, MMP-7 and laminin 5 γ 2 activity in multiple cancer cells, was observed after genistein administration which reduced cellular invasion and metastasis. Through exploitation of genistein's anti-metastatic properties, it could be possible to help treat invasive cancer phenotypes by reducing the initiation and activity of several specific MMPs that were upregulated in this process. As clinical trials on genistein

have already begun on specific cancers, research continues to expand and support the valuable effects that genistein could have through the diet in reducing invasion of cancer.

2.2.2 Genistein and cell microcolonization inhibition

In the previous section on genistein, research demonstrated genistein effects on MMPs that had the ability to work through multiple cell signaling pathways. In this section, genistein's mass effects on cell microcolonization pathways were explored. To begin, this review is from research on genistein's ability to reduce cell proliferation, inhibit cell cycle proteins that play a role in cell growth, and finally the reduction in apoptosis.

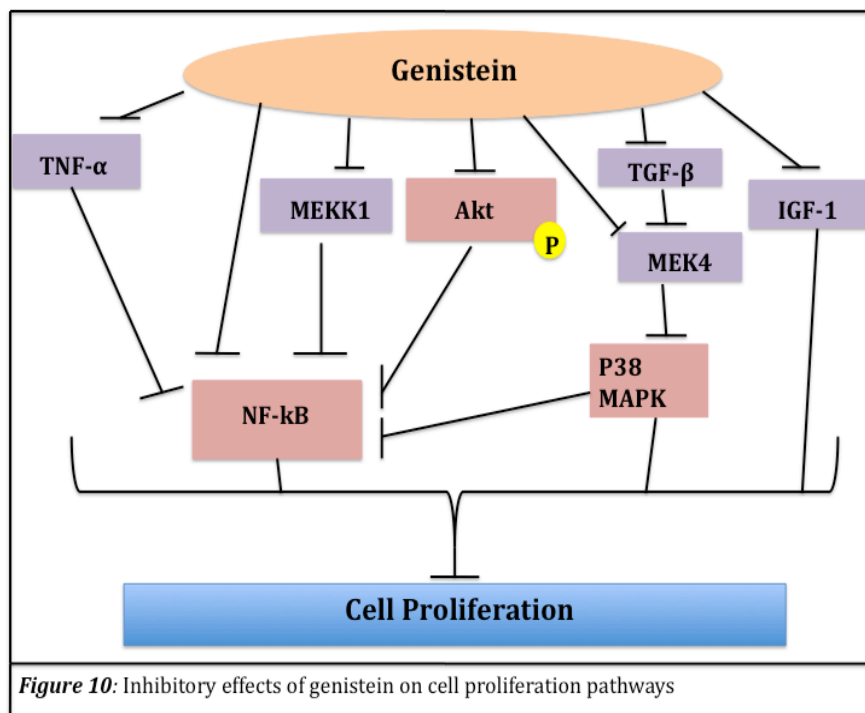
2.2.2.1 Genistein and cell proliferation

Genistein administration demonstrated effects on many regulators of cell proliferation. Cell growth, is essential for the cancer to establish a micro metastasis in the new secondary site. Review of literature reveals that genistein has a vast role in inhibiting several key pathways in cellular proliferation. Throughout this section, genistein's effects on NF-kB, Akt activation, MAPK pathway, IGF-1 mediated cellular proliferation and its estrogenic and non-estrogenic role will be discussed, followed by a brief overview of genistein's results documented from phase II clinical trials.

To begin, genistein, much like resveratrol, has demonstrated a strong influence over the NF-kB pathway. NF-kB is a key transcription factor that regulates the activation of several different genes that play a role in cellular proliferation and apoptosis. When activated, I κ B is phosphorylated, which releases NF-kB, and upon translocation to the nucleus it acts as a transcription factor (157). When genistein was administered to several different cancer cell lines (breast, prostate, head and neck squamous cell, and pancreatic),

DNA binding of NF- κ B was suppressed, reducing transcription and subsequent activation of NF- κ B regulated genes (157, 171-174).

In both PC3 and LNCaP prostate cancer cell lines, administration of 50 μ M concentrations of genistein over 48 hours, resulted in decreased NF- κ B



expression (172). These findings suggested that the p50 and p65 subunits of NF- κ B, that under normal conditions move into the nucleus and bind to the DNA, were inhibited from translocating and binding to the promoter site, thus inhibiting NF- κ B mediated effects on cell proliferation (Fig 10) (172).

Importantly, NF- κ B also displays increased expression in certain cancers, where the highly reactive oxygen species, H_2O_2 and cytokine, TNF- α , cause enhanced DNA binding of NF- κ B in the nucleus of these cells (174, 175). Treatment with genistein (50 μ M concentration for 24 hours) in prostate cancer cell lines and human myeloid leukemia cell lines, suppressed the DNA binding of NF- κ B in the nucleus of these cancer cells regardless of the stimulation from H_2O_2 and TNF- α (175). Thus, NF- κ B was found inhibited and does not translocate into the nucleus of the cell and facilitate transcription, even in the presence

of internal stimulators (175). Decreased NF-kB expression at this point is essential in reducing the production of proteins and activation of genes that aid in cell proliferation.

Another possible upstream mechanism for genistein's action has been proposed. A specific kinase, mitogen activated kinase kinase 1 (MEKK1), resides upstream of the kinases involved in NF-kB activation. MEKK1 works by phosphorylating IKK, which ultimately phosphorylates I κ B α , and releases NF-kB, and allows it to move into the nucleus to transcribe several genes (176, 177). Recent kinase assay trials have shown decreased kinase activity of MEKK1 with genistein treatment, and this could partially explain genistein's involvement in decreasing I κ B phosphorylation (16, 178).

Genistein has also been suggested to play an influential role in promoting antitumor activities in drug resistant forms of cancer. With the use of multiple chemotherapeutic agents such as cisplatin, gemcitabine and docetaxel, increased NF-kB expression was seen, leading to greater drug resistant forms of cancer (179-183). But, administration of genistein at certain concentrations that occurred before decreased amounts of chemotherapeutic agents were utilized in carcinoma states actually decreased NF-kB expression, which led to a more beneficial cancer fighting initiative (179, 180). Pre-treatment with various concentrations of genistein from 25 μ M, 30 μ M and 50 μ M for at least 24 hours, showed NF-kB inhibition and reduced cellular growth in *in vitro* cell lines (particularly multiple pancreatic cancer lines) (179-183). Similar results were demonstrated *in vivo* as well, with genistein (50 μ M) and lower doses of chemotherapeutic agents showing NF-kB inhibition (168, 179, 180).

In vivo investigation has also been performed on NF-kB activity, especially examining the role of genistein as an antioxidant and its ability to produce potent results

against oxidative stress and damage that occurs to human lymphocytes (175). During times of stress, disease, and cancer, the body produces TNF- α , a cytokine, as an immune mediated defense response. Typically, oxidative damage produces an enhanced NF-kB response, and mediates cellular events like proliferation. Human males treated with 50 mg of soy supplements for a period of 3 weeks showed a decrease in TNF- α mediated NF-kB activation, as well as a reduction in NF-kB DNA binding ability (175). These results were in agreement with previous *in vitro* studies on NF-kB and its ability to repress and mediate downstream effects like cellular growth.

Most of the NF-kB growth inhibition was seen at various micro molar concentrations in various cell lines. In breast cancer cells, a time and dose dependent increase in inhibition was seen from 5 to 50 μ M of genistein (184). Pancreatic cancer and nonsmall cell lung cancer also showed a reduction in NF-kB cell proliferative activity at 25 μ M of genistein (185). Prostate cancer showed inhibition of NF-kB at concentrations of 50 μ M of genistein (186, 187). Thus, NF-kB can be inhibited via different routes through genistein administration in multiple forms of cancer.

An important protein that regulates NF-kB activation is known to be Akt. Akt is a serine/threonine kinase that regulates cell proliferation and apoptosis (157). Akt is also an important initiator of cell proliferation via stimulating the mTOR pathway (157). It was hypothesized that cellular growth could be reduced via genistein treatment and that genistein inhibits Akt activation and causes subsequent inhibition of NF-kB and its cell growth targets (186). Several studies have shown similar results on the down-regulation of phosphorylated Akt after genistein treatment.

In prostate cancer cell lines, LNCaP cells displayed increased Akt activation with EGF treatment, while 50 μ M of genistein administration inhibited Akt phosphorylation and subsequent downstream activity (188). Inhibited phosphorylated Akt activity at the Ser⁴⁷³ residue upon 50 μ M of genistein treatment over a period of 24 hours was also observed in PC3 prostate cancer cells (186). Similar results were displayed in MDA-MB-231 and MCF-7 breast cancer cells. Treatment with 50 μ M of genistein for 48 hours resulted in a reduction of phosphorylated Akt Ser⁴⁷³ (184, 189). And again, this was seen with pancreatic cancer cell lines at 25 μ M genistein, and oral squamous cell carcinoma lines at 10 to 40 μ M of genistein for 48 hours (185, 190). Cervical cancer cells showed growth inhibition through decreased phosphorylated Akt at 20 and 60 μ M concentrations of genistein for a period of 48 hours (191). This reduction in Akt phosphorylation also displayed decreased DNA binding affinity of NF-kB, thus demonstrating the importance of Akt-NF-kB cross talk in cancer cells (184, 189).

In vivo studies on DMBA-induced mammary tumors in female Sprague dawley rats displayed inhibited phosphorylated Akt levels after genistein exposure (192). Genistein administration (500 mg/kg/day) occurred on postnatal days 22 through 28. Thus, downregulated Akt via genistein played an influential role on tumor formation and could have a beneficial role in reducing cellular proliferation in cancerous states by decreasing promotional activity attributed to activation of NF-kB (192).

Another important cellular pathway that enhanced cellular proliferation in cancer cells is the MAPK pathway. The MAPK pathway consists of kinases that subsequently activate each other which leads to final activation of ERK, JNK and p38 which ultimately turns on NF-kB and AP-1 expression as well as multiple other transcription factors that

lead to cellular growth (157). Genistein has been shown through cancer related studies to play a role in inhibiting different proteins of the MAPK pathway, which led to decreased growth.

In prostate cancer cell lines, genistein affected the transforming growth factor β (TGF- β) pathway that mediates MAPK. TGF- β , a secreted protein, is typically overexpressed in cancer, and has an effect on proliferation (193). TGF- β works to activate mitogen-activated protein kinase 4 (MEK4) which subsequently causes activation of MAPK p38 (157). MEK4, a tyrosine/threonine kinase, much like TGF- β , was shown to be increased in certain cancers (due to increased TGF- β secretion), including prostate, breast and pancreatic cancers (194, 195).

Prostate cancer cell lines, treated with 50 μ M genistein over a period of 23 hours displayed decreased levels of TGF- β induced phosphorylation of downstream effectors including p38 MAPK (196). This activity was observed further in other prostate cancer cell lines, where treatment with 50 μ M of genistein over 24 hours directly bound to and inhibited MEK4 activity, thus leading to reduced activation in the downstream effectors, p38 MAPK and down-regulated cellular proliferation (170). Genistein also induced these inhibitory effects at concentrations of 10 nM (160, 170, 193, 196). It appeared that lower concentrations of genistein were able to promote inhibitory effects through regulation of MEK4, upstream of p38 MAPK, suppressing the MAPK pathway (170, 193, 194, 197).

It has also been briefly demonstrated that an important growth factor, IGF-1, that advanced proliferation of cancer cells, was inhibited by genistein. IGF-1 tended to be increasingly produced during cancer, causing increased metastasis and growth.

Investigation of a prostate cancer model, the SV-40 Tag rat model *in vivo*, demonstrated a

growth inhibitory effect on prostate cancer cell growth when rats were fed 250 mg genistein per kg chow starting at birth to 12 weeks old (198). These genistein levels were equivalent to 2 μ M total genistein in the blood. Results demonstrated decreased expression of IGF-1 as well as decreased proliferation of the prostate cancer cells. Thus, showing that IGF-1 expression was additionally inhibited by genistein *in vivo* and this could be another important mechanism by which genistein exerted its anti-proliferative effects.

Literature shows that genistein plays a vast role in suppressing proliferation; and that this could be due to its similar structure to estrogen, and its ability to weakly mimic estrogen, as mentioned previously. As a phytoestrogen, genistein's phenolic pattern was similar to that of estrogen and for that reason can bind to the ER and consequently activating it (133). Genistein was able to modulate promoter sites via ER signaling including regulated binding of specific transcription factors like AP-1 to the promoter region (199). Thus, genistein was able to regulate the activation of specific transcription factors that played a role in cell growth during microcolonization.

Hence, it was important to view genistein's effects on proliferation when hormone (estrogen) dependent cells were present in specific cancers. These hormonally responsive cells tended to be seen in prostate cancers and breast cancers. It appeared that genistein exerted both agonistic and antagonistic effects upon being bound to the ER, depending on the estrogen concentration (200). If the estrogen within the environment was low (less than 10 μ M), genistein acted as an estrogenic agonist and caused growth, while high environmental estrogen (greater than 10 μ M) led to agonistic effects and reduced growth (200-202). Estrogen dependent breast cancer cells (MCF-7 and MDA-MB-231) displayed these same results, increased proliferation was seen at genistein concentrations of 10 nM

to 100 nM, and inhibited growth occurred at 20 μ M concentrations and above (201, 203, 204). Increased proliferation also occurred in *in vivo* models where MCF-7 cells were placed into athymic ovariectomized mice (28 days old) with genistein administration of 750 mg/kg (203). Genistein (125-1000 mg/kg, serum level of 0.39-3.36 μ M), caused enhanced growth of the hormone regulated breast tissue in animal models (203, 205, 206). Other models have demonstrated mice being fed less than 125 mg/kg of genistein or used a subcutaneous route of administration of genistein, which inhibited the proliferative effects (207). Thus, depending on dosage, genistein acted either as an agonist or antagonist of estrogen, inhibiting or enhancing the proliferative effects (203).

In non-hormone dependent cancers genistein inhibited cellular growth of cancer cells in an array of *in vitro* and *in vivo* models. In bladder cancer cell lines, growth was inhibited in a dose-dependent manner at genistein concentrations of 10-50 μ M over 48 hours (208). The study of bladder cancer *in vivo* in a mice orthotropic tumor model confirmed these results. An American Institute of Nutrition diet was utilized plus 0.14% of the diet being genistein, administered over a two-week period. The mice displayed decreased tumor growth and metastasis, with a 56% reduction in tumor size after genistein treatment (208).

Genistein administration on colon cancer cells also displayed a reduction in cancer growth. Treatment with 50 μ M of genistein *in vitro* led to an inhibition of cellular growth. In a mice model containing colon cancer cells that had produced lung metastases, a 44% reduction in lung metastases was observed following administration of 1 μ M of genistein (209). Additionally, human hepatocellular carcinoma cells evaluated *in vitro* showed that 10-20 μ g/mL of genistein used over 6 days, caused greater inhibition of tumor growth, up

to 80.1% (210). The *in vivo* model of hepatocellular carcinoma cells in an orthotopic murine model of nude mice also caused decreased metastases upon genistein administration (210). Here, 50 mg/kg of genistein was administered daily over a period of 20 days and tumor growth was observed to decline significantly after treatment. It was also noted, alongside a decrease in tumor size, there was also a decreased number of micro metastases present (210).

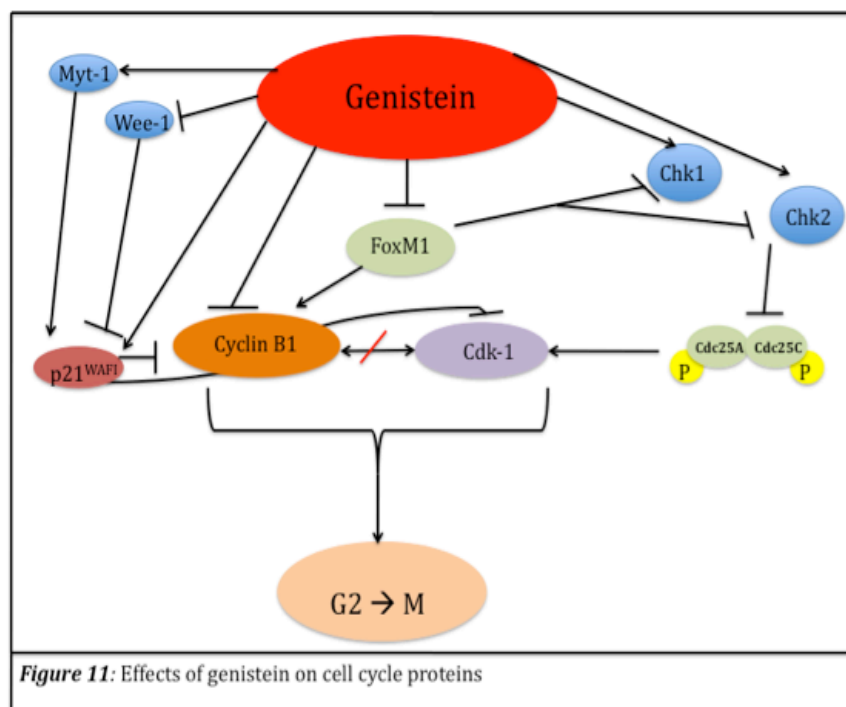
But, the *in vitro* and *in vivo* models were not the only examples of genistein use displaying inhibited cancer cell proliferation. Phase II clinical trials on progressive prostate cancer individuals have also demonstrated similar anti-proliferative results. Individuals with prostate cancer were tracked for cancer growth based on the amount of serum prostate-specific antigen (PSA) levels present in their system (211). As the prostate cancer progressed, PSA levels tended to increase over time, and is used as an indicator for cancer progression (211). In the clinical study, patients with prostate cancer were treated with soymilk daily at approximately 1 mg/kg/day of genistein consumption. The free genistein within the blood of these patients was approximately 44 nM. Upon treatment with genistein, a slowed rate of increase was observed in the PSA levels over time (around 20% increase in PSA) compared to the PSA rates prior to entering the study (around 56% increase in PSA) (211). Additionally, another Phase II clinical study examined genistein's effects on PSA levels in prostate cancer individuals. In this case, 6 mg/kg of genistein was administered to patients daily over a 6 month period, and 17% of these patients observed a 17% reduction in their PSA levels (212). Thus, these clinical trials suggest the beneficial effects of genistein on cancer in human subjects, with minimal side effects.

It is clear from the extensive research that genistein induced anti-proliferative effects on several different cancers (Fig 10). From these studies, it was shown that genistein affected the following proteins: NF- κ B, Akt, TGF- β , p38 MAPK, MEK4, the MAPK pathway, IGF-1, as well as having an effect on estrogen-dependent and estrogen-independent tissues. The *in vitro* concentrations that showed cancer cell inhibition ranged from 0.36 μ M to 60 μ M of genistein. The greatest effects appeared above 10 μ M. The *in vivo* concentrations ranged, depending on the animal model utilized, from 50-1000 mg/kg/day of genistein use. And finally, clinical trials showed growth inhibition effects at 1 to 6 mg/kg/day of genistein consumption. Thus, it is clear that genistein exerted important anti-proliferative effects on cancer cells through a variety of ways and mechanisms and this phytoestrogen would be important to explore further in advancing trials.

2.2.2.2 Genistein and cell cycle proteins

Genistein's vast effects were also observed in relation to the cell cycle. As discussed in the cell cycle proteins section of resveratrol, the cell cycle is tightly regulated by cellular proteins that include specific cdks and cyclins (213). The two main transitions that occur before progression through the cell cycle are the G1/S-phase checkpoint and the G2/M-phase checkpoint. The cell is allowed to progress through the first checkpoint, G1/S-phase when the following complexes are present; cyclin D-Cdk4/Cdk6, cyclin E-Cdk2 and cyclin A-Cdk2 (130, 213). The complex that is necessary for progression through the second transition phase, G2/M-phase, is the cyclin B-Cdk1 complex. These transition phases are important for regulation of the cell cycle normally, in cancer, continual division and proliferation occurs through perpetual progression through the cell cycle.

The concentrations of genistein that inhibited these cell cycle proteins varied from 5 to 200 μ M for several different cell lines, as documented in current research (214).



Numerous reports that shown that genistein decreased cell cycle progression at the G2/M transition phase in breast cancer cells as well as prostate cancer cells (215-217). Genistein reduced the enhanced activation of cyclin B1,

which normally allows the cells to progress through G2 and into M-phase (218). Cyclin B1 also acts by regulating the kinase action of Cdk2 and Cdc2 (cdk1), which help with progression through the G1/S-phase checkpoint and G2/M-phase checkpoint respectively. Genistein administration (50-200 μ M) altered activation states of these two kinases in PC-3-M prostate cancer cell lines (216).

Genistein has also displayed, at these concentration levels, inhibition of specific cell cycle regulators in ovarian cancer and neuroblastoma cell lines (219, 220). In neuroblastoma cells, 50 and 100 μ M of genistein caused activation of the tumor suppressor protein, checkpoint kinase 2 (Chk2), while ovarian cancer cells activated, similarly, Chk1 (219, 220). These two checkpoint kinases, cause dephosphorylation of two separate Cdc25 phosphatases (Cdc25A and Cdc25C) (219, 220). When this occurs, cyclin-dependent kinase

1 (CDK1 also known as Cdc2), is inactivated and thus does not allow the cdk-cyclin (Cyclin B1) complex to form and thereby fails to initiate movement from G2 into M phase (220). Similar results were found with hepatoma cell lines. Upon genistein (5 to 20 μ M) treatment to these cells, a dose-dependent decrease in Cdc2 activity and Cyclin B1 expression was observed (221). Similar results were obtained when genistein was used on other cancer cell lines (216, 217, 219-221).

