FLAVONOIDS AND CONNEXIN PROTEINS: GROWTH SUPPRESSION AND
PROGNOSTIC VALUE IN HUMAN BREAST CANCER

by

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Abstract

Connexin proteins form gap junctions permitting direct intercellular cytoplasmic exchange. Gap junctional intercellular communication (GJIC) is important for maintaining homeostasis and preventing cell transformation. Connexins may also have independent functions including tumor growth suppression. Most tumors express less connexins, have reduced GJIC, and have increased growth rates compared to nontumorigenic cells. Asian females, who consume foods rich in flavonoids have a 4 to 6-fold reduction in breast cancer incidence. The purpose of this study was to determine if common flavonoids, genistein and quercetin, increase connexin43 (Cx43), improve GJIC, and retard growth of metastatic human breast cancer cells (MDA-MB-231). Results demonstrated that genistein and quercetin are potential anti-breast cancer agents. Although flavonoid treatment did not improve GJIC, genistein (2.5 µg/ml, 5 µg/ml, 15 µg/ml) and quercetin (2.5 µg/ml, 5 µg/ml) increased Cx43 protein and suppressed MDA-MB-231 cell proliferation at concentrations which were not toxic to nontumorigenic breast cancer cells.

Reliable prognostic indicators of breast cancer include axillary nodal status, tumor size and histological grade. Additionally, estrogen receptor/progesterone receptor (ER/PR) status, and Ki67, an indicator of proliferative activity, are useful for predicting patient survival and relapse. Improved prognostic methods and a search for a more complete understanding of cancer biology inspires the identification of new molecular markers. Tissue microarrays, containing over 300 cases of invasive breast carcinoma were stained with Cx26, Cx32, and Cx43 antibodies. Connexin immunoreactivity was correlated with established immunohistochemical and histopathological breast cancer markers. Cx26, Cx32, and Cx43 did not correlate with tumor grade or tumor size. Cx32

inversely correlated with lymph node status ($P < 0.05$), and there was a positive

correlation between Cx43 and PR status ($P < 0.01$). Both Cx32 and Cx43 correlated

positively with ER status ($P < 0.01$), and Cx43 correlated negatively with Ki67

expression ($P < 0.01$). Cx26, Cx32, and Cx43 did not correlate with patient outcome. In

conclusion, connexin proteins did not appear to be reliable independent indicators of

breast cancer prognosis. However, Cx32 may protect against regional lymph node spread,

and Cx43 may counteract aggressively proliferating breast cancer.

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List of Abbreviations:

°C	Degrees Celcius
ANOVA	Analysis of variance
AP-1	Activator protein-1
BCA	Bicinchroninic acid
bFGF	Basic fibroblastic growth factor
BRMS1	Breast cancer metastasis suppressor 1
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Cdk 2	Cyclin-dependent kinase 2
c-erbB-2	Human epidermal growth factor receptor 2
5(6)-CFDA	5-(and -6) – carboxyfluorescein diacetate
CRS	Cancer Research Society
Cx	Connexin
Da	Daltons
DAPI	4 ,6-diamidino-2-phenylindole
dH_2O	Distilled water
ddH_2O	Distilled, deionized water
DCIS	Ductal carcinoma in situ
DDT	Dichlorodiphenyltrichloroethane
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E1	First extracellular loop of connexin proteins
E2	Second extracellular loop of connexin proteins
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
ER	Estrogen receptor
ERK	Estrogen receptor kinase
F-actin	Filamentous actin
FGFR	Fibroblastic growth factor receptor
G	Genistein
G1	Gap 1 phase of cell cycle
G2	Gap 2 phase of cell cycle
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GJIC	Gap junctional intercellular communication
H&E	Hemotoxylin and eosin
HER-2/neu	Human epidermal growth factor receptor-2
HGF/SF	Hepatic growth factor/scatter factor
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
IP_3	Inositol-1,4,5,-triphosphate
kD	kilodaltons
LDH	Lactate dehydrogenase

M	Mitosis phase of cell cycle (Introduction)/ complete growth medium (Figures)
MAPK	Mitogen-activated protein kinase
NFDM	Non-fat dry milk
NFkB	Nuclear factor kB
NSERC	Natural Sciences and Engineering Research Council
OVCAR-5	Human ovarian carcinoma cells
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PIP kinase	Phosphatidylinositol 4-phosphate 5-kinase
PKC	Protein kinase C
PR	Progesterone receptor
Q	Quercetin
S	DNA synthesis phase of cell cycle
SDS	Sodium dodecyl sulphate
SGLT1	Sodium-dependent glucose transporter
Skp2	S phase kinase associated protein 2
TGF-beta	Transforming growth factor-beta
TIMP-1	Tissue inhibitor of metalloproteinase-1
TMA	Tissue microarray
TPA	12-O-tetradecanoyl phorbol-13-acetate
Tris	Tris(hydroxymethanyl)aminomethane
Tween-20	Polyoxyethylene 20-sorbital monolaurate
µg/ml	Micrograms per milliliter
µl	Microlitre
µM	Micromolar
VEGF	Vascular endothelial growth factor

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CHAPTER 1

General Introduction

1.1   Introductory Statement:

Breast tumors are the most common type of cancer in Caucasian women living in Western nations (reviewed by Ganry, 2002). The lifetime risk of an American female developing breast cancer is approximately 1 in 8 (Ries et al., 2000). Similar breast cancer incidences have been reported in Western Europe. Interestingly, Asian women of Japan and China are approximately 5 times less likely to develop breast cancer than are white North American and Western European females (reviewed by Lambe et al., 2003).

Migration studies show that the breast cancer incidence in Asian women approaches that of American women after several generations of residence in the United States and adoption of the American diet (Ziegler et al., 1993). This suggests that the difference in breast cancer incidence between Asian and Western women is not genetic, but rather due to dietary or environmental factors. Asians consume approximately half the dietary fat that North Americans do (Roberts, 1988). Furthermore, the Asian diet is much higher in soy, fruit, and vegetable products compared the typical North American diet (reviewed by Messina, 1994). Soy products are rich in the isoflavone, genistein (Morton et al., 2002, Rice et al., 2001). Quercetin, a flavonol, is common to fruits and vegetables (reviewed by Murota and Terao, 2003; Wolffram et al., 2001). Quercetin and genistein have been shown to have potent antiproliferative effects on tumor cells by inducing apoptosis (Weber et al., 1997; Ying et al., 2002; Brownson et al., 2002), halting the cell cycle (Balabhadrapathruni et al., 2000; Ying et al., 2002), reducing the growth stimulatory effect of IP_3 (Weber et al., 1997) and tyrosine kinase (Brownson et al., 2002),

promoting differentiation (Jin and MacDonald, 2002), and interfering with estrogen (Brownson et al., 2002).

Most tumor cells express very low levels of connexin protein and/or exhibit impaired gap junctional intercellular communication (GJIC) (reviewed by Trosko and Ruch, 2002). Furthermore, dysfunctional GJIC is considered to be one of the earliest alterations associated with malignant transformation (Mehta et al., 1986; Mehta et al., 1996). The flavonoids, apigenin and tangeretin, have been shown to increase GJIC in rat liver epithelial cells (Chaumontet et al., 1997). These flavonoids may therefore have therapeutic implications for cancer. Specifically, increased GJIC may enhance intercellular exchange of growth regulatory signals and chemotherapeutic drugs.

In this thesis, I explored the effect of genistein and quercetin on the growth of a metastatic human breast cancer cell line (MDA-MB-231) through gap junctional mechanisms. Using tumor microarrays, I also investigated connexin proteins as prognostic indicators for breast cancer. I provide evidence that genistein and quercetin upregulate connexin43 (Cx43) and suppress proliferation MDA-MB-231 cells at concentrations which are not toxic to nontumorigenic human breast cells. I also present evidence that Cx43 is inversely correlated with the proliferative marker, Ki67, in human breast cancer tissue, and that Cx32 is inversely correlated with lymph node status.

1.2 **Flavonoids:**

Polyphenolic compounds are one of the largest and most ubiquitous group of plant metabolites. The phenylpropanoid pathway converts phenylalanine (reviewed by Winkel-Shirley, 1999) to more than 8000 of these plant-derived compounds. Polyphenolic structures have an aromatic ring bearing at least one hydroxyl group (Croft,

1998), and are very important in plant biology. Polyphenols protect plants from ultraviolet light, help ward off herbivores, and are important for plant growth and reproduction (reviewed by Winkel-Shirley, 1999).

There are 9 classes of polyphenols grouped by chemical structure: simple phenols, phenolic acids, acetophenones, hydroxycinnamic acids, naphthoquinones, xanthones, stilbenes, lignans, and flavonoids (reviewed by Ross and Kasum, 2002). Flavonoids are the largest class, and have a common structure of diphenylpropanes, consisting of two aromatic rings linked through 3 carbons (reviewed by Yang et al., 2001) (Figure 1.1). There are over 5000 flavonoids, which are divided into 6 groups based on heterocyclic C-ring variation (reviewed by Ross and Kasam, 2002). Flavonoid classes include: flavones, flavonols, isoflavones, flavanols, flavonones, and anthocyanins (reviewed by Ross and Kasam, 2002).

Flavonoids have interested botanists for years. However, recent attention has focused on the potential health benefits of flavonoids. Rusznyak and Szent-Gyorgyi (1936) first observed the biological activity of flavonoids, and proposed them to be plant-derived vitamins. Subsequently, the term "P-vitamins" was suggested for flavonoids (Kuo, 2001). Although this name was only used temporarily, the scientific literature reports that flavonoids have many biological activities. Flavonoids help fight bacterial and viral infections, and have vasodilatory, antioxidant, antitumor, and antiallergenic functions (reviewed by Bravo, 1998).

Oxidative damage is implicated in many chronic ailments, including cardiovascular disease (Kinugawa et al., 2003), diabetes (Brands et al., 2004), and cancer (Murtaugh et al., 2004). Reactive oxygen species, including peroxidases and malondialdehyde (reviewed by Ceconi, 2003) can damage DNA, protein and lipids

(reviewed by Ross and Kasum, 2002). The human body has natural antioxidants, such as superoxide dismutase and glutathione-S-transferase (McCord and Fridovich, 1969), enzymes that detoxify mutagenic xenobiotics and reduce hydrogen peroxide-mediated oxidative stress (reviewed by Ross and Kasum, 2002). However, these endogenous defenses are often overwhelmed (reviewed by Ross and Kasum, 2002). Researchers are therefore very interested in the antioxidant effect of flavonoids.

Structurally, flavonoids are ideal reducing agents. Flavonoids readily donate hydrogen atoms, quench oxygen radicals and delocalize unpaired electrons favorably throughout their conjugated structures (Lien et al., 1999). Furthermore, flavonoids can enhance the antioxidant activity of other molecules. In the presence of flavonoids, vitamin C and E inhibit plasma membrane lipid peroxidation (reviewed by Ross and Kasum, 2002). Furthermore, the flavonoids quercetin, myricetin, and fisetin increase the activity of glutathione-S-transferase (Fiander and Schneider, 2000). The biological effects of genistein and quercetin and other flavonoids are summarized in Table 1.1.

Antioxidant flavonols in green tea – epicatechin and gallocatechin – have been extensively studied regarding their ability to counteract breast tumor growth. Chen et al., (1998) reported that epigallocatechin gallate inhibited growth of human metastatic breast tumor cells (Hs578T). Sartippour et al. (2002) demonstrated that green tea inhibited the angiogenesis factor, vascular endothelial growth factor (VEGF), in MDA-MB-231 cells. Lastly, Kavanagh et al. (2001) reported that green tea decreased carcinogen-induced breast tumor burden in rats. Although oxidative damage contributes to cancer progression, it is important to consider that cancer is characterized by many factors. These include tissue invasion, metastasis, and uncontrolled cellular proliferation (reviewed by Hanahan and Weinberg, 2000).

Genistein and quercetin exhibit antiproliferative (Balabhadrapathruni et al., 2000; Choi et al., 2001), apoptotic (Katdare et al., 2002; Choi et al., 2001), and antioxidant effects (Ishige et al., 2001). These characteristics have sparked great interest among cancer researchers. Animal and human studies show that genistein is effective against developing mammary tumors, melanoma, colon (reviewed by Wang, 2000) and prostate cancer (Rao et al., 2002). Quercetin has been shown to inhibit the growth of cells lines derived from human cancers such as stomach, colon, prostate, and breast (reviewed by Soleas et al., 2002). Additionally, quercetin suppresses uterine and cervical cancer, melanoma, and intestinal tumor development in mice (reviewed by Soleas, 2002).

1.3   Flavonoid Consumption:

Flavonoid consumption varies according to diet and population. People living in the United States consume approximately 1 g of flavonoids daily (Kuhnau, 1976). This flavonoid consumption is high compared to Danish and Dutch people who consume 0.028 g/day of flavonols, flavones, and flavanones (Hertog et al., 1993), and Finnish men who ingest only 0.004 g of flavonoids daily (Knekt et al., 1997).

1.4   Quercetin and its Health Benefits:

Quercetin is the most abundant dietary flavonoid in Western diets, and ranks among the most potent flavonoids in terms of antioxidant and other health-promoting effects (Formica and Regelson, 1995). This flavonol is ubiquitously found in fruits and vegetables (Murota and Terao, 2003), and is most concentrated in onions, apples and tea (reviewed by Wolffram et al., 2002). Human daily intake of quercetin ranges from

10-20 mg (reviewed by Wolframm et al., 2002), and the concentration of quercetin in human blood plasma has reached 0.74 uM (reviewed by Ross and Kasum, 2002).

Additional to its antitumor effects, dietary quercetin is inversely correlated with myocardial infarction (Geleijnse et al., 2002; Knekt et al., 2002), and protects against heart disease by modulating low density lipoproteins (reviewed by Wang, 2000). Furthermore, quercetin may relieve diabetic symptoms by inhibiting sorbitol and polpol production (reviewed by Wang, 2000), causative agents associated with nerve, eye, and kidney damage.

1.5 Genistein and its Health Benefits:

Genistein is an isoflavone found exclusively in soy products such as tofu, miso, and aburaage. The Asian diet is rich in soy compared to the North American or European diet (Morton et al., 2002; Rice et al., 2001). Chinese and Japanese people consume 20-150 mg genistein daily (Barnes et al., 1995; Rice et al., 2001) and have peak plasma genistein concentrations between 1.08 and 2.40 uM (Hedlund et al., 2002) (Table 1.2).

Epidemiological studies report that regular isoflavone consumption reduces postmenopausal symptoms and bone loss (Greendale et al., 2002; Boulet et al., 1994). Furthermore, isoflavones are thought to reduce the incidence of breast cancer and cardiovascular disease (Glazier and Bowman, 2001).

1.6 Metabolism of Quercetin and Genistein:

Most dietary flavonoids are glycosylated (flavonoid glycosides), meaning that they have one or more simple sugars, such as glucose or galactose, attached to them. (reviewed by Murota, 2003) They commonly become altered during digestion. For

example, in common foods, such as onions and tea, quercetin is often in the form of quercetin-4'-O-glucoside and quercetin-3-glucoside (Graefe et al., 2002). These glycosides become metabolized to 3'-O-methyl quercetin, 4'-O-methyl quercetin, quercetin 7-O-beta-D-glucuronide, and quercetin aglycone (the non-sugar portion of quercetin, which is often considered pure quercetin) (Morland et al., 2003).

With the exception of fermented soy food, dietary genistein is conjugated to glucose, 6''-O-malonyl- or 6''-O-acetylglucose substituents (Barnes et al., 2003). There are many different genistein metabolites. The main hydroxylated genistein metabolite in humans is 3',4',5,7-tetrahydroxyisoflavone, and the main reduced form is dihydrogenistein (reviewed by Heinonen et al., 2003). Liver enzymes produce genistein 7-O-glucuronides (Zhang et al., 1999), and breast cancer cell lines, including MCF-7, ZR-75-1, BT-20, T47D, retain both genistein aglycone and genistein 7-sulphate (Peterson et al., 1998).

Cell research typically uses flavonoid aglycones. Because dietary flavonoids are typically glycosylated, it is important to consider the biological activity of flavonoid metabolites when comparing in vitro results to in vivo conditions. Studies comparing the potential health benefits of pure flavonoids to their metabolites have produced conflicting results. It appears that genistein metabolites retain antioxidant function (Widyarini et al., 2001). However, genistein 7-O-glucuronide, is weakly estrogenic compared to genistein aglycone, and may therefore have a proliferative effect on breast tumors. Furthermore, the same metabolite activates the immune system more effectively against tumor cells than genistein aglycone (Zhang et al., 1999). Some studies report that quercetin metabolites are more beneficial to one's health than quercetin aglycone (reviewed by

Murota, 2003), despite quercetin aglycone being a more potent antioxidant (Spencer et al., 2003).

1.7   Absorption and Cellular Action:

Apart from local gastrointestinal effects, flavonoids and/or their biologically active metabolites must be absorbed to produce a systemic effect. Some research shows that quercetin glucosides are more bioavailable than the free aglycone (Hollman et al. 1995 and 1999). A possible explanation for this finding is the involvement of the sodium-dependent glucose transporter (SGLT1). Located in the upper intestine, this cotransporter actively pumps sodium and glucose across the brush border membrane of enterocytes (Wright and Loo, 2000). Evidence indicates that SGLT1 also transports quercetin-3-glucoside across the intestinal brush border membrane (Wolffram et al., 2002). Once inside enterocytes, beta-glucosides hydrolyze quercetin glycosides forming free aglycone (reviewed by Wolffram, 2002). The free aglycone is then absorbed into the intestinal blood (reviewed by Murota, 2003). Alternatively, quercetin glucosides can be hydrolyzed on the mucosal surface of the intestine by the brush border membrane enzyme lactase phloridzin hydrolase (Day et al., 2000). However, SGLT1 transport and subsequent intracellular cleavage is thought to optimize absorption. This is because the aglycone is more lipophilic, and extracellular hydrolysis may allow quercetin to diffuse across the brush border membrane back into the mucosal solution (Wolffram et al., 2002).

Microflora in the small intestine hydrolyse genistein glycosides to free aglycones, which are reduced and demethylated (Day et al., 1998). Genistein aglycones and their metabolites are absorbed, and transported to the liver where microsomes catalyze the hydroxylation and conjugation of isoflavones to the more hydrophilic metabolites, such

as sulfonic or glucuronic acid conjugates. These are largely excreted in the urine, whereas a small portion of the absorbed gensitein enters the enterohepatic circulation (reviewed by Heinonen et al., 2003).

Both quercetin and genistein are hydrophobic molecules and are structurally similar to estradiol, and endogenous estrogen (Bhathena and Velasquez, 2002). Quercetin and genistein diffuse across the plasma membrane of target cells where they bind to two distinct estrogen receptors (ER): ER-alpha and ER-beta. Quercetin and genistein both bind with greater affinity to ER-beta, and are translocated inside the nucleus where they compete with 17beta-estradiol for binding to estrogen response elements. This modulates gene transcription (Bhathena and Velasquez, 2002). Estrogen is implicated in the development and progression of many breast cancers. Many treatments exploit the ER. Tamoxifen is an effective breast cancer treatment, which binds to the ER and their response elements, producing an antiestrogenic effect. Evidence shows that genistein and quercetin can have a similar effect. Genistein can block the ER (Kuiper et al., 1997) and quercetin binds with similar affinity as Tamoxifen to type II ER binding sites producing an antiproliferative effect in human breast (Markaverich et al., 1988), ovarian (Scambia et al., 1990), colorectal (Ranelletti et al., 1992), leukemic (Larocca et al., 1991), melanoma (Piantelli et al., 1995), and lung tumor cells (Caltagirone et al., 1997). Furthermore, quercetin can inhibit inducible nitric oxide synthase and cyclooxygenase-2 gene expression (Chen et al., 2001), opening the possibility that this flavonol may affect the production of other proteins.

1.8   <u>Genistein and Quercetin Cell Cycle Effects</u>:

Cell growth is carefully regulated by a series of checkpoints along the cell cycle, which help maintain a balance between cell proliferation and cell death or apoptosis. The 4 main stages of the cell cycle are: mitosis (M), DNA synthesis (S), and 2 gap phases (G1 and G2) interspersed between M and S. During G1, the cell commits to divide and grows in size, and G2 provides a safety gap, ensuring that DNA is replicated before mitosis. The p53 gene is often referred to as the guardian of the genome. It increases p21 expression in the face of DNA damage, halting the cell cycle between G2 and M, thereby preventing fixation of potentially carcinogenic mutations. Unfortunately, p53 is mutated in more than 50% of all cancers (reviewed by Fojo, 2002), contributing to abnormal replication. The tumor suppressive properties of genistein and quercetin have been extensively studied with respect to the cell cycle.

Genistein has been shown to halt the cell cycle between G2 and M and regulate cyclin B1 levels. Cyclin B1 accumulates in G2/M and assists in the formation of microtubule spindles and nuclear membrane breakdown, influencing cell division (Balabhadrapathruni et al., 2000). These effects have been observed in ER-negative breast cancer cells, an important finding given that ER-negative breast cancer patients respond poorly to established treatments such as chemotherapy and have a 5 year survival of 53% (International breast cancer study group, 1996). Genistein has also been shown to increase p53 protein levels in human breast cancer cells, contributing to apoptosis (Ying et al., 2002).

Quercetin has been shown to arrest cell division by elevating cyclin B1 levels and inhibiting cyclin-dependeint kinase 2 (cdk 2), an important protein permitting G1 and S phase progression (Choi et al., 2001). Quercetin can also increase p53 protein levels.

However, more intriguing is the ability of this flavonoid to directly upregulate p21 and halt the cell cycle regardless of any p53 mutation (Choi et al., 2001).

Quercetin and genistein have also been shown to downregulate *c-myc* and *ras* oncogenes, and have been reported to induce differentiation and apoptosis (Weber et al., 1997). Additionally, these two flavonoids affect important signaling cascades implicated in cancer. The activity of phosphatidylinositol (PI) and phosphatidylinositol 4-phosphate 5-kinase (PIP kinase) are elevated in many tumors, producing excess inositol-1,4,5,-triphosphate (IP$_3$), which has a proliferative effect on cells. Quercetin has been reported to inhibit PI and PIP kinases directly, and genistein has shown the same effect through tyrosine kinase inhibition (Weber et al., 1997). Genistein and quercetin have produced these effects at concentrations of 10-25 µM, which far exceed physiologically observed levels. The effects of genistein and quercetin on protein kinases are summarized on Table 1.3.

In summary, the antitumor mechanisms of genistein and quercetin are varied. Genistein and quercetin have antiestrogenic functions, and are growth inhibitory against ER-negative breast cancer. Furthermore, they have the ability to control the cell cycle at different stages suggesting that their effects may be synergistic, or that they may be used in conjunction with established cancer therapies to provide a more favorable outcome.

1.9   Gap Junctions, Connexins, and Cancer

Gap junction research arose when scientists began using electron microscopy to study the electrophysiology and cell ultrastructure of biological samples (Hertzberg and Bittar, 2000). A major controversy in the early neuroscience field was whether synaptic transmission was chemical or electrical. It was obvious that central nervous system

inhibition and neuromuscular transmission were chemically mediated. However, the same was not true for excitatory neuronal coupling.

