Cell Density-Dependent Changes in the Localization of the CD26 Protein in Colorectal Cancer Cells in Response to Flavonoid Treatment

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of my thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Colorectal cancer is the 3rd most common cancer worldwide and this rate of incidence is largely attributable to lifestyle factors such as the diet. A group of plant secondary metabolites called flavonoids has been found to exert various anticancer activities in colorectal cancer cell lines and is indeed thought to function similarly *in vivo*. The cell-surface enzymes CD26, CD38, and CD73 are present in colorectal cancers and their presence and activities in this context have the potential to be modulated by extracellular factors such as dietary flavonoids. The levels of CD26 protein in particular have been previously found to increase at the cell surface in HT-29 colorectal cancer cells following treatment with the flavonoid apigenin. Another extracellular factor which may alter the response of cancer cells is cell density. In this work, the role of apigenin was investigated first on the mRNA transcription of CD26, CD38, and CD73 in HT-29 cells cultured to increasing degrees of confluence. CD26 was identified as the most promising target because it revealed the greatest degree of mRNA variability. Next, the amount of CD26 mRNA and protein was quantified in HT-29 cells treated with apigenin and the related flavonoids genistein, kaempferol, and luteolin. Finally, the localization of CD26 protein was observed in these cells following flavonoid treatment. This investigation showed no consistent changes in either mRNA expression or whole cell protein abundance for CD26. However, there was a distinct change in cellular localization of CD26 in response to apigenin and genistein and this was seen particularly in colorectal cancer cells at low levels of confluence. The relocation of the CD26 protein may depend on particular features of the flavonoid structure. Furthermore this effect appears be modulated by a change in cell confluence. Therefore this study provides new insights with respect to the role of flavonoids in regulating CD26 in colorectal cancer cells.

Acknowledgements

I would like to dedicate this thesis to my grandmother Iulia Mocanu. Your selflessness, your endless love and support, and your delicious food helped me make it through graduate school. I cannot express my gratitude enough. I am grateful also to my awesome parents Carmen and Liviu for keeping my spirits high and always pushing me to better myself. Thank you also to my brother Cristian and to my grandfather Emil. Love you guys!

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List of Abbreviations

ADA	Adenosine deaminase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
BCL-2	B-cell lymphoma 2
CDK	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
DAPI	2-(4-amidinophenyl)-1H-indole-6-carboxamidine
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EAAT	Excitatory amino acid transporter
ECM	Extracellular matrix
ERβ	Estrogen receptor β
GLUT-1	Glucose transporter 1

GLUT-2	Glucose transporter 2
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MRP2	Multidrug resistance-associated protein 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
PBS	Phosphate-buffered saline
PBS Ca^{2+}/ Mg^{2+}	Phosphate-buffered saline with calcium and magnesium
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAse	Ribonuclease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGLT1	Sodium-dependent glucose transporter 1
TRPM2	Transient receptor potential cation channel M2

1 Introduction

1.1 Colon Cancer

Each year, over 1.2 million people worldwide are diagnosed with colon cancer and more than 600,000 die from this disease; it is the 3rd most common cancer and the 4th deadliest.[1, 2] Indeed, it accounts for 10% of all cancer-related mortality in Western countries.[2] Colon cancer is more common amongst older men and other risk factors include a family history of colon cancer, the excessive consumption of alcohol and processed meat, obesity, diabetes, smoking, low physical activity, and inflammatory bowel disease.[1, 2] It is particularly concerning that countries with a historically low prevalence of colon cancer are now experiencing an increase in its incidence.[1] This parallels their economic development and their consequent adoption of a Western lifestyle which predisposes them to this disease.[1] Some studies suggest that roughly 37% of colon cancers in women and 71% of those in men are preventable given a shift in lifestyle factors.[3, 4]

1.2 The Dietary Connection

In addition to exercise, hormone replacement therapy, aspirin use, and the surgical removal of precancerous lesions, some studies suggest that a dietary change is a means by which to prevent the onset of colon cancer.[1] Indeed, epidemiological studies demonstrate that fruit and vegetable intake are inversely associated with colon cancer risk.[2, 5] The diet is clearly important when one considers the direct and continuous interactions between dietary constituents and the colonic epithelium. Furthermore, the effects of dietary compounds on colon cancer cells may also exist in other cancers if they were to be exposed after systemic uptake. A subject of growing interest in the fields of colon cancer prevention and therapy is a group of plant secondary metabolites called flavonoids which share a 15-carbon 3-ringed structure.[6, 7]

1.3 Flavonoids

1.3.1 Apigenin

Apigenin (4′,5,7-trihydroxyflavone; Figure 1.1) is a flavonoid commonly found in the leaves of parsley and chamomile flowers.[8]

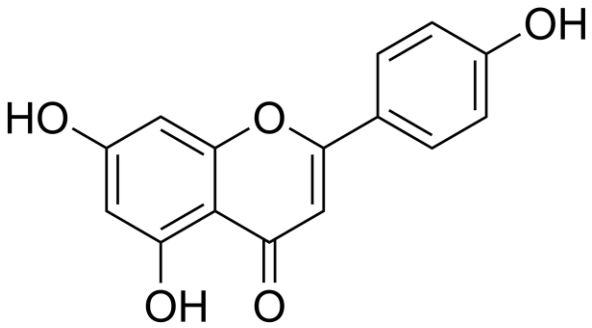


Figure 1.1. The structure of apigenin.[9]