Cyclin-CDKs were directly affected by genistein, but they were also indirectly affected by genistein via upregulation of certain CDK-inhibitors (CDKIs), which negatively controlled the cell cycle (157). Examples of CDKIs include p21^{WAF1}, p27^{KIP1}, p16^{INK4a} and in certain forms of cancer, increased expression of these proteins is seen in MDA-MB-231, MDA-MB-435, MCF-7 breast cancer lines, PC3, LNCaP prostate cancer lines, H460 and H322 non-small cell lung cancer lines and HN4 head and neck squamous cancer lines (16, 169, 171, 222-224). In MDA-MB-231 breast cancer cell lines, a dose dependent increase in p21^{WAF1} expression was observed with 5 to 30 μ M of genistein over 24 to 72 hours (171). p53 induced the expression of p21^{WAF1} in several cases, which led to the suppression of CDKs, thus inhibiting movement through the cell cycle and decreasing cellular growth at this step (171).

Genistein has also been shown to decrease cell cycle movement by interfering with signal transduction proteins that increased invasion of cancer cells. Importantly, in pancreatic and colon cancer cell lines (209, 225), genistein has been shown to activate the protein, Forkhead box protein M1 (FoxM1) which functions in cancer progression and is up regulated in several carcinomas. FoxM1 is important to the cell cycle, because of its ability to modulate several cell cycle regulators which include Cyclin B, Cdc25A and Cdc25B (225).

Poor prognosis of both breast and pancreatic cancer was seen with overexpression of FoxM1 (225). In pancreatic cancer cell lines, concentrations of 50 and 100 μM of genistein treatment over a period of 72 hours, showed inhibition of the cell cycle (225). Thus, genistein, reduced the expression of FoxM1, which inhibited transcription of important cell cycle regulators, sequestering the cells in the G1 or G2 phases of the cell cycle (16, 223, 225). In prostate cancer cell lines, TRAMP-C2, the involvement of two kinases (Myt-1 and Wee-1), that are upstream of the cell cycle regulators (Cyclin B1 and Cdc2), were shown to be altered upon genistein treatment: increased Myt-1 expression levels and decreased phosphorylation of Wee-1 (226). This caused a resulting increase in p21^{WAF1} levels, along with a decline in Cyclin B1 expression, and could be another mechanism through which genistein inhibits cell movement through the G2/M checkpoint (Fig 11) (226).

Throughout this section genistein demonstrated an important role in cell cycle regulation. Research has increased the understanding of genistein's role in down-regulating several key cell cycle regulators including chk1, chk2, cdc2, cdc25A, cdc25C, cyclin B1, p27^{WAF1}, FoxM1, Myt-1 and Wee-1 (130). Genistein's impact is on cancer cells is not limited to cell cycle regulation as mentioned above, but also research reveals its ability to induce apoptosis in cancer cells.

2.2.2.3 Genistein and apoptosis

Apoptosis is essential for the cancer cells to be eliminated from the environment and in this section, genistein's role in inducing apoptosis in cancer is discussed. Genistein demonstrated apoptotic effects on anti-apoptotic proteins, pro-apoptotic proteins, caspases, NF-kB and Akt. The concentrations that induced apoptosis *in vitro* (10-200 μM) were very similar to concentrations on genistein's anti-proliferative effects (130).

Apoptosis is regulated by a multitude of factors that include anti-apoptotic and pro-apoptotic proteins. Importantly, the Bcl-2 family including Bcl-2 and Bcl-xL are anti-apoptotic proteins that function in balance with two main pro-apoptotic proteins (Bax and Bak) and balance shifts in these two groups of protein determine the activation or inactivation of apoptosis (130). Current studies have shown a reduction in anti-apoptotic proteins, like Bcl-xL and Bcl-2 upon genistein treatment in several cancers. In MDA-MB-231 breast cancer cells treated with 15 to 30 μ M of genistein over 24-72 hours, decreased Bcl-2 expression was observed, causing 43% of cells to induce apoptosis (171). Bcl-2 is overexpressed in certain breast cancers, for example, MCF-7 breast cancer cell line, and increased apoptosis was observed with 10 and 20 μ M of genistein (227). Apoptosis was initiated at 25 to 50 μ M concentrations of genistein over 48 to 72 hours in head and neck squamous carcinoma cells (224). Bladder cancer cell lines also showed a marked reduction in Bcl-2 after 50 μ M use of genistein over a period of 48 hours (208). Additionally, Bcl-xL much like Bcl-2, showed decreased expression upon treatment of 25 and 50 μ M concentrations of genistein in MCF-7 breast cancer cells (228).

Bax, the pro-apoptotic protein, initiates apoptosis upon higher accumulation and decreased binding to Bcl2. Upon Bax accumulation, the ratio between Bax and Bcl-2 changes, cause mitochondrial membrane permeability, and cytochrome c release, caspase activation and apoptosis. Multiple studies have demonstrated increased Bax accumulation in cancer cells, which leads to these down stream signaling events. For example, in lung cancer cell lines, 30 and 50 μ M of genistein over 24 hours led to a 1.8 to 4 fold increase in Bax accumulation, which increased apoptosis (229). Similarly, prostate cancer cell lines treated with 50 μ M of genistein over 36 hours showed a 12-fold accumulation in Bax, also

initiating enhanced apoptotic action (230). Additionally, increased expression of Bax was seen at 50 μ M of genistein over 48 hours in bladder cancer cell lines (208). And, MDA-MB-231 breast cancer cell lines treated with 5, 15 or 30 μ M of genistein over 24 hours demonstrated a marked 11-fold increase in Bax levels compared to Bcl-2 (171). Thus, genistein has been shown to induce apoptosis by causing overexpression of Bax in cancer cells, which causes apoptosis.

Genistein (50 μ M) was shown to cause overexpression of another proapoptotic protein, Bak in MCF-7 breast cancer cells (228).

Another part of the apoptotic cascade is initiation of one of the main thiol proteases that aid in inducing apoptosis, caspase 3, which cleaved subsequent caspases involved in cellular death. In multiple prostate cancer cell lines, genistein induced caspase 3 mediated apoptosis at concentrations ranging from 20, 30 to 50 μ g/mL (188, 231). Concentrations of genistein greater than 50 μ g/mL initiated necrotic cell death (188, 231). In T lymphoma cells, genistein induced apoptosis in a time and dose-dependent fashion. Concentrations of genistein (15, 30 and 60 μ M) after 24 hours of treatment increased caspase 3 activity seven fold, which led to DNA fragmentation and induction of caspase 9 activity, overall increasing apoptosis in these cell lines (232, 233). Human hepatocellular cell lines treated with 25 and 50 μ M genistein also displayed an increase in caspase 3, 8 and 9 after the treatment period of 24 hours (234).

Apoptosis was elicited in other human colon adenocarcinoma cell lines after 4 days of being exposed to 60 and 150 μ M of genistein causing a 54-94% increase in apoptosis (235). Similar results were found in rat fetal nontransformed intestinal cell lines, upon 80 μ M genistein exposure over 48 hours led to a decrease in cell number (235). And finally,

exposure of 100 μ M of genistein over 48 hours in both colon cancer cells and human colon adenocarcinoma cells showed a reduction in cell number by 60 to 75% in these lines (235). Thus, these concentrations of genistein were beneficial in regulating the levels of pro-apoptotic proteins, anti-apoptotic proteins and caspase regulation in promoting apoptosis.

It is important to note the upstream regulators (Akt and NF- κ B) that promote cell survival and decrease the downstream regulators like Bak, Bax, caspase 3, caspase 8, and caspase 9. One of the key upstream regulators of apoptosis is Akt, which when activated, leads to decreased cellular death (236). Akt inactivates several proteins of the extrinsic and intrinsic pathways including Bad, caspase 9 and indirectly caspase 8 (236, 237). Akt is a cell survival protein, that is initiated through a growth factor mediated pathway and has been shown to be increased in cancer and diseased states (184).

Treatment with 40 μ M genistein on colon cancer cells showed a decrease in phosphorylated Akt after 6 hours, and caspase 3 was upregulated after 12 hours, thus apoptosis occurred after downregulation of Akt (236). Apoptosis was also initiated after exposure with 50 μ M genistein in a time-dependent manner in prostate cancer cell lines (186). It was observed, that after decreased activation of Akt was seen in prostate cancer cell lines, that Akt, when activated (as seen in normal cancer states) targeted several other proteins of the apoptosis mechanism (caspases, GSK-3, ceramide, NF- κ B) reducing apoptosis (186).

Similar results were found with MDA-MB-231 breast cancer cells, upon treatment with 50 μ M of genistein over a period of 48 hours, decreased Akt activation at the ser473 residue was observed (184). The induction of apoptosis was thought to be due to the 40% decrease in phosphorylation of the GSK fusion protein, that would normally help protect

against apoptotic action (184). Similarly, MCF-7 breast cancer cell lines (and MDA-MB-231 cell lines) after treatment with 50 μ M genistein, showed decreased Akt activation after 24 and 48 hours of treatment, increasing the mitochondrial dependent route of apoptosis in these cells (189). The activity of caspase 7 was increased, due to decreased Akt phosphorylation, also initiating a marked increase in apoptosis.

NF- κ B, a transcription factor, was altered by genistein treatment in cancer cells and induced apoptosis. NF- κ B, as mentioned in the cell proliferation section (2.1.2.1), was regulated by Akt phosphorylation and was involved in cross talk with Akt. NF- κ B was inhibited by genistein treatment, which was important as NF- κ B promoted anti-apoptotic factors. Importantly, NF- κ B regulates many cascades that lead to apoptosis such as endoplasmic reticulum (ER) stress-relevant regulators (GADD153, m-calpain, GRP78) and also reduces proteasome action (157). In MDA-MB-231 breast cancer cells, this cross talk between the two pathways was evident, as treatment with 50 μ M of genistein over 36 hours showed a 60% decrease in Akt phosphorylation which demonstrated reduced DNA binding affinity for NF- κ B activation (184). Increased apoptosis was observed when these two pathways were affected. In prostate cancer cells, 30 to 60 μ M concentrations of genistein showed increased apoptosis over a period of 72 hours, with at least a 4 fold increase in cell death (238).

Likewise, in androgen-sensitive and androgen-insensitive prostate cancer cell lines, 25-50 μ M genistein over 3 days inhibited NF- κ B effectively and induced apoptosis (172). Treatment with genistein *in vivo* decreased NF- κ B activity and increased apoptosis in male nude mice transfected with prostate cancer cells upon use of 0.43 mg/day genistein over a period of 3 days (238). Genistein acted by mediating the upstream activity of Akt, thus

causing decreased NF- κ B activation and increased apoptosis in cancer cells (184). But, NF- κ B activation was not only mediated through the Akt pathway, and thus could have other ways of being activated, but as demonstrated previously, genistein regulated different proteins of the NF- κ B signaling pathway which led to increased apoptosis over time.

Another important apoptotic regulator that is mediated by NF- κ B activation is p53. In this p53 pathway, activation and up regulation of p53 led to inhibition of anti-apoptotic proteins like Bcl-2, and increased activation of pro-apoptotic proteins including Apaf-1 and Bax, which caused apoptosis to occur via the intrinsic pathway (239). In MCF-7 breast cancer cell lines, 10 μ M of genistein significantly increased p53-dependent transcription, leading to increased apoptosis (239). In an *in vivo* study, mice were implanted with prostate cancer cells and administered a soy diet containing 812 mg genistein aglycone equivalents/kg over a two week period (240), showed that a soy-based diet increased p53 expression by up to 47%, and along with an increase in apoptosis ranging from 56%-83% (208). Similarly, *in vitro* studies on prostate cancer cells, treatment with 50 or 100 μ M genistein over 24 hours caused an 11.5 to 25 fold increase in p53 protein activation (208). Importantly, apoptosis was initiated after 12 hours of genistein treatment in these cell lines, which was consistent with activation of p53 and subsequent Bax initiation before apoptosis occurred (208).

It is hypothesized that genistein could have the ability to inhibit proteasome activity that would normally have led to degradation and down regulation of p53 activity (230). In prostate cancer cell lines an observed increase in apoptosis was seen after 100 μ M genistein exposure over a period of 12 to 24 hours (230). Coinciding with this, genistein up regulation of p53 was increased upon proteasome inhibition in the cancer cells, which led

to increased apoptosis as well (230, 231). Overall p53 mediated apoptotic effects were induced due to genistein treatment in multiple cancers (157, 230). In MDA-MB-231 breast cancer cell lines, 30 μ M concentrations of genistein over 72 hours showed a marked increase in apoptosis (171). But, in cell lines with a mutant p53 gene, when treated with 5, 15 or 30 μ M genistein over 72 hours showed decreased p53 expression (171). While a functional p53 gene was able to decrease the expression of Bcl-2 and induce activation of p21 and Bax, which initiated apoptosis, a mutated p53 gene could not necessarily induce apoptosis as efficiently with genistein administration in certain cancer cell via this route. But, p21 could still be upregulated and genistein initiated apoptosis via different routes, thus p53 is not the only mechanism through which genistein increased cell death was achieved in cancer (171).

These studies show that apoptosis was initiated via multiple routes through genistein action. Genistein had vast effects on cancer cells by effecting both proapoptotic proteins and anti-apoptotic protein levels, decreasing both phosphorylated Akt and NF-kB activation as well as up regulating p53. From the research gathered on genistein's role in programmed cell death it was observed that concentrations ranging from 10 to 200 μ M *in vitro* produced the greatest effects. Again, these findings suggest that genistein has the potential to be an effective therapeutic agent and utilizes multiple mechanisms to inhibit cancer progression.

2.2.3 Genistein and angiogenesis

The data above displayed genistein's vast effects on inhibiting cancer invasion, proliferation, cell cycle regulation, and apoptosis. But, it also had the capability of decreasing angiogenesis in cancer. Decreasing blood vessel density, vascularization,

endothelial cell growth and angiogenic facilitators are useful tools for decreasing cancer metastasis. Genistein's extensive anti-angiogenic effects were investigated by examining genistein's role in promoting angiogenic inhibitors, reducing pro-angiogenic factors, decreasing DNA binding activity of HIF-1 α , reducing VEGF expression, as well as shrinking the vessel formation necessary for angiogenesis.

A plant based diet, including compounds such as genistein, could be beneficial in inhibiting the growth of blood vessels to tumor sites and to limiting the growth of endothelial cells, which are required for new blood vessel formation. For example, endothelial cell growth was targeted in bovine brain capillary endothelial cells after exposure to 1 to 100 μ M genistein showed a dose dependent increase in growth inhibition of these cells 5 days post treatment (157, 241).

Potent inhibition of vascular endothelial cell growth was also seen, from the bovine adrenal cortex and aorta, neuroblastomas, rhabdomyosarcomas, and human retinoblastoma cells after genistein (5 and 50 μ M) treatment (241). Once endothelial cells proliferate they need to be able to move through the basement membrane by releasing proteolytic degradation enzymes and angiogenic facilitators that promote the process of angiogenesis. Use of 200 μ M genistein over 2 to 3 days on bovine microvascular endothelial cells (BME) showed a marked decrease in one of these angiogenic factors, bFGF. When bFGF was targeted by genistein, decreased migration of the endothelial cells necessary for angiogenesis occurred (241).

bFGF is not the only angiogenic factor that has a role in angiogenesis, there are several other factors, regulators and genes important in regulating and promoting the progression of angiogenesis. Other genes like uPAR and protease activated receptors (PAR-

2) are necessary for invasion and migration to blood vessels (167). Several others promote angiogenesis and invasion through a variety of mechanisms, VEGF, TGF- β , neuropilin, bone-derived growth factor (BPGF), lipophosphatidic acid (LPA), and thrombospondin (TSP). In prostate cancer cell lines, treatment with 30 and 50 μ M of genistein over a period of 6 to 72 hours led to down-regulation in several of these pro-angiogenic genes and proteins including uPAR, VEGF, neuropilin, TSP, BPGF, LPA, TGF- β 2, TSP-1, and PAR-2 (167, 242). Similarly, breast cancer cells at comparable concentrations of genistein (50 μ M over 72 hours) have demonstrated upregulation of the activating transcription factor 3 (ATF3), which induced increased gene expression of plasminogen activator inhibitor-1 (PAI-1) (162). This PAI-1 gene acts on tumors by reducing angiogenesis and metastasis. Human bladder cancer cells also displayed similar results on angiogenic factors, in particular, showing stimulatory effects on angiogenic inhibitors. Treatment with 5 to 10 μ g/ml of genistein over 12 hours led to a marked increase in angiostatin, endostatin and TSP-1 *in vitro* (243). Thus, genistein has been shown to regulate in multiple pathways, inhibiting angiogenic activators (uPAR, VEGF, neuropilin, TSP, BPGF, LPA, TGF- β 2, TSP-1, and PAR-2, bFGF), and increasing expression of angiogenic inhibitors (ATF3, PAI-1), changing the ratio of these two (activators and inhibitors) is known to regulate and inhibit the growth of pre-existing blood vessels. Hence, many genes were up-regulated or down-regulated by genistein, displaying its intricate role in angiogenic regulation.

One important regulatory process that genistein impaired effectively was VEGF expression (Fig 7). This is a key target in angiogenesis, as VEGF tended to facilitate angiogenesis in carcinomas. In pancreatic cancer cells, HIF-1 α (discussed in the resveratrol section), mediated VEGF expression in cancer cells during hypoxic states where a blood

supply rich in nutrients and oxygen are in demand (244). Genistein had the ability to act as a nonspecific inhibitor of HIF-1 α . Higher doses of genistein (100 to 250 μ M) during hypoxic states in pancreatic cancer cells showed a reduction in the DNA binding affinity of HIF-1 α , with a marked increase in VEGF suppression (244). A dose-dependent and time-dependent effect on decreased VEGF expression in pancreatic cells was seen with 10, 50, 100 and 250 μ M genistein treatment over 24 hours (244). Similarly, bladder cancer cells had decreased VEGF levels after 10 μ g/ml genistein treatment, with strong effects showing up after 3 hours of administration (243). Prostate cancer cells additionally had similar effects on both decreasing HIF-1 α binding affinity as well as decreasing VEGF expression (245). The expression of HIF-1 α was decreased upon 60 μ M genistein exposure over a period of 24 hours. Similarly, the downstream target, VEGF, also displayed a significant reduction in expression. Following treatment on prostate cancer cells both with and without radiation, genistein decreased blood vessel tube formation with 30 μ M concentrations of genistein over 24 hours (245). Hence, genistein treatment was a critical target of HIF-1 α , and acted through this factor to decrease VEGF and vessel formation, which are essential for angiogenesis progression.

Pancreatic cancer xenografts in a mouse model also showed similar results. Mice were injected with 1.3 mg (comparable to 100 μ M) genistein per day, and they showed a comparable decrease in VEGF expression as well as decreased microvessel density, which was normally attributed to angiogenic growth (244). Increased vessel density leads to an increased nutrient source for the tumor and greater growth and metastatic potential. Blood vessel growth and density was investigated in hepatocellular carcinoma *in vivo* studies where mice were injected with a xenograft transplant and administered 50 mg/kg

genistein daily over 15 days (246). Those treated with genistein had a significant decrease in microvessel density, a known marker for decreased angiogenesis, as well as a decrease in tumor volume by up to 20% that of the untreated genistein control (246). In bovine microvascular endothelial cells genistein use of 200 μ M over a period of 2-3 days showed decreased production of the capillary tube-like structures that led to microvessel density needed for angiogenesis to occur (241).

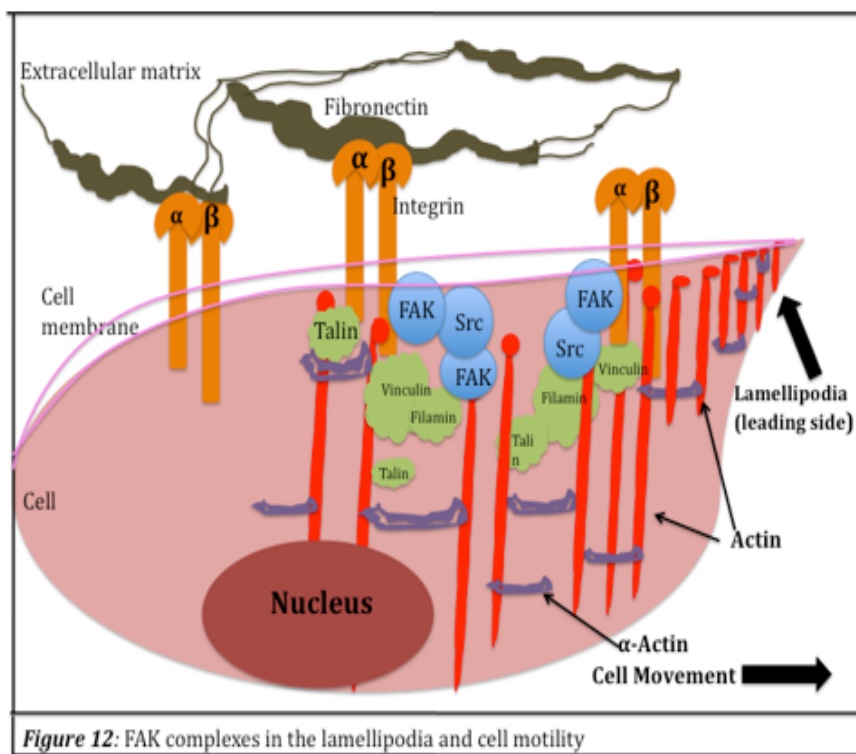
Furthermore, evaluation of angiogenesis and blood vessel growth was evaluated in bladder cancer models. An *in vivo* model of mice implanted with bladder cancer cells were fed either soy phytochemicals at 0.5% of the diet and genistein only at 0.14% of the diet over a 10 week period (208). Bladder cancer tumors in the mice models displayed a 35% to 50% reduction in the blood vessel density in the initial tumor site, coupled with a decrease in tumor size by 52% to 54% (208). Bladder cancer cells *in vitro* showed a reduction in VEGF mediated human umbilical endothelial cells (HUVECs), leading to a 69% reduction in tubules present in endothelial cell vessel formation with 5, 10, 20 and 50 μ M genistein (247). Hence genistein demonstrated a reduction in vessel formation, a sign of decreased angiogenesis occurring in cancer.