Specialized channels, directly connecting the cytoplasm of neighboring cells, were first identified in crayfish (Wiersma, 1947). Furshpan and Potter (1959) found that the remarkably fast excitatory nerve transmission required for crayfish escape responses was electrically mediated. At approximately the same time, electrical coupling was verified in the cardiac ganglion (Watanabe, 1958). Within several years, the transmission of electrical signals through specialized channels was verified in many species (reviewed by Gilula, 1990 and Goodenough, 1990).

In 1964, Loewenstein and Kanno showed that adjacent salivary glands, incapable of transmitting or generating electrical impulses, exchanged a small fluorescent molecule (fluorescein) and an electrical current between their membranes. Within the same year, similar electrical properties were observed in non-excitable glial cells of the leach (Kuffer and Potter, 1964).

Freeze-fracture electron microscopy significantly advanced the observation of these cell-cell channels now referred to as gap junctions. With the advent of new microscopic techniques, it became obvious that the direct transmission of current, hydrophilic dyes, and low-molecular weight metabolites occurred through gap junction pores. Gap junctions have now been discovered in almost all contacting metazoan cells where they function in embryogenesis, cell differentiation and growth, and coordinate cellular functions in tissues (reviewed by Peracchia, 1980).

1.10   Gap Junction Structure:

Gap junctions are protein channels that directly link neighboring cells, allowing the intercellular passage of cytoplasmic contents (Loewestein, 1979). The most recognizable feature of these "gap" junctional structures is an approximately 2-3 nm gap between membranes of apposed cells at focal plaque-like regions (Revel and Karnovsky, 1967). Oligomeric gap junction channels are formed by the end-to-end docking of two hemichannels, termed connexons, each of which is formed by a hexameric cluster of protein subunits called connexins. Freeze-fracture imaging permits detailed observation of the hydrophilic internal gap junction pore. At its narrowest point, the internal pore is approximately 15 nm in diameter and permits passage of molecules less than 1200 Da (reviewed by Goodenough and Paul, 2003).

Multiple connexins are often coexpressed in a single cell type, and there are different connexon arrangements. There exists homomeric connexons, consisting of identical connexin proteins, and heteromeric connexons formed by a variety of connexin proteins. Similarly, there are homotypic and heterotypic gap junction channels formed by identical and different hemichannels, respectively (reviewed by Kumar and Gilula, 1996).

Investigators have mapped heterotypic interactions within the connexin family. Some connexins, such as Cx31 and Cx40, are highly selective in their communication patterns, whereas others, such as Cx46, are rather promiscuous in heterotypic coupling. Most connexins, however, show intermediate behaviour, typically interacting with more closely related connexins (Cx32, Cx37, Cx43) (reviewed by Yeager and Nicholson, 2000). Homomeric/homotypic formation is the most common gap junction structure, underscoring the tendency for identical connexins to interact (Kumar and Gilula, 1996).

1.11   <u>Connexin Proteins</u>:

Over 20 different connexin genes have been identified. Connexin proteins were previously described by their tissue of origin. However, it has since been discovered that connexin proteins are not tissue specific. Instead, connexins are distributed throughout the body (reviewed by Beyer and Willecke, 2000). As a result, the operational nomenclature uses the generic term connexin for the protein family and a numerical suffix designating the predicted molecular mass in kilodaltons (kD) (reviewed by Beyer and Willecke, 2000). For example, the 43-kD connexin protein found in myocardium is called Cx43, and the 32-kD connexin protein from liver is termed Cx32.

Connexins are integral membrane proteins. Both the amino and carboxyl termini face the cytoplasm. Different connexin proteins contain many identical amino acids and there are regions of sequence homology. The two extracellular connexin loops, termed E1 and E2, contain three invariant cysteine residues (reviewed by Beyer and Willecke, 2000). These two regions are highly conserved among connexins. Evidence suggests that the E2 region of the extracellular loop is important for connexon docking (reviewed by Beyer and Willecke, 2000). Furthermore, E2 similarity among different connexins is an important determinant permitting heterotypic formations (reviewed by Laird and Saez, 2000). Identical connexins have conserved E2 sequences, likely explaining the predominance of homotypic formations.

Connexin proteins consist of 4 transmembrane spanning domains. Connexin regions associated with the plasma membrane consist of hydrophobic amino acid sequences. Two of these hydrophobic regions are located within highly conserved regions (reviewed by Beyer and Willecke, 2000). One of these transmembrane regions has an amphipathic amino acid sequence. This region lines the inside of the gap junction pore

along with the corresponding segments of the other five connexin proteins. Together these sequences form the hydrophilic pore of the connexon channel (reviewed by Laird and Saez, 2000).

Each connexin intracellular loop has a distinct amino acid sequence (Kumar and Gilula, 1996). The C-terminal domain is the site of greatest variation. Unique amino acid sequences are likely responsible for connexin-specific functions, including sensitivity to various stimuli, such as second messenger molecules, as well as the recruitment of other associated proteins within the gap junction complex (Toyofuku et al., 1998; Giepmans and Moolenaar, 1998; Kojima et al., 2001).

Cx26, Cx32, and Cx43 are the most intensely investigated connexins. The gene structure of these connexins is very similar. Each has two exons. The first exon contains only 5'-untranslated sequence, and the second exon contains the entire coding sequence as well as the 3'-untranslated region (reviewed by Beyer and Willecke, 2000).

The promoter and enhancer regions of Cx26, Cx32, and Cx43 genes lack TATA boxes, the region where transcription factors bind. Instead consensus sequences for nuclear factor *k*B (NF*k*B) have been identified. Subsequently, NF*k*B was discovered to be the transcriptional activator for connexin genes (reviewed by Beyer and Willecke, 2000).

Interestingly, estrogens have been shown to activate a Cx43 promoter-reporter gene construct expressed in HeLa cells (reviewed by Beyer and Willecke, 2000). Additionally, estrogen may have indirect effects on Cx43 through activation of the intermediate early genes *Fos*, *Jun*, or both, which bind to AP-1 sites within the promoter (reviewed by Beyer and Willecke, 2000). Quercetin and genistein have a similar structure and function to estrogen (Figure 1.1), supporting the possibility that these two flavonoids may induce Cx43 transcription.

1.12   Connexin Function Independent of Gap Junctional Intercellular Communication:

The effects of connexin expression have traditionally been attributed to gap junction coupling and the subsequent exchange of small molecules between cells. However, connexin proteins have also been shown to have effects independent of GJIC. Increased connexin expression in poorly coupled cell lines, such as C6 glioma, HeLa, and U373 glioblastoma cells, can enhance ATP release and subsequent calcium wave propagation independent of GJIC (Cotrina et al., 1998). Connexin proteins may also confer significant resistance to cell injury by processes independent of GJIC (Lin et al., 2003). Connexins can also regulate cell growth. Transfection of human Cx43 into human glioblastoma cell lines U251 and T98G profoundly reduced cell proliferation in monolayer culture and in nude mice. These results were not associated with GJIC, suggesting that Cx43 independently functions as a tumor suppressor gene (Huang et al., 1998).

The mechanisms by which connexin proteins suppress growth have been explored. p27 normally halts the cell cycle prior to S phase. Human tumors often express low levels of p27 compared to nontumorigenic cells, contributing to high proliferation rates (Zhang et al., 2003). Furthermore, low p27 protein levels have been correlated with high tumor grade and decreased survival (reviewed by Zhang et al., 2003). The Cx43 C-terminus has been shown to suppress the expression of S phase kinase associated protein 2 (Skp2), which in turn, suppresses p27 degradation (Moorby and Patel., 2001; Zhang et al., 2003). Therefore, Cx43 may help control proliferation rate and improve prognosis by maintaining p27 levels.

Gap junctional independent functions are not solely reserved to connexin proteins. Hemichannels also have functions independent of intercellular communication. Cx43 hemichannels can mediate calcium-regulated transmembrane NAD+ fluxes in mammalian cells (Bruzzone et al., 2001). Cx43 hemichannels inhibit apoptosis (Plotkin et al., 2003) and increase sensitivity to chemotherapeutic agents such as Taxol (Huang et al., 2001). Horizontal and bipolar cells in the retina of the eye interact to sharpen resolution and contrast in response to light stimuli (reviewed by Goodenough and Paul, 2003). Cx26 hemichannels are found exclusively on the dendrites of horizontal cells and do not couple with bipolar cells. Despite lack of gap junctional coupling, the flow of current through these hemichannels depolarizes the bipolar cell, opens calcium channels and increases glutamate release, thus sharpening vision (Janssen-Bienhold et al., 2001).

1.13   Connexin Trafficking, Modification, and Removal:

Connexins are processed by various organelles and assemble into hemichannels as they transport to the plasma membrane. Connexin trafficking and modification processes are not completely understood. However, a basic understanding is beginning to unfold. Most connexins cotranslationally insert into the endoplasmic reticulum. However, Cx26 can posttranslationally insert into the endoplasmic reticulum in its native structure (reviewed by Laird and Saez, 2000). Ribosomal signal peptidases cleave the connexin amino terminal, activating Cx26 (reviewed by Laird and Saez, 2000).

Following synthesis, connexins transport from the endoplasmic reticulum to the Golgi apparatus. The Golgi apparatus is a common site of protein modification, often dictating protein localization. The Golgi apparatus phosphorylates proteins, adds sugars such as N-acetylglucosamine, galactose, and sialic acid to proteins, or truncates

oligosaccharides already attached to proteins (Alberts et al., 1994). Connexins, however, lack extracellular sequences required for Golgi-mediated glycosylation. Although connexins are not glycosylated (reviewed by Laird and Saez, 2000), they are phosphorylated.

Connexin phosphorylation may occur in the Golgi apparatus or endoplasmic reticulum (reviewed by Laird and Saez, 2000). Alternatively, connexins may be phosphorylated by the interaction of protein kinase C (PKC), *src*, and mitogen-activated protein kinase (MAPK) in vesicles as they transit to the plasma membrane along microtubules (Lauf et al., 2002). One function of connexin phosphorylation may be to direct plasma membrane insertion. However, Cx26 remains unphosphorylated yet locates to the plasma membrane. Interestingly, excessive phosphorylation closes gap junctions and may be implicated in tumor development and progression (Warn-Cramer et al., 1998).

The precise location where connexins assemble into hemichannels remains unclear and may differ between connexins and even cell types. Possible sites include the endoplasmic reticulum, Golgi apparatus, or plasma membrane. Musil and Goodenough (1993) observed that Cx43 assembles into hemichannels in the trans-Golgi network. By contrast, Cx26 and Cx32 oligomerisation is thought to occur earlier in the secretory pathway, possibly within the endoplasmic reticulum (reviewed by Martin and Evans, 2004).

Once connexins oligermerize, hemichannels are carried in vesicles along microtubules to the plasma membrane. The vesicles fuse at nonspecific regions of the plasma membrane, and the hemichannels then move laterally, contacting established gap junction plaques at the plaque periphery (Lauf et al., 2002).

Gap junctions are dynamic structures. Connexins are in constant turnover, having a half-life of less than 5 hours (Laird et al., 1991). After forming at the periphery of gap junction plaques, connexons migrate to the center of the gap junction cluster where they are removed by endocytosis (Gaietta et al., 2002). Connexins produced by one cell can be internalized by the neighboring cell (reviewed by Laird and Saez, 2000). Once internalized, lysosomes and proteosomes degrade gap junctions (reviewed by Laird, 1996).

1.14   Gap Junction Selectivity and Function:

Gap junction pores are selectively permeable. The C-terminal region of connexins are sensitive to phosphorylation, which can lead to gap junction pore closure (Hossain and Boynton, 2000; Warn-Cramer et al., 1998). Furthermore, vertebrate gap junctions are voltage-gated (reviewed by Verselis and Veenstra, 2000).

Gap junctions function as homeostatic regulators. It has been hypothesized that cells exchange factors, which regulate growth, differentiation, and apoptosis through gap junction pores (reviewed by Trosko and Chang, 2001). Cyclic adenosine monophosphate (cAMP), an intracellular mediator of extracellular signals (Alberts et al., 1994), is transported through gap junction pores, and may function to amplify intercellular functions (Gilula et al., 1972; Tsien and Weingart, 1976). Another second messenger, IP3, which releases intracellular calcium and mediates cell signaling, passes through gap junction pores (Saez et al., 1989). Gap junctions may also be important for cell maturation as connexin-null mice exhibit significantly delayed differentiation (Furlan et al., 2001).

1.15   Gap Junctions in Cancerous cells:

GJIC has been implicated in the regulation of cell proliferation and differentiation (Loewenstein, 1979). It is therefore not surprising that most tumors exhibit dysfunctional GJIC (reviewed by Trosko and Ruch, 2002; Laird et al., 1999; Hanna et al., 1999; Umhauer et al., 2000). GJIC malfunction is one of the earliest alterations associated with malignant transformation (reviewed by Laird et al., 1999), and may be disrupted due to a number of mechanisms. Cx43, the most common connexin in tissues, is downregulated in most cancers including neuroblastoma, epithelial, endothelial, mesothelial, ovarian, breast, bladder, prostate, liver, and lung carcinomas (reviewed by Carystinos et al., 2001 and Laird et al., 1999). Connexin internalization, impeded connexin plasma membrane transportion, or lysosomal and proteosomal degradation may also adversely affect GJIC (reviewed by Carystinos et al., 2001).

Alternatively, carcinogens have been shown to block gap junction pores. 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) activates PKC (Rivedal and Opsahl, 2001), which in turn activates *src*, *ras*, and *raf*, stimulating MAPK's to hyperphosphorylate the connexin proteins. TPA has also been shown to physically block the gap junction pores independent of connexin hyperphosphorylation (Rivedal et al., 1994). Dichlorodiphenyltrichloroethane (DDT), blocks both Cx32 and Cx43-mediated GJIC, presumably giving rise to lung, pancreas, and liver cancers (reviewed by Trosko and Ruch, 2002).

Furthermore, growth factors such as the epidermal growth factor (EGF), platelet derived growth factor (PDGF), basic fibroblastic growth factor (bFGF) and hepatic growth factor/scatter factor (HGF/SF) inhibit GJIC (Yamasaki and Naus, 1996; Rivedal and Opsahl, 2001). The mechanism is the following: EGF binds to its receptor,

stimulating MAPK and *src*. Subsequently, connexin proteins become phosphorylated leading to gap junction pore closure (Loo et al., 1995; Kanemitsu et al., 1997). *Src* also influences Cx43 localization. Cells which overexpress *src* retain Cx43 intracellularly (de Feijter et al., 1996). Interestingly, almost all breast tumors overexpress *src* and approximately 60% overexpress epidermal growth factor receptor (EGFR). This combination especially encourages connexin internalization and hyperphosphorylation. The result is impaired exchange of homeostatic signals.

1.16   Connexins and Cancer Metastasis:

Reduced connexin expression and GJIC is associated with tumor phenotypes (reviewed by Naus, 2002 and by Carystinos et al., 2001). However, the influence of GJIC on metastasis is less clear. Some studies show that loss of GJIC contributes greatly to metastasis (Navolotski et al., 1997; Saunders et al., 2001), while others support that gap junctional coupling encourages metastasis, especially during extravasion and adhesion of cancerous cells to secondary sites (reviewed by Naus, 2002; El-Sabban and Pauli, 1991; El-Sabban and Pauli, 1994).

Support for the former is based on the idea that reduced GJIC leads to cell heterogeneity, an early step in transformation. When cells are no longer coupled, important growth regulatory and differentiation signals cannot be exchanged, leading to cell autonomy and dissociation. Once cells have detached, connexin upregulation and intercellular coupling may positively correlate with invasion (reviewed by Carystinos et al., 2001). Furthermore, it is unclear whether a change in connexin expression or GJIC is a cause or consequence of metastasis. For example, when the antimetastatic genes BRMS1 and TIMP-1 are overexpressed in MDA-MB-435 breast tumor cells, connexin-

mediated intercellular communication is restored between neighboring cells (Saunders et al., 2001).

Additionally, connexin expression appears to support melanoma metastasis (Saunders et al., 2001). Specifically, Cx26 is elevated in melanoma cells invading the dermis compared to those residing in the basal layer (Ito et al., 2000). Melanocyte transformation is also associated with a directional change in GJIC. Melanocytes normally communicate with keratinocytes. However, in their transformed state, the melanoma cells are coupled to each other and to the dermal layer (Hsu et al., 2000).

Connexin expression level may also be indicative of skin cancer stage. Cx26 and Cx43 are reduced during the initial skin carcinogenesis. However, Cx26 expression is restored in lymph node metastasis (Kamibayashi et al, 1995). This latter result suggests that connexin levels may be an important skin tumor diagnostic marker. Connexin proteins may also be important breast cancer markers. Laird et al. (1999) suggested that connexin expression may be a more accurate marker for breast cancer than more traditional prognostic indicators, such as ER, PR, c-erbB-2, and p53 status. This conclusion was based on the following two findings: Cx43 is downregulated at early breast cancer stages, and Cx43 gap junctions are absent regardless of ER status.

1.17   Cx26 and Cx43 Levels in Breast Cancer Progression:

Cx43 is the most abundant connexin in breast tissue (Jamieson et al., 1998). Cx26 has also been identified in breast tissue, but at significantly lower levels compared to Cx43 (Wilgenbus et al., 1992; Hirschi et al., 1996). In most tumors, Cx43 levels and

GJIC is inversely associated with tumor grade (reviewed by Laird, 1999). However, Jamieson et al. (1998) observed that Cx43 is upregulated in breast cancer progression. Some Cx43 was intercellular, but the majority of Cx43 was stromal or cytoplasmic (Jamieson et al., 1998). The same investigators observed that Cx26 may also contribute to breast cancer progression. As breast tissue progresses to grade II and grade III carcinomas, Cx26 levels increase. Similar to Cx43 localization, the majority of Cx26 protein was intracellular and did not form at the cell surface. It was hypothesized that increased Cx26 protein may impair GJIC between precancerous and non-cancerous tissue, contributing to transformation (Jamieson et al., 1998).

Wilgenbus et al. (1992) also studied the presence of Cx43, Cx26, and Cx32 in nontumorigenic and cancerous breast tissues. Immunoblot and immunocytochemistry showed that Cx43 was reduced in human breast cancer tissue compared to nontumorigenic breast tissue (Jamieson et al., 1998).

Importantly, both investigators failed to perform functional GJIC tests. Therefore, it is not possible to determine if the tumor characteristics were due to GJIC or due to the independent effects of connexin proteins.

Connexin Diseases and Cancer Incidence:

Connexin mutations are associated with several human diseases. Charcot-Marie-Tooth disease is an X-linked demyelinating neuropathy. Normally, Cx32 is localized to the paranodes and incisures of myelinating Schwann cells where they form gap junctions allowing ions and small molecules, important for signal transduction, across the myelin sheath. However, patients with Charcot-Marie-Tooth disease have dysfunctional Cx32 proteins (Wang et al., 2004).

Heart tissue relies on GJIC for the transport of ions, which mediate coordinated contractions. Cx43 is the most abundant connexin in cardiac tissue. However, Cx43 levels are commonly reduced in the left ventricle of patients with ischemic heart disease (Dupont et al, 2001). Enhanced GJIC can also contribute to cardiac dysfunction. Extra gap junction channels between the atrium and ventricle permit passage of contractile pulses without normal atrioventricular delay, contributing to re-entry arrythmias (Peters et al., 1994).

Diabetic cataractogenesis is characterized by dysfunctional gap junction channels. Furthermore, Cx26 mutations impair cochlear gap junctions, disrupting ion circulation and contributing to deafness (reviewed by Forge et al., 1999).

Interestingly, people with connexin diseases do not have higher incidences of cancer (Bone et al., 1997; Kelsell et al., 1997). One explanation may be that tissues commonly express more than one connexin. The existing normal connexin may therefore compensate for the dysfunctional connexin protein. Another possible explanation is that dysfunctional GJIC is not the sole cause of cancer development. Cancer is a multi-factorial disease. In addition to impaired GJIC, a cancerous cell exhibits the following characteristics: self-sufficient growth, evasion of apoptosis, insensitivity to anti-growth signals, sustained angiogenesis, limitless replication potential, and tissue invasion and metastasis (reviewed by Hanahan and Weinberg, 2000).

1.18   Molecular Markers for Breast Cancer:

Tumor size, histological grade, and axillary nodal status are currently used to stage breast cancer. Axillary nodal status is the most important prognostic indicator currently available, and decisions regarding adjuvant therapy are largely based on nodal

status. Tumors are graded by assessing the tubular arrangement of the carcinoma cells, the atypical nuclear appearance, and frequency of mitotic or hyperchromatic nuclear figures according to the method of Bloom and Richardson (Bloom and Richardson, 1957) and modified by Elston and Ellis (Elston and Ellis, 1991). Although tumor grading provides important prognostic information, it is not sufficiently accurate for predicting early relapse. Several other molecular markers, such as PR and ER status as well as the proliferative marker Ki67, and c-erbB-2 and p53, have therefore been investigated for prognostic value.

The PR is an ER response protein (Elledge et al., 2000). For 25 years, PR and ER values have been used to select patients who are likely to respond to tamoxifen treatment, one of the most effective breast cancer treatments (Ciacco and Elledge, 2000). However, their value as markers for breast cancer prognosis has only recently been studied. Two studies (Lamy et al., 2000; Chebil et al., 2003) showed that ER-positive postmenopausal women with high PR levels, who were treated with tamoxifen, have better survival rates compared to women with low PR levels. ER and PR-positive status is also inversely associated with nodal status, proliferative potential, and tumor grade (reviewed by Rogers et al., 2002), and positively associated with 5 year disease-free survival in breast tissue samples (Andronas et al., 2003). Furthermore, ER and PR-negative status has been associated with high risk of clinical breast cancer recurrence (Provenzano et al., 2003).