Apigenin is known to inhibit cell proliferation and cell cycle progression in oral squamous, esophageal, pancreatic, gastric, and colorectal cancer cell populations (its targets *in vitro* are presented below in Tables 1.1, 1.2, and 1.3 found in Sections 1.3.7, 1.3.8, and 1.3.9 respectively).[10-14] Lefort and Blay also showed that apigenin induced the upregulation of a cell-surface enzyme called CD26 and increased its anticancer functions.[15] The threshold concentrations required for these effects range between 1-10μM for a 48h treatment which is more readily achievable *in vivo* than the concentrations required to inhibit cell growth or induce apoptosis.[15] Furthermore, apigenin was also shown to potentiate the ability of the chemotherapeutic drug irinotecan to upregulate the CD26 protein by more than 30-fold.[15]

1.3.2 Genistein

Genistein (4',5,7-trihydroxyisoflavone; Figure 1.2) is an isomer of apigenin and can be found in soybeans.[16]

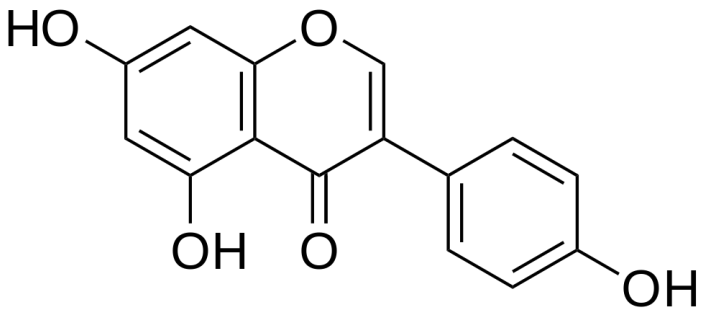


Figure 1.2. The structure of genistein.[17]

Genistein is one of the most common flavonoids in nature and consequently one of the most widely studied.[16] It has been found to inhibit cell growth and induce apoptosis in leukemias and cancers of the breast, prostate, colon, liver, lung, ovary, bladder, and brain.[16] Due to the ubiquity of soy in Asian diets and the status of genistein as the principal anticancer component of soy (its targets *in vitro* are presented below in Tables 1.1, 1.2, and 1.3), the consumption of dietary genistein is suspected to partly account for the differences in tumor incidence between Asian and Western countries.[18, 19] Whilst epidemiologic data on intake of purified genistein is understandably scarce, several studies have suggested that dietary soy intake reduces the risk of colorectal cancer in humans.[20-23]

1.3.3 Kaempferol

Kaempferol (3,4′,5,7-tetrahydroxyflavone; Figure 1.3) is another flavonoid similar in structure to apigenin and luteolin. Kaempferol can be found in broccoli, cabbage, tomatoes, and strawberries.[24]

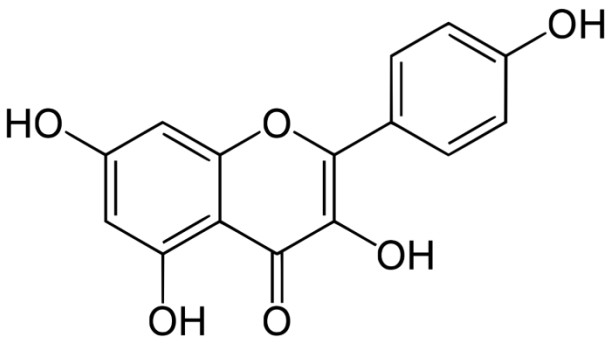


Figure 1.3. The structure of kaempferol.[25]

Like the other flavonoids, kaempferol can induce cell cycle arrest and apoptosis in cancer cells (its targets *in vitro* are presented below in Tables 1.1, 1.2, and 1.3). Being similar in molecular weight and having similar general biological effects to apigenin, it can nonetheless serve as a control substance in studies of CD26 due to its confirmed inability to affect CD26 protein levels or enzyme function within colon cancer cells.[15, 26]

1.3.4 Luteolin

Luteolin (3′,4′,5,7-tetrahydroxyflavone; Figure 1.4) is the primary metabolite of apigenin and as a result its activity within colon epithelial cells is a natural point of interest. Luteolin can be found in celery and in green peppers.[27]

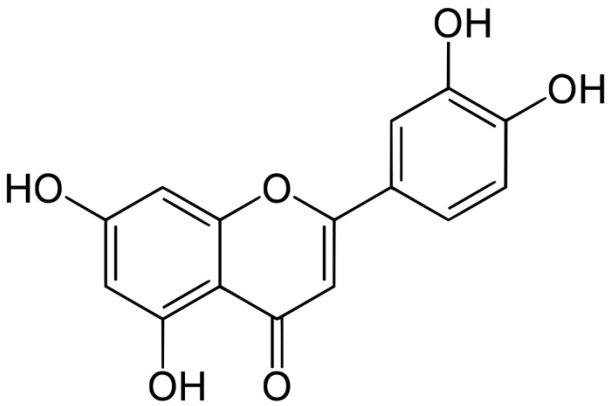


Figure 1.4. The structure of luteolin.[28] Luteolin has also been shown to be associated with cell proliferation, metastasis, and the induction of apoptosis within cancer cells (its targets *in*

vitro are presented below in Tables 1.1, 1.2, and 1.3). Although it can be isolated from blood plasma following a meal, studies have yet to determine whether the consumption of luteolin has a measurable effect on human cancers.[27]