From the research presented in this section, it was observed that genistein reduced angiogenesis through multiple mechanisms. It acted on promoting angiogenic inhibitors, reducing pro-angiogenic factors, as well as inhibiting DNA binding activity of HIF-1 α , reducing VEGF expression, and decreasing vessel formation necessary for angiogenesis to ensue. Genistein's anti-angiogenic effects were seen in genistein concentration ranges from 5 to 250 μ M *in vitro*, and 1.3 mg to 50 mg/kg *in vivo*. Hence, genistein could be an important inhibitor of angiogenesis in multiple cancers in humans.

2.2.4 Genistein and inhibition of cell migration and motility

Research has documented genistein's beneficial effects on cancer thus far, showing this compound was effective at inhibiting MMPs, cell microcolonization pathways, and angiogenesis. However, the essential factor that that could not be compensated for by other mechanisms, is cancer cell motility. Cancer cells increase their cell migratory potential through enhanced cell adhesion and an increase in important cellular complexes that aid in movement and further progression of cancer.

Adhesion and migration are important processes for cancer cells. Adhesion occurs when cells attach to the basement membrane, through structures such as integrins, found

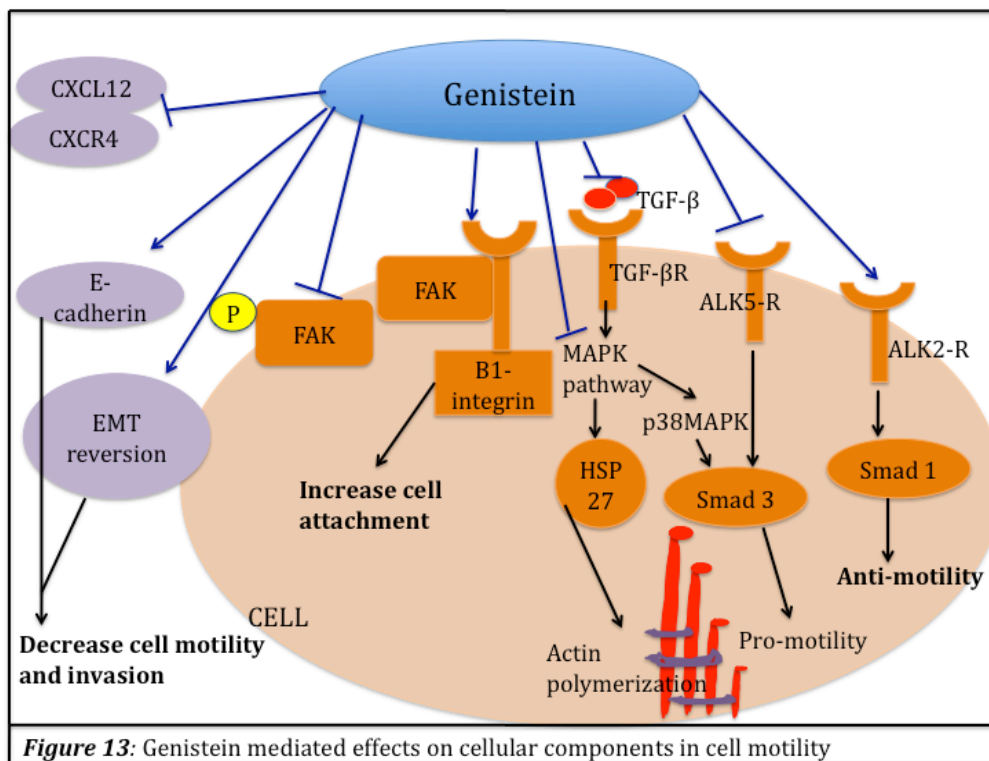


on the cell surface. During invasion, cancer cells have a loss of cell-to-cell attachment, and enhanced cell-to-membrane attachment, which influences their migratory potential. Attachment occurs through specific integrins and other

adhesion molecules that are expressed (typically on epithelial cell surfaces) that enable the cells to anchor efficiently onto the basement membrane. The transmembrane integrins are bound by the extracellular domain of the integrin to the basement membrane, and the opposite end of the integrin (the cytoplasmic domain) to the actin cytoskeletal filaments

within the cell (248, 249). This linkage is mediated through the use of cytoskeletal proteins vinculin, talin and filamin (157). The entire protein complex, which attaches the basement membrane to the cytoskeleton, forms a focal adhesion complex, and is usually exhibited at the leading edge of the cell at the lamellipodia where actin polymerization occurs.

Lamellipodia is an actin projection, which behaves similarly to a foot that pushes forward and aids in movement of the cell. The tail end of the cell retracts after actin reorganization occurs at the leading edge. The binding of several molecules that play a vital role in adhesion and migratory processes forms the entire focal adhesion complex which tethers the cell to the basement membrane. These proteins included focal adhesion kinase (FAK),



Src and paxillin (250) (Fig 12).

Genistein mediated its effects on the particular protein kinase FAK. In several cancers FAK is upregulated, causing

increased cell motility (157, 251-254) (Fig 12).

Genistein displayed, through multiple studies, inhibition of FAK as well as both increased and decreased cell adhesion (Fig 13). Initial cancer studies on genistein,

observed increased cell adhesion in certain instances, but data is limited due to the lack of research on its effects on cell adhesion in cancer. Hence, research investigated so far, show inconclusive evidence on cell adhesion regulated by genistein treatment. Though, inhibited cell adhesion was observed in hepatocarcinoma and pancreatic cancer cell lines at low micromolar concentrations, but allotted time could have been an issue in adhesiveness (210, 255). These results agreed with other research on genistein that documented decreased attachment of the cells coupled with decreased migration in invasive breast cancer cells (MDA-MB-231) (256). Decreased adhesion occurred through genistein's suppressive ability of NF- κ B and AP-1. Additionally, prostate cancer cells treated with physiological concentrations of genistein (1-10 μ M), reduced attachment and migration (255). On the other hand, increased adhesion was seen in prostate cancer cells that were orthotopically implanted into mice and fed 100-250 mg/kg of chow over a four week period, and they experienced a time and dose-dependent increase in cell adhesion (257) (Fig 13).

In vitro studies on prostate cancer cells also demonstrated a time and dose-dependent increase in cell adhesion upon low nanomolar concentrations of genistein (258). Further *in vitro* studies on prostate cancer cells, and to a lesser extent, breast cancer and melanoma cell lines upon treatment of 0.5 to 50 μ M genistein over 24 hours were shown to have increased adhesion (259). But, the increased cell attachment could have been due to a decrease that was observed in phosphorylated FAK, but still allowed greater FAK accumulation at the focal adhesion complex. Upon increased FAK levels, increased FAK complexing with the beta 1-integrin cell attachment proteins was observed, which led to enhanced expression of the β 1 integrin. This integrin is usually lost in the advanced cancer

state (258, 260). Thus when the integrin was intact, the cell adhered to the basement membrane and attachment increased. Though this was not the only mechanism that had an effect on adhesion and motility, preliminary studies are just being investigated and a further role of genistein on cell attachment are still under examination (Fig 13).

In metastasis adhesion is also influenced by chemokine receptors that act as sensory agents for the cancer cells. The cancer cells follow these chemokines to a new tissue or organ . These chemokine receptors are also important in adhesion of the cells to the new area (261). Genistein at concentrations ranging from 1-50 μ M in breast cancer cell lines over a period of 24 hours, showed a dose-dependent decrease in both the chemokine receptors CXCR4 and CXCL12 (261). A decreased number of receptors led to decreased adhesion at secondary and tertiary sites and a reduced migration of cells to the new metastatic areas (Fig 13).

In breast cancer cell lines, treatment with 1-50 μ M genistein over a 24 hour period showed decreased cell motility (259). Likewise, melanoma cancer cell lines have demonstrated a reduction in cell motility upon treatment with 20 to 50 μ M genistein over 24 hours (259). Prostate cancer cell lines also followed this general trend, and have demonstrated that with lower concentration of genistein (1 to 10 μ M) decreased cell motility (255). Interestingly, decreased cell motility was contact dependent in these prostate cell cultures. During invasion, tumor cells came in contact with multiple other cells, as they tended to crawl over the normal cells, due to their enhanced migratory state. In these prostate cancer cell lines, they demonstrated how genistein acted by inhibiting this cell-contact migration observed in invasion (255). The other mechanism that could

influence this reduction in cell motility was considered to be due to genistein regulated inhibition of important transcription factors, NF- κ B and AP-1 (259).

The decreased cell motility observed was in agreement, across several studies, that inhibited cell motility was linked with decreased phosphorylation of FAK. In a study on Chinese hamster ovary cells, overexpression of FAK was associated with an enhanced migratory phenotype (262). The autophosphorylation site of FAK, Y397, had also been shown to be a SRC binding site, and its activation was demonstrated in these ovary cells to be necessary for increased migration (262). Research thus far supported genistein's influential role on inhibiting FAK phosphorylation and hence reducing cell migration events in cancer progression. For example, in hepatocellular carcinoma cells, genistein (5 μ g/mL to 20 μ g/mL) has shown a time and dose-dependent increase in inhibition of FAK phosphorylation *in vitro* (210). Particularly, a reduction of 40% was observed on FAK phosphorylation after exposure with 5 μ g/mL genistein. In pancreatic cancer *in vivo* studies, mice orthotopically transplanted with pancreatic tumors were treated with 100-250 mg genistein/kg of chow over a four week time period and they also displayed a 12% reduction in FAK phosphorylation (257). Prostate cancer *in vitro* studies also demonstrated a reduction in the phosphorylation of FAK at low nanomolar concentrations (1-50 μ M over 24 hours) of genistein, and additionally, further prostate cancer studies showed genistein reducing the phosphorylation of FAK at the autophosphorylation site, Y387, which was needed for migration (258). These preliminary studies were in agreement that cell motility could be reduced with genistein treatment, as well as demonstrating an effect on cell adhesion. Both of these processes were mediated through the suppressive FAK

phosphorylation events induced by genistein, but more studies need to be done to understand more thoroughly the mechanism involved in cell motility (Fig 13).

Cell motility also occurred due to other enhanced cell signaling interactions that had an effect on cytoskeletal structures of the cell. One particular signaling pathway, that was discussed in the cell microcolonization section was the TGF- β induced pathway. This secreted protein led to activation of multiple signalers in the MAPK pathway, including a key tyrosine/threonine kinase, MEK4, p38 MAPK and mitogen-activated protein kinase-activated protein kinase 2 (MAP-KAPK2). Activation of these kinases led to an overall activated state of heat shock protein 27 (HSP27) that regulated the actin cytoskeleton of cells (194, 197).

The HSP27 protein is important for progression of cell motility, especially in cancer, where HSP27 is upregulated as seen in prostate cancer (263). HSP27 acts through increasing the F-actin polymerization, decreasing actin fragmentation and maintaining the focal contacts on the membrane (264-266). When HSP27 was de-phosphorylated, or inactivated, it did not allow for actin polymerization to occur through the process of actin capping. This was where a protein block was added to the end of the actin filament and did not allow the additional actin polymers to form (266). Genistein, as discussed in the previous microcolonization section, worked to inhibit MEK4 as well as p38 MAPK, which ultimately led to downregulation of HSP27 (170). In a mice prostate cancer *in vivo* model, treatment with 100 to 250 mg genistein/kg chow over a period of four weeks showed a decrease in the phosphorylation of p38 MAPK by 23% and a decrease in HSP27 phosphorylation by 12% (257). Other cancers treated with genistein supported these results, displaying decreased genistein-mediated HSP27 activation. In multiple prostate

cancer cell lines, treatment with 10 to 100 nM (up to 50 μ M) genistein caused suppression of TGF- β -MAPK2-mediated activation of HSP27 (193, 196). These concentration ranges of genistein that induced decreased HSP27 phosphorylation occurred at physiological concentrations (193, 196). This was only preliminary evidence that showed the possible role genistein had in decreasing phosphorylation of HSP27 and repressing cell motility, more research needs to be done to increase the support for genistein-mediated effect (Fig 13).

There was similar evidence that supported genistein's role in anti-cancer cell motility through another cell signaling mechanism, but again, the research was minimal and only gave a rough idea of genistein's mechanism of anti-motility action. The MEK4 pathway can interact with the endoglin pathway (157). This pathway includes the Smad family of transcription factors that, depending on their expression, could lead to increased or decreased cell motility patterns that mediate cell invasion responses (267). The understanding of this pathway has come from prior research done on prostate cancer. The general mechanism behind the endoglin-signaling pathway is that upon activation of the endoglin TGF- β type I receptor known as ALK-2, it subsequently activates the transcription factor Smad 1 (268, 269). Smad 1 plays an important role, promoting anti-motility effects (267). On the other side, the TGF- β /ALK5 (canonical pathway) receptor activates the transcription factor Smad 3, which is important in supporting pro-motility effects (267). Thus, cellular motility and subsequent invasion was regulated by the ratio of Smad 1 to Smad 3. Genistein was able to act on both parts of this endoglin pathway (267).

Firstly, genistein acted on the Smad 3 regulated portion of this pathway through suppressing MEK4 in prostate cancer cells. MEK4 had the ability to activate p38 MAPK

which interacted with Smad 3 and activated it (264). It has been shown, as mentioned earlier, that genistein inhibited p38 MAPK activation in prostate cancer cell lines (193). Hence, genistein had the potential to work by decreasing the pro-motility transcription factor Smad 3. The other linked signaling part of this pathway, Smad 1 was also influenced by genistein. Genistein acted, not through endoglin, but through the receptor ALK-2, in activating Smad 1, which led to increased transcription of anti-motility genes (269). Treatment of prostate cancer cells with 25 to 50 μ M genistein over 24 hours demonstrated genistein working through the ALK2 receptor as well as causing an increase in Smad1 promoter activity and Smad 1 expression which led to an inhibited cell motility phenotype (269, 270). Thus, with the use of genistein, Smad 1 was upregulated and Smad 3 was suppressed. Overall this led to a change in the ratio of these transcription factors, favoring Smad 1's anti-motility effects. Research is beginning to support genistein's anti-motility effects, but still more studies were required to provide stronger support for this mechanism (Fig 13).

TGF- β was not the only mechanism through which decreased cell motility occurred from genistein use. Other prostate cancer studies have shown genistein to play a role in increasing E-cadherin levels, which effected cell to cell binding (271). In prostate cancer cell lines, administration of 15 μ M of genistein over a time period of 24 hours, demonstrated an increase in E-cadherin expression. The cell lines also saw a decrease in vimentin, a protein that was utilized for increased invasion and metastasis during cancer. Alongside this increase in E-cadherin expression was also a reversal of the epithelial to mesenchymal transition (EMT) (271). This transition normally occurs in more progressed cancer states, where cells usually inhibit several proteins and regulators that can lead to

differentiated cell types (271). Thus increased E-cadherin levels and decreasing this EMT phenotype, showed that genistein decreased the invasive and metastatic phenotype in prostate cancer cells. Hence, genistein again appeared to act as a beneficial mechanism by which decreased motility and invasion in advanced cancer (Fig 13).

From this section it is clear that genistein was able to work through a variety of mechanisms to decrease cell motility and subsequent invasion of cancer cells. Genistein played a role in attachment, suppressing FAK-mediated migration, decreasing the activation of the TGF- β -MAPK signaling pathway to inhibit HSP27 and actin polymerization, working through the endoglin signaling pathway to decrease Smad3 and increased Smad1 to inhibit motility, as well as working independently of TGF- β to increase E-cadherin levels and cause a reversion of the EMT. Thus it was clear from the preliminary research on genistein and its effects on motility and attachment, that it would have a beneficial cancer fighting initiative at this point, but more studies need to be carried in order to support and better understand and clarify all of these mechanisms described in this section.

2.2.5 Conclusions on genistein

The expansive amounts of research presented above only begun to explore the multiple levels at which genistein action occurred. With genistein acting on several different layers of metastasis, it would be beneficial to continue to explore all the possible routes that genistein could potentially take advantage of to aid in reducing breast cancer metastasis.

Genistein acted at the level of MMP inhibition, modulating cell microcolonization, including cell proliferation, cell cycle proteins and apoptosis, reducing angiogenesis and

suppressing cell motility events in the metastatic cascade. The various molecular/cellular targets included, MMP-2, -7, -9, laminin 5 γ 2, COX-2, Akt, NF- κ B, AP-1, HIF-1 α , Cyclin B1, Cyclin D, p21^{WAF1}, CDK1, Bax, Bcl-2, Smad1, Smad3, p38 MAPK, MEK4, Endoglin, E-cadherin, CXCR4, CXCL12, FAK, Hsp27, VEGF, vessel density, angiogenic activators (uPAR, VEGF, neuropilin, TSP, BPGF, LPA, TGF- β 2, TSP-1, and PAR-2, bFGF), angiogenic inhibitors (ATF3, PAI-1), Apaf-1, p53, caspases, chk1, chk2, cdc2, cdc25A, cdc25C, FoxM1, Myt-1 and Wee-1. Hence, genistein worked through multiple pathways, proteins, and complexes and has great potential to treat the metastasized form of breast cancer.

3. Conclusions

3.1 Combinatory effects of genistein and resveratrol

Now that both resveratrol and genistein have been thoroughly examined for their role in inhibiting metastasis at multiple steps, it was essential to break down the key molecular processes and pathways being targeted by these agents. By doing so, it made the individual and combinatory effects of these compounds clearer. Examination ensued on resveratrol's individual protein and pathway effects, followed by genistein's key targets, and finally ending with the discussion of the potential combined effects of both resveratrol and genistein on the metastatic process. Specifically, these three subsections will discuss how these dietary compounds targeted multiple pathways within invasion, cell proliferation, apoptosis, angiogenesis, cell motility and migration (Fig 14).

Resveratrol, displayed from the research above (all references within the resveratrol section), widespread effects on the different cellular pathways that led to metastasis in several forms of cancer. This section summarized the key targets of resveratrol, and

examined those pathways and mechanisms that resveratrol had exploited individually compared to genistein action (Fig 14a). The initial section, that looked at the effects of resveratrol on invasion pertained to MMP-9 and MMP-2 expression that influenced the invasive phenotype of cancer cells.