Ki67 is a non-histone nuclear protein that is expressed throughout the S and G2/M phases of the cell cycle. It is not expressed during the G_0 resting phase. Ki67 has become the best-studied marker of tumor cell proliferation, and Ki67 is a reliable indicator of mitotic count, a simple method of determining proliferative rate, which correlates strongly with prognosis (Rogers et al., 2002). Ki67 also predicts histological grade,

axillary node status, and tumor recurrence. Five year and overall breast cancer survival is also associated with Ki67 (Rogers et al., 2002).

c-erbB-2 (also referred to as HER2) is a proto-oncogene, which produces a cytoplasmic membrane-associated tyrosine kinase. c-erbB-2 is overexpressed in approximately 30% of invasive breast cancers (Slamon et al., 1989; Press et al., 2002), where it activates the PI3K/Akt survival pathway, resulting in increased cell number through inhibition of apoptosis (Zhou and Hung, 2002). Elevated levels of c-erbB-2 also enhance MAPK activity (Waterman et al., 1999) and promote secretion of basement membrane degradative enzymes, such as the matrix metalloproteases, encouraging proliferation, invasiveness and a more metastatic phenotype (Tan et al., 1999). c-erbB-2 overexpression is also associated with poor breast cancer prognosis, especially for patients with node-positive tumors (Press et al., 1997; Cooke et al., 2001). Furthermore, c-erbB-2-positive breast cancer patients are more likely to experience relapse following surgery, possibly due to growth factors preferentially stimulating c-erbB-2-mediated proliferation (Tagliabue et al., 2002; Sieweke and Bissell, 1994). Lastly, c-erbB-2 overexpression is an independent predictor of disease-free survival (Dittadi et al., 1997; Riou et al., 2001; Ristimaki et al., 2002), and is positively associated with tumor grade (Gullick et al., 1991).

p53 halts the cell cycle in the presence of DNA damage (Lane, 2002). p53 mutations are found in approximately 20-40% of all breast carcinomas (Berard and Soussi, 1998; Soussi et al., 2000; Olivier et al., 2002; Beroud and Soussi, 2003), where they contribute to dysregulated cell cycle function and the acquisition of more mutations (Bray et al., 1998). p53 gene mutations appear to be an early event in breast carcinogenesis. Studies on microdissected breast tumors have shown that p53 mutations

can occur in ductal carcinoma in situ (DCIS) before the development of invasive breast cancer, and that the frequency of p53 mutation increases from near zero in low-grade DCIS to 30-40% in high-grade DCIS (Ho et al., 2000; Done et al., 2001). Furthermore, p53 mutations are more common among node-positive than node-negative breast cancer patients (Pharoah et al., 1999), and are associated with large, clinically advanced tumors (Pharoah et al., 1999). Lastly, breast cancer recurrence is more common among patients with p53 mutations compared to patients without p53 mutations (Norberg et al., 2001).

1.19   Connexins and Cancer Therapy:

Understanding that connexins may protect against cancer progression, investigators have introduced connexins into tumors to evaluate their therapeutic role. Retroviral vectors of Cx26 and Cx43 were successfully introduced into the human breast cancer cells MDA-MB-231, Hs578T, and HBL100. The connexins suppressed growth independent of GJIC by reducing fibroblastic growth factor receptor (FGFR) expression and possibly affecting the expression of other proteins involved in tumor progression (Qin et al., 2002). Cx26 and Cx43 were also transfected into MDA-MB-435 cells. Differentiation capacity and growth regulation increased, normalizing tumor cell behaviour (Hirschi et al., 1996). Additionally, Cx43 transfected into osteosarcoma cells inhibited cell cycle transition from G1 to S phase, suppressing cell proliferation (Zhang et al., 2001). Fernstrom et al. (2002) transfected human ovarian carcinoma cells with Cx43. The cells exhibited reduced growth rate and decreased expression of P-glycoprotein, a protein that pumps chemotherapy drugs out of the cell and sequesters it from the nuclear DNA. Furthermore, the growth of C6 glioma cells was suppressed following Cx43 cDNA

transfection (Zhu et al., 1991 and 1992). Therefore, introducing connexins into tumors represents a potential means of controlling growth and enhancing drug therapy.

1.20 Thesis Outline:

This thesis is divided into several sections. Chapter 2 tests the hypothesis that genistein and quercetin suppress growth of metastatic human breast cancer cells (MDA-MB-231) by regulating Cx43 expression and GJIC activity. This chapter is supplemented by 2 appendixes. Appendix 1 demonstrates E-cadherin expression in MDA-MB-231 cells. Appendix 2 explores the possibility that filamentous actin (F-actin) may be involved in the genistein and quercetin-mediated morphological change of MDA-MB-231 cells. Chapter 3 of this thesis tests the hypothesis that Cx26, Cx32, and Cx43 are reliable breast cancer prognostic indicators. Together these results may provide new breast cancer therapies and present more evidence regarding the relationship between connexin proteins and GJIC in tumors.

Figure 1.1. Basic flavonoid structure. This schematic diagram of the basic
flavonoid structure was adapted from Ross and Kasum (2002). Flavonoids are
ideal reducing agents. Their conjugated structure allows them to readily donate
hydrogen atoms and delocalize unpaired electrons making them potent
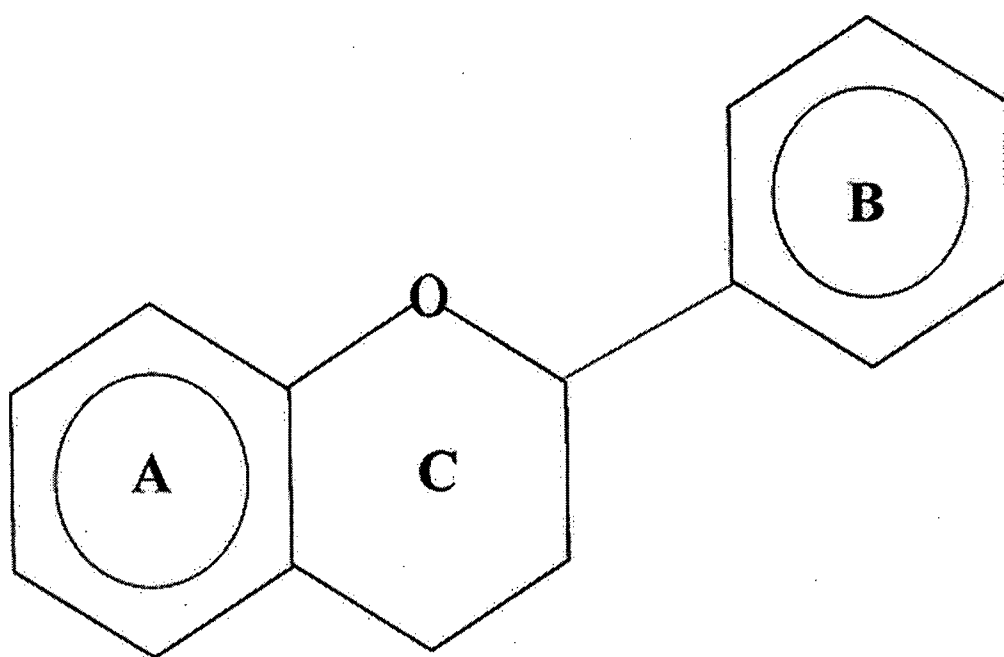antioxidants.

Table 1.1. Biological effects genistein and quercetin and other flavonoids

Flavonoids in general:

Biological effects	Reference
Antibacterial, antiviral, antioxidant, antitumor, antiallergenic, , antidiarrheal, antiulcer functions	Bravo (1998)
Enhance antioxidant effect of vitamin C and E	Ross and Kasum (2002)
Increase activity of glutathione-S-transferase (myricetin, fisetin)	Fiander and Schneider (2000)
Inhibit growth of Hs578T cells (epigallocatechin gallate)	Chen et al. (1998)
Inhibit VEGF in MDA-MB-231 cells (angiogenesis factor) (green tea extract and epigallocatechin-3-gallate)	Sartippour et al. (2002)
Reduce carcinogen-indiced breast tumor burden in rats (green tea extract: epigallocatechin-3-gallate, epigallocatechin, epicatechin gallate, epicatechin, gallocatechin, catechin, gallocatechin gallate)	Kavanagh et al. (2001)

Genistein:

Biological effects	Reference
Antiproliferative, apoptotic functions in HER-2/neu oncogene expressing human breast epithelial cells	Katare et al. (2002)
Relieves postmenopausal symptoms	Greendale et al. (2002)
Reduce incidence of breast cancer and cardiovascular disease	Glazier and Bowman (2001)
Regulates cyclin B1, and halts the cell cycle between G2 and M	Balabhadrapathruni et al. (2000)
Increase p53 levels in human breast cancer cells in T47D cells	Ying et al. (2002)
Inhibits PI and PIP kinases indirectly through tyrosine kinase inhibition in OVCAR-5 and MDA-MB-435 cells	Weber et al. (1997)
Blocks the ER	Kuiper et al. (1997)

Quercetin:

Biological effects	Reference
Antiproliferative, apoptotic in MCF-7 cells	Choi et al. (2001)
Increase activity of glutathione-S-transferase	Fiander and Schneider (2000)
Inhibit growth of human stomach, colon, prostate, breast cancer cells	Soleas et al. (2002)
Inhibit growth of uterine, cervical, intestinal tumors and melanomas in mice	Soleas et al. (2002)
Consumption inversely associated with myocardial infarction	Gelejinse et al. (2002); Knekt et al. (2002)
Relieve diabetic symptoms such as eye, kidney, and nerve damage	Wang et al. (2000)
Elevates cyclin B1, inhibits cdk 2, arrests cell cycle between G1 and S in MCF-7	Choi et al. (2001)
Increase p53 and p21 levels in MCF-7 cells	Choi et al. (2001)
Inhibits PI and PIP kinases directly in OVCAR-5 and MDA-MB-435 cells	Weber et al. (1997)
Binds to type II ER sites with similar affinity as Tamoxifen in human breast cells	Markaverich et al. (1988)

Table 1.2. Plasma concentrations of genistein in Japanese and Chinese people consuming a regular diet. This table was adapted from Hedlund et al. (2003).

	n	Plasma conc. (μM)	
		Mean	High
Japanese			
Genistein	14	0.28	2.40
Equol	14	0.006	0.022[a]
Daidzein	14	0.11	0.90
Chinese			
Genistein	12	0.34	1.08
Equol	53	0.016	0.50
Daidzein	53	0.12	1.70

Table 1.3. Genistein and quercetin-induced protein kinase inhibition
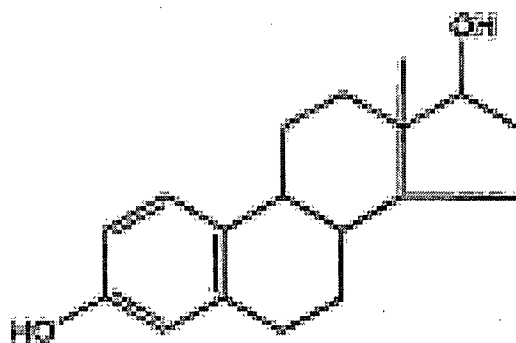
Genistein:

Protein Kinase Effect	Reference
Inhibits PDGF kinase activity in rat hepatic stellate cells	Liu et al. (2002)
Inhibits cdk 2 kinase activity (cyclin-dependent kinase subfamily – cell cycle control) in rat hepatic stellate cells	Liu et al. (2002)
Inhibits ERK kinase activity (MAP kinase subfamily) in rat hepatic stellate cells	Liu et al. (2002)
Inhibits EGF kinase activity (tyrosine kinase) in B-cell precursor leukemia cells	Uckun et al. (1995)
Inhibits protein histidine kinase activity	Dixon and Ferreira (2002)
Inhibits CD-19-associated tyrosine kinase activity (results in death of human B-cell precursor leukemia cells) in B-cell precursor leukemia cells	Uckun et al. (1995)
Inhibits TGF-beta1 serine and threonine kinase activity	Kim et al. (1998)

Quercetin:

Protein Kinase Effect	Reference
Inhibits tyrosine kinase activity in rat mammary tumors	Ferry et al. (1996); Levy et al. (1984)
Reduces protein kinase C activity in murine melanoma B16-BL6 cells	Zhang et al. (2004)
Inhibition of *Raf*, MAPK, Elk-1 and Akt-1 phosphorylation in human PC-3 prostate cancer cells	Huynh et al. (2003)
Inhibits Akt/protein kinase B, and ERK in primary cortical neurons	Spencer et al. (2003)
Blocks EGFR-signaling pathway via tyrosine kinase inhibition in pancreatic tumor cells	Lee et al. (2002)

Figure 1.2. Structure of 17beta-estradiol, and quercetin and genistein aglycones. These diagrams were adapted from Bhathena and Velasquez (2002), and Pan et al. (2002). Quercetin and genistein are structurally similar to 17beta-estradiol, and are able to bind to estrogen response elements, affecting gene transcription. Quercetin and genistein are also hydophobic and are thus able to enter cells by diffusing across the plasma membrane.

17beta-estradiol
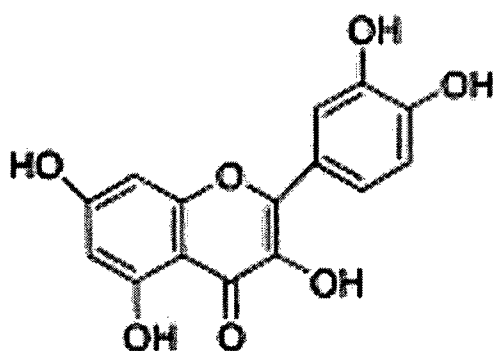
Quercetin
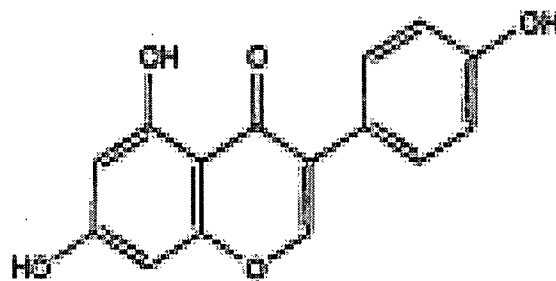
Genistein

Figure 1.3. Relationship between connexin proteins, connexons, and gap junctional intercellular channels. This diagram was adapted from Goodenough and Paul (2003). There is a small extracellular "gap" which separates opposed plasma membranes. Each gap junction forms a hydrophilic axial channel, which directly interconnects the cytoplasm of opposed cells.

CHAPTER 2

Genistein and Quercetin Increase Connexin43 Protein and Suppress Growth of MDA-MB-231 Cells

2.1 Introduction:

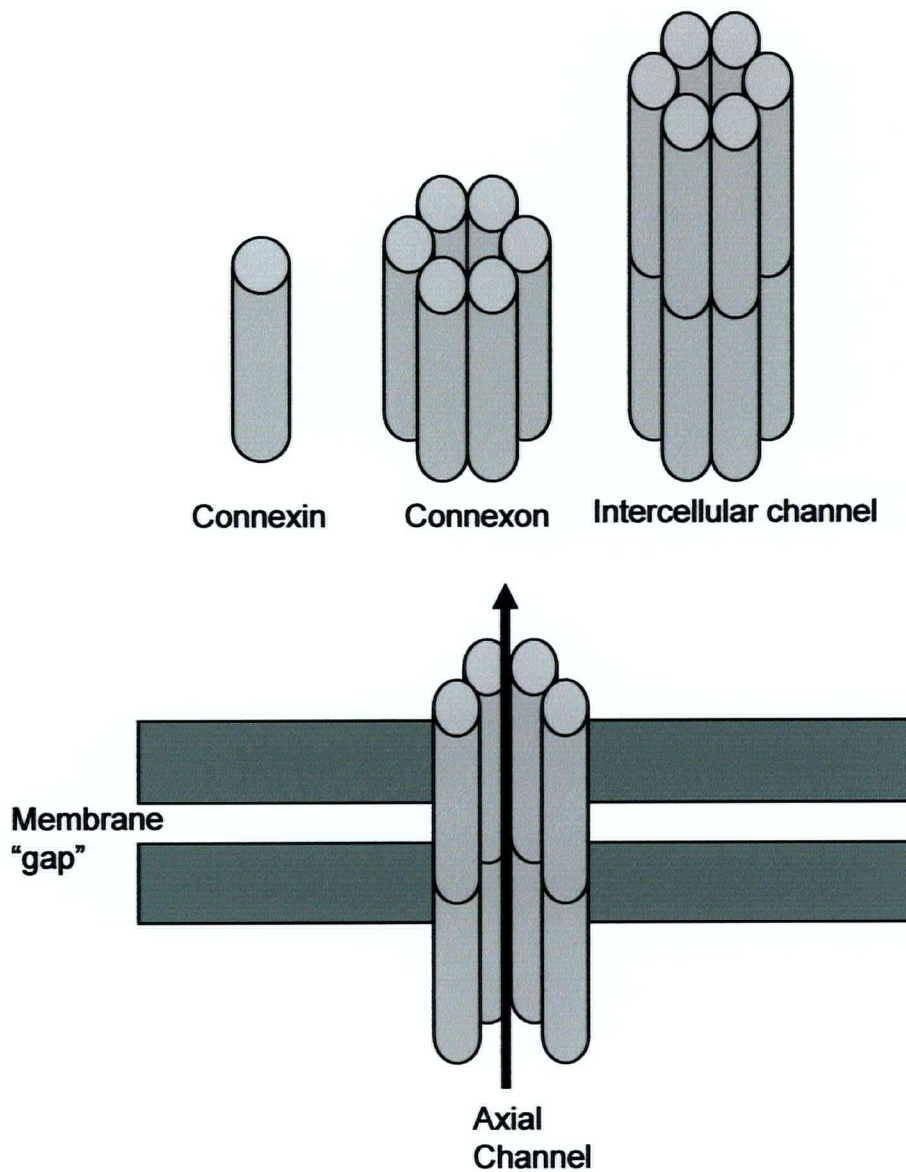MDA-MB-231 cells are a metastatic human breast cancer cell line. These cells typically do not communicate through gap junction pores and express very low levels of Cx43 (Qin et al., 2002). Furthermore, these cells rapidly divide and are ER-negative. Connexin upregulation and improved GJIC are potential routes for cancer therapy. Improved GJIC may improve delivery of chemotherapeutic agents (reviewed by Chipman et al., 2003), and elevated connexin proteins may regulate growth independent of GJIC (Qin etal., 2002). The flavonoids, epicatechin gallate, apigenin, and tangeretin have been shown to maintain GJIC in the presence of tumor promoters (Ale-Agha et al., 2002, Chaumontet et al., 1997). There is also evidence that genistein and quercetin suppress tumor growth (Weber et al., 1997, Ying et al., 2002, Brownson et al., 2002). This study employed immunocytochemistry, Western blot analysis, and proliferative analyses to determine if genistein and quercetin regulated Cx43 levels, GJIC, and suppressed the growth of metastatic human breast cancer cells.

Discovering a definitive mechanism by which flavonoids prevent or treat breast cancer could have significant implications for breast cancer patients and for people genetically at risk for the disease. If genistein and quercetin suppress tumor growth, increase Cx43 expression and improve GJIC, a potent synthetic flavonoid could be designed, which stimulates gap junction genes. The beneficial results may include a synergistic action with established chemotherapeutic agents and an effective treatment for

breast cancer patients without the debilitating side effects of surgical and radiation

treatments.

2.2   Materials and Methods:

2.2.1   Treatment Chemicals:

The flavonoids, genistein (98% HPLC from soybean) and quercetin (98% HPLC) were purchased from Sigma-Aldrich (St. Louis, MO). The flavonoids were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to 15 mg/ml, shielded from light, and stored at -20°C until used to treat cells. In all experiments, cells were treated with genistein (0.5 μg/ml, 2.5 μg/ml, 15 μg/ml), quercetin (0.5 μg/ml, 2.5 μg/ml, 5 μg/ml, 15 μg/ml) in complete medium for 72 hours. The medium was replaced every 24 hours during this treatment period. Treatment controls consisted of complete medium only and vehicle control [complete medium supplemented with 0.1% (v/v) DMSO].

2.2.2   Cell Culture:

MSTV1-7 [gift from Dr. Cal Roskelley, UBC, Vancouver, BC, Canada; originally obtained from American Type Culture Collection (ATCC)], a nontumorogenic human breast cell line was used as a positive control for Cx43 expression and GJIC. MSTV1-7 cells were also used to determine if genistein and/or quercetin treatments were toxic to nontumorigenic breast cells. Hs578T cells (gift from Dr. Dale Laird, UWO, London, ON, Canada; ATCC) are a human breast tumor cell line, which express abundant Cx43 and readily communicate through gap junctions. Hs578T cells were used as a positive control for Cx43 immunoblotting and GJIC. Experiments were performed on a metastatic human breast tumor cell line, MDA-MB-231 (gift from Dr. Dale Laird; ATCC). This cell line typically expresses very low levels of Cx43 and a low level of GJIC.

MSTV1-7 cells were grown in Dulbecco's modified Eagle's Nutrient Mixture F-12 Ham medium (Sigma-Aldrich) supplemented with 5% (v/v) fetal bovine serum

(Invitrogen, Carlsbad, CA), 1% (v/v) L-glutamine (Invitrogen), 0.1% (v/v) penicillin-streptomycin (Invitrogen), and 0.1% (v/v) insulin (Invitrogen). MDA-MB-231 cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, and 0.1% (v/v) penicillin-streptomycin. Hs578T cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum, 2% (v/v) L-glutamine, and 0.1% (v/v) penicillin-streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% carbon dioxide.

2.2.3   Immunocytochemistry:

Cells were grown to confluence on 12 mm coverslips (Fischer Scientific; Hampton, NH) in 35 mm plates. Following treatment, the cells were then fixed with 80% methanol at -20°C for 20 minutes. The fixative was aspirated and the cells were stored at 4°C overnight. Following rehydration in phosphate buffered saline (PBS; pH 7.4) for 15 minutes, the cells were blocked for non-specific antibody binding with 10% (v/v) normal goat serum and 1% (w/v) bovine serum albumin (BSA) in PBS for 30 minutes. The cells were incubated with goat anti-rabbit Cx43 antibody (1:400 dilution; Sigma-Aldrich) in PBS with 1% (w/v) BSA for 1 hour at room temperature. Cells were then washed 3 times 10 minutes with PBS and incubated with Alexa-Fluor 568 goat anti-rabbit IgG secondary antibody (1:500 dilution; Molecular Probes, Eugene, OR) in PBS with 1% (w/v) BSA for 1 hour at room temperature. Cells were then washed 3 times 10 minutes with PBS, quickly rinsed with distilled, deionized water (ddH$_2$0) to remove PBS crystals and then mounted with Vectashield medium (Vector Laboratories Inc., Burlingame, CA) to preserve the fluorescent signal.

Controls for immunocytochemistry consisted of PBS incubation in place of Cx43 primary and secondary antibody (autofluorescent control), affinity purified rabbit IgG in place of the Cx43 primary antibody (Cx43 primary antibody control), and PBS in place of secondary antibody (secondary antibody control). After sealing the coverslips with nail polish, the fluorescent immunoreaction was visualized with a Zeiss Axiophot photomicroscope (Carl Zeiss, Thornwood, NY) equipped with filters for FITC (excitation 470 nm/ emission 525 nm) and Alexa 568 (excitation 546 nm/ emission 590 nm). Differential interference contrast (DIC) and fluorescent images were viewed using the 40x objective (oil). Fluorescent images were captured at an exposure of 490ms using Zeiss Axioplan 4 software (Carl Zeiss). The percentage of cells expressing Cx43 following treatment with 15 µg/ml genistein and 5 µg/ml quercetin was determined (Table 2.1). For this purpose, 214 genistein treated cells, and 156 quercetin treated cells were counted from random fields.

2.2.4   Immunocytochemistry combining Cx43, Ki67, DAPI:

Ki67, Cx43, and 4 ,6-diamidino-2-phenylindole (DAPI) were combined in immunocytochemistry to test for the growth suppressive effects of genistein and quercetin and to quantify the proliferative activity in Cx43 expressing cells. The same procedure as mentioned above for Cx43 immunocytochemistry was performed with the following alterations. Primary antibody incubation consisted of mouse anti-human Ki67 antibody (1:500 dilution; Sigma-Aldrich) combined with goat anti-rabbit Cx43 antibody (1:400 dilution; Sigma-Aldrich). Secondary antibody incubation consisted of Alexa-Fluor 568 goat anti-rabbit IgG (1:500; Molecular Probes) for Cx43 and Alexa-Fluor 488 goat anti-mouse IgG (1:500; Molecular Probes) for Ki67. Cells were incubated for 5 minutes

with DAPI (1:34 000; Molecular Probes) before mounting the coverslips. The same immunocytochemistry controls were used as for Cx43 immunocytochemistry except that mouse IgG was used to control for the primary Ki67 antibody.