1.3.5 Flavonoids Crossing the Intestinal Epithelium and Entering the Plasma Membrane

Flavonoids are usually found and consumed in the glycoside form which is more water soluble than their aglycone form.[29] Once they reach the small intestine, sodium-dependent glucose transporter 1 (SGLT1) can transfer the glycosides across the apical surface of the epithelial cells.[30] Flavonoid glycosides can also pass through the epithelium using the glucose transporters GLUT-1 and GLUT-2.[29] Cytosolic β-glucosidases can then cleave off this sugar moiety after which the flavonoid agylcone can remain within the cell or enter the systemic circulation.[30] However, this method of absorption is rendered inefficient by the multidrug resistance-associated protein 2 (MRP2) which can export the flavonoid glycoside back into the lumen of the small intestine.[30] Within the lumens of both the small and large intestines, brush border β-glucosidases can cleave the flavonoid aglycone from the glycosylated form after which it can be absorbed by the epithelial cells.[30] Once the aglycone reaches the liver, it can be glucuronidated, sulfated, or undergo oxidative metabolism prior to excretion.[30]

Flavonoid aglycones can also directly enter plasma membranes because they are hydrophobic and therefore tend to accumulate in membranes, especially lipid rafts.[29] The structure of flavonoids determines how extensively they penetrate through the plasma membrane.[31] The polar phosphate groups of the membrane make hydrogen bonds with hydroxyl groups, sugar moieties, and sulfate groups.[31] This may also affect the orientation of the flavonoid and whether it can interact with lipid rafts. Flavonoids may affect the membrane fluidity of

cancer cells to different degrees and this phenotypic effect has been hypothesized to result in different effects on the metastatic properties of cancer cells *in vitro*.[32-34]

1.3.6 The Complexity of Molecular Signaling Due to Flavonoids

Flavonoids modulate a wide variety of signaling pathways within the cytosol of the cell. These govern critical cell functions such as metabolism, cell growth and progression through the cell cycle, apoptosis, autophagy, cell differentiation, and cancer cell metastasis.[11, 35-50] The effects of the flavonoids discussed above on these important cell processes within cancer cells have been summarized in Tables 1.1, 1.2, and 1.3. The signaling due to flavonoids can be complex and initiate branching pathways of regulation. For example, one group found that apigenin binds to ribosomal protein S9, inhibiting its activity in both HT-29 and SW620 colon carcinoma cells.[51] In turn this may affect the transcription of a number of proteins downstream and it raises the possibility that other flavonoids may have the same effect.

1.3.7 Flavonoids and Cancer Cell Cycle Arrest/ Apoptosis

Cancer cells have altered cell cycle dynamics: they experience both an increase in positive growth signaling and a decrease in those signaling pathways which would normally prevent unchecked growth.[52] Cyclins and cyclin-dependent kinases (CDK) are key regulators of cell division, each having a different role depending on the stage of the cell cycle as well as the type of cell in question.[52, 53] They generally form complexes to carry out their functions, which are counteracted by CDK inhibitors such as p21.[53] Apoptosis is a controlled death process normally used to remove unwanted cells from an organism.[54] It can be triggered by a number of pathways, all of which result in the activation of caspase proteins.[54] Caspases are proteases that, when activated, help dismantle a wide variety of cellular structures.[54] Apoptotic events are further modulated by anti-apoptotic proteins such as B-cell lymphoma-2 (BCL-2) and BCL-XL

and pro-apoptotic proteins such as BAX or BAD.[54] The effects of flavonoids on cancer cell cycle arrest and apoptosis and the overall significance of these effects are summarized in Table 1.1 below.

Table 1.1. The effects of flavonoid treatment on cell cycle arrest or apoptosis.

Cell Line Cell Type	Flavonoid Treatment	Effect	Significance	Citation
T24 Bladder Carcinoma	40μM Apigenin	• ↓ Bcl-xl • ↓ Bcl-2 • ↑ Bax • ↑ Bad • ↑ Caspase-3	• Apoptosis via the activation of caspase 3, the downregulation of the anti-apoptotic proteins Bcl-xl and Bcl-2, and the upregulation of the pro-apoptotic proteins Bax and Bad	35
HepG2 Liver Carcinoma	40μM Apigenin	• ↑ Bax • ↓ Bcl-2 • ↑ Cleaved PARP • ↑ Cleaved caspase-9	• Inhibition of autophagy further increased the expression of the apoptotic proteins Bax, cleaved PARP, and cleaved caspase 9 • Inhibition of autophagy further decreased the expression of the anti-apoptotic protein Bcl-2	36
HCT116 Colorectal Carcinoma	50μM Apigenin	• ↓ Pro-caspase 8 • ↓ Pro-caspase 9 • ↓ Pro-caspase 3 • ↑ $p21^{waf1}$ • ↑ p53 • ↓ Cdc25c • ↓ CDK1 • ↓ Cyclin B1	• Cell cycle arrest in G2/M phase via upregulation of p53 and $p21^{waf1}$ and downregulation of Cdc25c, CDK1, and cyclin B1 • Apoptosis via caspase protein activation (cleavage of pro-caspases 3, 8, and 9)	37
MDA-MB-231 Breast Carcinoma	50μM Apigenin	• ↓ Cyclin A • ↓ Cyclin B • ↓ CDK1 • ↑ $p21^{waf1}$	• Cell cycle arrest in G2/M phase via downregulation of cyclin A, cyclin B, and cyclin-dependent kinase-1 (CDK1), as well as upregulation of $p21^{waf1}$	38