Through extensive research, resveratrol demonstrated inhibition of HRG- β , a growth factor that activated the HER-2 receptor. This growth factor was upregulated in a certain percentage of cancers. Upon being activated, HRG- β increased MAPK expression, particularly ERK1/2 expression, which resulted in an increased invasive potential of the cancer cells through MMP-9 induced expression. Resveratrol also inhibited the transcription factor, NF- κ B, which was upregulated, or constitutently expressed, in many cancers. NF- κ B played an important role in downstream effects on invasion, including activation of COX-2 and SPARC. COX-2 expression acted on MMP-9 and promoted its expression. Consequently, SPARC activation induced MMP-9 and MMP-2 expression. Cancer cells also increased the expression of other pathways, including the PI3K/Akt pathway, through a multitude of ways. This included, a mutant PTEN tumor suppressor gene, or upstream overexpression. Resveratrol acted by inhibiting PI3K and the Akt pathway, which led to a reduction in IGF-1 downstream of this pathway. Decreased expression of IGF-1 caused inhibited MMP-9 and MMP-2 activation. Resveratrol also inhibited Talin, a cytoskeletal protein aided in production of FAK complexes, leading to cell adhesion and migration. Finally invasion was inhibited through resveratrol's ability to activate cdc42, which decreased the conversion of filopodia to lamellipodia, causing a reduction in migration of the cancer cells. Hence, it was clear from these effects that resveratrol alone

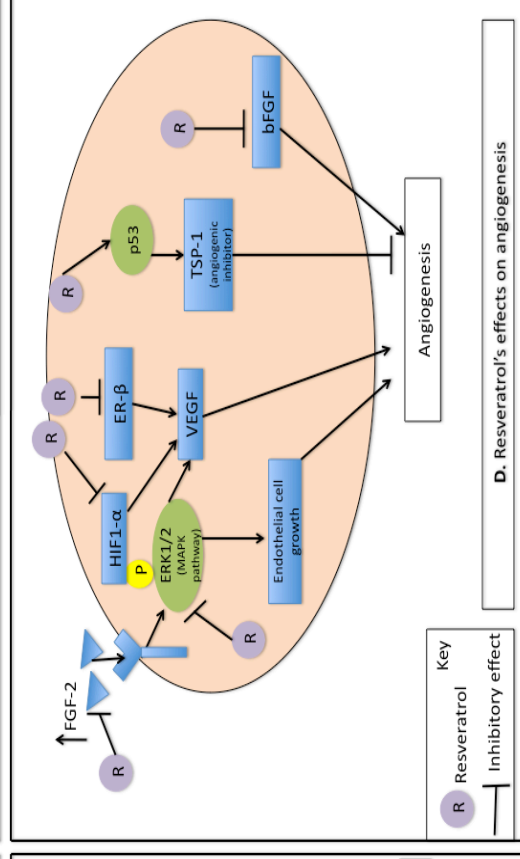
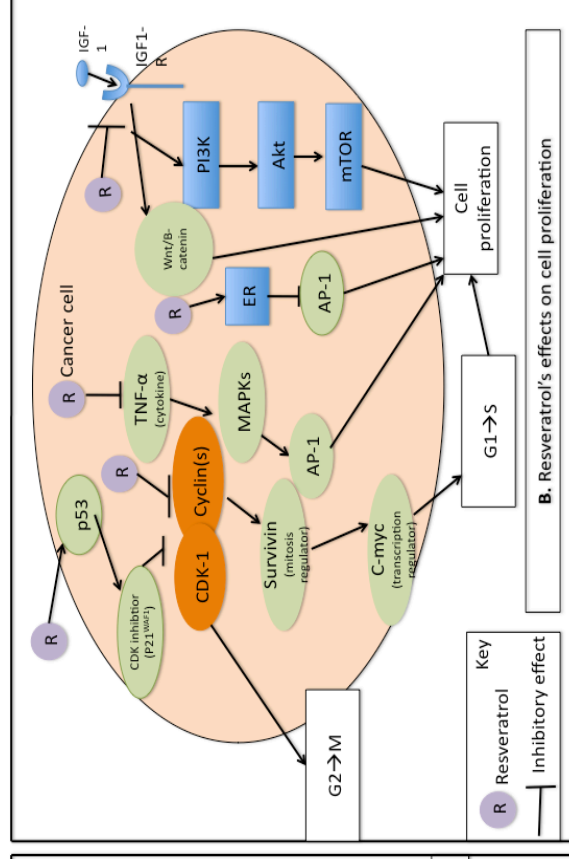
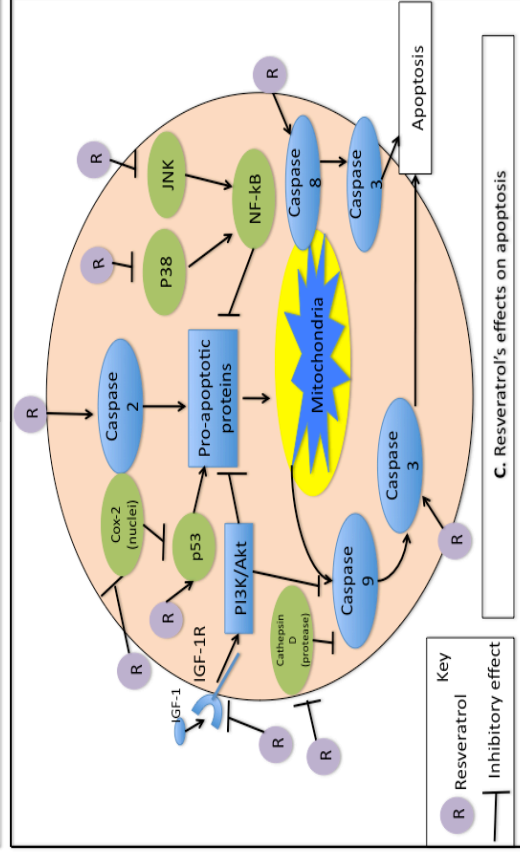
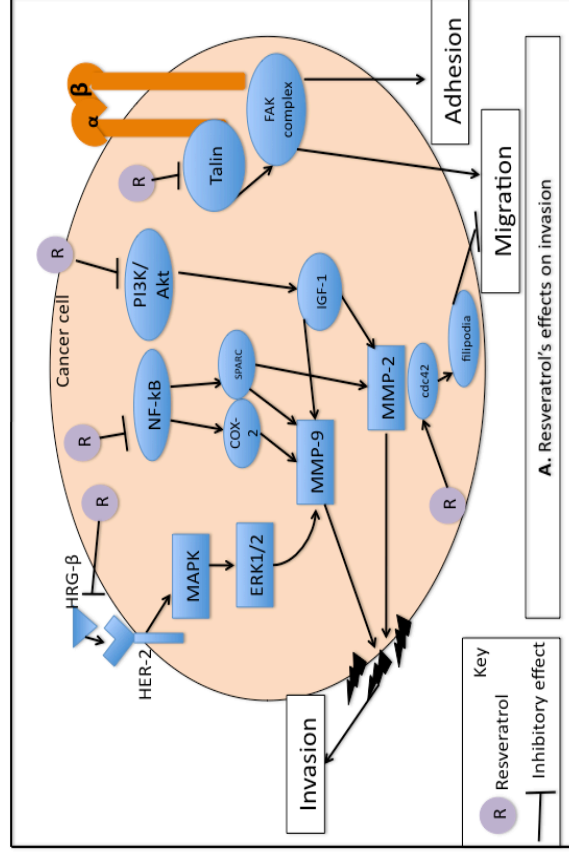
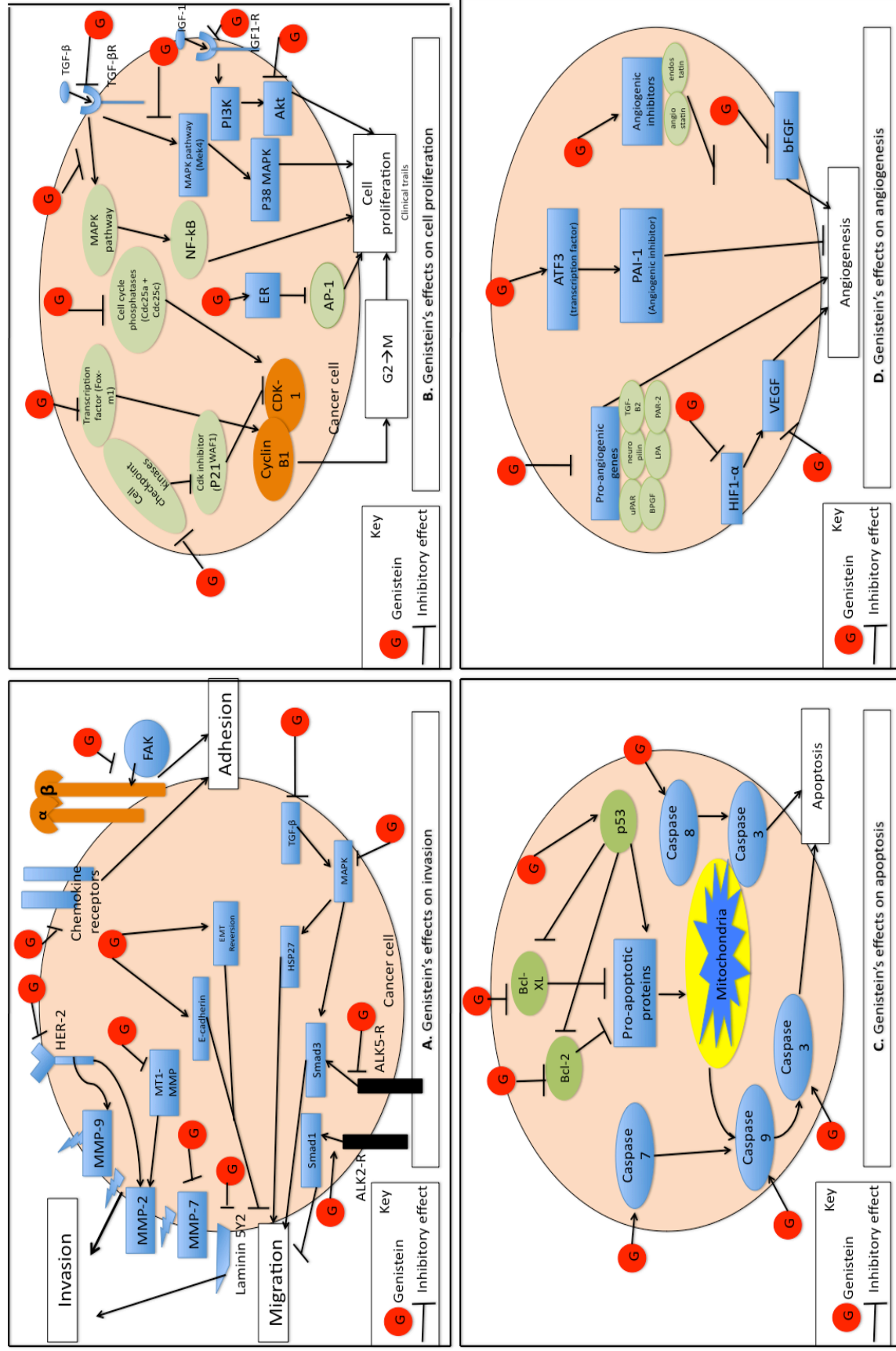


Figure 14a: Effects of resveratrol on metastasis



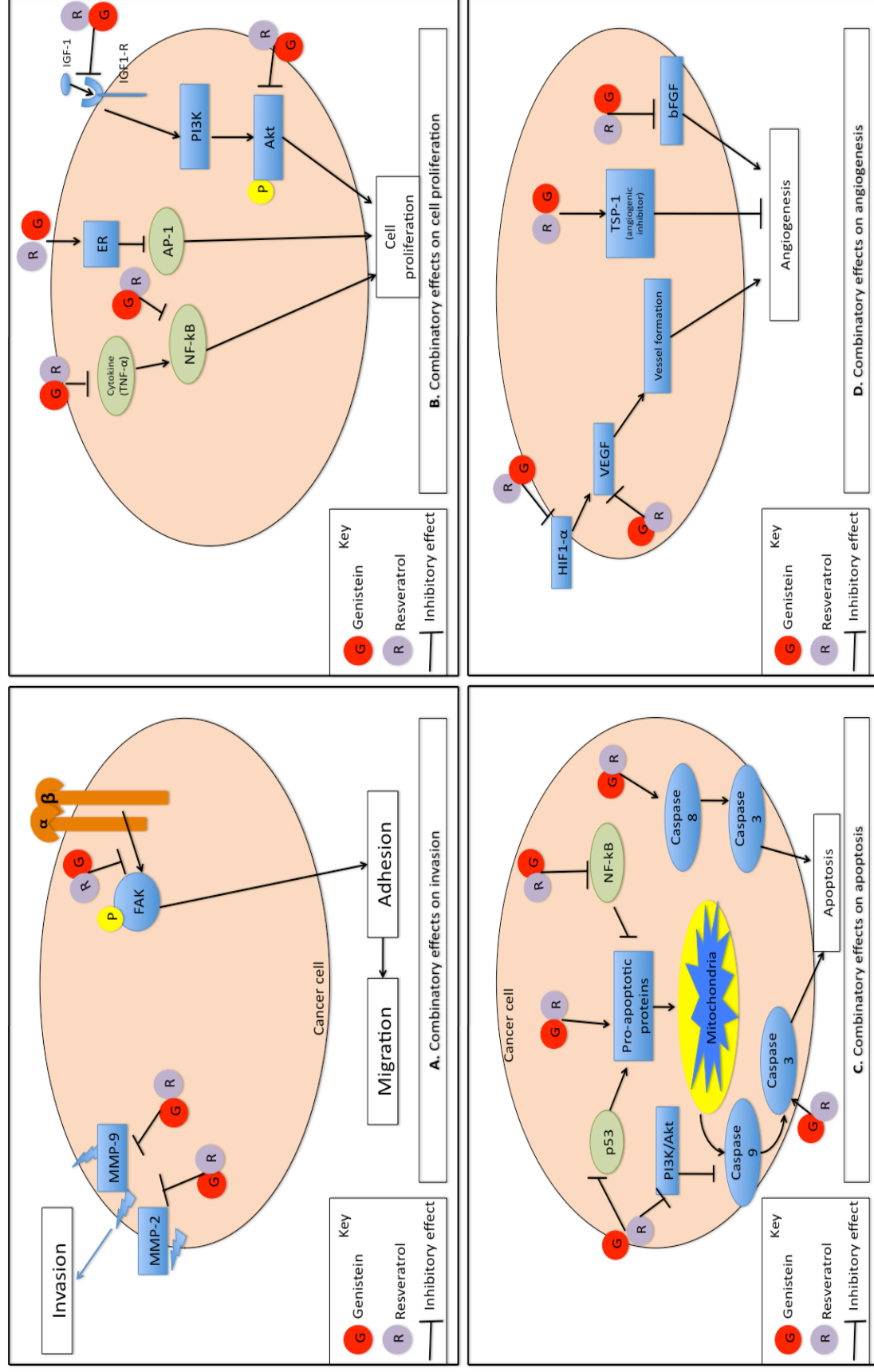
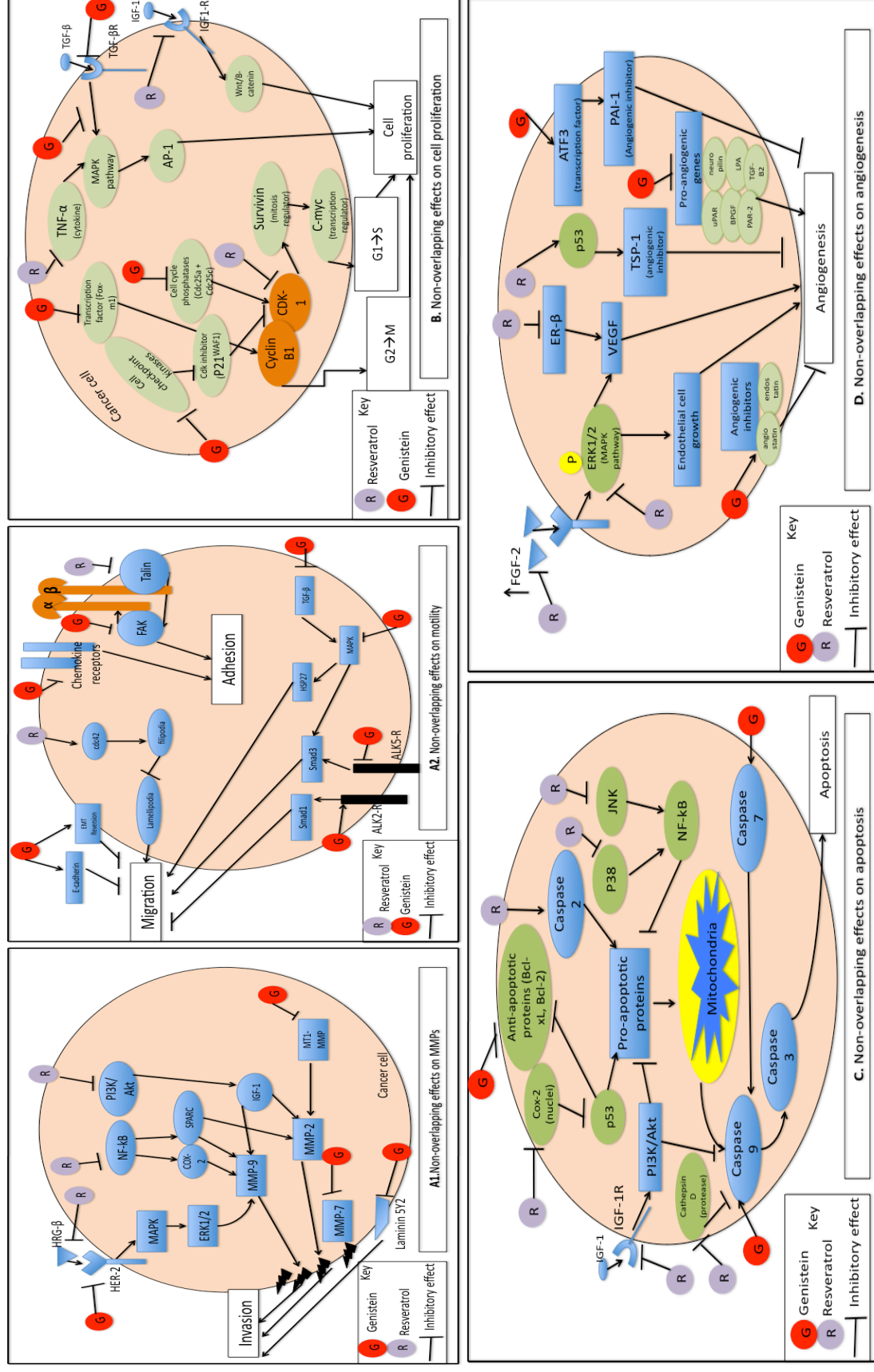


Figure 14c: Combinatory effects of genistein and resveratrol on metastasis



has the potential to reduce adhesion, migration and invasion of breast cancer cells through multiple routes.

Breast cancer was also influenced by resveratrol at the cell proliferation step. Resveratrol displayed anti-proliferative effects on cancer cells through several pathways. For example, resveratrol treatment caused inhibition of CDK-1, which was a kinase, that complexes with cyclins. When cdk-cyclin were activated, survivin, a protein activated by cell progression through the G2 to M phase, was increased. Increased production of survivin acted on cellular MYC, increasing the production of this transcription factor, enhancing expression of genes that included those that progressed the cell through G1 to S phase and caused increased proliferation. Thus upstream inhibition of CDK-1, led to inhibition of the cell cycle checkpoint. Resveratrol also inhibited TNF- α production, which was an inflammatory cytokine. TNF- α was overproduced in several cancers, which caused strong activation of certain parts of the MAPK pathway, including JNK and MEK. JNK and MEK expression caused over activation of the transcription factor, AP-1, which has enhanced cellular proliferation.

Resveratrol also reduced proliferation through inhibition of the IGF-1 receptor. As mentioned, IGF-1 expression was increased during cancer, which activated two main pathways involved in growth. First, IGF-1 activated β -catenin, part of the cadherin complex that helped anchor proteins and regulated proliferation. When IGF-1 expression was induced, Wnt was also activated, causing decreased degradation of β -catenin, and increased activation of cell proliferation associated genes. The other pathway that was induced through IGF-1 expression

was the PI3K/Akt pathway, when it was activated it led to downstream expression of mTOR, a protein kinase associated with cell proliferative effects. Thus resveratrol demonstrated regulation of multiple pathways associated with cancer cell proliferation.

Resveratrol treatment has also acted on apoptotic pathways, causing increased cell death in cancer cells. As mentioned in the anti-proliferative effects, resveratrol has inhibited the IGF-1R, which when activated, led to enhanced activation of the PI3K/Akt pathway. When this pathway was turned on, it blocked caspase 9 activation, reducing apoptosis. Cancer cells also induced COX-2 expression within the entire cell, while resveratrol treatment showed an increased expression of COX-2 localized in the nuclei of cells. The nuclei accumulation of COX-2 increased activated p53 in cancer cells, which enhanced the expression of pro-apoptotic proteins, and initiated the intrinsic apoptotic pathway. Resveratrol, additionally, increased activated caspase 2, which initiated the expression of pro-apoptotic proteins.

Another level of apoptosis resveratrol acted at was the MAPK pathway. This compound inhibited two key proteins in the MAPK pathway, JNK and p38. Both of these increased NF-kB expression, which led to the production of genes that reduced the amount of pro-apoptotic protein expression and decreased apoptosis. Finally, resveratrol inhibited apoptosis through increasing production of cathepsin D, an aspartic protease that led to increased caspase 9 expression and induced apoptosis via this route as well.

Furthermore, resveratrol displayed anti-angiogenic properties in cancer. Resveratrol caused inhibition of the overexpressed growth factor, FGF-2, that was activated by cancer cells. When FGF-2 was bound to its receptor, it induced phosphorylation of the MAPK pathway, specifically ERK1/2. Resveratrol inhibited this step through decreasing activation of ERK1/2. ERK1/2, when activated by cancer, initiated endothelial cell growth and increased VEGF expression, both of which, propagated angiogenesis to occur. Resveratrol also inhibited the estrogen receptor beta (ER- β) induced transcription of VEGF. And, resveratrol demonstrated increased p53 expression, which was normally inhibited or mutated by cancer. When p53 was activated it caused increased TSP-1 production, an adhesive glycoprotein that reduced angiogenesis through multiple ways, including decreasing migration of endothelial cells (Fig 14a).

Next, examination ensued on the dietary compound, genistein. Again, this sub-section will display the general pathways and mechanisms that genistein exploited, which led to decreased metastasis during cancer (references can be found in the main genistein section of this paper). Genistein inhibited invasion of cancer cells through multiple routes (Fig 14b). Genistein research observed a reduction in the expression of multiple MMPs (including MMP-2 and MMP-9) upon treatment, which limited the invasiveness of cancer cells. Genistein also inhibited HER-2 (the growth factor receptor), which was one of the ways decreased expression of MMPs occurred. The HER-2 receptor was part of the epidermal growth factor family of receptors, and was over-activated in many cancers, thus genistein worked by inhibiting activation of it. Genistein also repressed MT1-MMP expression, which was

an MMP containing transmembrane domain at the cell surface, when activated it induced MMP-2 expression and caused subsequent degradation of the membrane. Genistein additionally acted on the invasive phenotype of cancer cells through a direct reduction in MMP-7 expression, and decreased laminin 5 γ 2 attachment. This particular laminin, an essential scaffolding protein of the basement membrane, played an important role in invasion. Genistein also inhibited the chemokine receptors, which were present at the secondary and tertiary sites of metastasis and aided in the adhesion of cells to new areas and tissues. Genistein worked transiently by increasing the binding of FAK to the β 1-integrin, which caused increased adhesiveness and attachment of the cells, which could play an unknown role in invasion at this point. Cancer cells also increased the production of TGF- β , a secreted protein that acted as a signaling molecule for a multitude of metastatic events. Importantly, it activated the MAPK pathway, which initiated the expression of HSP27. HSP27 worked in a variety of ways, including maintenance of actin filaments and FAK complexes, stabilizing and also increasing migration. Genistein displayed inhibition of TGF- β signaling at the level of TGF- β expression as well as decreased MAPK activation. MAPK signaling cross talked with Smad 3, a polypeptide that activated and induced migration of cells. Genistein inhibited Smad 3 through its receptor ALK5-R, and on the other hand, activated the ALK2-R receptor, leading to increased Smad 1 production. Smad 1 mediated the signals that reduced cellular migration. Genistein also demonstrated the ability to inhibit migration through increased E-cadherin production, which allowed cell-to-cell contacts to occur and led to a reduction in the incidence of migration. And finally, genistein played a role

in activating the EMT reversion, which also decreased the migration and invasion of cancer cells.