The degree of Ki67 expression, and hence proliferative activity, was scored in the following manner according to fluorescent intensity: 0, no Ki67 immunoreactivity/no proliferation; 1, weak Ki67 immunoreactivity/low level of proliferation; 2, intermediate Ki67 immunoreactivity/intermediate level of proliferation; 3, high Ki67 immunoreactivity/high level of proliferation. All samples were scored by randomly selecting 20 fields of view and without knowledge of the treatment conditions. In total, 206 genistein treated cells, 180 quercetin treated cells, and 388 untreated cells were counted. Chi-squared analysis was performed on the results to determine if there was a significant difference in proliferative activity between treatment conditions. Immunocytochemistry was repeated on 7 independent MDA-MB-231 cultures.

2.2.5   Protein Isolation and Western blot analysis of Cx43:

a. *Protein isolation*

Following one rinse with PBS, cells were scraped off tissue culture plates in lysis buffer [10% (v/v) glycerol (Fischer Scientific), 1% (v/v) Nonidet P-40 (Sigma-Aldrich), 0.1% (v/v) sodium dodecyl sulfate (SDS) (Fischer Scientific; Hampton, NH), 0.5 M NaCl, 5 mM Tris (pH 8.0), and protease inhibitor cocktail tablets (Complete, Mini; Roche, Indianapolis, IN)] on ice. DNA in the lysate was sheared using 22 and 27-gauge needles (Beckton Dickinson; Franklin Lakes, NJ). Total protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce-BioLynx, Rockford, IL).

b. *Western Blot analysis of Cx43*:

Each protein sample was diluted in 10 times sample buffer [50% (v/v) 2-mercaptoethanol (Fischer Scientific), 20% (v/v) glycerol, 5% (v/v) bromophenol blue (Fisher Scientific), 5% (v/v) SDS] to a final concentration of 200μg for MDA-MB-231 cells, 100 μg for Hs578T cells, and 200 μg for MSTV1-7 cells. The samples were heated at 95°C for 90 seconds. 40 μl of each sample was loaded in parallel with 10 μl of molecular weight markers (Bio-Rad Lab., Hercules, CA) on a 10% SDS-polyacrylamide gel electrophoresis [10% (w/v) acrylamide (acrylamide:bis-acrylamide, 30:1; Fischer Scientific), 1% (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.001% (v/v) N,N,N',N'-tetramethylethylenediamine electrophoresis grade (Fischer Scientific), 0.4 M Tris-HCl (pH 8.8)]. The samples were run on the gel using electrophoresis buffer [1% (w/v) SDS, 25 mM Tris-HCl (pH 8.3), 200 mM glycine] at 100 volts until bromophenol blue reached the bottom of the gel. The gel was then placed in transfer buffer [25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% (v/v) methanol] for 10 minutes. Protein was transferred to a nitrocellulose membrane (Bio-Rad Lab.) at 100 volts for 1 hour.

The nitrocellulose membrane was washed once with PBS and blocked in 0.02% (v/v) polyoxyethylene 20-sorbital monolaurate (Tween-20; Fischer Scientific), 5% (w/v) non-fat dry milk (NFDM) in PBS for 1 hour at room temperature. The blocking solution was discarded and the membrane was incubated overnight at 4°C in goat anti-rabbit Cx43 antibody [1:1 000 dilution in PBS with 1% (w/v) NFDM; Sigma-Aldrich]. The membrane was rinsed 3 times 10 minutes each in PBS and then bathed in secondary goat anti-rabbit antibody tagged with horseradish peroxidase [1:5 000 dilution in PBS with 1% (w/v) NFDM; Cedarlane, Hornby, ON, Canada] for 1 hour at room temperature. Following 3

times 10 minute rinses in PBS, the membrane was incubated in Supersignal West Pico

Luminol Enhancer solution (Pierce-BioLynx; Brockville, ON), wrapped in a single layer

of saran and exposed to X-ray film (Kodak Scientific Imaging; Rochester, NY). To

normalize protein loading, the membrane was reprobed with mouse anti-rabbit

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:20 000 dilution in

PBS with 1% (w/v) NFDM; Research Diagnostics Inc., Minneapolis, MN) overnight at

4°C and then incubated in secondary goat anti-mouse antibody tagged to horseradish

peroxidase [1:10 000 dilution in PBS with 1% (w/v) NFDM; Cedarlane].

Cx43 was identified by comparing protein bands to the molecular weight marker.

Nonphosphorylated Cx43 is identified as one band at the molecular weight of 43 kDa.

Phosphorylated Cx43 isoforms are identified by 1 to 4 bands at higher apparent molecular

weight compared to nonphosphorylated Cx43 (Laird et al., 1991).

Western blot analysis was performed on 3 independent MDA-MB-231 cultures.

2.2.6   Measurement of Gap Junctional Coupling by Preloading and Scrape Loading:

a. *Preloading:*

Cells were grown to confluence on 60 mm plates (two plates for each treatment

condition; one donor plate and one recipient plate). Following aspiration of treatment

medium, donor cells were preloaded with dye solution [5 uM calcein-AM (Molecular

Probes) and 10 μM DiI (Sigma-Aldrich) in an isotonic (0.3 M) glucose solution] for 20

minutes in a humidified incubator (37°C, 5% carbon dioxide/95% air). Subsequently, the

dye solution was aspirated from the donor plate, and the cells rinsed twice with glucose

solution, trypsinized [0.25% trypsin (Invitrogen)], and suspended in 5 ml complete

medium. The donor cells were then seeded onto the recipient (unlabeled) cells at a 1:500

ratio. Cells were maintained in the humidified incubator (6 hours for MDA-MB-231 and MSTV1-7 cells, and 1.5 hours for Hs578T cells) and then examined using the aforementioned photomicroscope, capture software, and FITC and rhodamine filters (excitation 570 nm/ emission 590 nm).

The theory behind this preloading technique is the following: DiI is a lipophilic dye that labels the plasma membrane of the cell, thus marking the donor cells. In contrast, calcein has a molecular weight of 622 Daltons and is thus able to pass through gap junctions. GJIC was measured by the passage of calcein from donor cells to the underlying recipient cells. Only attached donor cells, which were coupled to at least 1 recipient cell, were examined.

b. Scrape Loading:

Scrape loading is a qualitative measure of GJIC. It is not as specific as the preloading method because it does not attribute dye transfer to one cell. This technique was used to verify preloading results. Confluent cells on a 60mm plate were scraped with a razor blade in the presence of dye solution {[5 uM 5-(and -6) – carboxyfluorescein diacetate (5(6)-CFDA; Molecular Probes)]} and 10 µM dextran-tagged rhodamine (Sigma-Aldrich) in complete medium]} for 10 minutes at room temperature. Cut cells take up the dye solution and quickly heal. Dextran-tagged rhodamine (10 000 Daltons) is retained within the recipient cells. 5(6)-CFDA is small enough to pass through functional gap junctions. The extent of gap junctional coupling is determined by measuring the spread of 5(6)-CFDA from the cut line. Images were captured using the aforementioned photomicroscope, capture system, and FITC and rhodamine filters.

GJIC was evaluated by preloading and scrape loading techniques on 3 independent MDA-MB-231 cell cultures.

2.2.7 Cell Count:

Approximately 275 000 MDA-MB-231 cells were seeded onto 3 plates (10% confluence) for each treatment condition (genistein or quercetin, complete medium only, or 0.1% (v/v) DMSO vehicle control). The next day (day 1), an extra set of cells in complete medium were trysinized [trypsin-EDTA, Invitrogen], and suspended in 5ml medium. 500µl of this cell suspension was added to 20ml of isotonic solution (Beckman Coulter Inc., Fullerton, CA). After mixing thoroughly, cells were counted using the Beckman Coulter Counter (Beckman Coulter Inc.) 3 times for each plate. After correcting for the dilution factor, the total cell number was determined. The day 1 count served as the number of cells prior to genistein and quercetin treatment. On days 1 – 3, the remaining plates were treated with genistein and quercetin, complete medium only, or 0.1% (v/v) DMSO vehicle every 24 hours for 72 hours. After the treatment period, the cells were counted in the aforementioned manner. Cell count data was repeated on 3 independent MDA-MB-231 cultures. Each time, 3 plates were used for each treatment condition.

Cell confluence is a confounding factor affecting cell proliferation. Because it is not possible to seed the exact number of cells onto each plate across trials, separate trials were not combined. Instead, results of each trial were observed for the same trend, and 1 trial was chosen as a representative. The statistical significance of this trial was compared

to statistical significance of the combined trials with cell counts normalized to the 0.1% (v/v) DMSO vehicle. The representative trial and normalized combined trials were statistically the same.

Data was analyzed using one-way analysis of variance (ANOVA). The treatment conditions were compared against the 0.1% (v/v) DMSO vehicle control using the Dunnett multiple comparisons test.

2.2.8 <u>Cytotoxicity test</u>:

Lactate dehydrogenase (LDH), a stable cytosolic enzyme, is released upon cell lysis, and is used as a measure of cytotoxicity. Released LDH in culture supernatants was measured with a 30 minute coupled enzymatic assay using the CytoTox-96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). The enzymatic reaction converts tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells.

Treatment medium was extracted from cells after each day of the 3 day treatment period. This extract contained the treatment-induced LDH. 10X lysis buffer (Promega) was added to an equal volume of treatment medium remaining on the cells (15 µl lysis solution per 100 µl medium) and incubated at 37˚C for 45 minutes. This extract represented the total LDH (LDH released following genistein and quercetin treatment plus cellular LDH).

For each treatment condition and each treatment day, 50 µl of treatment-induced LDH and 50 µl of total LDH was added in triplicate to wells of a 96-well plate. 50 µl of the "reconstituted substrate mix" was added to each well of the enzymatic assay plate. The plate was covered, protected from light, and incubated at room temperature for 30

minutes. 50 µl of the "stop solution" was added to each well, and the absorbance was recorded at 490 nm. Flavonoid-induced cytotoxicity was determined by dividing the treatment-induced LDH reading by the total LDH reading. These results were then normalized to the 0.1% (v/v) DMSO vehicle condition.

Data was analyzed using one-way ANOVA. The treatment conditions were compared against the 0.1% (v/v) DMSO vehicle using the Dunnett multiple comparisons test. This cytotoxicity procedure was repeated on extracts from 3 different cultures for both MDA-MB-231 and MSTV1-7 cells.

2.3   Results:

2.3.1   *Genistein and Quercetin Up-Regulate Cx43 in MDA-MB-231 cells*:

MDA-MB-231 cells are a metastatic human breast tumor line, which express a very low level of Cx43 protein, and do not form functional gap junctions. Western blot analysis was used to determine the relative amount of Cx43 produced following flavonoid treatment. It was also used to determine if genistein and quercetin affected the phosphorylation state of Cx43. The amount of total protein loaded into each well was determined by probing with an antibody against GAPDH, a glycolytic enzyme which is constitutively expressed in virtually all cells at high levels.

Compared to the 0.1% (v/v) DMSO vehicle and complete medium only condition, nonphosphorylated Cx43 protein levels increased following genistein and quercetin treatment (Figure 2.1). The flavonoids did not appear to induce phosphorylation of Cx43 protein. Interestingly, observing Figure 2.1 (A), treatment with physiological levels of genistein (0.5 µg/ml) up-regulated Cx43. Cx43 levels increased further following 2.5 µg/ml genistein treatment. However, higher genistein concentrations (15 µg/ml) did not raise Cx43 levels beyond that observed at 2.5 µg/ml. Figure 2.1 (B) supports the results of Figure 2.1(A): genistein treatment increased Cx43 proteins levels. The genistein-induced Cx43 upregulation was less dramatic compared to Figure 2.1 (A), suggesting that there is inter-passage variation in protein expression following genistein treatment.

Quercetin treatment, at concentrations of 2.5 µg/ml and 5 µg/ml, consistently up-regulated Cx43 protein compared to the 0.1% (v/v) DMSO vehicle and complete medium only condition (Figure 2.1 C and D). Additionally, Figure 2.1 (C) shows a slight increase in Cx43 following 72 hours treatment with 0.5 µg/ml quercetin. Interestingly, treatment

with 15 µg/ml quercetin did not significantly up-regulate Cx43 protein compared to the controls (Figure 2.1 C and D).

2.3.2 *Genistein and Quercetin Effects on Cx43 Localization in MDA-MB-231 cells*:

Immunocytochemistry was performed to verify Western blot results and to determine intracellular localization of Cx43. Cx43 localized to the plasma membrane in nontumorigenic human breast cells (MSTV1-7) is shown as a positive control (Figure 2.2 A). Compared to the untreated cells, treatment with 2.5 µg/ml and 15 µg/ml genistein resulted in an apparent increase in Cx43 protein (Figure 2.2 E and F). This result is consistent with the Cx43 expression observed by Western blot analysis. Furthermore, genistein, at these treatment concentrations, resulted in Cx43 localized to the plasma membrane.

All quercetin treatment concentrations (0.5 µg/ml, 2.5 µg/ml, 5 µg/ml, 15 µg/ml) produced more Cx43 protein compared to untreated cells (Figure 2.3 C-F) Furthermore, the amount of Cx43 appeared to increase as the concentration of quercetin increased. These observations contrast with the Western blot analyses, which did not show an increase in Cx43 protein following treatment with 15 µg/ml quercetin. Therefore, immunoblot analysis may not be as sensitive as immunocytochemistry in identifying Cx43 protein. Interestingly, quercetin treatment failed to localize Cx43 protein to the plasma membrane. Instead, cells treated with quercetin retained Cx43 in the perinuclear region.

Genistein and quercetin treatment failed to induce Cx43 expression in all cells, suggesting that MDA-MB-231 cells are heterogeneous with respect to protein expression. Approximately 10% of the cells produced Cx43 protein following either genistein or

quercetin treatment (Table 2.1). Controls for primary and secondary antibody specificity, and autofluorescence indicate that the Cx43 antibody used for immunocytochemistry was specific (Figure 2.2 G-I).

2.3.3 *Genistein and Quercetin Do Not Improve GJIC in MDA-MB-231 Cells*:

The effect of 72 hours treatment with genistein and quercetin on GJIC in MDA-MB-231 cells was studied using both the scrape loading and preloading dye transfer technique. MDA-MB-231 cells, treated with 0.1% (v/v) DMSO vehicle or complete medium only, did not exhibit GJIC (Figure 2.4-2.7). Furthermore, genistein and quercetin treatment did not increase GJIC. Extra care was taken to observe preloaded cells in contact with surrounding cells because cell density was greatly reduced following 72 hours treatment with 15 μg/ml genistein and 5 μg/ml quercetin.

Positive controls for GJIC included MSTV1-7 and Hs578T cells (Figure 2.4 A-F). GJIC was particularly apparent in Hs578T cells. The gap junction permeable dye (calcein for the preloading technique, and carboxyfluoresceine for the scrape loading technique) spread to cells beyond the ones directly coupled to the preloaded cell signifying a high degree of coupling (Figure 2.4 F).

2.3.4 *Genistein and Quercetin Reduce MDA-MB-231 Cell Proliferation*:

Cell count analysis was performed to determine if genistein and quercetin treatment suppressed growth of MDA-MB-231 cells (Figure 2.8). There was no significant difference in cell number between the complete medium only condition and the 0.1% (v/v) DMSO vehicle. Comparing flavonoid treated groups to the 0.1% (v/v) DMSO vehicle, 2.5 μg/ml ($P < 0.05$) and 15 μg/ml ($P < 0.01$) genistein, and 2.5 μg/ml (P

< 0.05), 5 µg/ml (*P* < 0.01), and 15 ug/ml (*P* < 0.01) quercetin significantly reduced cell number. 0.5 µg/ml genistein and quercetin did not significantly reduce cell number compared to the 0.1% (v/v) DMSO vehicle condition.

Cell proliferation was also measured directly by immunocytochemistry (Figure 2.9). Ki67 expression in MDA-MB-231 cells was scored following 72 hours treatment with 15 µg/ml genistein, 5 µg/ml quercetin, or 0.1% (v/v) DMSO vehicle. All cells, which received 0.1% (v/v) DMSO vehicle treatment, expressed some level of Ki67, and therefore were dividing (Figure 2.9 C). Of the vehicle control treated cells, 36.6% were highly proliferating. Comparatively, only 3.3% of cells were highly proliferating and 55.6% were non-dividing following 5µg/ml quercetin treatment. Approximately 15% of cells treated with 15 µg/ml genistein (Figure 6B) were highly proliferating, and 26% were non-dividing (Table 2.2). These results support the cell count results: namely genistein (*P* < 0.0001) and quercetin (*P* < 0.0001) reduce proliferation of MDA-MB-231 cells compared to control conditions.

Cx43 staining was observed in conjunction with Ki67 and DAPI staining to determine if genistein and quercetin-induced Cx43 contributed to the reduced cell proliferation (Figure 2.9 D-G). MDA-MB-231 cells, which expressed Cx43 following 15µg/ml genistein or 5µg/ml quercetin treatment did not show reduced proliferation compared to cells which did not produce Cx43 (Table 2.3).

2.3.5 *Fifteen µg/ml Quercetin is Cytotoxic to MDA-MB-231 and MSTV1-7 cells*:

During the first 24 hours of treatment, a significantly greater amount of LDH was released from MDA-MB-231 cells (*P* < 0.05) treated with 15 µg/ml quercetin compared to the 0.1% (v/v) DMSO vehicle (Figure 2.10). This suggests that 15 µg/ml quercetin had

a toxic effect on MDA-MB-231 cells during this treatment period. Furthermore, 15 µg/ml quercetin induced significantly greater LDH release from MSTV1-7 cells ($P < 0.01$) compared to the 0.1% (v/v) DMSO vehicle throughout the entire 72 hour treatment period (Figure 2.11). This suggests that 15 µg/ml quercetin is cytotoxic to this type of nontumorigenic human breast cell. No other treatment concentrations (2.5 µg/ml genistein, 15 µg/ml genistein, or 5 µg/ml quercetin) were toxic to MDA-MB-231 or MSTV1-7 cells during any period of the 72 hour treatment duration. Interestingly, 2.5 µg/ml genistein induced significantly less LDH release from MSTV1-7 cells ($P < 0.05$) during the first 24 hours of treatment compared to the 0.1% (v/v) DMSO vehicle. The same protective effect was observed with 2.5 µg/ml genistein on MDA-MB-231 cells ($P < 0.01$) during the last 24 hours of the treatment period. Therefore, the significant reduction in MDA-MB-231 cell number and reduced proliferation cannot be attributed to genistein or quercetin toxicity.

It is interesting that 15 µg/ml quercetin did not increase Cx43 protein levels in MDA-MB-231 cells whereas 2.5 µg/ml and 5 µg/ml quercetin did when measured by Western blot analysis. The toxic effect of 15 µg/ml quercetin on MDA-MB-231 cells may explain this effect. However, this explanation does not account for the Cx43 protein observed by immunocytochemistry in MDA-MB-231 cells treated with 15 µg/ml quercetin.

Figure 2.1. Western blot analysis showing relative amount of Cx43 following genistein and quercetin treatment. Cells were treated for 72 hours, replacing the treatment medium every 24 hours. Control cells were grown in complete medium (M) and 0.1% (v/v) DMSO. DMSO was used to maintain genistein (G) and quercetin (Q) in solution. All treatment concentrations were in µg/ml. Example G0.5 = 0.5µg/ml genistein. All samples were loaded onto the gel at 200 µg/lane, except for Hs578T cells, which were loaded at 25 µg/lane. Hs578T cells express abundant Cx43. This cell line acts as a positive control for Cx43 protein. Western blots were repeated on extracts from 4 different genistein and quercetin treated cultures. Two Western blot results for each flavonoid treatment are shown above. These blots are representative of the 4 separate experiments.

All treatment concentrations of genistein, including 0.5 µg/ml, increased Cx43 protein compared to the 0.1% (v/v) DMSO vehicle and complete medium control conditions (**A**). **B** supports the general results of **A**. Physiological observed levels of genistein (0.5 µg/ml), as well as 2.5 µg/ml and 15 µg/ml increased Cx43 protein.

Quercetin, at concentrations of 2.5 µg/ml and 5 µg/ml, consistently increased Cx43 protein compared to the 0.1% (v/v) DMSO vehicle and complete medium controls (**C, D**). 15µg/ml quercetin did not increase Cx43 protein by Western blot analysis.

A

Cx43

GAPDH

M DMSO G0.5 G2.5 G5 G7 G10 G15 G20

B

Cx43

GAPDH

M DMSO G0.5 G2.5 G15 Hs578T MSTV1-7

C

M DMSO Q0.5 Q2.5 Q5 Q15 MSTV1-7 Hs578T
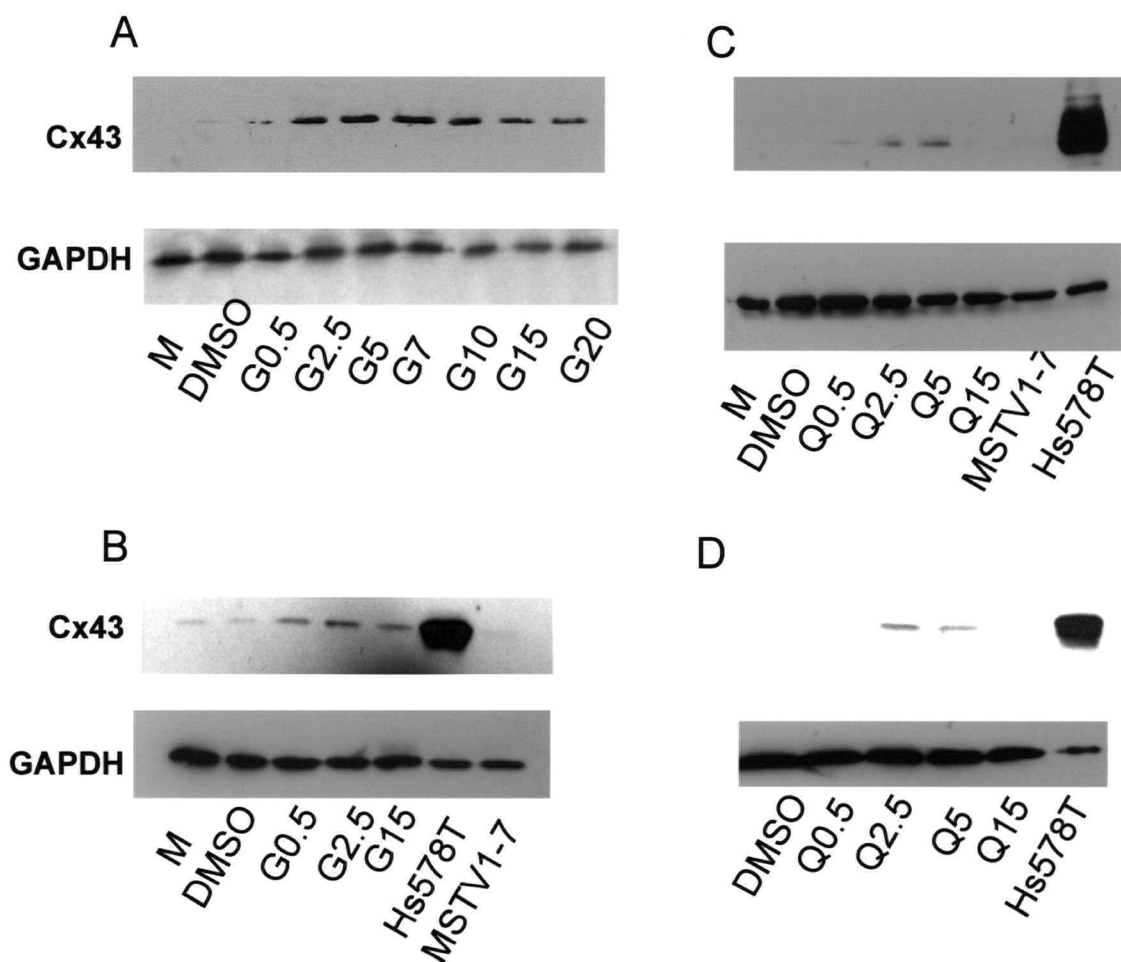
D

DMSO Q0.5 Q2.5 Q5 Q15 Hs578T

Figure 2.2. Immunocytochemistry analysis showing Cx43 localization following genistein treatment. MDA-MB-231 cells were treated every 24 hours for 72 hours with 0.1% (v/v) DMSO vehicle, complete medium, or 0.5 µg/ml, 2.5 µg/ml, 15 µg/ml genistein. Nontumorigenic human breast cells (MSTV1-7) show Cx43 plaques at the plasma membrane (arrows, **A**). Treatment with 2.5 µg/ml and 15 µg/ml genistein resulted in Cx43 localization at the plasma membrane (**E, F**). This is evident by punctuate Cx43 staining at the plasma membrane. The effect was not evident in every cell, suggesting a heterogeneous cell population with respect to Cx43 expression and/or response to genistein treatment (Table 2.1).