KYSE-510 Esophageal Carcinoma	80μM Apigenin	• ↑ p63 • ↑ p73 • ↓ p53 • ↑ Cleaved caspase 3 • ↑ $p21^{waf1}$ • ↓ Cyclin B1	• Upregulation of p63 and p73 protein induced $p21^{waf1}$ transcription and cleavage of caspase 3 • Cell cycle arrest in G2/M phase via $p21^{waf1}$ upregulation and cyclin B1 downregulation • Apoptosis via cleavage of caspase 3	11
HT-29 Colorectal Carcinoma	200μM Genistein	• ↑ Caspase-3 • ↑ Caspase-8 • ↑ Bax • ↓ Bcl-2	• Apoptosis via the activation of caspase 3 and caspase 8, the downregulation of the anti-apoptotic protein Bcl-2, and the upregulation of the pro-apoptotic protein Bax	39
A2780/ CP70 Ovarian Carcinoma	40μM Kaempferol	• ↑ Phosphorylated Chk2 • ↑ Phosphorylated Cdc25c • ↑ p21 • ↑ Phosphorylated CDK1 • ↑ DR5 • ↑ Fas • Increased enzyme activity of caspase-3/7, caspase-8, and caspase-9	• Cell cycle arrest in G2/M phase via Chk2/Cdc25C/Cdc2 and Chk2/p21/Cdc2 pathways • Activation of Chk2 by kaempferol in turn inactivated the G2/M phase- associated proteins Cdc25C and Cdc2 • Apoptosis via the DR5/FADD/Caspase 8 pathway • Upregulation of DR5 increased the activation of Caspase-3/7 and Caspase-8	40
HT-29 Colorectal Carcinoma	60μM Kaempferol	• ↓ CDK2 • ↓ CDK4 • ↓ Cyclin D1 • ↓ Cyclin E • ↓ Cyclin A • ↓ Cyclin B1 • ↓ Cdc25C	• Cell cycle arrest in G1 phase via downregulation of cyclin D1, E, A, and CDK2/4 • Cell cycle arrest in G2/M phase via downregulation of Cdc25C and cyclin B1	41
KYSE-510	80μM Luteolin	• ↑ p63 • ↑ p73 • ↓ p53 • ↑ Cleaved caspase 9	• Upregulation of p63 and p73 protein induced $p21^{waf1}$ transcription and cleavage of caspase 3 and caspase 9 • Luteolin found to be more	11

Esophageal Carcinoma		• ↑ Cleaved caspase 3 • ↑ $p21^{waf1}$ • ↓ Cyclin B1	cytotoxic than apigenin • Cell cycle arrest in G2/M phase via $p21^{waf1}$ upregulation and cyclin B1 downregulation • Apoptosis via cleavage of caspase 3 and caspase 9	

The symbol "↑" indicates an increase in whole cell protein (or the indicated activity), or upregulation, whereas the symbol "↓" indicates a decrease in whole cell protein (or the activity), or downregulation.

Flavonoids are able to induce cell cycle arrest and apoptosis in many cancer cell lines. Cell cycle arrest can be induced at the G1/S checkpoint through the downregulation of cyclins D1, E, and A as well as the downregulation of the cyclin-dependent kinases CDK2 and CDK4.[41] More frequently, however, the cell cycle is arrested at the G2/M checkpoint by the downregulation of cyclin B1 and CDK1, and the altered expression of the cyclin-dependent kinase regulating proteins Cdc25c, p21, and p53.[11, 37, 38, 40, 41] Flavonoids can also trigger apoptosis through the increased expression of the apoptotic proteins Bax and Bad, the decreased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl, and the activation of Caspase-3, -7, -8, and -9.[11, 35-37, 39, 40]

1.3.8 Flavonoids and the PI3K/Akt/mTOR Pathway

The PI3K/Akt/mTOR pathway is involved with a broad range of upstream regulators and downstream targets; therefore it affects a broad range of cellular processes including cell survival, cell cycle progression, apoptosis, autophagy, metabolism, and cancer cell metastasis.[55] It may also be able to modulate the presence of cell surface proteins like CD26, CD38, and CD73 by altering the activity of a number of transcription factors which regulate gene expression.[55] Various components of this pathway, including p-85α, Akt1, Akt2, p-mTOR and p-p70S6K, have been found in colorectal cancer cells compared with normal colonic tissue

belonging to the same patients.[56] The effects of flavonoids on the PI3K/Akt/mTOR pathway and the overall significance of these effects are summarized in Table 1.2 below.

Table 1.2. The effects of flavonoid treatment on the PI3K/Akt/mTOR pathway.