Genistein was observed to display a pivotal role in decreasing proliferation in cancer. Genistein acted through the following mechanisms to cause anti proliferative effects. First, genistein increased expression of the cyclin-cdk inhibitor, p21^{WAF1}. Genistein did this by regulating Wee-1 and myt-1 which acted upstream of p21. The increased expression of p21 decreased cyclin-cdks, reducing G2-M cell cycle progression, inhibiting proliferation of cancer cells. Cancer cells also increased the expression of proto-oncogenes, such as Fox-m1, a transcription factor that played a pivotal role in cell cycle progression. Genistein inhibited the enhanced expression of Fox-m1, which reduced its downstream effects, including the activation of cyclin-cdk complexes that caused cell cycle progression and proliferation. Protein kinases also promoted the cell cycle, such as chk1 and chk2, which phosphorylated the phosphatases, cdc25a and cdc25c activating CDK-1 and initiating cell cycle progression. Genistein has demonstrated inhibition of chk1 and chk2 activation that led to decreased downstream signaling events that promoted proliferation. Genistein also acted on TGF- β in this pathway and inhibited it. TGF- β activated different MAPKs, including Mekk1 and Mek4. Genistein acted on the activation point of both of these protein kinases, inhibiting them. Increased expression of Mekk1 led to NF-kB activation and transcription of genes pertinent to cell proliferation. On the other hand, Mek4 activation led to p38 MAPK expression and induction of proliferation as well. Hence, genistein acted at multiple points in these pathways to inhibit proliferation.

Genistein acted through several different mechanisms to increase apoptosis in cancer cells. Genistein worked independently to activate caspase 7, and initiated apoptosis via this route. Genistein also activated caspase 9 and induced apoptosis that way as well. Genistein worked upstream of these caspases at the level of the pro-apoptotic proteins. Antiapoptotic proteins such as Bcl-2 are up regulated in cancer. When Bcl-2 was present in larger quantities, as seen in cancer, it sequestered pro-apoptotic proteins like Bax and Bad, and allowed them to release cytochrome c and subsequently failed to activate the rest of the apoptotic cascade. Genistein also reduced the expression of another anti-apoptotic protein, Bcl-xL, which had the same effect as Bcl-2 on apoptosis. Genistein acted at another level, by activating p53, which was normally inhibited by cancer cells. Increased expression of p53 led to a reduction in Bcl-2 and Bcl-xL production, and increased pro-apoptotic proteins that activated the apoptotic cascade. Hence, genistein acted again at multiple levels to initiate cellular death in these cancer cells.

Genistein also demonstrated decreased angiogenesis in cancer cells. The mechanism through which genistein acted was via multiple different pathways. Genistein inhibited a multitude of pro-angiogenic genes and factors that are up regulated by cancer cells. These included, uPAR, neuropilin, TGF- β 2, BPGF, LPA, and PAR-2. Increased production of these factors promoted angiogenesis. Genistein also worked to activate angiogenic inhibitors that cancer cells repressed, including angiostatin and endostatin. When the inhibitors were present in greater quantities they caused a reduction in angiogenesis. Genistein also activated Cyclic AMP-dependent transcription factor (ATF3), which was normally inhibited by cancer

cells. ATF3 activation directed an increase in the expression of Plasminogen activator inhibitor-1 (PAI-1). PAI-1, a serine-protease inhibitor worked to reduce blood coagulation and ultimately inhibited angiogenesis. Hence, genistein functioned at multiple points to stop the formation of a blood supply and nutrients to the tumor cells (Fig 14b).

These results suggested that resveratrol and genistein acted independently on invasion, proliferation, apoptosis, angiogenesis and motility in cancer (Fig 14a&b); but it was also important to explore those shared pathways and proteins that genistein and resveratrol could exploit together in cancer (Fig 14a). If genistein and resveratrol each inhibited the same pathway or mechanism in cancer cells, they could lead to a magnitude of greater inhibition at these targets, and thus, an increased repression of certain pathways. This next section will demonstrate where genistein and resveratrol could potentially work together to reduce cancer at the areas of invasion, motility proliferation, apoptosis, and angiogenesis.

To begin, genistein and resveratrol have already been shown to act on specific MMPs, which could reduce invasion. Both resveratrol and genistein have displayed decreased MMP-9 expression, as well as MMP-2 expression. Diminished expression of these two MMPs, which were normally increased by breast cancer, could inhibit the breakdown of the basement membrane and cause a reduction in the invasive potential of cancer cells. Resveratrol and genistein could also work in combination to repress the phosphorylation of FAK. When FAK was activated it led to greater adhesion of cancer cells coupled with an increased migratory process.

Thus, combinatory effects of genistein and resveratrol could potentially stop invasion and migration of breast cancer.

Resveratrol and genistein also displayed the potential to work together and reduce cancer metastasis at the level of cell proliferation, apoptosis and angiogenesis. At these points, both resveratrol and genistein demonstrated inhibition of multiple pathways that will be discussed throughout the remainder of this section.

1. Both resveratrol and genistein have exhibited p53 activation and expression, which was normally reduced by cancer cells. Thus combined treatment may lead to a more substantial activation of p53. This tumor suppressor gene demonstrated two ways in which it worked, the first was through its ability to inhibit proliferation, and secondly, to induce apoptosis. Resveratrol and genistein studies have both demonstrated an increase in the production of p21^{WAF1} in cancer cells. P21, as mentioned, was an inhibitor of cdk-cyclins, and thus repressed cell cycle progression and proliferation. These two dietary compounds, through their ability to increase p53 expression, which could conduct the expression of pro-apoptotic proteins, have the potential to increase apoptosis. These compounds may also act together directly on the expression of pro-apoptotic proteins, like Bax and Bad, to increase their production, leading to their accumulation in the mitochondria, followed by initiation of the intrinsic caspase cascade.

2. Resveratrol and genistein could also work at the level of NF-kB, which they both demonstrated individual effects on in prior research, to decrease its expression and subsequent transcription of genes essential for cell proliferation. Resveratrol and

genistein have the potential to act upstream of NF- κ B, at the level of TNF- α production, which was normally increased by cancer cells. The combination of these two compounds could reduce TNF- α expression, which, in the case of cancer, increased NF- κ B activation and led to further proliferation. The two dietary agents, by their propensity to decrease expression of NF- κ B, could allow pro-apoptotic proteins to accumulate, thus allowing them to build up and initiate apoptosis. It has been observed that genistein and resveratrol alone activated the estrogen receptor, and thus together, due to structural similarity between the two and estrogen, they could act as an ER antagonist to reduce the production of the transcription factor, AP-1. Therefore, decreasing cell proliferation through the ER as well.

3. The combinatory effects of these two may potentially lead to inhibition of IGF-1R, and hence its activation, which in cancer, it tended to have increased binding and activation due to enhanced IGF-1 production by the cells.

4. Downstream effects of IGF-1R included activation of PI3K, which led to phosphorylation of Akt to enhance cell proliferation. Resveratrol and genistein displayed individual effects on the reduction of Akt phosphorylation, and hence, the combination of these two could play a more extensive role in inhibiting this proliferative pathway. Cancer cells, when they caused overactivation of the PI3K/Akt pathway, reduced the production of caspase 9, leading to inhibition of apoptosis as well. These two dietary compounds could directly inhibit activation of this pathway, allowing caspase 9 to be activated and cell death to occur.

5. Resveratrol and genistein have also demonstrated potential in activating the intrinsic and extrinsic apoptotic routes through working directly on the caspases.

Resveratrol and genistein alone possessed the ability to activate caspase 3, the executioner caspase, which increased apoptosis of cancer cells. Which means together these compounds could act at the level of caspase 3 as well. Both of these compounds have also displayed activating properties towards caspase 8 followed by caspase 3, through a mitochondrial-independent route, that could lead to induction of apoptosis.

6. The final effects of both genistein and resveratrol individually, were that they displayed inhibition within the angiogenic pathway. Both resveratrol and genistein, thus, have the propensity to inhibit HIF- α , which was normally produced by cancer cells in a hypoxic state, causing increased VEGF expression, HUVECs and vessel formation for angiogenesis to occur. These two compounds could also act directly on VEGF expression, which could repress angiogenesis through decreasing endothelial vessel formation, and endothelial cell growth.

7. Genistein and resveratrol alone displayed an increase in TSP-1 production, which as mentioned, was a protein that inhibited neovascularization and angiogenesis. Hence, displaying another potential way these compounds could work in combination to decrease angiogenesis. These two compounds could also act at another level, targeting other growth factors, such as bFGF, and inhibiting it, which under normal expression, mediated the formation of new blood vessels. Hence, these two compounds could have the ability to act at multiple levels to decrease angiogenesis (Fig 14c & d).

The combination of genistein and resveratrol has the potential to lead to decreased metastasis at the levels of invasion, motility, proliferation, apoptosis, and

angiogenesis. After the research on the individual effects of genistein and the individual effects of resveratrol were displayed, plus the overlap in the processes and mechanisms that both genistein and resveratrol shared were identified, the combination of these two compounds showed promise that when used simultaneously, they could reduce metastasis in breast cancer. When inhibition at many levels occurred upon treatment with either of these compounds, the potential for a greater reduction in metastasis at each step of this process could be heightened if these two compounds were combined as a therapeutic treatment. Thus, the combined treatment of resveratrol and genistein could have greater effects on prevention and treatment of metastasis by working at multiple layers, than either of these dietary compounds alone.

3.2 Future studies

Research in the field of cancer biology as in any other medical or health related field begins with a specific research hierarchy. This hierarchy includes *in vitro* cell culture studies, *in vivo* animal studies, and finally clinical trials. It is important to start with *in vitro* trials, to show if a relationship exists between the combinatory dietary agents and metastasis in breast cancer cells. If a relationship is occurring, showing that the combinatory effects do in fact, regulate different targets of metastasis, and these results are replicated from different labs, then animal studies are undertaken. Therefore starting with the cell culture lines and working up to further animal and clinical trials in humans makes the most sense.

Since cell culture studies are the initial step before a therapeutic agent can progress to the translational level, this thesis will discuss possible future studies

that can be undertaken in the field of cell culture studies to study the combinatory effects of genistein and resveratrol on inhibiting breast cancer progression. Based on the results from the previous section, it is clear that resveratrol and genistein have the propensity to work at multiple different layers of metastasis individually. But, this sheds light on a gap in the current research, which is examining the potential of genistein and resveratrol in combination to study whether the dual interaction of these two would lead to a greater inhibition in the steps of breast cancer metastasis. Identifying these current gaps in research is essential in building a strong foundation for this research. To begin, both resveratrol and genistein demonstrate inhibitory effects through similar and different mechanisms on cdk-cyclins, transcription factors, and growth pathways. But, when these two are present together, the results are unknown. Thus, we can hypothesize that they may lead to a reduction in cell growth through utilizing the same pathways as well as inhibiting others unique to that dietary agent. There is also a gap occurring in regards to genistein and resveratrol, in combination, initiating apoptosis. Although, they have shown effects by directly and indirectly activating the intrinsic and extrinsic routes of apoptosis, there is no current evidence to support the two compounds working together to reduce cell death. A hypothesis to test this idea can be that these two compounds may work together to activate the intrinsic and extrinsic pathways and mechanisms sufficient to induce apoptosis in breast cancer cells. Thirdly, there is a gap in research on the ability of genistein and resveratrol to reduce cell motility in breast cancer cells. It is observed that cell motility can be reduced individually by these compounds through inhibition of Focal adhesions and

cell structural proteins. But again, there is no research to back up the motility effects of these dietary compounds working together. A logical hypothesis to follow from the data presented is that genistein and resveratrol, when combined, may lead to a greater reduction in cell motility through a decrease in cytoskeletal protein interactions. The final gap in research to explore at this point is the ability of genistein and resveratrol to work together to effectively lower invasion in breast cancer cells. Even though both of these compounds have seen a reduction in the expression of MMPs and invasion in prior studies, there is no evidence to support that this is the case when the compounds are combined. Hence, the hypothesis for cell invasion states that genistein and resveratrol, working in combination, may inhibit MMP expression and decrease cancer cell invasiveness. Since research needs to explore the therapeutic ability of these two compounds in combination, future studies should initiate with a focus on the role of genistein and resveratrol in these four main areas of metastasis. Herein, the future studies proposed are; the effect of a combination of genistein plus resveratrol on cell proliferation, apoptosis, cell motility and invasion.

Study one: Combinatory effects on cell proliferation

Study one will explore the effect of a combination of genistein plus resveratrol on cell proliferation. To test the hypothesis that using a combinational treatment of both resveratrol and genistein on breast cancer cells may help prevent and reduce breast cancer metastasis through inhibiting cell proliferation. Tumor growth is an important part of cancer, by promoting unregulated growth due to the cancer's ability to evade inhibitory growth signals. Since growth can be regulated

through dietary agents like genistein and resveratrol (Fig 14), it is important to consider these compounds combined as potential therapeutic option.

Study one will focus on using a suitable breast cancer cell line that has an invasive and metastatic phenotype. This portion of study one will focus on discussing the experimental set up for testing the combined treatment on breast cancer cells. Since previous experiments have an array of concentrations they use for genistein and resveratrol (<1 μM up to >250 μM), an MTT assay will need to be performed to determine the cytotoxicity of the combined treatment of genistein and resveratrol. This can determine the dose-dependent curve that may give the desired combined physiological concentration of these compounds to use on further experiments. The cell viability assay (MTT assay) works by determining the cytotoxic effects of the combined treatment of genistein and resveratrol, in this case. Breast cancer cells will need to be treated with a control such as 0.1% DMSO in the medium, as well as a positive control, such as Doxorubicin (an anthracycline antibiotic) that is an inhibitor of proliferation. In all assays performed, triplicates will be used, and allotted a minimal time period of 72 hours, which is necessary for testing the combined resveratrol and genistein model at the dose-dependent concentrations. After the given time, MTT reagent will be added to the cells, which is a dye that will measure the proportional number of viable cells based on fluorescence intensity. Only living cells will take up the MTT dye. Absorbance's can be assessed, and the percentage of viable cells for the treatment and controls will be calculated from the findings. The results to expect include a time and dose-dependent decrease in breast cancer cell survival in the treatment groups

containing both resveratrol and genistein. The physiological combined concentration (resveratrol plus genistein) that is not statistically significantly different from the control will be used for further experiments.

Once the ideal combined concentration is figured out, the next step is to understand how these two compounds work together on cell proliferation. So, the next experimental set up includes the use of the flow cytometry method. The flow cytometry experiment will give data on how the combined treatment of these compounds may potentially cause an increase in growth arrest in the cancer cells. The experimental conditions will use the designated concentration of the combined treatment. From here, the treatment will be dissolved in a reagent such as dimethyl sulfoxide (DMSO), and the control, in this case, may be DMSO concentrations. At different time points, such as, 0, 24, 48 and 72 hours, the cells may be harvested (216). Thawed cells will then treated with propidium iodide and BrdU, to stain for the presence of DNA content, and sent through the flow cytometer column for DNA content detection.

The results to expect for experiment one show a time-dependent increase in the number of cells arrested in the G2/M phase as well as a possible increase in the number of cells arrested in the sub-G1 phase. These results are consistent with the logic that genistein and resveratrol lead to growth arrest through several different pathways, inhibiting the cells in G2/M phase and to a lesser degree, even G1/S phase. This shows that the cells reduced dividing ability and thus, growth, upon combined treatment, and may be further pushed into apoptosis at this time. These findings may confirm the notion that combining genistein and resveratrol as a

treatment on breast cancer cells leads to a reduction in cell proliferation, in this case, through inhibiting cell cycle progression.

Study two: Combinatory effects on apoptosis

Study two, would logically follow from study one on cell proliferation, with an experiment to test if the breast cancer cells are exhibiting a greater degree of apoptosis after combined treatment with resveratrol plus genistein compared to the control which may be sodium carbonate (Na_2CO_3). Na_2CO_3 is usually a choice of control because it is a stronger base, and has the ability to regulate and stabilize the pH. Because both of these compounds can effectively induce apoptosis in cancer cells through regulating the extrinsic and intrinsic pathways of cell death, the results to expect may display a greater degree of apoptosis within those breast cancer cells that utilize this treatment.

An experimental approach to test for apoptosis occurring in breast cancer cells is to perform DNA laddering on an agarose gel. This protocol requires the combined designated concentration of the compounds being administered to the breast cancer cells over a period of 24, 48 and 72 hours. A suitable control for this experiment may be a reagent such as sodium carbonate (Na_2CO_3) as mentioned. This experiment works by taking small fragments of oligonucleosomal DNA, extracting them, running them on a gel, and separating them out by gel electrophoresis. Visualization of the isolated DNA will occur, followed by checking the results for laddering in the DNA, which will show fragments on the gel compared to the control, this fragmentation being representative of apoptosis occurring.

To further detect any apoptotic events occurring in the cancer cells, Annexin V and flow cytometry will be performed. After treatment and a designated time period as well as use of a control such as DMSO (used in prior experiments), the cells are harvested. Treatment with propidium iodide (PI) and annexin V occurs, followed by cell sorting through the flow cytometer to detect cell viability depending on the stain measurements. PI and annexin V binds to dead cells (apoptotic and necrotic), and annexin V alone will bind to early apoptotic cells. When the cells are passed through the flow cytometer they are sorted based on the presence or absence of PI and annexin V.

For this design, the results to expect from these experiments include those cells treated with the combinational treatment of genistein plus resveratrol to have an increase in laddering in a time-dependent manner, compared to the control, thus applicable to increased apoptosis occurring. The results to expect on the flow cytometry design are an increase in the number of apoptotic cells upon use of the combinational treatment compared to the control. Both genistein and resveratrol may potentially work together to increase the amount of apoptosis through a variety of cellular mechanisms, including transcription factors, tumor suppressor genes, and pro-apoptotic factors. Inducing death in the cancer cells is important, so that the cancer cells, with their unregulated behavior and evasion of cell signals, don't continue to persist and lead to continuous cancer cell survival.

Study three: Combinatory effects on cell motility

Study three that may be performed will be to determine the effect of genistein and resveratrol on metastasis by examining cancer cell motility. Once anti-

proliferative and apoptotic effects are observed in the breast cancer cells, the migratory process must be investigated. This is an important process as the movement of cells is essential for the cancer to initiate spread by leaving the site of origin and moving throughout the body to other locations, a main mechanism of metastasis. Motility structures in the cell such as actin reorganization structures and cytoskeletal protein structures are essential for the cancer to expand beyond its horizons to other areas. Both genistein and resveratrol display adhesive and anti-migratory effects by effecting multiple protein interactions and pathways within this process. Hence, exploring the combinatory action of these agents is imperative for understanding their role in breast cancer metastasis.

The experimental approach to test for this will be a cell motility assay, to check if treatment with resveratrol and genistein may inhibit actin cytoskeletal structures, an important feature of metastasis, leading to a decrease in cell migration/motility. The cell motility assay to use for this is a wound-healing assay. The breast cancer cells will be seeded in plates for a period of time (24, 48 to 72 hours). To simulate wounding the monolayer of cells, use of a pipette tip will occur to make a straight scratch across the cells. The control in this case will be the untreated cells and the experimental are the cells treated with the combinatory concentration. Photographs will document the cells moving across the wounded boundary over this time, and allow a count to be taken of the number of cells migrating. It may also be beneficial to stain the cells after this process for actin, and see if there is a decrease in the presence of actin in the treated cells.

The expected results for this process may show a decrease in the number of cells migrating across the wound in the treatment group, this will demonstrate a decrease in migration potential through compound use. There may also be a decrease in the presence of actin in the cells treated with the combination of compounds. Individually genistein and resveratrol inhibit many of the cytoskeletal processes occurring in the cancer cells, including regulating FAKs and actin polymerization, thus combining them in a treatment may lead to these same results and a greater reduction in cell motility. These two compounds may retain the ability to reduce actin polymerization at the leading end of the cancer cells as well.

Study four: Combinatory effects on cell invasion

Study four, the final study proposed here, will observe cell invasiveness in breast cancer cells after treatment with genistein and resveratrol. Previous findings from above show a decrease in MMP expression by resveratrol and genistein individually. When combined, the potential to cause a greater reduction in the expression of several MMPs is predicted. A decrease in the amount of MMPs being released, reduces the enzymatic breakdown of the basement membrane, therefore, not allowing the breast cancer cells to invade into the bloodstream.

The experimental approach for testing invasion will be to utilize a cell invasion assay. The breast cancer cells, after treatment with combined genistein and resveratrol, will be suspended in the upper boyden chamber, that will be maintained with a matrigal coating. On the other side of the chamber is a chemoattractant (that attracts the cancer cells). The control will lack the addition of the combined treatment. After a given time period, the cells that invade through the

matrigal coating, which is the simulated basement membrane, are stained and quantified as the number of invading cells.