There is no observable increase in punctuate membrane staining following 0.1% (v/v) DMSO vehicle treatment (**C**) compared to the complete medium control (**B**). Therefore, the DMSO in the flavonoid treatment does not, by itself, induce the modified Cx43 staining (**E, F**). This effect is likely attributable to the flavonoid. However, because the flavonoid cannot be administered without DMSO, it is not possible to rule out any effects due to flavonoid-DMSO interaction. Cx43 antibody controls are shown (**G-I**).
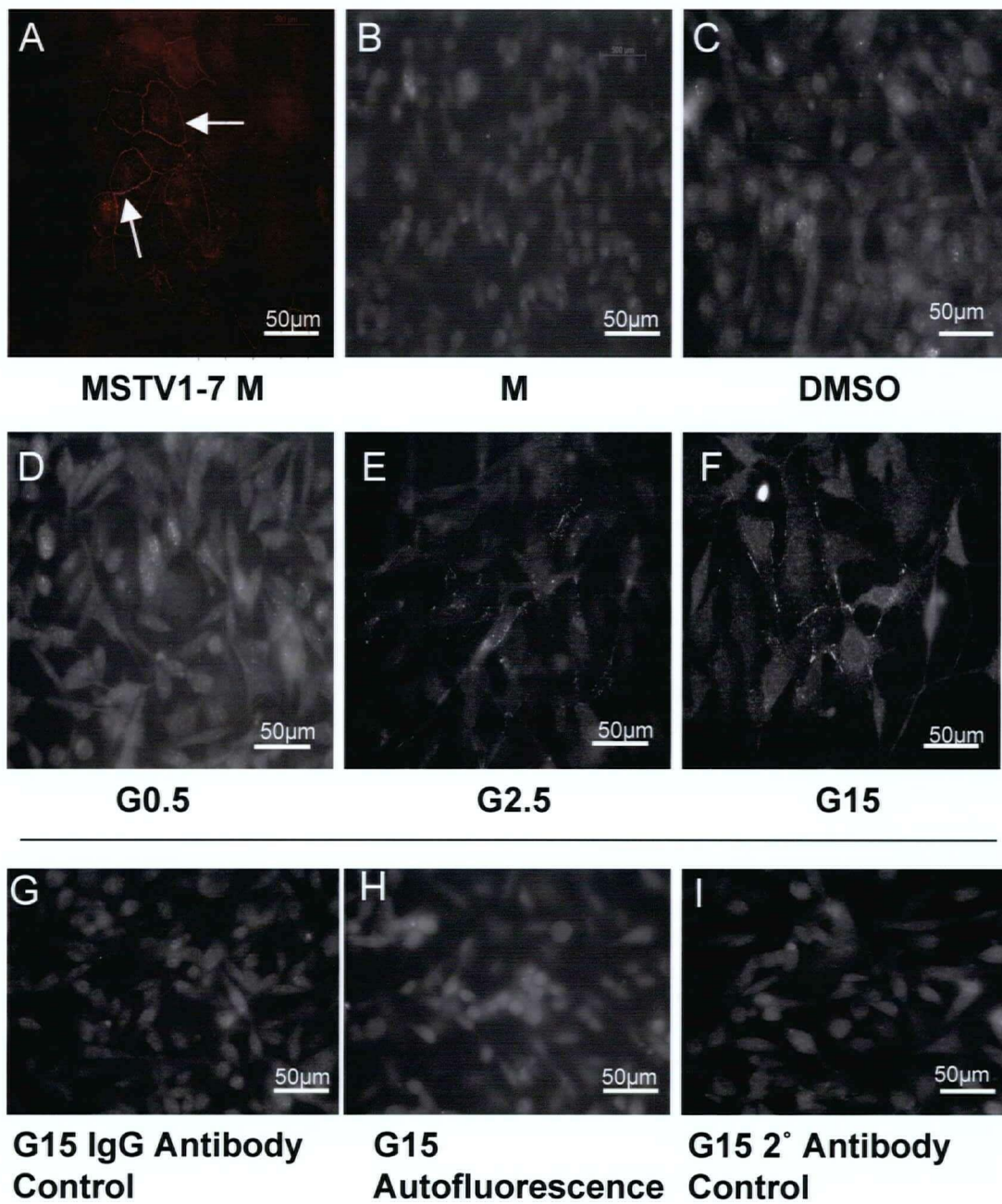
MSTV1-7 M

M

DMSO

G0.5

G2.5

G15

G15 IgG Antibody
Control

G15
Autofluorescence

G15 2˚ Antibody
Control

Figure 2.3. Immunocytochemistry analysis showing Cx43 location following quercetin treatment. MDA-MB-231 cells were treated with 0.5 μg/ml, 2.5 μg/ml, 5 μg/ml, and 15μg/ml quercetin for 72 hours replacing the treatment medium every 24 hours. Quercetin increased Cx43 at all treatment concentrations tested compared to the 0.1% (v/v) DMSO vehicle condition (**B**). Quercetin appeared to retain Cx43 protein in the perinuclear space of MDA-MB-231 cells (**C-F**). Cx43 staining was not apparent in every cell, suggesting a heterogeneous cell population with respect to Cx43 expression and/or quercetin response (Table 2.1). The medium only condition (**B**) and antibody controls (**G-I**) are shown in Figure 2.2, along with the positive control for Cx43-membrane associated plaques (arrows, **A**).
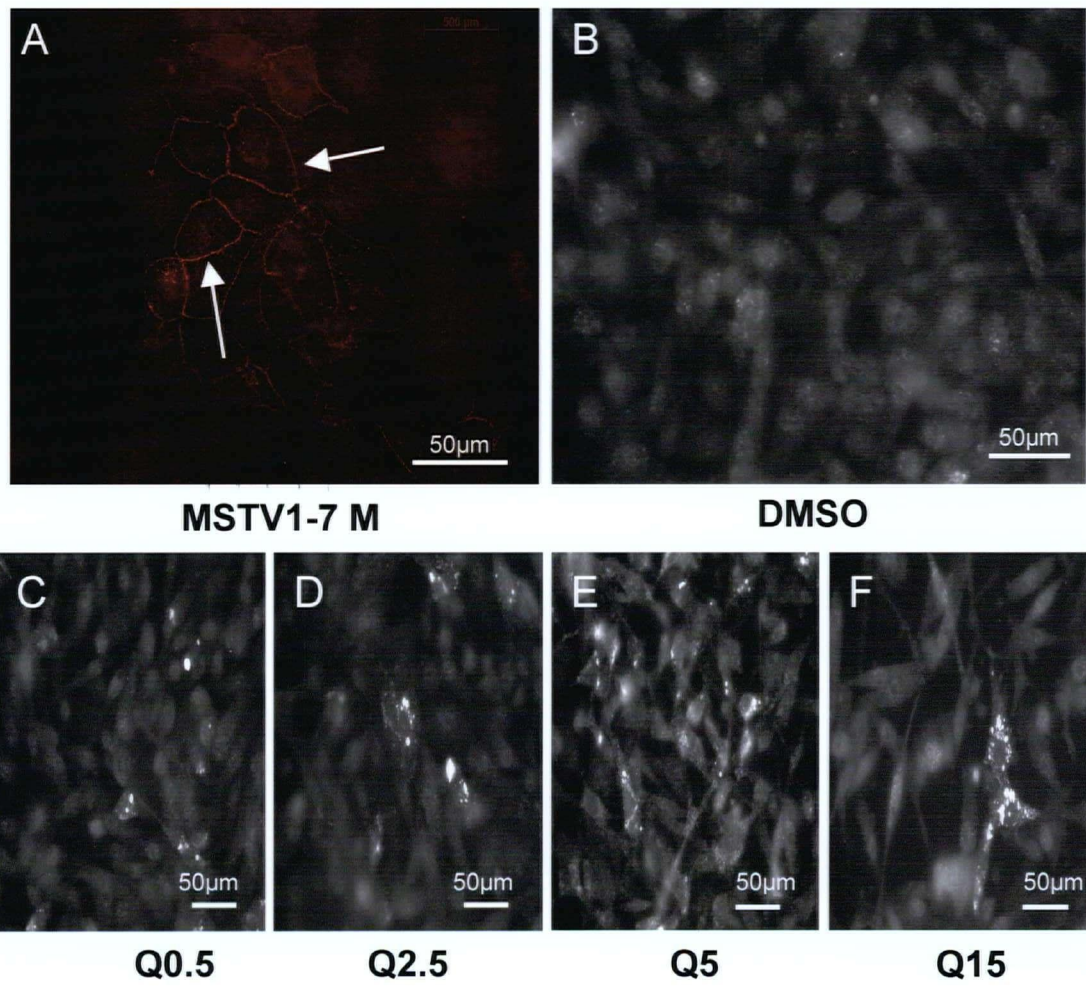
MSTV1-7 M   **DMSO**

Q0.5   **Q2.5**   **Q5**   **Q15**

Table 2.1. Proportion of MDA-MB-231 Cells which Produced Cx43 following Genistein and/or Quercetin Treatment (computed from Table 2.2)

15 µg/ml Genistein [% Cx43 (+)]	5 µg/ml Quercetin [% Cx43 (+)]	Genistein and Quercetin [Total % Cx43 (+)]
10.7	7.8	9.6

Figure 2.4. Effect of genistein on GJIC in MDA-MB-231 cells as measured by the preloading technique. Nontumorigenic breast cells (MSTV1-7) and Hs578T cells grown in complete medium act as positive controls for dye transfer (**A-F**). Calcein passed from the donor cell to surrounding recipient cells (arrows, **C, F**). The effect was especially profound in Hs578T cells.

72 hours of genistein treatment (**J-L**) did not increase GJIC in MDA-MB-231 cells compared to the 0.1% (v/v) DMSO vehicle condition (**G-I**). Donor cells were labeled with calcein (green; gap junction permeable) and DiI (red; lipophilic membrane dye) and then seeded onto recipient cells. Both donor and recipient cells received the same treatment. DIC images (**A, D, G, J**) demonstrate the confluent monolayer of recipient cells.
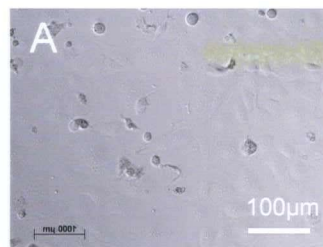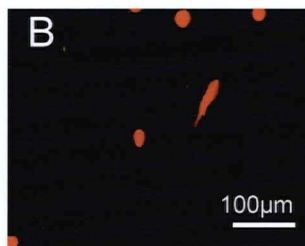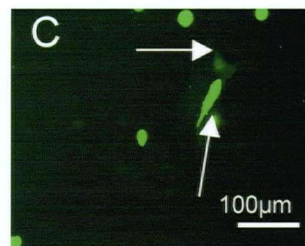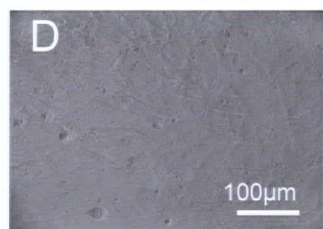
MSTV1-7 M

MSTV1-7 M

MSTV1-7 M

Hs578T M
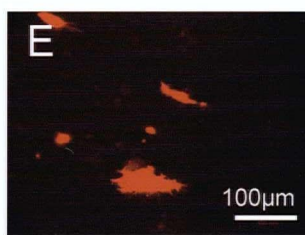
Hs578T M
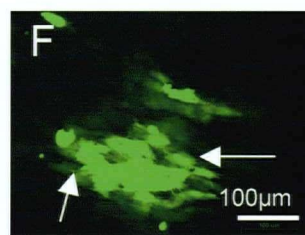
Hs578T M
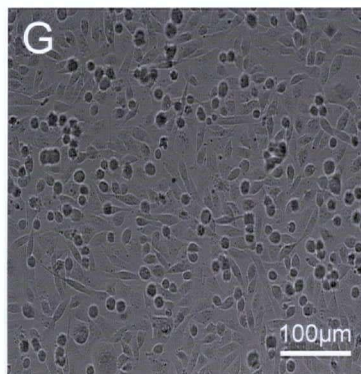
DMSO
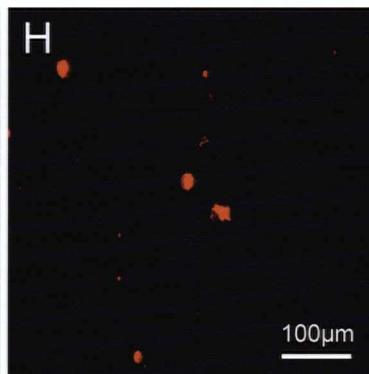
DMSO
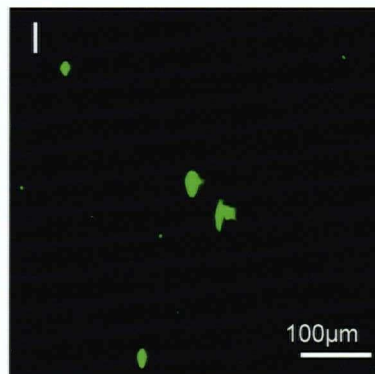
DMSO
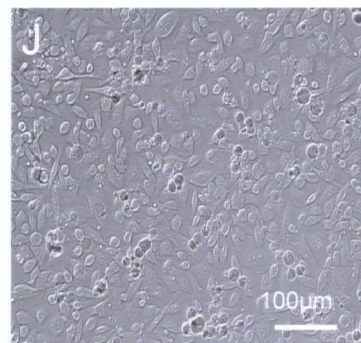
G2.5
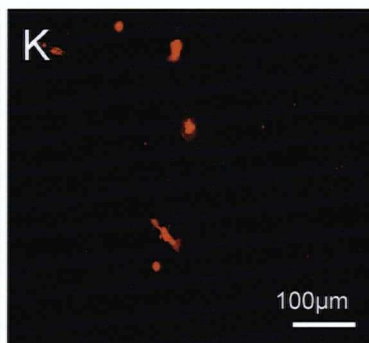
G2.5
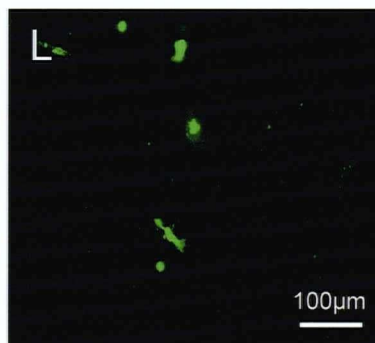
G2.5

Figure 2.5. Effect of genistein on GJIC in MDA-MB-231 cells using the scrape loading

technique. 72 hours of genistein treatment (2.5 µg/ml and 15 µg/ml) (**D-H**) did not

increase GJIC in MDA-MB-231 cells compared to the 0.1% (v/v) DMSO vehicle

condition (**A-C**). These results support the preloading observations (Figure 2.4).

Scraped cells were incubated in 5-(and -6)-carboxyfluoresceine diacetate (green) and

dextran-tagged rhodamine (red). Carboxyfluoresceine is gap junction permeable whereas

dextran-tagged rhodamine is not.

Hs578T cells are shown as a positive control for GJIC observed by the scrape loading

technique (**I, J**). Carboxyfluoresceine migrated to surrounding cells (arrow, **J**). The

background control for carboxyfluoresceine (green) is shown (**K**).

DMSO

DMSO

DMSO

G2.5

G2.5

G15

G15

G15

DMSO Hs578T

DMSO Hs578T

Background control for 5(6)-CFDA
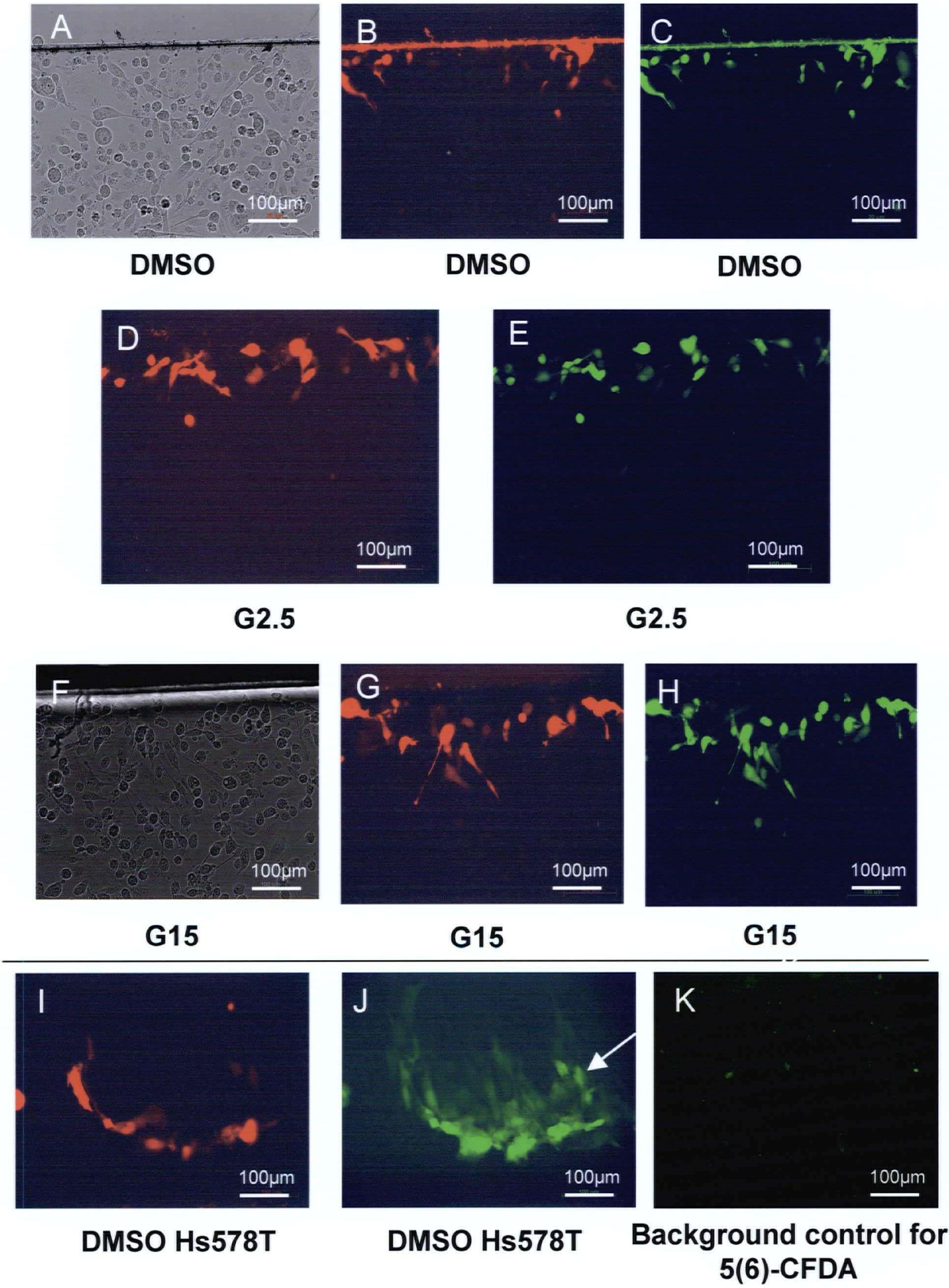
Figure 2.6. Effect of quercetin on GJIC in MDA-MB-231 cells using the preloading technique. 72 hours of quercetin treatment (2.5 µg/ml and 5 µg/ml) (**A-F**) did not increase GJIC in MDA-MB-231 cells compared to the 0.1% (v/v) DMSO vehicle condition. Calcein (green; gap junction permeable) did not transfer from the DiI (red; lipophilic membrane dye) labeled donor cells to surrounding recipient cells. The 0.1% (v/v) DMSO vehicle condition is shown in Figure 2.4 (**G-I**). The images of the positive control (MSTV1-7; **A-C**: Hs578T; **D-F**) for GJIC measured by the preloading technique are shown in Figure 2.4.