Cell Line / Cell Type	Flavonoid Treatment	Effect	Significance	Citation
HepG2 / Liver Carcinoma	20μM Apigenin	• ↓ Phosphorylated PI3K • ↓ Phosphorylated Akt • ↓ Phosphorylated mTOR • ↑ LC3-I • ↑ LC3-II	• Accumulation of LC3-I and LC3-II proteins indicate that autophagy has been triggered. • Autophagy was found to be a protective response to prevent apoptosis. • Apigenin's anti-proliferative and apoptotic effects were dependent on the inhibition of the PI3K/Akt/mTOR signaling pathway.	36
HCT-116 / Colorectal Carcinoma	50μM Apigenin	• ↓ Phosphorylated Akt but not total Akt • ↓ Phosphorylated p70 S6 kinase • ↓ Phosphorylated 4E-BP1	• Inhibition of Akt/mTOR signaling pathway leads to autophagosome formation • Apigenin induces β-catenin degradation in lysosomes which inhibits Wnt/ β-catenin signaling	42
HEK293 / Human Embryonic Kidney	5μM Genistein	• ↓ Phosphorylated Akt	• Resulted in decrease in phosphorylation of GSK-3β	43
HeLa / Cervical Carcinoma	25μM Genistein	• ↓ Phosphorylated Akt • ↓ Phosphorylated p70 S6 kinase • ↓ Phosphorylated mTOR	• Genistein helped decrease cell proliferation	44

HEK293 Human Embryonic Kidney	10μM Kaempferol	• ↑ Phosphorylated Akt	• No effect on GSK-3β phosphorylation	43
SK-HEP-1 Liver Carcinoma	75μM Kaempferol	• ↓ Phosphorylated Akt • ↓ Phosphorylated mTOR • ↑ LC3-I • ↑ LC3-II	• Inhibition of Akt/mTOR signaling pathway leads to autophagosome formation • Accumulation of LC3-I and LC3 II proteins indicate that autophagy has been triggered.	45
U251MG & U87MG Glioblastoma	20μM Luteolin	• ↓ Phosphorylated IGF-1R • ↓ Phosphorylated Akt • ↓ Phosphorylated mTOR • ↓ MMP-9 • ↓ MMP-2 • ↑ TIMP-1 • ↑ TIMP-2	• Reduction in phosphorylated insulin-like growth factor 1 receptor (IGF-1R) led to downstream reduction in phosphorylated Akt and mTOR • Reduction in phosphorylated Akt and mTOR led to downregulation of metalloproteinase (MMP)-2 and MMP-9 and upregulation of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 • This resulted in the inhibition of cell migration	46
JAR & JEG-3 Placental Carcinoma	20μM Luteolin	• ↓ Phosphorylated Akt • ↓ Phosphorylated p70 S6 kinase • ↓ Phosphorylated mTOR	• Inhibition of PI3K/Akt/MTOR signaling pathway led to inhibition of cell proliferation • Downregulation of mTOR resulted in a decrease in downstream gene transcription	47

The symbol "↑" indicates an increase in whole cell protein (or the indicated activity), or upregulation, whereas the symbol "↓" indicates a decrease in whole cell protein (or the activity), or downregulation.

Flavonoids can affect the PI3K/Akt/mTOR pathway at several points. Flavonoid treatment can prevent the phosphorylation and activation of PI3K, which subsequently inhibits

the phosphorylation and activation of the downstream proteins Akt and mTOR.[36] Alternatively, the inactivation of Akt and mTOR can be induced by the inactivation of IGF-1R.[46] A reduction in mTOR activation results in the decreased phosphorylation of p70 s6 kinase, a transcription factor, and consequently reduced gene transcription and cell proliferation.[42, 44, 47] The inhibition of Akt/mTOR signalling can also trigger autophagic processes as evidenced by increases in the protein levels of LC3-I and LC3-II.[36, 45] Additionally, Akt/mTOR inhibition can also downregulate the metalloproteinases MMP-2 and MMP-9 and upregulate the metalloproteinase inhibitors TIMP-1 and TIMP-2, resulting in decreased cell migration.[46]

1.3.9 Flavonoids and the Wnt Pathway

The Wnt signaling pathway controls embryonic development and regulates functions such as cell proliferation, cell survival, and cell differentiation.[57] The Wnt signaling pathway is one that is often found to be mutated in cancers.[58] For example, colon cancers have been noted to have accumulated nuclear β-catenin as well as mutations in APC, Axin 1, and Axin 2.[57] Indeed, mutations in the Wnt pathway are the cause of 90% of all colon cancers.[59] Although complex, these mutations have been generalized as a general upregulation of oncogenes and downregulation of tumor suppressors found in the Wnt pathway.[58] Additionally, several transcription factors are modulated by the Wnt pathway and in turn these could affect the production of cell-surface proteins such as CD26, CD38, and CD73.[59] The effects of flavonoids on the Wnt pathway and the overall significance of these effects are summarized in Table 1.3 below.

Table 1.3. The effects of flavonoid treatment on the Wnt pathway.

Cell Line Cell Type	Flavonoid Treatment	Effect	Significance	Citation
SW480 Colorectal Carcinoma	20μM Apigenin	• ↓ Nuclear but not cytosolic β-catenin • ↓ Axin2 • ↓ C-myc • ↓ Cyclin D1	• Prevented entry of β-catenin into the cell nucleus • Decreased the expression of the Wnt downstream target genes Axin2, C-myc, Cyclin D1	[48]
HCT116 Colorectal Carcinoma	50μM Apigenin	• ↓ Cytoplasmic and nuclear β-catenin • ↓ Cyclin D1 • ↓ c-Myc • ↓ Axin2 • Induction of autophagosome formation • Reduced cell proliferation	• Inhibition of Wnt/ β-catenin signaling via β-catenin degradation • Inhibition of transcription of the Wnt target genes cyclin D1, c-Myc, and Axin2	[42]
HEK293 Human Embryonic Kidney	5μM Genistein	• ↓ Phosphorylated GSK-3β • ↓ Nuclear β-catenin • Decreased β-catenin/Tcf complex formation • Decreased binding of β-catenin/Tcf complex to DNA • ↓ Axin2 • ↓ C-myc • ↓ Cyclin D1	• Inhibition of Wnt/ β-catenin signaling via decrease in β-catenin\Tcf complex formation and DNA binding • Inhibition of transcription of the Wnt target genes cyclin D1, c-Myc, and Axin2	[43]
HEK293	5μM Kaempferol	• Decreased β-catenin/Tcf complex formation	• Inhibition of Wnt/ β-catenin signaling via decrease in β-catenin\Tcf complex formation and DNA binding	[43]