The results to expect for this experiment may demonstrate a decrease in the invasive potential of the breast cancer cells after using a combination of both genistein and resveratrol. Thus, the treated group will display less cells invading through the matrigal coating compared to the control. Genistein and resveratrol display the propensity to decrease invasion through reducing MMP expression. The MMPs are a main regulator of basement membrane breakdown that lead to greater invasiveness. A greater inhibition, hence, is expected at this point when a combination of these agents is used.

After cell proliferation, apoptosis cell migration and invasion are measured, experimental designs including microarray experiments may be useful to view the expression of specific genes that may be up or down-regulated based on combined genistein and resveratrol treatment. After looking at factors such as this, and having several labs support the data and results, *in vivo* experiments on the combined treatment of genistein and resveratrol and how they can affect metastasis in a breast cancer transfected mouse model should follow.

Overall, the multiple studies that may show promise in the future, and have been examined throughout this section, include the combinatory effects of genistein and resveratrol on cell proliferation, apoptosis, motility and invasion. After performing *in vitro* studies, *in vivo* studies in animals can be carried out on the combinatory potential of these compounds. Long term goals of research, if combined treatments prove successful on preventing and treating metastasized

breast cancer, include the initiation of clinical trials on humans, as well as looking at even more combinations of dietary agents to work collectively with breast cancer therapies already in place. Overall, the combinatory effects of genistein and resveratrol show promise in aiding and preventing breast cancer metastasis, and at this time further research should commence on combinatory use of these dietary compounds.

REFERENCES

1. **Society AC** 2010 Cancer Facts and Figures 2010. In. Atlanta, GA: American Cancer Society
2. **Sun L, Lin SS, Zhao RP, Yu BY, Yuan ST, Zhang LY** 2010 The Saponin Monomer of Dwarf Lilyturf Tuber, DT-13, Reduces Human Breast Cancer Cell Adhesion and Migration during Hypoxia via Regulation of Tissue Factor. *Biological & Pharmaceutical Bulletin* 33:1192-1198
3. **Meyer T, Marshall JF, Hart IR** 1998 Expression of alpha v integrins and vitronectin receptor identity in breast cancer cells. *British Journal of Cancer* 77:530-536
4. **Felding-Habermann B, O'Toole TE, Smith JW, Fransvea E, Ruggeri ZM, Ginsberg MH, Hughes PE, Pampori N, Shattil SJ, Saven A, Mueller BM** 2001 Integrin activation controls metastasis in human breast cancer. *Proceedings of the National Academy of Sciences of the United States of America* 98:1853-1858
5. **Taherian A, Li XL, Liu YQ, Haas TA** 2011 Differences in integrin expression and signaling within human breast cancer cells. *Bmc Cancer* 11
6. **Mego M, Mani SA, Cristofanilli M** 2010 Molecular mechanisms of metastasis in breast cancer-clinical applications. *Nature Reviews Clinical Oncology* 7:693-701
7. **Boonrao M, Yodkeeree S, Ampasavate C, Anuchapreeda S, Limtrakul P** 2010 The inhibitory effect of turmeric curcuminoids on matrix metalloproteinase-3 secretion in human invasive breast carcinoma cells. *Archives of Pharmacal Research* 33:989-998
8. **Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N** 2000 Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Research* 2:252-257
9. **Mitchison TJ, Cramer LP** 1996 Actin-based cell motility and cell locomotion. *Cell* 84:371-379
10. **Azios NG, Krishnamoorthy L, Harris M, Cubano LA, Cammer M, Dharmawardhane SF** 2007 Estrogen and resveratrol regulate rac and cdc42 signaling to the actin cytoskeleton of metastatic breast cancer cells. *Neoplasia* 9:147-158
11. **Liu YX, Ji R, Li J, Gu Q, Zhao X, Sun T, Wang JJ, Li JB, Du QY, Sun B** 2010 Correlation effect of EGFR and CXCR4 and CCR7 chemokine receptors in predicting breast cancer metastasis and prognosis. *Journal of Experimental & Clinical Cancer Research* 29:9
12. **Aggarwal BB, Shishodia S** 2006 Molecular targets of dietary agents for prevention and therapy of cancer. *Biochemical Pharmacology* 71:1397-1421
13. **Gee MS, Procopio WN, Makonnen S, Feldman MD, Yeilding NM, Lee WMF** 2003 Tumor vessel development and maturation impose limits on the effectiveness of anti-vascular therapy. *American Journal of Pathology* 162:183-193

14. **Friedl P** 2004 Prespecification and plasticity: shifting mechanisms of cell migration. *Current Opinion in Cell Biology* 16:14-23
15. **Hidalgo M, Eckhardt SG** 2001 Development of Matrix Metalloproteinase Inhibitors in Cancer Therapy. *Journal of the National Cancer Institute* 93:178-193
16. **Banerjee S, Li Y, Wang Z, Sarkar FH** 2008 Multi-targeted therapy of cancer by genistein. *Cancer Letters* 269:226-242
17. **Gupta SC, Kim JH, Prasad S, Aggarwal BB** 2010 Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer and Metastasis Reviews* 29:405-434
18. **Kerbel R, Folkman J** 2002 Clinical Translation of Angiogenesis Inhibitors. *Nature Reviews* 2:12
19. **Normanno N, Morabito A, De Luca A, Piccirillo MC, Gallo M, Maiello MR, Perrone F** 2009 Target-based therapies in breast cancer: current status and future perspectives. *Endocr Relat Cancer* 16:675-702
20. **Gong C, Yao HR, Liu QA, Chen JQ, Shi JW, Su FX, Song EW** 2010 Markers of Tumor-Initiating Cells Predict Chemoresistance in Breast Cancer. *Plos One* 5:11
21. **Sartippour MR, Shao ZM, Heber D, Beatty P, Zhang LP, Liu CH, Ellis L, Liu W, Go VL, Brooks MN** 2002 Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *Journal of Nutrition* 132:2307-2311
22. **Athar M, Back JH, Kopelovich L, Bickers DR, Kim AL** 2009 Multiple molecular targets of resveratrol: Anti-carcinogenic mechanisms. *Archives of Biochemistry and Biophysics* 486:95-102
23. **Saiko P, Szakmary A, Jaeger W, Szekeres T** 2008 Resveratrol and its analogs: Defense against cancer, coronary disease and neurodegenerative maladies or just a fad? *Mutation Research-Reviews in Mutation Research* 658:68-94
24. **Bishayee A** 2009 Cancer Prevention and Treatment with Resveratrol: From Rodent Studies to Clinical Trials. *Cancer Prevention Research* 2:409-418
25. **Goswami SK, Das DK** 2009 Resveratrol and chemoprevention. *Cancer Letters* 284:1-6
26. **Harikumar KB, Aggarwal BB** 2008 Resveratrol - A multitargeted agent for age-associated chronic diseases. *Cell Cycle* 7:1020-1035
27. **Fontecave M, Lepoivre M, Elleingand E, Gerez C, Guittet O** 1998 Resveratrol, a remarkable inhibitor of ribonucleotide reductase. *Febs Letters* 421:277-279
28. **Sun NJ, Woo SH, Cassady JM, Snapka RM** 2003 DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia* (vol 61, pg 363, 1998). *Journal of Natural Products* 66:734-734
29. **Bharat A, Shishir S** 2005 Resveratrol. In: *Resveratrol in Health and Disease*: CRC Press; 1-15
30. **Pan M-H, Lin Y-T, Lin C-L, Wei C-S, Ho C-T, Chen W-J** 2011 Suppression of Heregulin-beta 1/HER2-Modulated Invasive and Aggressive Phenotype of

- Breast Carcinoma by Pterostilbene via Inhibition of Matrix Metalloproteinase-9, p38 Kinase Cascade and Akt Activation. Evidence-Based Complementary and Alternative Medicine:1-12
31. **Banerjee S, Bueso-Ramos C, Aggarwal BB** 2002 Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: Role of nuclear factor-kappa B, cyclooxygenase 2, and matrix metalloproteinase 9. *Cancer Research* 62:4945-4954
 32. **Aggarwal BB** 2004 Nuclear factor-kappa-B: The enemy within. *Cancer Cell* 6:203-208
 33. **Kundu JK, Surh YJ** 2004 Molecular basis of chemoprevention by resveratrol: NF-kappa B and AP-1 as potential targets. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* 555:65-80
 34. **Tang F, Su Y, Chen N, Hsieh H, Chen K** 2008 Resveratrol inhibits migration and invasion of human breast-cancer cells. *Mol Nutr Food Res* 52:683-691
 35. **Saikali Z, Setya H, Singh G, Persad S** 2008 Role of IGF-1/IGF-1R in regulation of invasion in DU145 prostate cancer cells. *Cancer Cell International* 8
 36. **Zhang D, Samani AA, Brodt P** 2003 The role of the IGF-I receptor in the regulation of matrix metalloproteinases, tumor invasion and metastasis. *Hormone and Metabolic Research* 35:802-808
 37. **Li D, Chen X, Yu H** 2011 Resveratrol Inhibits MMP-2 Expression of Hepatoma in Nude Mice. *Journal of Animal and Veterinary Advances* 10:33-37
 38. **Gagliano N, Moscheni C, Torri C, Magnani I, Bertelli AA, Gioia M** 2005 Effect of resveratrol on matrix metalloproteinase-2 (MMP-2) and Secreted Protein Acidic and Rich in Cysteine (SPARC) on human cultured glioblastoma cells. *Biomedicine & Pharmacotherapy* 59:359-364
 39. **Benitez DA, Pozo-Guisado E, Alvarez-Barrientos A, Fernandez-Salguero PM, Castellon EA** 2007 Mechanisms involved in resveratrol-induced apoptosis and cell cycle arrest in prostate cancer-derived cell lines. *Journal of Andrology* 28:282-293
 40. **Guvakova MA, Adams JC, Boettiger D** 2002 Functional role of alpha-actinin, PI 3-kinase and MEK1/2 in insulin-like growth factor I receptor kinase regulated motility of human breast, carcinoma cells. *Journal of Cell Science* 115:4149-4165
 41. **Gehm BD, McAndrews JM, Chien PY, Jameson JL** 1997 Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proceedings of the National Academy of Sciences of the United States of America* 94:14138-14143
 42. **Levenson AS, Gehm BD, Pearce ST, Horiguchi J, Simons LA, Ward JE, Jameson JL, Jordan VC** 2003 Resveratrol acts as an estrogen receptor (ER) agonist in breast cancer cells stably transfected with ER alpha. *International Journal of Cancer* 104:587-596
 43. **Vyas S, Asmerom Y, De Leon DD** 2005 Resveratrol regulates insulin-like growth factor-II in breast cancer cells. *Endocrinology* 146:4224-4233

44. **Kai L, Samuel SK, Levenson AS** 2010 Resveratrol enhances p53 acetylation and apoptosis in prostate cancer by inhibiting MTA1/NuRD complex. *International Journal of Cancer* 126:1538-1548
45. **Sun CY, Hu Y, Liu XY, Wu T, Wang YD, He WJ, Wei WN** 2006 Resveratrol downregulates the constitutional activation of nuclear factor-kappa B in multiple myeloma cells, leading to suppression of proliferation and invasion, arrest of cell cycle, and induction of apoptosis. *Cancer Genetics and Cytogenetics* 165:9-19
46. **Manna SK, Mukhopadhyay A, Aggarwal BB** 2000 Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: Potential role of reactive oxygen intermediates and lipid peroxidation. *Journal of Immunology* 164:6509-6519
47. **Holmes-McNary M, Baldwin AS** 2000 Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the I kappa B kinase. *Cancer Research* 60:3477-3483
48. **Karin M, Liu ZG, Zandi E** 1997 AP-1 function and regulation. *Current Opinion in Cell Biology* 9:240-246
49. **Suganuma M, Okabe S, Marino MW, Sakai A, Sueoka E, Fujiki H** 1999 Essential role of tumor necrosis factor alpha (TNF-alpha) in tumor promotion as revealed by TNF-alpha-deficient mice. *Cancer Research* 59:4516-4518
50. **Natarajan K, Singh S, Burke TR, Grunberger D, Aggarwal BB** 1996 Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proceedings of the National Academy of Sciences of the United States of America* 93:9090-9095
51. **Egan LJ, Mays DC, Huntoon CJ, Bell MP, Pike MG, Sandborn WJ, Lipsky JJ, McKean DJ** 1999 Inhibition of interleukin-1-stimulated NF-kappa B RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity. *Journal of Biological Chemistry* 274:26448-26453
52. **Hwang D, Jang BC, Yu G, Boudreau M** 1997 Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide - Mediation through both mitogen-activated protein kinase and NF-kappa B signaling pathways in macrophages. *Biochemical Pharmacology* 54:87-96
53. **von Knethen A, Callsen D, Brune B** 1999 Superoxide attenuates macrophage apoptosis by NF-kappa B and AP-1 activation that promotes cyclooxygenase-2 expression. *Journal of Immunology* 163:2858-2866
54. **Pozo-Guisado E, Lorenzo-Benayas MJ, Fernandez-Salguero PM** 2004 Resveratrol modulates the phosphoinositide 3-kinase pathway through an estrogen receptor alpha-dependent mechanism: Relevance in cell proliferation. *International Journal of Cancer* 109:167-173
55. **Vanamala J, Reddivari L, Radhakrishnan S, Tarver C** 2010 Resveratrol suppresses IGF-1 induced human colon cancer cell proliferation and elevates apoptosis via suppression of IGF-1R/Wnt and activation of p53 signaling pathways. *Bmc Cancer* 10

56. **Jiang H, Shang X, Wu H, Gautam S, Al-Holou S, Li C, Kuo J, Zhang L, Chopp M** 2009 Resveratrol downregulates PI3K/Akt/mTOR signaling pathways in human U251 glioma cells. *J Exp Ther Oncol* 8:25-33
57. **Yuan ZQ, Sun M, Feldman RI, Wang G, Ma XL, Jiang C, Coppola D, Nicosia SV, Cheng JQ** 2000 Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 19:2324-2330
58. **Arboleda MJ, Lyons JF, Kabbinavar FF, Bray MR, Snow BE, Ayala R, Danino M, Karlan BY, Slamon DJ** 2003 Overexpression of AKT2/protein kinase B beta leads to up-regulation of beta 1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. *Cancer Research* 63:196-206
59. **Ruggeri BA, Huang LY, Wood M, Cheng JQ, Testa JR** 1998 Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas. *Molecular Carcinogenesis* 21:81-86
60. **Murray AW** 2004 Recycling the cell cycle: Cyclins revisited. *Cell* 116:221-234
61. **Alao JP** 2007 The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Molecular Cancer* 6
62. **Pozo-Guisado E, Alvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B, Fernandez-Salguero PM** 2002 The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. *Biochemical Pharmacology* 64:1375-1386
63. **Kundu JK, Chun KS, Kim SO, Surh YJ** 2004 Resveratrol inhibits phorbol ester-induced cyclooxygenase-2 expression in mouse skin: MAPKs and AP-1 as potential molecular targets. *Biofactors* 21:33-39
64. **Zhou J-h, Cheng H-y, Yu Z-q, He D-w, Pan Z, Yang D-t** 2011 Resveratrol induces apoptosis in pancreatic cancer cells. *Chinese Medical Journal* 124:1695-1699
65. **Ahmad N, Adhami VM, Afaq F, Feyes DK, Mukhtar H** 2001 Resveratrol causes WAF-1/p21-mediated G(1)-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. *Clinical Cancer Research* 7:1466-1473
66. **Shih A, Davis FB, Lin HY, Davis PJ** 2002 Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. *Journal of Clinical Endocrinology & Metabolism* 87:1223-1232
67. **Kim AL, Zhu YC, Zhu HJ, Han L, Kopelovich L, Bickers DR, Athar M** 2006 Resveratrol inhibits proliferation of human epidermoid carcinoma A431 cells by modulating MEK1 and AP-1 signalling pathways. *Experimental Dermatology* 15:538-546
68. **Goga A, Yang D, Tward AD, Morgan DO, Bishop JM** 2007 Inhibition of CDK1 as a potential therapy for tumors over-expressing MYC. *Nature Medicine* 13:820-827
69. **Zhang P, Li H, Wu M-L, Chen X-Y, Kong Q-Y, Wang X-W, Sun Y, Wen S, Liu J** 2006 c-Myc downregulation: a critical molecular event in resveratrol-

- induced cell cycle arrest and apoptosis of human medulloblastoma cells. *Journal of Neuro-Oncology* 80:123-131
70. **Mohan J, Gandhi AA, Bhavya BC, Rashmi R, Karunakaran D, Indu R, Santhoshkumar TR** 2006 Caspase-2 triggers Bax-Bak-dependent and -independent cell death in colon cancer cells treated with resveratrol. *Journal of Biological Chemistry* 281:17599-17611
 71. **Verhagen AM, Vaux DL** 2002 Cell death regulation by the mammalian IAP antagonist Diablo/Smac. *Apoptosis* 7:163-166
 72. **Wei MC, Zong WX, Cheng EHY, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacCregor GR, Thompson CB, Korsmeyer SJ** 2001 Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. *Science* 292:727-730
 73. **LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D, Ashkenazi A** 2002 Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nature Medicine* 8:274-281
 74. **Haupt S, Berger M, Goldberg Z, Haupt Y** 2003 Apoptosis - the p53 network. *Journal of Cell Science* 116:4077-4085
 75. **Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P, Blenis J, Tschopp J** 2000 TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nature Cell Biology* 2:241-243
 76. **Pozo-Guisado E, Merino JM, Mulero-Navarro S, Lorenzo-Benayas MJ, Centeno F, Alvarez-Barrientos A, Salguero PMF** 2005 Resveratrol-induced apoptosis in MCF-7 human breast cancer cells involves a caspase-independent mechanism with downregulation of Bcl-2 and NF-kappa B. *International Journal of Cancer* 115:74-84
 77. **Agarwal C, Sharma Y, Agarwal R** 2000 Anticarcinogenic effect of a polyphenolic fraction isolated from grape seeds in human prostate carcinoma DU145 cells: Modulation of mitogenic signaling and cell-cycle regulators and induction of G1 arrest and apoptosis. *Molecular Carcinogenesis* 28:129-138
 78. **Dorrie J, Gerauer H, Wachter Y, Zunino SJ** 2001 Resveratrol induces extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in acute lymphoblastic leukemia cells. *Cancer Research* 61:4731-4739
 79. **Sareen D, van Ginkel PR, Takach JC, Mohiuddin A, Darjatmoko SR, Albert DM, Polans AS** 2006 Mitochondria as the primary target of resveratrol-induced apoptosis in human retinoblastoma cells. *Investigative Ophthalmology & Visual Science* 47:3708-3716
 80. **Sareen D, Darjatmoko SR, Albert DM, Polans AS** 2007 Mitochondria, calcium, and calpain are key mediators of resveratrol-induced apoptosis in breast cancer. *Molecular Pharmacology* 72:1466-1475
 81. **Alonso M, Tamasdan C, Miller DC, Newcomb EW** 2003 Flavopiridol induces apoptosis in glioma cell lines independent of retinoblastoma and p53 tumor suppressor pathway alterations by a caspase-independent pathway. *Molecular Cancer Therapeutics* 2:139-150

82. **Diaz GD, Li QJ, Dashwood RH** 2003 Caspase-8 and apoptosis-inducing factor mediate a cytochrome c-independent pathway of apoptosis in human colon cancer cells induced by the dietary phytochemical chlorophyllin. *Cancer Research* 63:1254-1261
83. **Scarlatti F, Sala G, Somenzi G, Signorelli P, Sacchi N, Ghidoni R** 2003 Resveratrol induces growth inhibition and apoptosis in metastatic breast cancer cells via de novo ceramide signaling. *Faseb Journal* 17:2339-+
84. **Tang HY, Shih A, Cao HJ, Davis FB, Davis PJ, Lin HY** 2006 Resveratrol-induced cyclooxygenase-2 facilitates p53-dependent apoptosis in human breast cancer cells. *Molecular Cancer Therapeutics* 5:2034-2042
85. **Bhardwaj A, Sethi G, Vadhan-Raj S, Bueso-Ramos C, Takada Y, Gaur U, Nair AS, Shishodia S, Aggarwal BB** 2007 Resveratrol inhibits proliferation, induces apoptosis, and overcomes chemoresistance through down-regulation of STAT3 and nuclear factor-kappa B-regulated antiapoptotic and cell survival gene products in human multiple myeloma cells. *Blood* 109:2293-2302
86. **Li ZG, Hong T, Shimada Y, Komoto I, Kawabe A, Ding Y, Kaganai J, Hashimoto Y, Imamura M** 2002 Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. *Carcinogenesis* 23:1531-1536
87. **Tessitore L, Davit A, Sarotto I, Caderni G** 2000 Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and p21(CIP) expression. *Carcinogenesis* 21:1619-1622
88. **Pirola L, Froejdoe S** 2008 Resveratrol: One molecule, many targets. *Life* 60:323-332
89. **de la Lastra CA, Villegas I** 2005 Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. *Molecular Nutrition & Food Research* 49:405-430
90. **Tsujii M, Dubois RN** 1995 ALTERATIONS IN CELLULAR ADHESION AND APOPTOSIS IN EPITHELIAL-CELLS OVEREXPRESSING PROSTAGLANDIN-ENDOPEROXIDE-SYNTHASE-2. *Cell* 83:493-501
91. **Tsujii M, Kawano S, Tsuji S, Sawaoko H, Hori M, DuBois RN** 1998 Cyclooxygenase regulates angiogenesis induced by colon cancer cells (vol 93, pg 705, 1997). *Cell* 94
92. **Nikolic D, van Breemen RB** 2001 DNA oxidation induced by cyclooxygenase-2. *Chemical Research in Toxicology* 14:351-354
93. **Tanoue T, Moriguchi T, Nishida E** 1999 Molecular cloning and characterization of a novel dual specificity phosphatase, MKP-5. *Journal of Biological Chemistry* 274:19949-19956
94. **Theodosiou A, Smith A, Gillieron C, Arkinstall S, Ashworth A** 1999 MKP5, a new member of the MAP kinase phosphatase family, which selectively dephosphorylates stress-activated kinases. *Oncogene* 18:6981-6988
95. **Nonn L, Duong D, Peehl DM** 2007 Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells. *Carcinogenesis* 28:1188-1196