A Q2.5

B Q2.5

C Q2.5

D Q5

E Q5
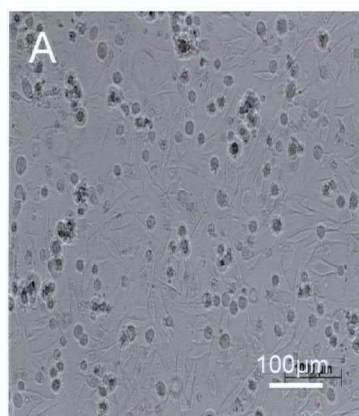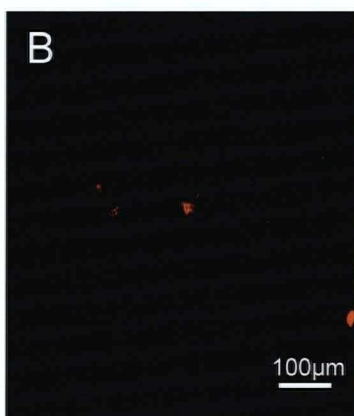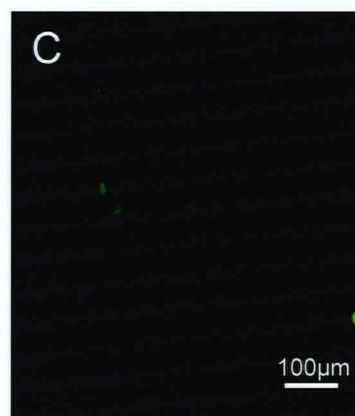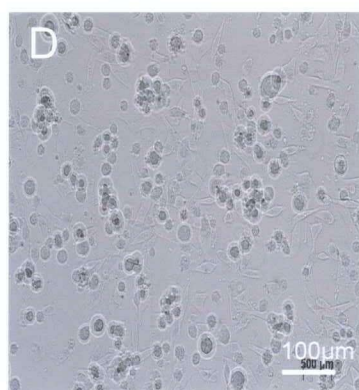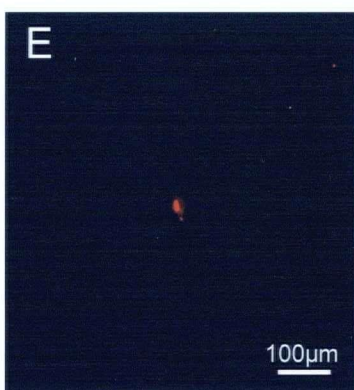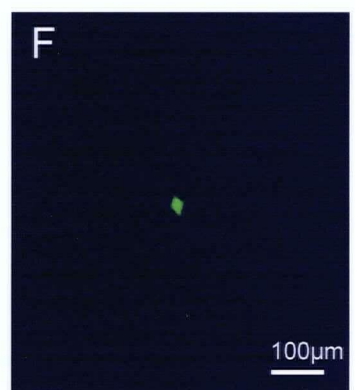
F Q5

Figure 2.7. Effect of quercetin on GJIC in MDA-MB-231 cells using the scrape loading technique. Considering the background control for carboxyfluoresceine (Figure 2.5 **K**), 72 hours of quercetin treatment (2.5 µg/ml and 5 µg/ml) did not improve GJIC in MDA-MB-231 cells compared to the 0.1% (v/v) DMSO vehicle condition. Carboxyfluoresceine (green; gap junction permeable) did not pass from the dextran-tagged rhodamine (red; gap junction impermeable) labeled donor cells to surrounding recipient cells. These results support the preloading results for quercetin treatment. The 0.1% (v/v) DMSO vehicle condition is shown in Figure 2.5 (**A-C**). The positive control for scrape loading is shown in Figure 2.5 (**I, J**).

A

Q2.5

B

Q2.5

C

Q2.5

D

Q5

E

Q5

F

Q5

100μm
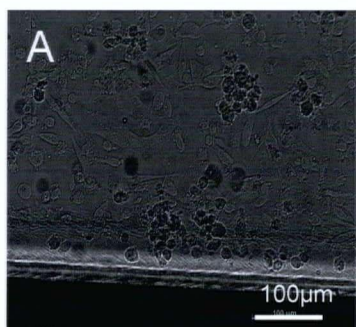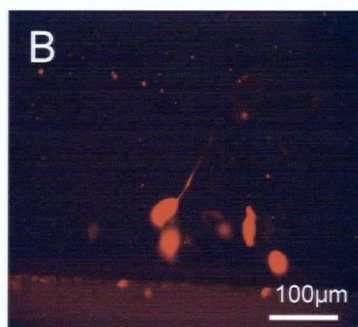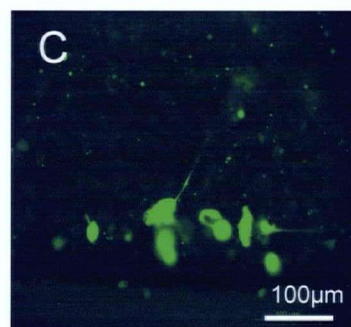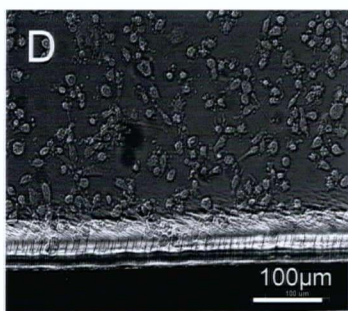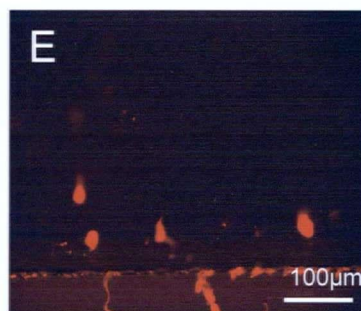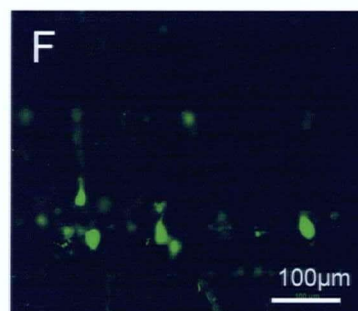
Figure 2.8. Growth suppressive effect of genistein and quercetin on MDA-MB-231 cells as measured by cell count. Flavonoid treatment reduced the MDA-MB-231 cell number compared to the complete medium and 0.1% (v/v) DMSO vehicle conditions. Cells were seeded at low density and treated every 24 hours for 72 hours with 0.5 µg/ml, 2.5 µg/ml, 15 µg/ml genistein or 0.5 µg/ml, 2.5 µg/ml, 5 µg/ml, 15 µg/ml quercetin. Cell number was significantly reduced following 2.5 ug/ml (*, $P < 0.05$) and 15 µg/ml (**, $P < 0.01$) genistein, and 2.5 µg/ml (*, $P < 0.05$), 5 µg/ml (**, $P < 0.01$), and 15 µg/ml (**, $P < 0.01$) quercetin compared to the 0.1% (v/v) DMSO vehicle condition.

Cell Number vs Flavonoid Treatment
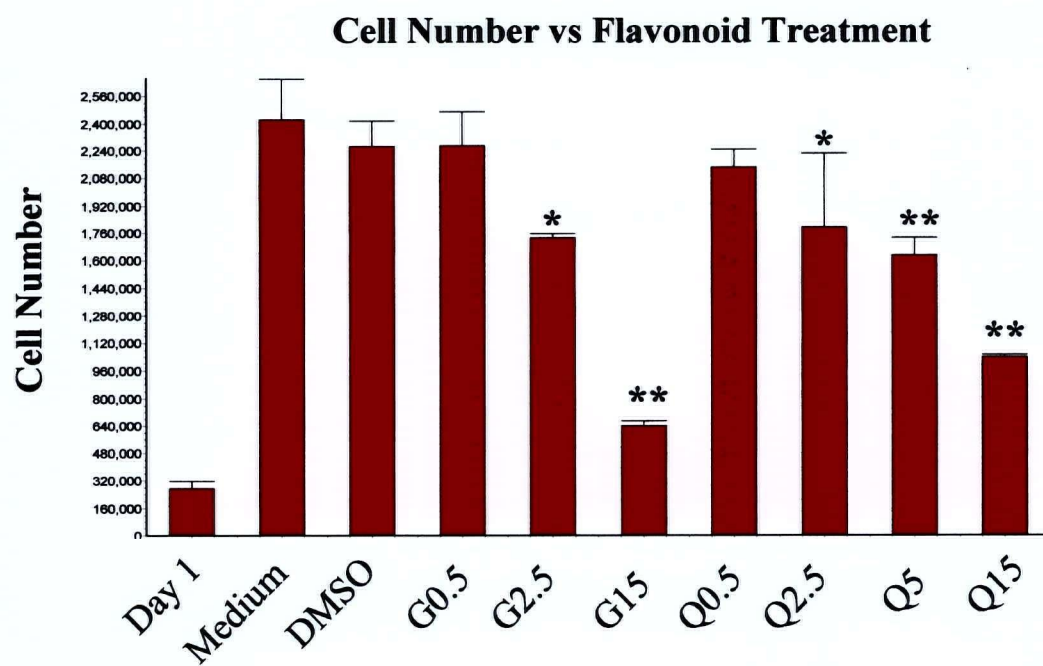
Figure 2.9. Effect of genistein and quercetin, and Cx43 on MDA-MB-231 cell proliferation. MDA-MB-231 cells, treated for 72 hours with 5 µg/ml quercetin, 15 µg/ml genistein, and 0.1% DMSO vehicle, were triple labeled with Ki67 (green), Cx43 (red), and DAPI (blue). The fraction of cells expressing various intensity levels of Ki67 (0, 1, 2, 3; arrows with corresponding scores) were counted to determine the effect of flavonoids on proliferation (**A-C**). The results are summarized in Table 2.2: quercetin and genistein treatment significantly reduced cell proliferation ($P < 0.0001$).

Ki67 and Cx43 labeling were analyzed to determine if there was any association between Cx43 expression and proliferation (**D-G**). There was no significant difference in proliferation between cells which produced Cx43 and cells which did not produce Cx43 following flavonoid treatment (Table 2.3). Furthermore, the results are not consistent. **C** shows highly proliferating cells with Cx43, and **D** shows non-dividing cells with Cx43. Antibody controls for Ki67 and Cx43 (**H-I**).

Q5 Ki67/DAPI   G15 Ki67/DAPI   DMSO Ki67/DAPI

G15
Ki67/Cx43/DAPI

G15
Ki67/Cx43/DAPI

Q5
Ki67/Cx43/DAPI

Q5
Ki67/Cx43/DAPI

Ki67 MIgG with DAPI   Cx43 RIgG
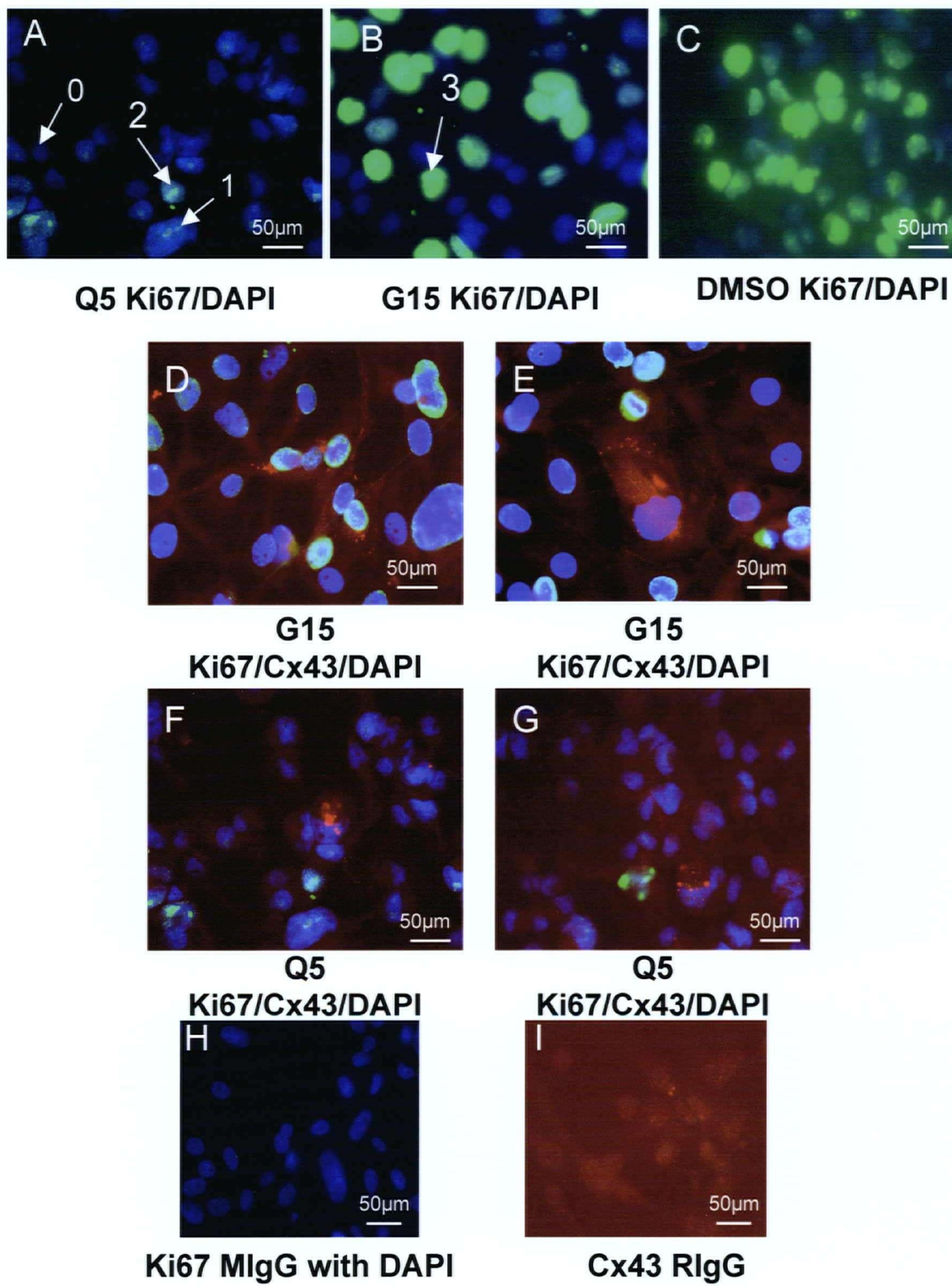
Table 2.2. Ki67 scores of flavonoid treated cells: effect of genistein and quercetin on cell proliferation

Ki67 Score*	15 µg/ml Genistein (number and % cells)	5 µg/ml Quercetin (number and % cells)	0.1% (v/v) DMSO (number and % cells)
0	54 (22.8)	100 (55.6)	0 (0)
1	80 (33.9)	50 (27.8)	156 (40.2)
2	42 (17.8)	24 (13.3)	90 (23.2)
3	30 (12.7)	6 (3.3)	142 (36.6)

*Ki67 staining was scored on a scale of 0-3: 0, negative (no proliferation); 1, weak positive; 2, intermediate positive; 3, strong positive (high proliferation)

Table 2.3. Ki67 scores of flavonoid –induced Cx43 expressing MDA-MB-231 cells

Ki67 Score	15 µg/ml Genistein (number of cells)	15 µg/ml Genistein (number of cells)	5 µg/ml Quercetin (number of cells)	5 µg/ml Quercetin (number of cells)	0.1% (v/v) DMSO (number of cells)	0.1% (v/v) DMSO (number of cells)
	Cx43 (+)	Cx43 (-)	Cx43 (+)	Cx43 (-)	Cx43 (+)	Cx43 (-)
0	4	50	10	90	0	0
1	6	74	4	46	0	156
2	12	30	0	24	0	90
3	0	30	0	6	0	142

Figure 2.10. Cytotoxic effect of genistein and quercetin on MDA-MB-231 cells. MDA-MB-231 cells were treated with 2.5 µg/ml and 15 µg/ml genistein and 5 µg/ml and 15 µg/ml quercetin every 24 hours for 72 hours. Each day the treatment medium was extracted and the cells lysed to determine whether or not genistein and quercetin were toxic to cells. The LDH released upon flavonoid treatment was compared to the LDH released following 0.1% (v/v) DMSO vehicle treatment.

15 µg/ml quercetin was toxic to MDA-MB-231 cells during the first 24 hours of the treatment period (*$P < 0.05$) (A). No other genistein or quercetin treatment concentrations were toxic to MDA-MB-231 cells throughout the 72 hour treatment period. Interestingly, during the last 24 hours of the treatment duration, 2.5 µg/ml genistein induced significantly less LDH from MDA-MB-231 cells compared to the 0.1% (v/v) DMSO vehicle condition (**$P < 0.01$) (C).

A

Day 1 MDA-MB-231 Relative Cytotoxicity

B

Day 2 MDA-MB-231 Relative Cytotoxicity

C

Day 3 MDA-MB-231 Relative Cytotoxicity
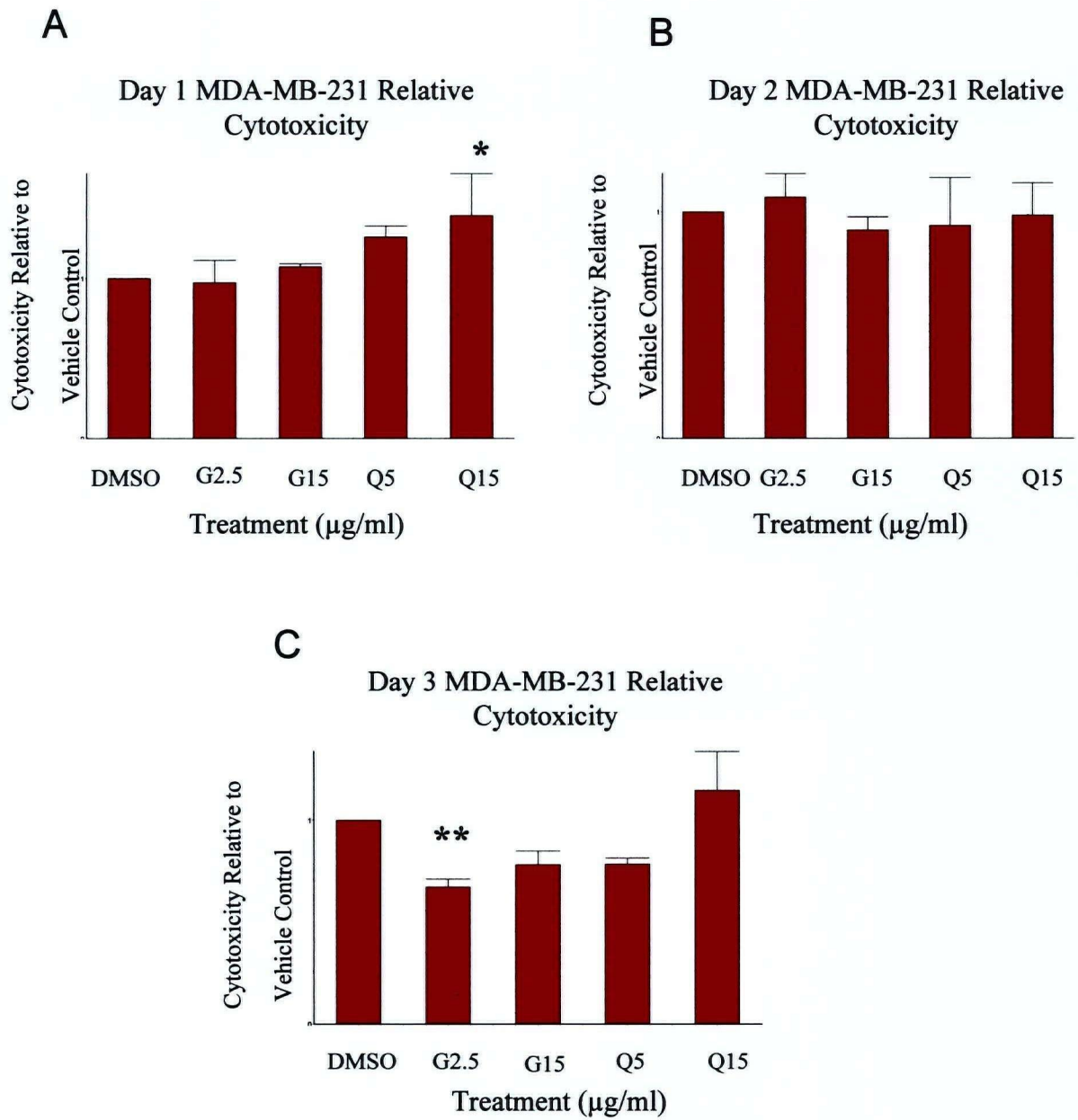
Figure 2.11. Cytotoxic effect of genistein and quercetin on MSTV1-7 cells. MSTV1-7 cells were treated with 2.5 μg/ml and 15 μg/ml genistein and 5 μg/ml and 15 μg/ml quercetin every 24 hours for 72 hours. Each day the treatment medium was extracted and the cells lysed to determine whether or not genistein and quercetin were toxic to cells. The LDH released upon flavonoid treatment was compared to the LDH released following 0.1% (v/v) DMSO vehicle treatment.

15 μg/ml quercetin was toxic to nontumorigenic breast cells throughout the entire treatment period (**$P < 0.01$) (A-C). Interestingly, during the first 24 hours of the treatment duration, 2.5 μg/ml genistein induced significantly less LDH release from MDA-MB-231 cells compared to the 0.1% (v/v) DMSO vehicle condition (*$P < 0.05$) (A).

A

Day 1 MSTV1-7 Relative Cytotoxicity
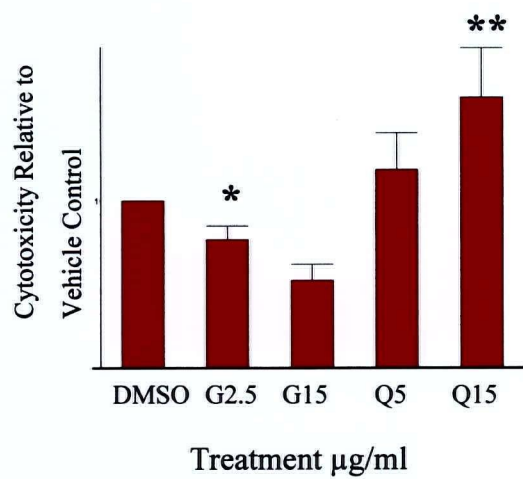
B

Day 2 MSTV1-7 Relative Cytotoxicity
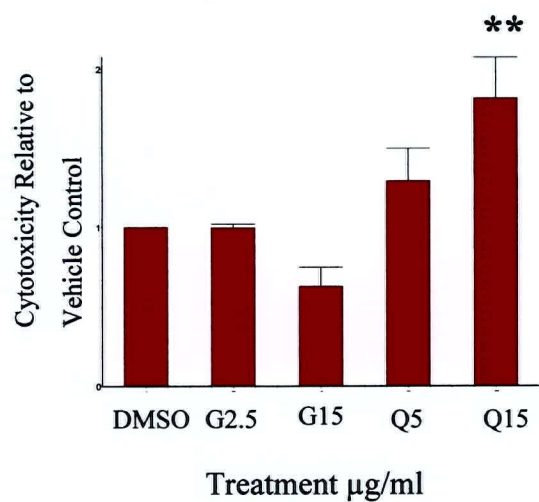
C

Day 3 MSTV1-7 Relative Cytotoxicity
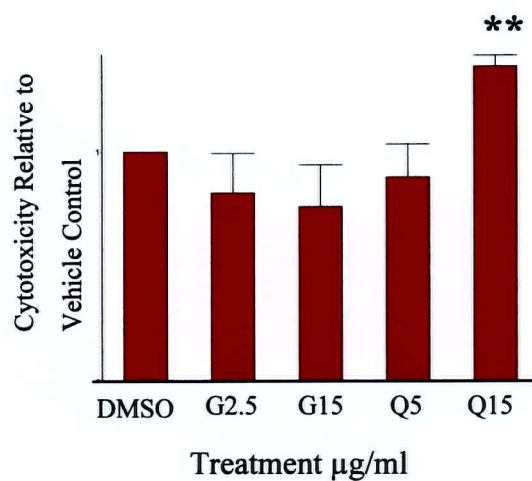
2.4   Discussion:

Breast cancer is the second most frequent cause of death among North American women (reviewed by Lacey et al., 2002). Approximately, 1 out of 8 American females will develop breast cancer throughout their lifetime, and 1 out of 30 North American females will die from the disease (Ries et al, 2000). Current treatments for breast cancer include hormone therapy, radiotherapy, chemotherapy, and surgical tumor removal (reviewed by Shenkier et al., 2004). Many of these treatments have significant physical and psychological side effects. For example, tamoxifen, considered to be a very effective adjuvant hormonal therapy for ER-positive breast cancer patients, has been associated with increased risk of endometrial cancer and thromboembolic disorders (reviewed by Morse et al., 2003). Chemotherapy is associated with physical side effects including immunosuppression, hair loss, nausea, fatigue, diarrhea, weight gain, constipation, and mucositis (reviewed by Morse et al., 2003). Chemotherapy has also been associated with cognitive deficits including impaired memory, mental flexibility, attention, reaction time, visuo-spacial memory, motor and verbal function (reviewed by Morse et al., 2003). In addition to side effects, not all therapies ensure a cure.