Human Embryonic Kidney		• Decreased binding of β-catenin/Tcf complex to DNA • ↓ Axin2 • ↓ C-myc • ↓ Cyclin D1	• Inhibition of transcription of the Wnt target genes cyclin D1, c-Myc, and Axin2	
N/A	Apigenin, Luteolin, Kaempferol	• Inhibited GSK-3β activity by binding its docking cavity • IC_{50} Apigenin: 1.91±0.06μM • IC_{50} Luteolin: 1.51±0.05μM • IC_{50} Kaempferol: 3.47±0.11μM	• GSK-3β phosphorylates numerous Wnt pathway molecules, including β-catenin, LRP5, and LRP6	[49]

The symbol "↑" indicates an increase in whole cell protein (or the indicated activity), or upregulation, whereas the symbol "↓" indicates a decrease in whole cell protein (or the activity), or downregulation.

Flavonoids inhibit the Wnt signaling pathway in numerous ways. Primarily, flavonoids act to alter the metabolism of β-catenin. This can be achieved through the increased degradation of whole cell β-catenin, by preventing the entry of cytosolic β-catenin into the nucleus, a decrease in the binding of β-catenin to the TCF transcription factor, and the decreased binding of this complex to DNA.[42, 43, 48] This in turn results in the reduced transcription of the Wnt pathway target genes cyclin D1, c-Myc, and Axin2.[42, 43, 48] Flavonoids may also directly bind a regulator of the Wnt pathway, GSK3β, and inhibit its activity.[49] In these ways flavonoids may be able to diminish the increased proliferative signaling of the Wnt pathway frequently seen in colorectal cancers.[57-59]

1.3.10 Epigenetic Effects of Flavonoids

It is possible that flavonoids affect such a broad range of intercellular signaling pathways because they also act as epigenetic modulators. Flavonoids can alter the enzyme activity as well as the abundance of proteins associated with DNA methylation or histone acetylation.[60] In PC-3 and 22Rv1 human prostate cancer cells, apigenin decreased both the enzyme activity and the protein expression of histone deacetylase proteins (HDAC); consequently this increased the acetylation of histones H3 and H4.[61] Kaempferol in particular inhibits the enzymatic activity of all 11 known human HDAC proteins, which in turn increases the acetylation of histone H3 in HepG2 and Hep3B human hepatoma cells as well as in HCT-116 human colon carcinoma cells.[62] Genistein was found to decrease the enzyme activity and protein levels of DNA methyltransferases (DNMT) and HDACs in the HeLa human cervical cancer cell line.[63] Additionally, genistein decreased the global DNA methylation levels, DNMT activity, and levels of DNMT1 mRNA and protein in MCF-7 and MDA-MB-231 human breast cancer cells.[64]

It is evident that, although they function similarly, the flavonoids can exert different epigenetic effects which may be further modified depending on the specific properties of the cell line being observed. Apigenin and luteolin were both found to reduce the enzyme activity of DNMTs extracted from the esophageal cancer cell line KYSE 510.[65] However, luteolin was the more effective substance, having inhibited over 50% of DNMT activity at a concentration of 50μM.[65] In another study, apigenin decreased the protein expression of DNMT1, DNMT3a, and DNMT3b as well as HDAC 1-8 in mouse skin epidermal JB6 P+ cells.[66] In HCT116 cells, luteolin had a similar function: it also decreased the protein expression of DNMT1, DNMT3a, and DNMT3b but only decreased the expression of HDAC proteins 1, 2, 3, 6, and 7.[67]

Most importantly, flavonoids have been observed to epigenetically regulate the transcription of genes associated with cancer. In MDA-MB-231 breast cancer cells, apigenin induced an increase in $p21^{WAF1/CIP1}$ transcription by significantly inhibiting HDAC activity and inducing histone H3 acetylation.[38] Treatment with genistein was found to increase the DNA methylation of *Sfrp2*, *Sfrp5* and *Wnt5a*, thereby suppressing the expression of their gene products.[68] This was achieved in conjunction with a reduction in histone H3 acetylation at the promoter regions of these genes; this reduced the binding ability of RNA polymerase II.[68] Kaempferol was found to decrease DACT2 methylation by binding DNMT1, and the resultant increase in DACT2 protein inhibited the expression of β-catenin which decreased the proliferation and migration of HCT116, HT-29, and YB5 colorectal cancer cell lines.[50]

Dietary exposure to flavonoids during fetal development may also epigenetically "program" the individual as an adult. Agouti mice exposed to genistein only as prenates had significantly increased erythropoiesis and granulopoiesis as adults, and also experienced a hypermethylation of specific genes.[69] As a result 20% of 21,742 gene products in the bone marrow were found to be differentially expressed.[69]

1.4 Targets of Interest

Described below are three cell surface proteins whose presence and activity within HT-29 colorectal cancer cells may be altered following flavonoid exposure: CD26, CD38, and CD73. The expression of these proteins is found in colorectal cancers and changes upon the development of metastasis, which suggests that they may either impede or advance this process.[70-73] CD26 and CD38 are both highly expressed within the immune system and may exert similar functions within colorectal cancer cells.[74, 75] CD26 and CD73 have opposing

functions in the metabolism of the immunosuppressant adenosine; the former helps degrade it whereas the latter allows for its production.[73, 76] Of these, CD26 is the principal target of interest.