96. **Signorelli P, Ghidoni R** 2005 Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *Journal of Nutritional Biochemistry* 16:449-466
97. **Shankar S, Singh G, Srivastava RK** 2007 Chemoprevention by resveratrol: molecular mechanisms and therapeutic potential. *Frontiers in Bioscience* 12:4839-4854
98. **Chan JY, Phoo MS, Clement M-V, Pervaiz S, Lee SC** 2008 Resveratrol displays converse dose-related effects on 5-fluorouracil-evoked colon cancer cell apoptosis - The roles of caspase-6 and p53. *Cancer Biology & Therapy* 7:1305-1312
99. **Lin H-Y, Sun M, Tang H-Y, Simone TM, Wu Y-H, Grandis JR, Cao HJ, Davis PJ, Davis FB** 2008 Resveratrol causes COX-2-and p53-dependent apoptosis in head and neck squamous cell cancer cells. *Journal of Cellular Biochemistry* 104:2131-2142
100. **Alkhalaf M** 2007 Resveratrol-induced apoptosis is associated with activation of p53 and inhibition of protein translation in T47D human breast cancer cells. *Pharmacology* 80:134-143
101. **Testa JR, Bellacosa A** 2001 Commentary - AKT plays a central role in tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* 98:10983-10985
102. **Jiang H, Zhang L, Kuo J, Kuo K, Gautam SC, Groc L, Rodriguez AI, Koubi D, Hunter TJ, Corcoran GB, Seidman MD, Levine RA** 2005 Resveratrol-induced apoptotic death in human U251 glioma cells. *Molecular Cancer Therapeutics* 4:554-561
103. **Castillo-Pichardo L, Martinez-Montemayor MM, Martinez JE, Wall KM, Cubano LA, Dharmawardhane S** 2009 Inhibition of mammary tumor growth and metastases to bone and liver by dietary grape polyphenols. *Clinical & Experimental Metastasis* 26:505-516
104. **Liaudet-Coopman E, Beaujouin M, Derocq D, Garcia M, Glondou-Lassis M, Laurent-Matha V, Prebois C, Rochefort H, Vignon F** 2006 Cathepsin D: newly discovered functions of a long-standing aspartic protease in cancer and apoptosis. *Cancer Letters* 237:167-179
105. **Vyas S, Asmerom Y, De Leon DD** 2006 Insulin-like growth factor II mediates resveratrol stimulatory effect on cathepsin D in breast cancer cells. *Growth Factors* 24:79-87
106. **Gescher AJ, Steward WP** 2003 Relationship between mechanisms, bioavailability, and preclinical chemopreventive efficacy of resveratrol: A conundrum. *Cancer Epidemiology Biomarkers & Prevention* 12:953-957
107. **Yu H, Chun-yan S, Jing H, Liu H, Lu Z, Zhang-bo C** 2007 Antimyeloma effects of resveratrol through inhibition of angiogenesis. *Chinese Medical Journal* 120:1672-1677
108. **Trapp V, Parmakhtiar B, Papazian V, Willmott L, Fruehauf JP** 2010 Anti-angiogenic effects of resveratrol mediated by decreased VEGF and increased TSP1 expression in melanoma-endothelial cell co-culture. *Angiogenesis* 13:305-315

109. **Dameron KM, Volpert OV, Tainsky MA, Bouck N** 1994 CONTROL OF ANGIOGENESIS IN FIBROBLASTS BY P53 REGULATION OF THROMBOSPONDIN-1. *Science* 265:1582-1584
110. **Tokunaga T, Nakamura M, Oshika Y, Tsuchida T, Kazuno M, Fukushima Y, Kawai K, Abe Y, Kijima H, Yamazaki H, Tamaoki N, Ueyama Y** 1998 Alterations in tumour suppressor gene p53 correlate with inhibition of thrombospondin-1 gene expression in colon cancer cells. *Virchows Archiv-an International Journal of Pathology* 433:415-418
111. **Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L** 2006 VEGF receptor signalling - in control of vascular function. *Nature Reviews Molecular Cell Biology* 7:359-371
112. **Li X, Claesson-Welsh L, Shibuya M** 2008 VEGF receptor signal transduction. *Angiogenesis: in Vitro Systems* 443:261-284
113. **Brakenhielm E, Cao RH, Cao YH** 2001 Suppression of angiogenesis, tumor growth, and wound healing by resveratrol, a natural compound in red wine and grapes. *Faseb Journal* 15:1798-+
114. **Garvin S, Ollinger K, Dabrosin C** 2006 Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo. *Cancer Letters* 231:113-122
115. **Issbrucker K, Hippenstiel S, Vosswinkel R, Suttorp N, Clauss M** 2003 p38 MAP kinase - a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability. *Faseb Journal* 17:A1242-A1242
116. **Lin MT, Yen ML, Lin CY, Kuo ML** 2003 Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src-dependent vascular endothelial cadherin tyrosine phosphorylation. *Molecular Pharmacology* 64:1029-1036
117. **Tseng E, Scott-Ramsay EA, Morris ME** 2004 Dietary organic isothiocyanates are cytotoxic in human breast cancer MCF-7 and mammary epithelial MCF-12A cell lines. *Experimental Biology and Medicine* 229:835-842
118. **Price DJ, Miralem T, Jiang SX, Steinberg R, Avraham H** 2001 Role of vascular endothelial growth factor in the stimulation of cellular invasion and signaling of breast cancer cells. *Cell Growth & Differentiation* 12:129-135
119. **von Marschall Z, Cramer T, Hoecker M, Burde R, Plath T, Schirner M, Heindenreich R, Breier G, Riecken EO, Wiedenmann B, Rosewicz S** 2000 De novo expression of vascular endothelial growth factor and its receptors in human pancreatic cancer: Evidence for a novel autocrine mitogenic loop. *Gastroenterology* 118:A447-A447
120. **Brem S, Cotran R, Folkman J** 1972 TUMOR ANGIOGENESIS A QUANTITATIVE METHOD FOR HISTOLOGIC GRADING. *Journal of the National Cancer Institute* 48:347-356
121. **Tseng SH, Lin SM, Chen JC, Su YH, Huang HY, Chen CK, Lin PY, Chen Y** 2004 Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. *Clinical Cancer Research* 10:2190-2202

122. **Lu RQ, Serrero G** 1999 Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. *Journal of Cellular Physiology* 179:297-304
123. **Kimura Y, Okuda H** 2001 Resveratrol isolated from *Polygonum cuspidatum* root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma-bearing mice. *Journal of Nutrition* 131:1844-1849
124. **Busquets S, Ametller E, Fuster G, Olivan M, Raab V, Argiles JM, Lopez-Soriano FJ** 2007 Resveratrol, a natural diphenol, growth in an experimental reduces metastatic cancer model. *Cancer Letters* 245:144-148
125. **Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR** 2003 Cell migration: Integrating signals from front to back. *Science* 302:1704-1709
126. **Azios NG, Dharmawardhane SF** 2005 Resveratrol and estradiol exert disparate effects on cell migration, cell surface actin structures, and focal adhesion assembly in MDA-MB-231 human breast cancer. *Neoplasia* 7:128-140
127. **Sakamoto S, McCann RO, Dhir R, Kyprianou N** 2010 Talin1 Promotes Tumor Invasion and Metastasis via Focal Adhesion Signaling and Anoikis Resistance. *Cancer Research* 70:1885-1895
128. **McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC** 2005 The role of focal-adhesion kinase in cancer. A new therapeutic opportunity. *Nature Reviews Cancer* 5:505-515
129. **Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y** 2004 Role of resveratrol in prevention and therapy of cancer: Preclinical and clinical studies. *Anticancer Research* 24:2783-2840
130. **Pavese JM, Farmer RL, Bergan RC** 2010 Inhibition of cancer cell invasion and metastasis by genistein. *Cancer and Metastasis Reviews* 29:465-482
131. **Notarnicola M, Messa C, Orlando A, D'Attoma B, Tutino V, Rivizzigno R, Caruso MG** 2008 Effect of genistein on cholesterol metabolism-related genes in a colon cancer cell line. *Genes and Nutrition* 3:35-40
132. **Fanti P, Monier-Faugere MC, Geng Z, Schmidt J, Morris PE, Cohen D, Malluche HH** 1998 The phytoestrogen genistein reduces bone loss in short-term ovariectomized rats. *Osteoporosis International* 8:274-281
133. **Matsumura A, Ghosh A, Pope GS, Darbre PD** 2005 Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* 94:431-443
134. **Kuiper G, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA** 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138:863-870
135. **Messina MJ, Persky V, Setchell KDR, Barnes S** 1994 SOY INTAKE AND CANCER RISK - A REVIEW OF THE IN-VITRO AND IN-VIVO DATA. *Nutrition and Cancer-an International Journal* 21:113-131

136. **Xiao CW, Mei J, Wood CM** 2008 Effect of soy proteins and isoflavones on lipid metabolism and involved gene expression. *Frontiers in Bioscience-Landmark* 13:2660-2673
137. **Bouker KB, Hilakivi-Clarke L** 2000 Genistein: Does it prevent or promote breast cancer? *Environmental Health Perspectives* 108:701-708
138. **Adlercreutz H** 1990 WESTERN DIET AND WESTERN DISEASES - SOME HORMONAL AND BIOCHEMICAL-MECHANISMS AND ASSOCIATIONS. *Scandinavian Journal of Clinical & Laboratory Investigation* 50:3-23
139. **Severson RK, Nomura AMY, Grove JS, Stemmermann GN** 1989 A PROSPECTIVE-STUDY OF DEMOGRAPHICS, DIET, AND PROSTATE-CANCER AMONG MEN OF JAPANESE ANCESTRY IN HAWAII. *Cancer Research* 49:1857-1860
140. **Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM** 1991 CANCERS OF THE PROSTATE AND BREAST AMONG JAPANESE AND WHITE IMMIGRANTS IN LOS-ANGELES-COUNTY. *British Journal of Cancer* 63:963-966
141. **Hwang YW, Kim SY, Jee SH, Kim YN, Nam CM** 2009 Soy Food Consumption and Risk of Prostate Cancer: A Meta-Analysis of Observational Studies. *Nutrition and Cancer-an International Journal* 61:598-606
142. **Lee HP, Gourley L, Duffy SW, Esteve J, Lee J, Day NE** 1991 DIETARY-EFFECTS ON BREAST-CANCER RISK IN SINGAPORE. *Lancet* 337:1197-1200
143. **Wu AH, Ziegler RG, Horn Ross PL, Nomura AMY, West DW, Kolonel LN, Rosenthal JF, Hoover RN, Pike MC** 1996 Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiology Biomarkers & Prevention* 5:901-906
144. **Bohe G, Sansbury LB, Albert PS, Cross AJ, Kahle L, Ashby J, Slattery ML, Caan B, Paskett E, Iber F, Kikendall JW, Lance P, Daston C, Marshall JR, Schatzkin A, Lanza E** 2008 Dietary flavonoids and colorectal adenoma recurrence in the polyp prevention trial. *Cancer Epidemiology Biomarkers & Prevention* 17:1344-1353
145. **Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hamalainen E, Hasegawa T, Okada H** 1991 URINARY-EXCRETION OF LIGNANS AND ISOFLAVONOID PHYTOESTROGENS IN JAPANESE MEN AND WOMEN CONSUMING A TRADITIONAL JAPANESE DIET. *American Journal of Clinical Nutrition* 54:1093-1100
146. **Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinai N** 2000 Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. *Journal of Nutrition* 130:2243-2250
147. **Horiuchi T, Nakamura T, Onouchi T, Ito H, Orimo H** 2000 Effect of soy protein on bone metabolism in postmenopausal Japanese women. *Journal of Bone and Mineral Research* 15:S292-S292
148. **Nagata C, Inaba S, Kawakami N, Kakizoe T, Shimizu H** 2000 Inverse association of soy product intake with serum androgen and estrogen concentrations in Japanese men. *Nutrition and Cancer-an International Journal* 36:14-18

149. **Nagata C, Kabuto M, Kurisu Y, Shimizu H** 1997 Decreased serum estradiol concentration associated with high dietary intake of soy products in premenopausal Japanese women. *Nutrition and Cancer-an International Journal* 29:228-233
150. **Nagata C, Shimizu H, Takami R, Hayashi M, Takeda N, Yasuda K** 2000 Relations of insulin resistance and serum concentrations of estradiol and sex hormone-binding globulin to potential breast cancer risk factors. *Japanese Journal of Cancer Research* 91:948-953
151. **Nagata C, Takatsuka N, Kawakami N, Shimizu H** 2000 Association of diet with the onset of menopause in Japanese women. *American Journal of Epidemiology* 152:863-867
152. **Takatsuka N, Nagata C, Kurisu Y, Inaba S, Kawakami N, Shimizu H** 2000 Hypocholesterolemic effect of soymilk supplementation with usual diet in premenopausal normolipidemic Japanese women. *Preventive Medicine* 31:308-314
153. **Pugazhendhi D, Watson KA, Mills S, Botting N, Pope GS, Darbre PD** 2008 Effect of sulphation on the oestrogen agonist activity of the phytoestrogens genistein and daidzein in MCF-7 human breast cancer cells. *Journal of Endocrinology* 197:503-515
154. **Murata M, Midorikawa K, Koh M, Umezawa K, Kawanishi S** 2004 Genistein and daidzein induce cell proliferation and their metabolites cause oxidative DNA damage in relation to isoflavone-induced cancer of estrogen-sensitive organs. *Biochemistry* 43:2569-2577
155. **Thomas DB, Karagas MR** 1987 CANCER IN 1ST AND 2ND GENERATION AMERICANS. *Cancer Research* 47:5771-5776
156. **Messina M, Bennink M** 1998 Soyfoods, isoflavones and risk of colonic cancer: a review of the in vitro and in vivo data. *Baillieres Clinical Endocrinology and Metabolism* 12:707-728
157. **Banerjee S, Li YW, Wang ZW, Sarkar FH** 2008 Multi-targeted therapy of cancer by genistein. *Cancer Letters* 269:226-242
158. **Vihinen P, Ala-aho R, Kahari VM** 2005 Matrix metalloproteinases as therapeutic targets in cancer. *Current Cancer Drug Targets* 5:203-220
159. **WojtowiczPraga SM, Dickson RB, Hawkins MJ** 1997 Matrix metalloproteinase inhibitors. *Investigational New Drugs* 15:61-75
160. **Huang XK, Chen S, Xu L, Liu YQ, Deb DK, Platanias LC, Bergan RC** 2005 Genistein inhibits p38 map kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells. *Cancer Research* 65:3470-3478
161. **Kumi-Diaka JK, Hassanhi M, Merchant K, Horman V** 2006 Influence of genistein isoflavone on matrix metalloproteinase-2 expression in prostate cancer cells. *Journal of Medicinal Food* 9:491-497
162. **Lee W-Y, Huang S-C, Tzeng C-C, Chang T-L, Hsu K-F** 2007 Alterations of metastasis-related genes identified using an oligonucleotide Microarray of genistein-treated HCC1395 breast cancer cells. *Nutrition and Cancer-an International Journal* 58:239-246

163. **Kousidou OC, Mitropoulou TN, Roussidis AE, Kletsas D, Theocharis AD, Karamanos NK** 2005 Genistein suppresses the invasive potential of human breast cancer cells through transcriptional regulation of metalloproteinases and their tissue inhibitors. *International Journal of Oncology* 26:1101-1109
164. **Puli S, Lai JCK, Bhushan A** 2006 Inhibition of matrix degrading enzymes and invasion in human glioblastoma (U87MG) Cells by isoflavones. *Journal of Neuro-Oncology* 79:135-142
165. **Skogseth H, Follestad T, Larsson E, Halgunset J** 2006 Transcription levels of invasion-related genes in prostate cancer cells are modified by inhibitors of tyrosine kinase. *Apmis* 114:364-371
166. **El Touny LH, Banerjee PP** 2009 Identification of a Biphasic Role for Genistein in the Regulation of Prostate Cancer Growth and Metastasis. *Cancer Research* 69:3695-3703
167. **Li YW, Sarkar FH** 2002 Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. *Cancer Letters* 186:157-164
168. **Li YW, Kucuk O, Hussain M, Abrams J, Cher ML, Sarkar FH** 2006 Antitumor and antimetastatic activities of docetaxel are enhanced by genistein through regulation of osteoprotegerin/receptor activator of nuclear factor-kappa B (RANK)/RANK ligand/MMP-9 signaling in prostate cancer. *Cancer Research* 66:4816-4825
169. **Li YW, Bhuiyan M, Sarkar FH** 1999 Induction of apoptosis and inhibition of c-erbB-2 in MDA-MB-435 cells by genistein. *International Journal of Oncology* 15:525-533
170. **Xu L, Ding Y, Catalona WJ, Yang XJ, Anderson WF, Jovanovic B, Wellman K, Killmer J, Huang X, Scheidt KA, Montgomery RB, Bergan RC** 2009 MEK4 Function, Genistein Treatment, and Invasion of Human Prostate Cancer Cells. *Journal of the National Cancer Institute* 101:1141-1155
171. **Li YW, Upadhyay S, Bhuiyan M, Sarkar FH** 1999 Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. *Oncogene* 18:3166-3172
172. **Davis JN, Kucuk O, Sarkar FH** 1999 Genistein inhibits NF-kappa B activation in prostate cancer cells. *Nutrition and Cancer-an International Journal* 35:167-174
173. **Alhasan SA, Ensley JF, Sarkar FH** 2000 Genistein induced molecular changes in a squamous cell carcinoma of the head and neck cell line. *International Journal of Oncology* 16:333-338
174. **Natarajan K, Manna SK, Chaturvedi MM, Aggarwal BB** 1998 Protein tyrosine kinase inhibitors block tumor necrosis factor-induced activation of nuclear factor-kappa B, degradation of I kappa B alpha, nuclear translocation of p65, and subsequent gene expression. *Archives of Biochemistry and Biophysics* 352:59-70
175. **Davis JN, Kucuk O, Djuric Z, Sarkar FH** 2001 Soy isoflavone supplementation in healthy men prevents NF-kappa B activation by TNF-alpha in blood lymphocytes. *Free Radical Biology and Medicine* 30:1293-1302

176. **Karin M, Delhase M** 2000 The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Seminars in Immunology* 12:85-98
177. **Lee FS, Peters RT, Dang LC, Maniatis T** 1998 MEKK1 activates both I kappa B kinase alpha and I kappa B kinase beta. *Proceedings of the National Academy of Sciences of the United States of America* 95:9319-9324
178. **He H, Chen L, Zhai M, Chen JZS** 2009 Genistein Down-regulates The Constitutive Activation of Nuclear Factor-kappa B in Human Multiple Myeloma Cells, Leading to Suppression of Proliferation and Induction of Apoptosis. *Phytotherapy Research* 23:868-873
179. **Banerjee S, Zhang YX, Ali S, Bhuiyan M, Wang ZW, Chiao PJ, Philip PA, Abbruzzese J, Sarkar FH** 2005 Molecular evidence for increased antitumor activity of gemcitabine by genistein in vitro and in vivo using an orthotopic model of pancreatic cancer. *Cancer Research* 65:9064-9072
180. **Banerjee S, Zhang Y, Wang Z, Che M, Chiao PJ, Abbruzzese JL, Sarkar FH** 2007 In vitro and in vivo molecular evidence of genistein action in augmenting the efficacy of cisplatin in pancreatic cancer. *International Journal of Cancer* 120:906-917
181. **Sarkar FH, Li YW** 2006 Using chemopreventive agents to enhance the efficacy of cancer therapy. *Cancer Research* 66:3347-3350
182. **Mohammad RM, Banerjee S, Li YW, Aboukameel A, Kucuk O, Sarkar FH** 2006 Cisplatin-induced antitumor activity is potentiated by the soy isoflavone genistein in BxPC-3 pancreatic tumor xenografts. *Cancer* 106:1260-1268
183. **Li Y, Ellis K-L, Ali S, El-rayes BF, Nedeljkovic-Kurepa A, Kucuk O, Philip A, Sarkar FH** 2004 Apoptosis-inducing effect of chemotherapeutic agents is potentiated by soy isoflavone genistein, a natural inhibitor of NF-kappaB in BxPC-3 pancreatic cancer cell line. *Pancreas* 28:90-95
184. **Gong LJ, Li YW, Nedeljkovic-Kurepa A, Sarkar FH** 2003 Inactivation of NF-kappa B by genistein is mediated via Akt signaling pathway in breast cancer cells. *Oncogene* 22:4702-4709
185. **El-Rayes BF, Ali S, Ali IF, Philip PA, Abbruzzese J, Sarkar FH** 2006 Potentiation of the effect of erlotinib by genistein in pancreatic cancer: The role of Akt and nuclear factor-kappa B. *Cancer Research* 66:10553-10559
186. **Li YW, Sarkar FH** 2002 Inhibition of nuclear factor kappa B activation in PC3 cells by genistein is mediated via Akt signaling pathway. *Clinical Cancer Research* 8:2369-2377
187. **Li YW, Sarkar FH** 2002 Gene expression profiles of genistein-treated PC3 prostate cancer cells. *Journal of Nutrition* 132:3623-3631
188. **Oh HY, Leem J, Yoon SJ, Yoon S, Hong SJ** 2010 Lipid raft cholesterol and genistein inhibit the cell viability of prostate cancer cells via the partial contribution of EGFR-Akt/p70S6k pathway and down-regulation of androgen receptor. *Biochemical and Biophysical Research Communications* 393:319-324
189. **Ferenc P, Solar P, Kleban J, Mikes J, Fedorcko P** 2010 Down-regulation of Bcl-2 and Akt induced by combination of photoactivated hypericin and