ER status is one of the most valuable predictive and prognostic factors in the clinical management of breast cancer. Many breast cancer therapies focus on inhibiting the ER in an attempt to control proliferation. Unfortunately, one-third of breast cancer patients lack ER, and these patients respond very poorly to chemotherapy and endocrine therapy (reviewed by Sommer and Faqua, 2000). Therefore, more effective preventative and therapeutic strategies are needed for breast cancer.

Increased connexin expression and improved GJIC may represent a new, effective therapeutic approach to breast cancer management. GJIC is important for growth

regulation. Dysfunctional GJIC, whether caused by connexin protein degradation (reviewed by Carystinos et al., 2001) or physical gap junction pore closure (Rivedal and Opsahl, 2001; reviewed by Trosko and Ruch, 2002), has been associated with metastatic breast cancer (Saunders et al., 2001). Additionally, dysfunctional GJIC has been associated with cell heterogeneity and is one of the earliest alterations associated with malignant transformation (reviewed by Laird, 1999). Furthermore, connexin proteins have been known to have antitumor functions independent of GJIC. Connexin upregulation has been associated with improved response to chemotherapy agents such as etoposide, paclitaxel, and doxorubicin (reviewed by Chipman et al., 2003). Furthermore, Qin et al. (2002) transfected human breast cells with Cx26 and Cx43. The result was suppressed tumor growth independent of GJIC. It was hypothesized that the connexin proteins disrupted the interaction between fibroblast growth factor and its receptor, reducing mitogenic activity (Qin et al., 2002).

The flavonoids, genistein and quercetin, have been reported to induce apoptosis and halt the cell cycle in several types of tumors including breast, prostate, colon, and lung (reviewed by Knekt, 1997). Although apigenin and tangeretin have been reported to counteract GJIC inhibition in rat liver epithelial cells (Chaumontet et al., 1997), the antitumor effects of genistein and quercetin, through gap junctional mechanisms, is a novel investigation. This study aimed to determine if genistein and quercetin reduce proliferation of MDA-MB-231 cells by regulating Cx43 levels and GJIC activity.

Cx43 is the most common connexin protein in breast cells where it is found in the lateral and basal surfaces of myoepithelial cells (Jamieson et al., 1998). An inverse relationship has been reported between the amount of Cx43 protein and tumor severity. Most high grade and metastatically aggressive tumors have relatively low levels of Cx43

(reviewed by Laird, 1999; Saunders et al., 2001). Furthermore, Wilgenbus (1992) reported that human breast cancer tissue tends to have lower levels of Cx43 compared to nontumorigenic human breast tissue.

MDA-MB-231 cells are a highly metastatic human breast tumor cell line, which express extremely low levels of Cx43 protein. Although apigenin and tangeretin treatment have increased Cx43 in rat liver epithelial cells (Chaumontet et al. 1994 and 1997), this is the first study to investigate the effect of genistein and quercetin on Cx43 protein in tumor cells. Genistein produced the greatest response in Cx43 expression at a concentration of 2.5 μg/ml, and quercetin maximally stimulated Cx43 protein at 5 μg/ml. Interestingly, 0.5 μg/ml genistein (0.26 μM) increased Cx43 protein. This result is significant because it represents the average genistein plasma concentration (0.28 μM) reported in Japanese men (Hedlund et al., 2003) (Table 1.2). The breast cancer incidence among Asian females is 4-6 times lower than among North American females. Asians consume a diet rich in genistein (reviewed by Messina, 1994) and migratory studies demonstrate that breast cancer incidence among Asians dramatically increases when they move to North America (Ziegler et al., 1993). Therefore, it is possible that the high consumption of genistein among Asians contributes to their lower breast cancer incidence (Lambe et al. 2003).

The mechanism by which genistein and quercetin elicit Cx43 upregulation requires further study. Genistein and quercetin are structurally similar to estrogen (Figure 1.1). These two flavonoids have been reported to bind to ER-alpha and beta, translocate to the nucleus and affect gene transcription through estrogen response elements (Bhathena and Velasquez, 2002). However, MDA-MB-231 cells are ER-negative. Therefore genistein and quercetin must affect Cx43 expression by a mechanism

independent of the ER. The lipophilic nature of genistein and quercetin is a possible mechanism. Lipophilic molecules are able to traverse the plasma membrane, and once inside the cell, genistein and quercetin may activate proteins involved in Cx43 transcription or directly stimulate Cx43 promoter regions.

Gap junction formation requires that connexin proteins be located at the plasma membrane. There was no evidence of plasma membrane-associated Cx43 in untreated MDA-MB-231 cells; only some diffuse cytoplasmic Cx43 immunoreactivity. However, following treatment with 2.5 µg/ml and 15 µg/ml genistein, Cx43 appeared at cell borders. By contrast, Cx43 did not appear localized to the plasma membrane following quercetin treatment. Instead, the Cx43 in quercetin treated MDA-MB-231 cells concentrated near the nucleus in the region typically occupied by the Golgi apparatus and late endosomes.

Genistein and quercetin may influence Cx43 localization by affecting the trafficking of connexin proteins. Connexin proteins are produced in the endoplasmic reticulum, and then become phosphorylated either in the Golgi apparatus (reviewed by Laird and Saez, 2000) or by protein interaction in vesicles as they transit along microtubules to the plasma membrane (Lauf et al., 2002). It has been hypothesized that one function of connexin phophorylation is to direct plasma membrane insertion (Warn-Cramer et al., 1998). Immunoblot analysis in this study showed no evidence of Cx43 phosphorylation following genistein or quercetin treatment. This suggests that genistein-mediated Cx43 plasma membrane localization must occur by a mechanism other than connexin phosphorylation. The mechanism by which quercetin impedes the transport of Cx43 to the plasma membrane remains to be determined. It may be that quercetin

interferes with the transport of connexin proteins from the Golgi apparatus along microtubules.

Few studies have investigated the ability of flavonoids to direct Cx43 plasma membrane insertion. Ale-Agha et al. (2002) demonstrated that 75 μg/ml genistein counteracted the tumor promoter induced internalization of Cx43 in rat liver epithelial cells. Chaumontet et al. (1994) also used rat liver epithelial cells to show that 7 μg/ml apigenin and 10 μg/ml tangeretin localized Cx43 to cell-cell contact regions.

Tumor promoting agents such as TPA and DDT inhibit GJIC and can give rise to cancers (reviewed by Rivedal, 2001). Studies have shown the ability of flavonoids to counteract tumor promoter-induced reduction in GJIC. (-)Epicatechin, a flavonoid found in cocoa and green tea, is able to maintain GJIC in the presence of TPA in rat liver epithelial cells (Ale-Agha et al., 2002). Sai et al. (2000) demonstated that green tea protects against pentachlorophenol-induced inhibition of GJIC in mice. Without using a tumor promoter in this study, it was found that neither genistein nor quercetin enhanced GJIC in MDA-MB-231 cells. The finding that genistein localized Cx43 to the plasma membrane but did not increase GJIC suggests that there must be factors other than aberrant Cx43 localization, which impede GJIC in MDA-MB-231 cells.

Cells must be held in close proximity to form functional gap junction pores. E-cadherin is an important intercellular adhesion molecule and is considered to be the most promising candidate for cell-cell interaction during gap junction formation (Fujimoto et al., 1997). Interestingly, MDA-MB-231 cells do not express E-cadherin (Appendix 1). This may represent one explanation for the lack of GJIC in this cell line.

Studies have reported the ability of genistein and quercetin to halt the cell cycle, induce apoptosis, and thus regulate cell population (Balabhadrapathruni et al., 2000, Ying

et al., 2002, Choi et al., 2001, Weber et al., 1997). In this study, cell count analysis showed that genistein and quercetin treatment reduced MDA-MB-231 cell number compared to control conditions. 15 µg/ml genistein and 5 µg/ml and 15 µg/ml quercetin produced the greatest reductions in cell number. Except for 15 µg/ml quercetin, this reduction in cell number was independent of cytotoxicity. Cell count analyses were also performed on genistein and quercetin treated Hs578T cells. Although genistein and quercetin did not affect Cx43 expression or GJIC in this cell line, a similar reduction in cell number occurred.

Genistein and quercetin treated MDA-MB-231 cells were stained with DAPI, Cx43, and the proliferative marker Ki67. This experiment was performed to determine if genistein and quercetin inhibited proliferation, and to determine whether or not Cx43 contributed to a reduced growth rate. Results showed that genistein and quercetin treated MDA-MB-231 cells exhibited a significant reduction in cell proliferation, supporting cell count analyses. Cells expressing Cx43 following quercetin treatment showed a trend towards reduced proliferation. However, cells expressing Cx43 after genistein treatment showed a trend towards increased cell proliferation. These inconclusive results may be explained by the fact genistein and quercetin were used to increase Cx43 expression in this study. Genistein and quercetin are known to affect cell growth alone and therefore represent confounding variables. In order to make definitive statements regarding the growth suppressive effects of Cx43, MDA-MB-231 cells should be transfected with Cx43 DNA alone.

Genistein and quercetin are non-steroidal plant derived compounds, which possess estrogenic activity. Therefore, there has been concern regarding the possible hormonal effects of genistein and quercetin-based products. Few studies have

investigated the safety of genistein and quercetin consumption. However, Miniello et al. (2003) concluded that consumption of genistein-based infant formulas produced no short-term or long-term reproductive problems in humans. Bloedon et al. (2002) observed that purified genistein consumption, at concentrations up to 16 mg/kg body weight, had minimal clinical toxicity in postmenopausal women. In vitro studies have used lower treatment concentrations of quercetin compared to genistein (Chaumontet et al., 1997), suggesting that quercetin may be more toxic than genistein.

Cytotoxicity assays performed on MDA-MB-231 cells and MSTV1-7 cells, treated with genistein and quercetin, demonstrated that quercetin is more cytotoxic than genistein. Over a 3 day treatment period, genistein (0.5 µg/ml, 2.5 µg/ml, 15 µg/ml) was not toxic to either the nontumorigenic MSTV1-7 cells or metastatic MDA-MB-231 human breast cancer cells. However, 15 µg/ml quercetin consistently produced a toxic reaction in both these cell lines. Interestingly, 15 µg/ml quercetin did not enhance Cx43 expression in MDA-MB-231 cells, suggesting that the toxic effects interfered with the transcription or translation of Cx43. Although studies need to be performed in vivo, the finding that genistein and quercetin suppressed cell growth at concentrations not toxic to a nontumorigenic breast cells is important. It suggests that genistein and quercetin could potentially be used as a therapy for breast cancer patients with minimal side effects.

In summary, genistein and quercetin may represent a new treatment method for breast cancer. Genistein and quercetin suppress proliferation of MDA-MB-231 cells at concentrations, which are not toxic to nontumorigenic human breast cells. The suppressed proliferation is independent of GJIC, and may involve Cx43 acting in synergy with genistein and quercetin.

2.4.1 Future Directions:

This study gives rise to several interesting research ideas. First, it would be interesting to transfect MDA-MB-231 cells with Cx43 DNA to observe the growth suppressive effects of Cx43. Secondly, genistein and quercetin upregulate Cx43, but the mechanism is unknown. Connexin genes are not mutated in cancers (reviewed by Chipman et al., 2003) suggesting that cancerous cells may have non-responsive connexin promoter regions or dysfunctional transcriptional activators and inhibitors. To determine if flavonoids stimulate connexin promoter sequences, luciferase could be attached to truncated connexin promoter regions. Luciferase activity could be monitored to determine the target site of flavonoid action. Furthermore, to verify direct interaction between the flavonoid and promoter region, DNA footprinting or pull-down experiments using cell lysates could be performed. (Seoane et al., 2002). If no direct interaction is confirmed, this would suggest Cx43 upregulation through modulation of transcriptional inhibitors and activators. Thirdly, given that genistein and quercetin exert growth suppressive effects, it would be interesting to perform gene array studies to identify the growth regulatory proteins affected by genistein and quercetin. Fourthly, E-cadherin is an intercellular adhesion molecule important for the formation of functional gap junctions. There is also evidence that E-cadherin expression is required to transport Cx43 to the cell membrane (Fujimoto et al., 1997). It would be interesting to transfect MDA-MB-231 cells with E-cadherin, treat the cells with genistein and quercetin, and observe if functional gap junctions form. Increased GJIC could have clinical relevance for breast cancer therapy through improved chemotherapy drug delivery. Fifthly, given that this study involved metastatic human breast cells, it would be interesting to observe if genistein and quercetin affect the invasive and migratory nature of MDA-MB-231 cells.

Matrigel, a basement membrane model derived from a murine tumor (Kleinman et al.,
1986) could be used to measure cell invasion, and a Boyden chamber could be used to
measure cell migration as described by Korah et al. (2000). Lastly, the in vivo effect of
genistein and quercetin in tumor models needs to be explored.

CHAPTER 3

Tissue Microarray Analysis of Cx26, Cx32, Cx43 Expression and its Prognostic Significance in Human Breast Cancer

3.1   Introduction:

Breast cancer is one of the most common malignancies and accounts for more than 15% of all female cancer deaths (Lacey et al., 2002). Current prognostic indicators exist including axillary nodal status, histological grade, and tumor size (Rogers et al., 2002). More recently, estrogen receptor (ER) and progesterone receptor (PR) status (Lamy et al., 2000; Chebil et al., 2003), along with the proliferative marker, Ki67 have been used as prognostic breast cancer indicators (reviewed by Rogers et al., 2002). Histopathological (nodal status, histological tumor grade, tumor size) and immunohistochemical (ER/PR status, Ki67, HER-2/neu) breast cancer markers are important in predicting patient survival and relapse (Andronas et al., 2003; Bouzubar et al., 1989). Furthermore, many treatment decisions rely upon breast cancer markers (Rogers et al., 2003). Current breast cancer treatments include hormone therapy, radiotherapy, chemotherapy, and surgical tumor removal. All these treatments have significant physiological and psychological side effects. Furthermore, they do not ensure a cure. The search for improved treatments and a more complete understanding of cancer behaviour inspires the discovery of new molecular markers.

Connexin proteins traditionally form gap junction pores. It is believed that growth regulatory signals, including hormones and differentiation factors pass through gap junction pores (Fernstrom et al., 2002; Furlan et al., 2001; reviewed by Trosko and Chang, 2001). Impaired GJIC has been associated with tumor phenotype (reviewed by Naus, 2002 and by Carystinos et al., 2001). Additionally, connexin proteins have been

reported to have functions independent of GJIC. Specific to breast cancer, Cx43 and Cx26 were transfected into cultured human breast cancer cells (MDA-MB-435), resulting in improved differentiation and growth regulation capacity (Hirshi et al., 1996). Qin et al. (2002) also transfected human breast cancer cells (MDA-MB-231) with Cx43. The result was suppressed tumor growth independent of GJIC (Qin et al., 2002).

Few studies (Jamieson et al., 1998; Wilgenbus et al., 1992) have observed the presence and distribution of connexin proteins in nontumorigenic and breast tumor tissues. These studies have been compromised by a relatively small sample size. For example, Jamieson et al. (1998) examined the immunocytochemical expression of Cx26 and Cx43 in 11 benign breast lesions, 1 mucoid and 1 lobular carcinoma, and 27 invasive carcinomas of no special histological type. Comparatively, this present study investigated Cx26, Cx32 and Cx43 expression in over 300 human breast tissue samples.

The purpose of this study was to determine if connexin proteins are reliable markers for breast cancer behaviour. After scoring tissue sections for the abundance of connexin proteins, statistical analysis was performed by correlating connexin protein levels with patient survival, tumor grade, tumor size, lymph node status and previously established immunohistochemical markers such as p53, ER/PR status, Ki67 and c-erbB-2 expression. If connexin proteins are found to have a protective role in breast cancer progression then this may lead to new therapeutic methods and a more complete understanding of breast tumor behaviour.

3.2   Materials and Methods:

3.2.1   *Tissue Source and Preparation*:

Paraffin embedded, form fixed tissue blocks were obtained from 481 breast cancer patients at Vancouver General Hospital between 1974 and 1995 for tissue microarray (TMA) construction. The tissue blocks were sectioned and stained with hemotoxylin and eosin (H&E), and then graded according to the Nottingham modification of the Scarth, Bloom Richardson method (Elston and Ellis, 1991) prior to TMA construction. Then representative areas of invasive carcinomas were marked on both the slide and matching paraffin tissue block for TMA construction.

3.2.2   *TMA Construction*:

TMAs were obtained in collaboration with Dr. David Huntsman at the Genetic Pathology Evaluation Center in Vancouver General Hospital, British Columbia. Representative areas of breast carcinomas were selected and marked on the H&E slide. The corresponding tissue block, used to create the H&E slide, was identified and primed for TMA construction. A tissue arraying instrument (Beecher Instruments, Silver Springs, MD) was used to create the TMAs as described by Parker et al. (2002). Briefly, the instrument was used to create holes in the recipient block with defined array coordinates. A solid stylet was used to transfer the tissue cores into the recipient block. Two 0.6mm-diameter tissue cores were taken from each breast cancer case. Three composite high-density TMA blocks were designed, and serial 4μm sections were cut with a Leica microtome and transferred to adhesive-coated slides.

3.2.3 *Immunohistochemistry Protocol*:

Citrate buffer (pH 6.0) was prepared by mixing 18 ml of 0.1 M citrate acid with 82 ml of 0.1 M sodium citrate and bringing the volume to 1 litre with distilled water (dH$_2$O). The citrate buffer was preheated to 60-90°C while the slides were deparaffinized and rehydrated by the following procedure: xylene incubation 3 times for 5 minutes, 100% absolute alcohol incubation twice for 3 minutes, 95% ethanol incubation for 3 minutes, 80% ethanol incubation for 3 minutes, followed by a 2 minute rinse with ddH$_2$0. The slides were immediately placed in the heated citrate buffer for 30 minutes for antigen retrieval. The citrate buffer was then cooled to room temperature for 20 minutes. The slides were removed and then rinsed with phosphate buffered saline (PBS; pH 7.0) 3 times for 5 minutes. In order to block endogenous peroxide activity, the slides were incubated with 3% (v/v) hydrogen peroxide for 30 minutes, followed by 3 times 5 minute rinses with PBS.

The sections were incubated for 30 minutes with 1% bovine serum albumin (w/v) (BSA) in PBS. The blocking solution was drained from the sections and the primary antibodies, diluted with 1% BSA (w/v) in PBS, were incubated overnight in a sealed chamber at 4°C. The primary antibodies used are listed on Table 3.1. To control for antibody specificity, a negative control (no primary antibody) and a positive control (adult rat brain) was used for each connexin protein.

Following overnight incubation, the sections were rinsed with PBS 3 times for 5 minutes, and then incubated in universal biotinylated secondary antibody (Dako, Glostrup, Denmark) for 30 minutes in a sealed chamber at room temperature. Following 3 times 5 minute rinses with PBS, the sections were incubated in strepavidin-horseradish peroxidase reagent (Dako) for 30 minutes in a sealed chamber, and then rinsed again with

PBS. The sections were then stained for 11 minutes with Nova red (Vector Laboratories

Inc., Burlingame, CA), rinsed with dH$_2$O twice for 5 minutes, counterstained with

hematoxylin, and then aggressively washed with tap water. The sections were dipped in

2% sodium bicarbonate for 30 seconds, rinsed with tap water, and then dehydrated

through graded ethanol, xylene, and mounted in resinous mounting medium.

Table 3.1. Antibodies applied to TMAs

Antibody	Clone	Supplier	Dilution
Connexin26	Monoclonal	Zymed	1:250
Connexin32	Polyclonal	Sigma	1:1000
Connexin43	Polyclonal	Sigma	1:2000

Zymed, San Francisco, CA
Sigma, St. Louis, MO
Antibody concentrations were optimized based on staining adult rat brain tissue, a positive control for
Cx26, Cx32, and Cx43

3.2.4 *Scoring of Connexin Immunohistochemical Reaction and Data Analysis*:

TMA sections were scored semiquantitatively based upon the proportion of tumor

cells stained and the staining intensity. The scoring system used for Cx43 and Cx26 was

as follows: 0, negative; 1, weak positive immunoreactivity or strong staining in <10% of

tumor cells; 2, moderate positive immunoreactivity or strong focal reaction in 10%-50%

of tumor cells; 3, strong positive immunoreactivity in >50% of tumor cells (Figure 3.1).

Due to reduced antibody specificity, sections stained with Cx32 were scored as follows:

0, negative; 1, weak positive immunoreactivity or strong staining in <10% of tumor cells;

2, strong positive immunoreactivity in >50% of tumor cells. All samples were evaluated

and scored simultaneously by a pathologist and a graduate student without knowledge of

the patient's outcome information. Only representative tissue cores containing at least

100 tumor cells were scored. In total, over 300 tissue sections were scored each for Cx26,

Cx32, and Cx43.

3.2.5   *Data Analysis*:

All scores were entered into a standardized electronic spreadsheet (Excel for Microsoft Windows, Redmond, WA) as described by Liu etal. (2002). The spreadsheets were then processed by using the software TMA-Deconvoluter 1.06, Cluster and TreeView programs adapted for TMS analysis. The processed score data were then analyzed with SPSS for Windows statistical software (SPSS version 11; SPSS, Chicago, IL).

The Spearman's rho correlation test was used to perform correlation analysis between connexins and tumor grade, lymph node status, tumor size, patient outcome (disease specific and overall survival), Ki67, p53, PR and ER status, c-erbB-2, and between Cx26, Cx32, and Cx43. Results were considered statistically significant when $P < 0.05$. The Kaplan-Meier method was used to perform survival analysis. Statistical significance between survival curves was assessed by the log-rank test.

3.3   **Results**:

3.3.1   *Correlations between connexins and tumor grade, lymph node status, twenty-year patient survival, Ki67, PR and ER status, and c-erbB-2 expression*

There was a significant positive correlation between Cx26 and Cx43 ($P < 0.05$), and between Cx32 and Cx43 ($P < 0.01$). However, there was no significant correlation between the presence of Cx26 and Cx32.

There were no statistically significant correlations between any of the connexin proteins investigated and tumor grade, tumor size, or p53 and c-erbB-2 expression.