1.4.1 CD26

CD26 (Figure 1.5) is an outward-facing 110kDa transmembrane enzyme that cleaves N-terminal dipeptides in which proline or alanine are in the penultimate position.[77] It is found on T-cells, on the epithelial cells of the kidney, gastrointestinal tract, and bile duct, and its soluble form can be found in the plasma.[74] Its substrates include a number of chemokines implicated in cell regulation, most notably the chemotactic compounds RANTES and CXCL12.[76] By binding adenosine deaminase (ADA), CD26 is indirectly involved in the breakdown of adenosine, a molecule which stimulates colorectal cancer cell growth, increases the migratory response of colon cancer cells to CXCL12 by upregulating its receptor CXCR4, and acts as an immunosuppressant which inhibits the cytotoxic activities of NK and T cells present within the tumor microenvironment.[78-80] Lastly, CD26 degrades the extracellular matrix (ECM) proteins fibronectin and collagen and thereby potentiates cell adhesion, migration, and invasion.[77]

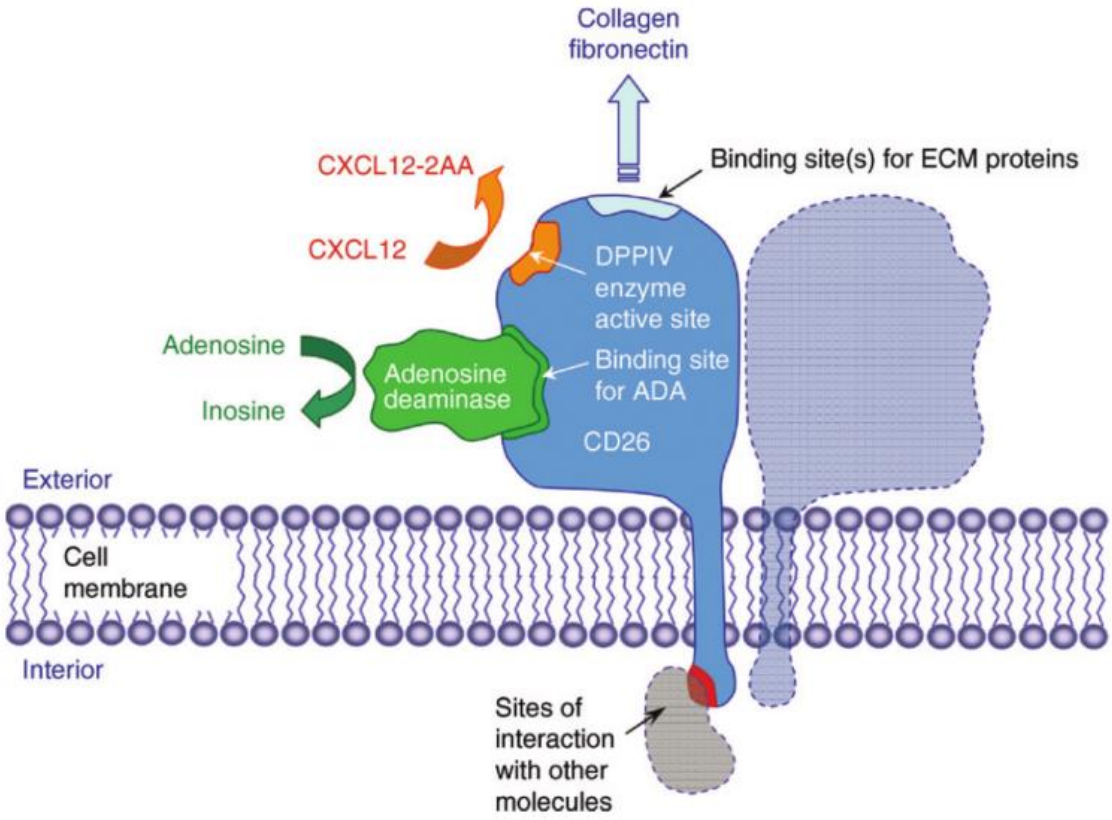


Figure 1.5. Transmembrane CD26 has numerous functions.

Its extracellular domain binds adenosine deaminase, degrades CXCL12, and binds extracellular matrix proteins including collagen and fibronectin. Its intracellular domain interacts with cytosolic proteins.[81]

1.4.1.1 Cytoplasmic Processing of CD26

Certain amino acid residues are important for the folding and transport of CD26. The location of specific prolines in the transmembrane domain of the CD26 peptide confer the appropriate conformation and hydrophobicity to allow the protein to move into the endoplasmic reticulum membrane.[82] Mutating these residues causes the ejection of CD26 peptide from the membrane and its subsequent degradation in the cytosol.[82] CD26 peptide conformation is also important for its leaving the endoplasmic reticulum; substituting Asp599 for Ala599 generated a

conformational change in the protein which increased its retention in the endoplasmic reticulum and subsequent degradation.[83]