- genistein in human breast cancer cells. *Journal of Photochemistry and Photobiology B-Biology* 98:25-34
190. **Park S-J, Kim M-J, Kim Y-K, Kim S-M, Park J-Y, Myoung H** 2010 Combined cetuximab and genistein treatment shows additive anti-cancer effect on oral squamous cell carcinoma. *Cancer Letters* 292:54-63
 191. **Kim S-H, Kim S-H, Kim Y-B, Jeon Y-T, Lee S-C, Song Y-S** 2009 Genistein Inhibits Cell Growth by Modulating Various Mitogen-Activated Protein Kinases and AKT in Cervical Cancer Cells. *Natural Compounds and Their Role in Apoptotic Cell Signaling Pathways* 1171:495-500
 192. **Peng J-H, Zhu J-D, Mi M-T, Li F-J, Cai L, Dong J-Z, Zhang H-X, Zhao Y, Xue R-L** 2010 Prepubertal genistein exposure affects erbB2/Akt signal and reduces rat mammary tumorigenesis. *European Journal of Cancer Prevention* 19:110-119
 193. **Xu L, Chen S, Bergan RC** 2006 MAPKAPK2 and HSP27 are downstream effectors of p38MAP kinase-mediated matrix metalloproteinase type 2 activation and cell invasion in human prostate cancer. *Oncogene* 25:2987-2998
 194. **Lotan TL, Lyon M, Huo D, Taxy JB, Brendler C, Foster BA, Stadler W, Rinker-Schaeffer CW** 2007 Up-regulation of MKK4, MKK6 and MKK7 during prostate cancer progression: an important role for SAPK signalling in prostatic neoplasia. *Journal of Pathology* 212:386-394
 195. **Wang L, Pan Y, Dai JL** 2004 Evidence of MKK4 pro-oncogenic activity in breast and pancreatic tumors. *Oncogene* 23:5978-5985
 196. **Xu L, Bergan RC** 2006 Genistein inhibits matrix metalloproteinase type 2 activation and prostate cancer cell invasion by blocking the transforming growth factor beta-mediated activation of mitogen-activated protein kinase-activated protein kinase 2-27-kDa heat shock protein pathway. *Molecular Pharmacology* 70:869-877
 197. **Wagner EF, Nebreda AR** 2009 Signal integration by JNK and p38 MAPK pathways in cancer development. *Nature Reviews Cancer* 9:537-549
 198. **Harper CE, Cook LM, Patel BB, Wang J, Eltoum IA, Arabshahi A, Shirai T, Lamartiniere CA** 2009 Genistein and Resveratrol, Alone and in Combination, Suppress Prostate Cancer in SV-40 Tag Rats. *Prostate* 69:1668-1682
 199. **El Touny LH, Banerjee PP** 2007 Genistein induces the metastasis suppressor kangai-1 which mediates its anti-invasive effects in TRAMP cancer cells. *Biochemical and Biophysical Research Communications* 361:169-175
 200. **Messina M, McCaskill-Stevens W, Lampe JW** 2006 Addressing the soy and breast cancer relationship: Review, commentary, and workshop proceedings. *Journal of the National Cancer Institute* 98:1275-1284
 201. **Lucki NC, Sewer MB** 2011 Genistein Stimulates MCF-7 Breast Cancer Cell Growth by Inducing Acid Ceramidase (ASAH1) Gene Expression. *Journal of Biological Chemistry* 286:19399-19409
 202. **Rajah T, Du N, Drews N, Cohn R** 2009 Genistein in the Presence of 17 β -Estradiol Inhibits Proliferation of ERB Breast Cancer Cells. *Pharmacology* 84:68-73

203. **Hsieh CY, Santell RC, Haslam SZ, Helferich WG** 1998 Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo. *Cancer Research* 58:3833-3838
204. **van Duursen MBM, Nijmeijer SM, de Morree ES, de Jong PC, van den Berg M** 2011 Genistein induces breast cancer-associated aromatase and stimulates estrogen-dependent tumor cell growth in in vitro breast cancer model. *Toxicology* 289:67-73
205. **Santell RC, Chang YC, Nair MG, Helferich WG** 1997 Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. *Journal of Nutrition* 127:263-269
206. **Ju YH, Allred CD, Allred KF, Karko KL, Doerge DR, Helferich WG** 2001 Physiological concentrations of dietary genistein dose-dependently stimulate growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted in athymic nude mice. *Journal of Nutrition* 131:2957-2962
207. **Hilakivi-Clarke L, Andrade JE, Helferich W** 2010 Is Soy Consumption Good or Bad for the Breast? *Journal of Nutrition* 140:2326S-2334S
208. **Singh AV, Franke AA, Blackburn GL, Zhou JR** 2006 Soy phytochemicals prevent orthotopic growth and metastasis of bladder cancer in mice by alterations of cancer cell proliferation and apoptosis and tumor angiogenesis. *Cancer Research* 66:1851-1858
209. **Ogasawara M, Matsunaga T, Suzuki H** 2007 Differential effects of antioxidants on the in vitro invasion, growth and lung metastasis of murine colon cancer cells. *Biological & Pharmaceutical Bulletin* 30:200-204
210. **Gu Y, Zhu C-F, Dai Y-L, Zhong Q, Sun B** 2009 Inhibitory effects of genistein on metastasis of human hepatocellular carcinoma. *World Journal of Gastroenterology* 15:4952-4957
211. **Pendleton JM, Tan WW, Anai S, Chang M, Hou W, Shiverick KT, Rosser CJ** 2008 Phase II trial of isoflavone in prostate-specific antigen recurrent prostate cancer after previous local therapy. *Bmc Cancer* 8
212. **Devere White RW, Hackman RM, Soares SE, Beckett LA, Li Y, Sun B** 2004 Effects of a genistein-rich extract on PSA levels in men with a history of prostate cancer. *Urology* 63:259-263
213. **Malumbres M, Barbacid M** 2009 Cell cycle, CDKs and cancer: a changing paradigm. *Nature Reviews Cancer* 9:153-166
214. **Ramos S** 2007 Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *Journal of Nutritional Biochemistry* 18:427-442
215. **Cappelletti V, Fioravanti L, Miodini P, Di Fronzo G** 2000 Genistein blocks breast cancer cells in the G(2)M phase of the cell cycle. *Journal of Cellular Biochemistry* 79:594-600
216. **Choi YH, Lee WH, Park KY, Zhang LJ** 2000 p53-independent induction of p21 (WAF1/CIP1), reduction of cyclin B1 and G2/M arrest by the isoflavone genistein in human prostate carcinoma cells. *Japanese Journal of Cancer Research* 91:164-173
217. **Yan G-R, Xiao C-L, He G-W, Yin X-F, Chen N-P, Cao Y, He Q-Y** 2010 Global phosphoproteomic effects of natural tyrosine kinase inhibitor, genistein, on signaling pathways. *Proteomics* 10:976-986

218. **Choi YH, Zhang LJ, Lee WH, Park KY** 1998 Genistein-induced G2/M arrest is associated with the inhibition of cyclin B1 and the induction of p21 in human breast carcinoma cells. *International Journal of Oncology* 13:391-396
219. **Ouyang G, Yao L, Ruan K, Song G, Mao Y, Bao S** 2009 Genistein induces G2/M cell cycle arrest and apoptosis of human ovarian cancer cells via activation of DNA damage checkpoint pathways. *Cell Biology International* 33:1237-1244
220. **Ismail IA, Kang K-S, Lee HA, Kim J-W, Sohn Y-K** 2007 Genistein-induced neuronal apoptosis and G2/M cell cycle arrest is associated with MDC1 up-regulation and PLK1 down-regulation. *European Journal of Pharmacology* 575:12-20
221. **Su SJ, Chow NH, Kung ML, Hung TC, Chang KL** 2003 Effects of soy isoflavones on apoptosis induction and G2-M arrest in human hepatoma cells involvement of caspase-3 activation, Bcl-2 and Bcl-X-L downregulation, and Cdc2 kinase activity. *Nutrition and Cancer-an International Journal* 45:113-123
222. **Davis JN, Singh B, Bhuiyan M, Sarkar FH** 1998 Genistein-induced upregulation of p21(WAF1), downregulation of cyclin B, and induction of apoptosis in prostate cancer cells. *Nutrition and Cancer-an International Journal* 32:123-131
223. **Lian FR, Bhuiyan M, Li YW, Wall N, Kraut M, Sarkar FH** 1998 Genistein-induced G(2)-M arrest, p21(WAF1) upregulation, and apoptosis in a non-small-cell lung cancer cell line. *Nutrition and Cancer-an International Journal* 31:184-191
224. **Alhasan SA, Pietrasczkiewicz H, Alonso MD, Ensley J, Sarkar FH** 1999 Genistein-induced cell cycle arrest and apoptosis in a head and neck squamous cell carcinoma cell line. *Nutrition and Cancer-an International Journal* 34:12-19
225. **Wang Z, Ahmad A, Banerjee S, Azmi A, Kong D, Li Y, Sarkar FH** 2010 FoxM1 is a Novel Target of a Natural Agent in Pancreatic Cancer. *Pharmaceutical Research* 27:1159-1168
226. **El Touny LH, Banerjee PP** 2006 Identification of both Myt-1 and Wee-1 as necessary mediators of the p21-independent inactivation of the Cdc-2/cyclin B1 complex and growth inhibition of TRAMP cancer cells by genistein. *Prostate* 66:1542-1555
227. **Tophkhane C, Yang S, Bales W, Archer L, Osunkoya A, Thor AD, Yang X** 2007 Bcl-2 overexpression sensitizes MCF-7 cells to genistein by multiple mechanisms. *International Journal of Oncology* 31:867-874
228. **Po LS, Wang TT, Chen ZY, Leung LK** 2002 Genistein-induced apoptosis in MCF-7 cells involves changes in Bak and Bcl-x without evidence of anti-oestrogenic effects. *British Journal of Nutrition* 88:463-469
229. **Lian FR, Li YW, Bhuiyan M, Sarkar FH** 1999 p53-independent apoptosis induced by genistein in lung cancer cells. *Nutrition and Cancer-an International Journal* 33:125-131
230. **Kazi A, Daniel KG, Smith DM, Kumar NB, Dou QP** 2003 Inhibition of the proteasome activity, a novel mechanism associated with the tumor cell

- apoptosis-inducing ability of genistein. *Biochemical Pharmacology* 66:965-976
231. **Kumi-Diaka J, Sanderson NA, Hall A** 2000 The mediating role of caspase-3 protease in the intracellular mechanism of genistein-induced apoptosis in human prostatic carcinoma cell lines, DU145 and LNCaP. *Biology of the Cell* 92:595-604
 232. **Baxa DM, Luo XX, Yoshimura FK** 2005 Genistein induces apoptosis in T lymphoma cells via mitochondrial damage. *Nutrition and Cancer-an International Journal* 51:93-101
 233. **Buchler P, Gukovskaya TS, Mouria T, Buchler MC, Buchler MW, Friess H, Pandol TJ, Reber HA, Hines OJ** 2003 Prevention of metastatic pancreatic cancer growth in vivo by induction of apoptosis with genistein, a naturally occurring isoflavonoid. *Pancreas* 26:264-273
 234. **Jin C-Y, Park C, Kim G-Y, Lee S-J, Kim W-J, Choi YH** 2009 Genistein enhances TRAIL-induced apoptosis through inhibition of p38 MAPK signaling in human hepatocellular carcinoma Hep3B cells. *Chemico-Biological Interactions* 180:143-150
 235. **Chen AC, Donovan SM** 2004 Genistein at a concentration present in soy infant formula inhibits Caco-2BBE cell proliferation by causing G2/M cell cycle arrest. *Journal of Nutrition* 134:1303-1308
 236. **Nakamura Y, Yogosawa S, Izutani Y, Watanabe H, Otsuji E, Sakai T** 2009 A combination of indol-3-carbinol and genistein synergistically induces apoptosis in human colon cancer HT-29 cells by inhibiting Akt phosphorylation and progression of autophagy. *Molecular Cancer* 8
 237. **Datta SR, Dudek H, Tao X, Masters S, Fu HA, Gotoh Y, Greenberg ME** 1997 Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91:231-241
 238. **Raffoul JJ, Banerjee S, Singh-Gupta V, Knoll ZE, Fite A, Zhang H, Abrams J, Sarkar FH, Hillman GG** 2007 Down-regulation of apurinic/aprimidinic endonuclease 1/redox factor-1 expression by soy isoflavones enhances prostate cancer radiotherapy in vitro and in vivo. *Cancer Research* 67:2141-2149
 239. **Sakamoto T, Horiguchi H, Oguma E, Kayama F** 2010 Effects of diverse dietary phytoestrogens on cell growth, cell cycle and apoptosis in estrogen-receptor-positive breast cancer cells. *Journal of Nutritional Biochemistry* 21:856-864
 240. **Zhou JR, Yu LY, Zhong Y, Nassr RL, Franke AA, Gaston SM, Blackburn GL** 2002 Inhibition of orthotopic growth and metastasis of androgen-sensitive human prostate tumors in mice by bioactive soybean components. *Prostate* 53:143-153
 241. **Fotsis T, Pepper M, Adlercreutz H, Hase T, Montesano R, Schweigerer L** 1995 GENISTEIN, A DIETARY INGESTED ISOFLAVONOID, INHIBITS CELL-PROLIFERATION AND IN-VITRO ANGIOGENESIS. *Journal of Nutrition* 125:S790-S797
 242. **Li YW, Che MX, Bhagat S, Ellis KL, Kucuk O, Doerge DR, Abrams J, Cher ML, Sarkar FH** 2004 Regulation of gene expression and inhibition of

- experimental prostate cancer bone metastasis by dietary genistein. *Neoplasia* 6:354-363
243. **Su SJ, Yeh TM, Chuang WJ, Ho CL, Chang KL, Cheng HL, Liu HS, Hsu PY, Chow NH** 2005 The novel targets for anti-angiogenesis of genistein on human cancer cells. *Biochemical Pharmacology* 69:307-318
 244. **Buchler P, Reber HA, Buchler MW, Friess H, Lavey RS, Hines OJ** 2004 Antiangiogenic activity of genistein in pancreatic carcinoma cells is mediated by the inhibition of hypoxia-inducible factor-1 and the down-regulation of VEGF gene expression. *Cancer* 100:201-210
 245. **Singh-Gupta V, Zhang H, Banerjee S, Kong D, Raffoul JJ, Sarkar FH, Hillman GG** 2009 Radiation-induced HIF-1 alpha cell survival pathway is inhibited by soy isoflavones in prostate cancer cells. *International Journal of Cancer* 124:1675-1684
 246. **Gu Y, Zhu CF, Iwamoto H, Chen JS** 2005 Genistein inhibits invasive potential of human hepatocellular carcinoma by altering cell cycle, apoptosis, and angiogenesis. *World J Gastroenterol* 11:6512-6517
 247. **Guo Y, Wang S, Hoot DR, Clinton SK** 2007 Suppression of VEGF-mediated autocrine and paracrine interactions between prostate cancer cells and vascular endothelial cells by soy isoflavones. *Journal of Nutritional Biochemistry* 18:408-417
 248. **Yamada KM** 1997 Integrin signaling. *Matrix Biology* 16:137-141
 249. **Giancotti FG, Ruoslahti E** 1999 Transduction - Integrin signaling. *Science* 285:1028-1032
 250. **Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, Schlaepfer DD** 2000 FAK integrates growth-factor and integrin signals to promote cell migration. *Nature Cell Biology* 2:249-256
 251. **Liu YQ, Kyle E, Patel S, Housseau F, Hakim F, Lieberman R, Pins M, Blagosklonny MV, Bergan RC** 2001 Prostate cancer chemoprevention agents exhibit selective activity against early stage prostate cancer cells. *Prostate Cancer and Prostatic Diseases* 4:81-91
 252. **Tremblay L, Hauck W, Aprikian AG, Begin LR, Chapdelaine A, Chevalier S** 1996 Focal adhesion kinase (pp125(FAK)) expression, activation and association with paxillin and p50(CSK) in human metastatic prostate carcinoma. *International Journal of Cancer* 68:164-171
 253. **Hiscox S, Jordan NJ, Morgan L, Green TP, Nicholson RI** 2007 Src kinase promotes adhesion-independent activation of FAK and enhances cellular migration in tamoxifen-resistant breast cancer cells. *Clinical & Experimental Metastasis* 24:157-167
 254. **Meng XN, Jin Y, Yu Y, Bai J, Liu GY, Zhu J, Zhao YZ, Wang Z, Chen F, Lee KY, Fu SB** 2009 Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion. *British Journal of Cancer* 101:327-334
 255. **Miekus K, Madeja Z** 2007 Genistein inhibits the contact-stimulated migration of prostate cancer cells. *Cellular & Molecular Biology Letters* 12:348-361

256. **Valachovicova T, Slivova V, Bergman H, Shuherk J, Sliva D** 2004 Soy isoflavones suppress invasiveness of breast cancer cells by the inhibition of NF-kappa B/AP-1-dependent and -independent pathways. *International Journal of Oncology* 25:1389-1395
257. **Lakshman M, Xu L, Ananthanarayanan V, Cooper J, Takimoto CH, Helenowski I, Pelling JC, Bergan RC** 2008 Dietary genistein inhibits metastasis of human prostate cancer in mice. *Cancer Research* 68:2024-2032
258. **Bergan R, Kyle E, Nguyen P, Trepel J, Ingui C, Neckers L** 1996 Genistein-stimulated adherence of prostate cancer cells is associated with the binding of focal adhesion kinase to beta-1-integrin. *Clinical & Experimental Metastasis* 14:389-398
259. **Farina HG, Pomies M, Alonso DF, Gomez DE** 2006 Antitumor and antiangiogenic activity of soy isoflavone genistein in mouse models of melanoma and breast cancer. *Oncology Reports* 16:885-891
260. **Sawai H, Okada Y, Funahashi H, Matsuo Y, Takahashi H, Takeyama H, Manabe T** 2005 Activation of focal adhesion kinase enhances the adhesion and invasion of pancreatic cancer cells via extracellular signal-regulated kinase-1/2 signaling pathway activation. *Molecular Cancer* 4
261. **Hsu EL, Chen N, Westbrook A, Wang F, Zhang R, Taylor RT, Hankinson O** 2009 Modulation of CXCR4, CXCL12, and Tumor Cell Invasion Potential In Vitro by Phytochemicals. *J Oncol* 2009:491985
262. **Cary LA, Chang JF, Guan JL** 1996 Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *Journal of Cell Science* 109:1787-1794
263. **Cornford PA, Dodson AR, Parsons KF, Desmond AD, Woolfenden A, Fordham M, Neoptolemos JP, Ke YQ, Foster CS** 2000 Heat shock protein expression independently predicts clinical outcome in prostate cancer. *Cancer Research* 60:7099-7105
264. **Benndorf R, Hayess K, Ryazantsev S, Wieske M, Behlke J, Lutsch G** 1994 PHOSPHORYLATION AND SUPRAMOLECULAR ORGANIZATION OF MURINE SMALL HEAT-SHOCK PROTEIN HSP25 ABOLISH ITS ACTIN POLYMERIZATION-INHIBITING ACTIVITY. *Journal of Biological Chemistry* 269:20780-20784
265. **Lavoie JN, Gingrasbreton G, Tanguay RM, Landry J** 1993 INDUCTION OF CHINESE-HAMSTER HSP27 GENE-EXPRESSION IN MOUSE CELLS CONFERS RESISTANCE TO HEAT-SHOCK - HSP27 STABILIZATION OF THE MICROFILAMENT ORGANIZATION. *Journal of Biological Chemistry* 268:3420-3429
266. **Lavoie JN, Hickey E, Weber LA, Landry J** 1993 MODULATION OF ACTIN MICROFILAMENT DYNAMICS AND FLUID-PHASE PINOCYTOSIS BY PHOSPHORYLATION OF HEAT-SHOCK PROTEIN-27. *Journal of Biological Chemistry* 268:24210-24214
267. **Hayes SA, Huang XK, Kambhampati S, Platanias LC, Bergan RC** 2003 p38 MAP kinase modulates Smad-dependent changes in human prostate cell adhesion. *Oncogene* 22:4841-4850

- 268. **Liu YQ, Jovanovic B, Pins M, Lee C, Bergan RC** 2002 Over expression of endoglin in human prostate cancer suppresses cell detachment, migration and invasion. *Oncogene* 21:8272-8281
- 269. **Craft CS, Romero D, Vary CPH, Bergan RC** 2007 Endoglin inhibits prostate cancer motility via activation of the ALK2-Smad1 pathway. *Oncogene* 26:7240-7250
- 270. **Craft CS, Xu L, Romero D, Vary CPH, Bergan RC** 2008 Genistein induces phenotypic reversion of endoglin deficiency in human prostate cancer cells. *Molecular Pharmacology* 73:235-242
- 271. **Zhang L-l, Li L, Wu D-p, Fan J-h, Li X, Wu K-j, Wang X-y, He D-l** 2008 A novel anti-cancer effect of genistein: reversal of epithelial mesenchymal transition in prostate cancer cells. *Acta Pharmacologica Sinica* 29:1060-1068