However, there was a trend for breast tumors overexpressing c-erbB-2 to have strong Cx32 staining. There was a significant negative correlation ($P < 0.05$) between Cx32 and lymph node status. Also, there was a significant positive correlation between Cx43 and PR expression ($P < 0.01$). Furthermore, both Cx32 and Cx43 correlated positively with ER status ($P < 0.01$). Cx26 protein negatively correlated with ER status ($P < 0.05$).

Importantly, there was a significant negative correlation ($P < 0.01$) between Cx43 protein and Ki67 expression. This latter result suggests that Cx43 is associated with reduced tumor cell proliferation. The above mentioned results are summarized in Table 3.2. No staining pattern (negative, weak, intermediate, strong) of any connexin protein investigated (Cx26, Cx32, Cx43) correlated with patient outcome (Figure 3.2).

Figure 3.1. Images of human breast tumor tissue stained with Cx26, Cx32, and Cx43

antibodies. Breast tumor tissue was sampled from patients at Vancouver Hospital

between 1974 and 1995. Connexin expression was scored semiquantitatively on a scale of

0-3 for Cx26 and Cx43. Due to reduced staining specificity, Cx32 expression was scored

on a scale of 0-2. The details of scoring criteria are stated "Methods and Materials"

section of this chapter under the heading *Scoring of Connexin Immunohistochemical*

Reaction and Data Analysis. Briefly, for Cx26 and Cx43, 0 = negative, 1 = weak positive,

2 = intermediate positive, 3 = strong positive. For Cx32, 0=negative, 1 = intermediate

positive, 2 = strong positive.

Cx26
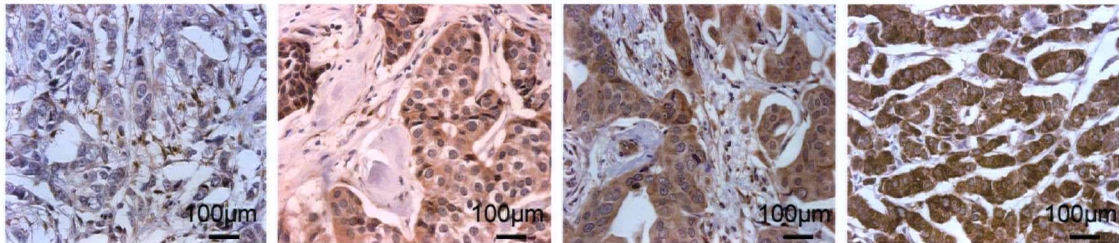
| Negative | Weak positive | Intermediate positive | Strong positive |

Cx32
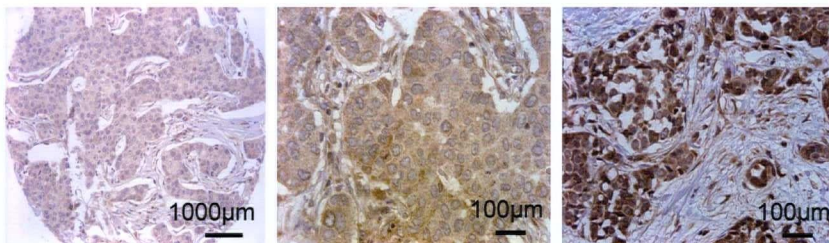
| Negative | Intermediate positive | Strong positive |

Cx43

| Negative | Weak positive | Intermediate positive | Strong positive |

A
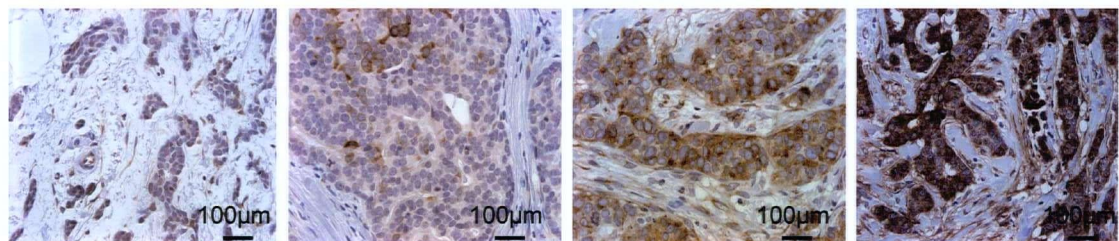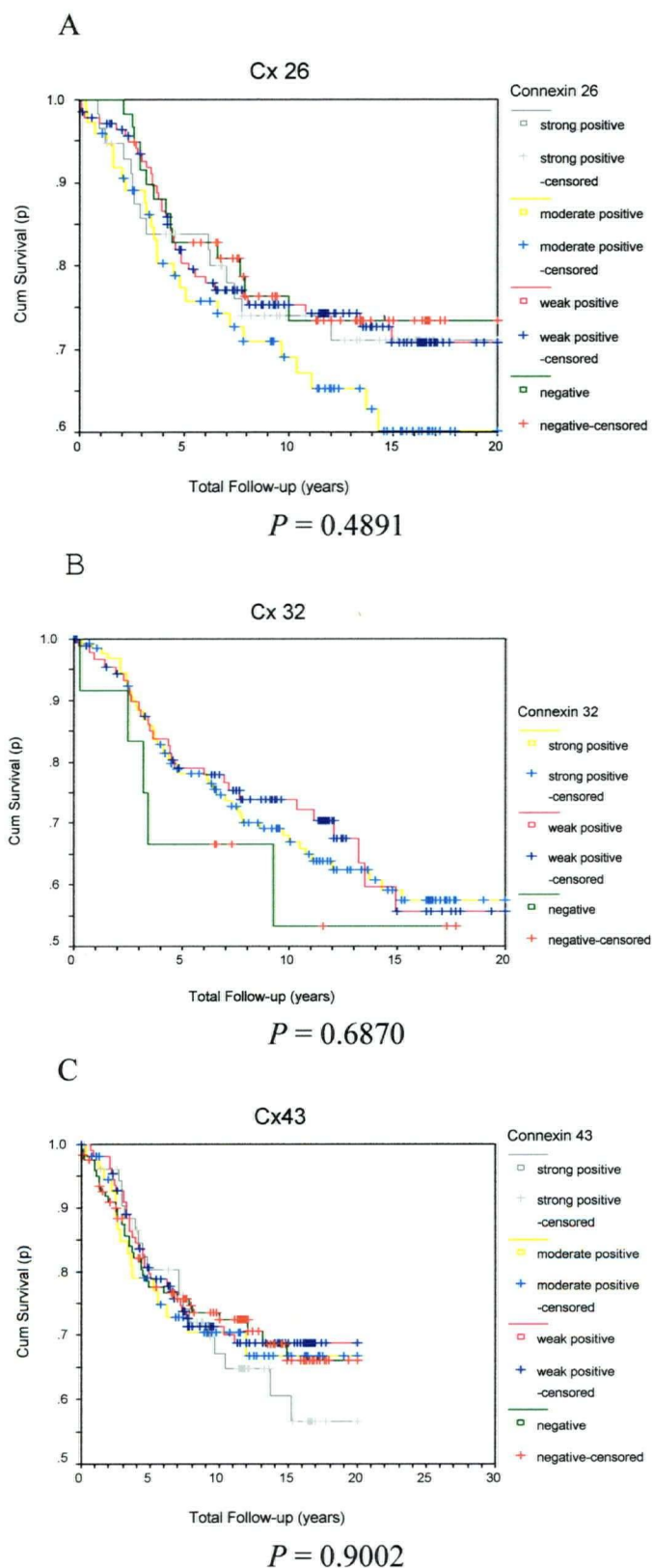
Figure 3.2: Prognostic significance (patient outcome) of Cx26, Cx32, and Cx43 analyzed by the Kaplan-Meier survival analysis, log-rank test. Cx26 (A), Cx32 (B), Cx43 (C).

Table 3.2. Correlations between prognostic indicators for breast tumors

		Connexin26	Connexin32	Connexin43
Connexin26	Correlation Coefficient Sig. (2-tailed) N	1.000 349	0.090 0.188 214	0.134* 0.016 326
Connexin32	Correlation Coefficient Sig. (2-tailed) N	0.090 0.188 214	1.000 251	0.339** 0.000 238
Connexin43	Correlation Coefficient Sig. (2-tailed) N	0.134* 0.016 326	0.339** 0.000 238	1.000 363
PR	Correlation Coefficient Sig. (2-tailed) N	-0.027 0.637 307	0.052 0.437 222	0.169** 0.002 323
Tumor grade	Correlation Coefficient Sig. (2-tailed) N	0.049 0.364 346	0.063 0.322 249	-0.046 0.385 361
Lymph Node Status	Correlation Coefficient Sig. (2-tailed) N	-0.014 0.807 310	-0.136* 0.044 221	-0.030 0.587 320
ER	Correlation Coefficient Sig. (2-tailed) N	-0.129* 0.030 283	0.217** 0.002 204	0.168** 0.004 297
p53	Correlation Coefficient Sig. (2-tailed) N	0.061 0.310 276	0.020 0.784 195	-0.079 0.181 289
Ki67	Correlation Coefficient Sig. (2-tailed) N	0.012 0.845 288	-0.077 0.263 211	-0.191** 0.001 304
Tumor size	Correlation Coefficient Sig. (2-tailed) N	0.001 0.980 298	-0.062 0.374 207	0.058 0.308 306
c-erbB-2	Correlation Coefficient Sig. (2-tailed) N	0.17 0.758 318	0.122 0.066 229	-0.069 0.213 329

**Correlation is significant at the 0.01 level (2-tailed).
*Correlation is significant at the 0.05 level (2-tailed).

3.4   Discussion:

Connexin proteins form hydrophilic protein channels called gap junctions, which directly link neighboring cells, and permit the intercellular passage of cytoplasmic contents (Loewenstein, 1979). GJIC has been hypothesized to regulate growth, differentiation, and apoptosis (reviewed by Trosko and Chang, 2001). It is therefore, not surprising that GJIC has been implicated in tumor initiation and progression (reviewed by Laird et al., 1999). Reduced GJIC can lead to cell heterogeneity and contribute to cell transformation. GJIC has also been inversely correlated with breast cancer metastasis (reviewed by Carystinos et al., 2001).

Connexin proteins can have antitumor functions independent of GJIC. Cx43 was shown to reduce proliferation in human glioblastoma cells without increasing GJIC (Huang et al., 1998). Cx26 and Cx43 have also been shown to reduce growth of human breast cancer cells by disrupting the interaction between fibroblastic growth factor and its receptor (Qin et al., 2002). Furthermore, Cx43 is downregulated at various stages of breast cancer progression including ductal carcinomas in situ, infiltrating ductal carcinomas, and infiltrating lobular carcinomas (Jamieson et al., 1998). These observations have inspired investigation into whether or not connexin proteins are reliable prognostic indicators for breast cancer.

Three connexin proteins have been reported in human breast tissue: Cx43, Cx26, and Cx32 (Pozzi et al., 1995). Cx43 is the most common connexin protein in breast tissue (Jamieson et al., 1998). It is found most frequently in the basal cells, and in myoepithelial cells (Jamieson et al., 1998). Cx26 has been identified between luminal cells in major ducts, and to a lesser extent in alveolar/lobular structures and in breast epithelium (Jamieson et al., 1998; Pozzi et al., 1995). Immunohistochemistry has shown Cx32 in

rodent and human mammary glands (Pozzi et al., 1995). Cx32 has also been observed co-localized with Cx26 in mammary glands of nonpregnant humans (Pozzi et al., 1995). Interestingly, Saunders et al. (2001) reported that a metastatic human breast tumor cell line (MDA-MB-435) expressed Cx32, but that the same cells, transfected with the metastasis suppressor gene, BRMSI did not (Saunders et al., 2001). Although more studies are needed, this suggests that Cx32 contributes to metastatic potential.

Axillary lymph node status is a major indicator of breast cancer stage, and is considered the most important prognostic factor for breast cancer survival (Voordeckers et al., 2004). Furthermore, lymph node status is also considered when deciding on adjuvant therapy (reviewed by Rogers et al., 2002). Currently, surgical biopsy of the axillary region is used to evaluate lymph node status (Ranger and Mokbel, 2003). However, this procedure is burdened by significant immediate and delayed complications such as lymphodema, paraesthesia, pain, and restriction of arm motion (Trifiro et al., 2004).

Tumor size and histological grade are also reliable indicators of breast cancer prognosis. Wieland et al. (2004) reported that 81% of patients with breast tumors less than or equal to 3 cm in diameter survived 5 years compared to 45% of patients with tumors greater than 3 cm in diameter. Guerra et al. (2003) reported that, in females with nonmetastatic invasive ductal carcinoma, histological grade significantly predicted 18-year overall and disease-free survival (Guerra et al., 2003). Furthermore, tumor size, combined with lymph node status, is an accurate indicator of regional relapse (Carter etal., 1989). Regional relapse, itself, predicts survival rates (Wieland et al., 2004). Wieland et al. (2004) reported that the 5 year survival rates of patients with and without regional recurrence were 39% and 87% respectively.

Although less invasive means of measuring these histopathological factors for breast cancer are currently being investigated (Snelling et al., 2004; Ranger and Mokbel, 2003; Catzeddu et al., 2004), there is interest in identifying molecular markers which predict these factors. In this study, Cx32 was the only connexin protein investigated, which showed a favorable, negative correlation with lymph node status. Tumors with relatively high amounts of Cx32 tended to be lymph node negative. Although more studies are needed, this suggests that Cx32 may play a role in preventing the spread of breast cancer to regional lymph nodes. Because lymph node status is a reliable indicator of patient survival, ideally one would expect to observe a favorable correlation between Cx32 protein and patient longevity. However, this was not the case. None of the connexin proteins investigated in this study predicted patient outcome. This lends support to the idea that cancer is a multi-factorial and largely unpredictable disease.

None of the connexin proteins investigated in this study correlated significantly with tumor grade. These results indirectly contrast the findings of Jamieson et al. (1998), who reported that a greater proportion of grade III breast tumors expressed Cx43 and Cx26 compared to grade II breast tumors. However, Jamieson et al. (1998) used 40 breast tissue samples of various histological grades, a relatively small sample size compared to over 300 breast tumor samples used in this present study. Therefore, based on the results of this present TMA study, connexin proteins do not appear to be reliable indicators of breast tumor histological grade.

There was no correlation between Cx26, Cx32, or Cx43 with tumor size in this study. However, Cx43 showed a very strong negative correlation with Ki67. Ki67 is a very reliable indicator of cell proliferation, and its expression in breast cancer samples correlates with mitotic activity, recurrence rates after mastectomy, and survival

(Bouzubar et al., 1989). The strong, negative correlation between Cx43 and proliferative rate bears more significance than the insignificant correlation between Cx43 and tumor size. Although tumor size has been traditionally used to predict prognosis, proliferative rate is a better indicator of the aggressive nature of tumors (Thomssen et al., 2003), and may therefore more reliably predict patient outcome. Although this study suggests that Cx43 protein has a suppressive effect on breast tumor growth rate, more studies are needed to determine whether or not this effect is independent of GJIC.

Cx26, Cx32, and Cx43 were correlated with other immunohistochemical tumor markers such as p53, ER and PR status, and c-erbB-2. p53 is often referred to as the "guardian of the genome" for its ability to halt the cell cycle in the presence of DNA damage (Lane, 2002). Mutations in the p53 tumor suppressor gene are the most common molecular abnormalities observed in human cancers (reviewed by Rogers et al., 2002). Mutated p53 contributes to a loss of cell cycle regulation and has been associated with poor breast cancer prognosis (Bray et al., 1998). None of the connexin proteins investigated in this study correlated with p53 expression.

ER and PR status has traditionally been used to select patients suitable for tamoxifen treatment (Ciacco and Elledge, 2000). Breast cancer patients who are ER and PR-positive tend to have a more favorable nodal and tumor grade status. Furthermore, their tumors tend to proliferate more slowly (Jarvinen et al., 2000). Cx43 and Cx32 showed a strong, positive correlation with ER status. There was also a strong positive correlation between Cx43 and PR status. To our knowledge, this is the first study observing the direct correlation between connexin proteins and ER/PR status in breast cancer tissue. Past research has shown that estrogen-mediated activation of ER-alpha suppresses GJIC and Cx43 expression, resulting in endometrial tumor progression (Saito

et al., 2004). Zhao et al. (1996) observed that progestin reduces transcription of Cx43 in myometrial cells through a mechanism independent of the PR.

c-erbB-2 is an oncogene. It encodes a transmembrane tyrosine kinase growth factor receptor, which is amplified in some breast tumors and is positively related to relapse and poor prognosis (Slamon et al., 1987). None of the connexin proteins investigated in this study significantly correlated with c-erbB-2 expression in human breast tumor tissue. However, high levels of Cx32 protein tended to occur in breast tumors with high expression of c-erbB-2 ($P = 0.066$). This observation is interesting as Pozzi et al. (1995) suggested that Cx32 may contribute to metastasis.

In summary, connexin proteins do not appear to be reliable independent markers for breast cancer prognosis. None of the connexin proteins investigated in this study predicted patient survival or histological tumor grade. However, high levels of Cx32 expression correlated with negative lymph node status. Although more studies are needed, this suggests that Cx32 may protect against the regional spread of breast cancer. Importantly, Cx43 showed a strong, negative correlation with Ki67, a reliable indicator of proliferative activity. Although no causal relationship has been proven, this suggests that Cx43 protein may counteract aggressively proliferating breast cancer. The results of this study have clinical relevance. Cx43 and Cx32 could be introduced into breast cancer patients as an adjuvant therapy to control tumor growth rate and lymph node spread. Future studies observing the influence of connexin proteins on tumor characteristics should differentiate between GJIC effects and the independent function of connexin proteins.

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Figure Appendix 1.1. E-cadherin expression in MDA-MB-231 cells. E-cadherin

immunocytochemistry and Western blot analyses were performed on untreated MDA-

MB-231 cells. Untreated MSTV1-7 cells were used as a positive control for E-cadherin

expression. For immunocytochemistry, cells fixed with 80% methanol for 20 minutes at

-20°C were incubated with a monoclonal E-cadherin antibody (gift from Dr. Roskelley,

Dept of Anatomy and Cell Biology, UBC, Vancouver, Canada; originally obtained from

Transduction Laboratories, NH) diluted 1:500 in 1% BSA in PBS for 1 hour followed by

1 hour incubation with goat anti-mouse Alexa 568 secondary antibody (Molecular

Probes) (1:500). For immunoblot analysis, protein was loaded at 150μg/lane, and probed

with monoclonal E-cadherin antibody (1:15 000) for 1 hour. The blot was then incubated

for 1 hour with horseradish peroxidase and then developed.

MSTV1-7 cells clearly express E-cadherin as observed by Western blot (**A**) and

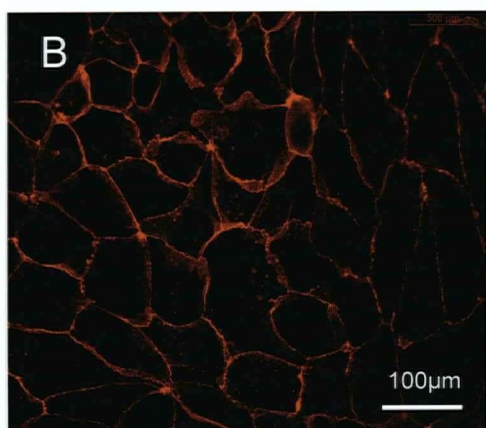immunocytochemistry (**B**) analysis. By contrast, MDA-MB-231 cells do not express E-

cadherin (**C**).
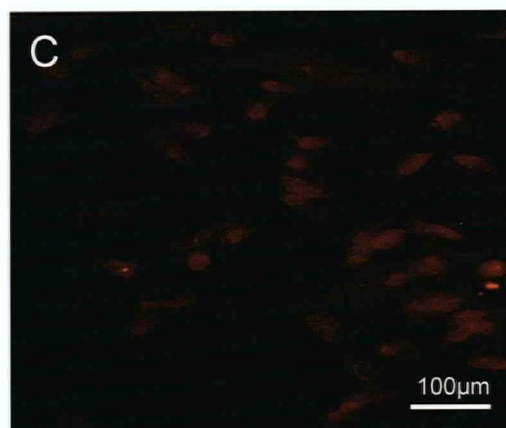
A

E-cad

MSTV1-7 DMSO

MSTV1-7 M

231 M

MSTV1-7 M E-cad

MDA-MB-231 M E-cad

Appendix 2

Genistein and Quercetin Induced Morphological Change in MDA-MB-231 cells

Background:

MDA-MB-231 cells appeared more flat following 72 hours treatment with genistein and quercetin. The purpose of this brief investigation was to determine if F-actin, an important component of the cytoskeletal network (Taylor and Taylor, 1994) was involved in this morphological change.

Materials and Methods:

The treatment protocols and cell fixation procedures are described in chapter 2 of this thesis. Alexa Fluor 488 Phalloidin, dissolved in 1.5ml methanol (Molecular Probes), was used to stain F-actin in MDA-MB-231 cells. The methanol was completely evaporated at room temperature using air circulation prior to being diluted 1:20 in 1% (w/v) BSA/PBS. Cells were incubated in phalloidin for 10 minutes, washed 3 times 10 minutes in PBS, and then mounted with Vectashield mounting medium (Vector Laboratories Inc.)

Results:

DIC and fluorescent microscopy were used to observe cell morphology and the organization of F-actin following treatment of MDA-MB-231 cells with genistein and quercetin. Figure Appendix 2.1 clearly shows that following genistein and quercetin treatment, MDA-MB-231 cells appeared flatter compared to the 0.1% (v/v) DMSO vehicle treated cells (**A-D**). Filamentous actin (F-actin) is an important component of the

cytoskeleton and influences cell shape. F-actin was located at the cell border of nontumorigenic human breast cells, which appeared flat (**E**). Although genistein and quercetin treatments flattened out MDA-MB-231 cells, F-actin did not appear to be the main structure involved. Genistein and quercetin treatments did not change F-actin staining compared to the 0.1% (v/v) DMSO vehicle condition (**F-J**).

Conclusions:

Nontumorigenic breast cells typically appear flat and adhered to one another. It is believed that cell-cell and cell-surface adhesion are important for growth regulation. By contrast, the growth of many cancer cells is anchorage-independent (reviewed by Ben-Ze'ev, 1997). The metastatic human breast cells used in this study (MDA-MB-231) appeared round or dome-shaped when untreated. However, following 72 hours of genistein or quercetin treatment, the MDA-MB-231 cells dramatically flattened out. F-actin is an important component of the cytoskeletal network, which profoundly influences cell shape (reviewed by Jamora and Fuchs, 2002). This brief study showed no change in F-actin structure following genistein or quercetin treatment. Therefore, F-actin does not appear to be the main mechanism responsible for the morphological change observed.

However, F-actin alone does not maintain cell shape. Cell morphology depends on the interaction between many cytoskeletal components including vinculin, alpha and beta-catenin, alpha-actin, and intercellular adhesion proteins such as E-cadherin. Therefore, future studies investigating the morphological effects of genistein and quercetin need to investigate all components of the cytoskeletal network.

Figure Appendix 2.1. Effect of genistein and quercetin on MDA-MB-231 morphology

DMSO

G15

Q2.5

Q5

MSTV1-7 M

DMSO

G2.5

G15

Q2.5

Q5