CD26 is then processed in the Golgi apparatus before it reaches its destination on the plasma membrane of the cell. In Caco-2 colon cancer cells, it was found that CD26 was glycosylated and formed homodimers in the Golgi apparatus.[84] While glycosylation was not a prerequisite to the formation of homodimers in these cells, it was found that, in Caco-2 cells, CD26 needed to be inside the Golgi for it to dimerize.[84] Results are contradictory, however, depending on the cell type being investigated. One study used Chinese hamster ovary cells transfected with rat CD26 protein: the mutation of the Asn319 residue, an N-glycosylation site on the extracellular part of CD26, to Gln319 prevented the dimerization of CD26.[85] This in turn eliminated the cell surface expression and enzymatic activity of CD26.[85] It was retained in the cytoplasm and its degradation was faster than that of other mutants or the wild-type protein.[85] The mutation of other N-glycosylation sites affected the protein in a similar fashion but to a diminished degree, suggesting that specific N-glycosylation sites are critical to proper CD26 trafficking.[85]

The glycosylation of CD26 also determines the location to which the protein will be distributed within the cell. In Caco-2 cells, it was found that inhibiting O-linked glycosylation induced a shift from CD26 appearing predominantly at the apical surface to being almost 40% basolateral.[86] Inhibiting N-linked glycosylation, however, resulted in CD26 being equally distributed between the apical and basolateral membranes of Caco-2 cells.[86] In HT-29 cells, N-glycosylation was more important for the apical sorting of CD26 although both N-and O-glycosylation were found to be necessary for apical sorting to be effective.[86] Sialylation, a type

of glycosylation, has also been found to control apical targeting: its inhibition in MDCK and Caco-2 cells causes CD26 to be primarily secreted to the basolateral membranes.[87]

The structure of CD26 itself may also carry targeting information that determines the location to which the protein is to be sent. In canine kidney MDCK cells transfected with rat liver CD26, it was found that the extracellular domain of CD26 contains information that allows the protein to be sorted to the apical side whereas the transmembrane and cytoplasmic domains contain basolateral targeting sites.[88]

1.4.1.2 Transporting CD26 to the Plasma Membrane

From the Golgi apparatus, CD26 is transported to the plasma membrane although the exact process by which this is done has yet to be elucidated. In Caco-2 cells, the adenylyl cyclase activator forskolin reduced 50% of CD26 at the apical surface without changing the total amount of CD26.[89] The CD26 was found to accumulate in vesicles containing lamp-1, a lysosomal membrane protein, but not in the Golgi apparatus or Golgi-associated structures.[89] CD26 was also located in these structures in non-treated cells, indicating that they represent a post-transcriptional stage of intercellular transport.[89] Further studies using this cell line showed that forskolin treatment reduced the expression of CD26 and two other proteins decreased throughout the entire cell surface, suggesting that a shared path of transport was affected.[90] Endocytosis was found to be unaffected by this treatment demonstrating that the shuttling of CD26 to the cell surface was all that was affected.[90]

Intercellular signaling may also affect the distribution of CD26. HepG2 hepatocytes were found to require E-cadherin/ β-catenin-associated adherens junctions in order to shift basolateral CD26 to the apical side of the cells.[91]

In the plasma membrane, CD26 binds to cholesterol-rich lipid microdomains, also known as lipid rafts.[86] Glycosylation seems to be necessary here as well; inhibiting N- and O-linked glycosylation almost entirely inhibited this interaction in both HT-29 and Caco-2 cells.[86] Furthermore, inhibiting N- and O-linked glycosylation inhibited to a greater degree the association of CD26 with lipid microdomains in HT-29 than in Caco-2 cells.[86]

The binding of CD26 to lipid rafts seems to have a great effect on its downstream activities and capabilities. In human Jurkat T-cells, the localization of CD26 to lipid rafts was determined to be necessary for downstream signaling events to occur.[92] In the murine bone marrow cell line S17, it was found that purified lipid rafts constituted almost 75% of all CD26 enzymatic activity.[93] Indeed, it was found that the Km of CD26 was an order of magnitude smaller in the cell membranes than in the soluble forms of CD26 in FF18 and GR murine liver cells, SF murine skin cells, and FDC-P1 myeloid precursor cells.[93]

It can be postulated that CD26 interacts with other lipid raft-bound proteins upon stimulation. Indeed, in T-cell lipid rafts, CD26 binds to Caveolin-1, which in turn recruits a complex of other proteins that together induce cell proliferation and NF-kB activation.[94] CD26 has also been found to translocate to the cell nucleus via caveolin-dependent endocytosis.[95] There, CD26 can interact with genes, and this has been seen to lead to the repression of POLR2A which caused a reduction in cell proliferation.[95] These pathways may also exist in some colon cancers: Caveolin-1 mRNA was found in HT-29, HCT-116, and Caco-2 cells but Caveolin-1 protein was only found in HT-29 and HCT-116 cell lines.[96] The presence of Caveolin-1 was found to be proportional to the growth rate of the cells.[96] The expression of Caveolin-1 in HT-29 and DLD-1 colon carcinoma cells reduced the probability of tumor formation *in vivo*.[97]

Furthermore, Caveolin-1 protein levels were found to be reduced in human colon tumor epithelium as compared to normal colon epithelium for 10/15 patients tested.[97]

1.4.1.3 CD26 and Cancer

The relationship between CD26 activity and cancer growth varies depending on the type of cell being investigated. Compared to the normal cells of origin, CD26 expression is decreased in melanoma and hepatocellular carcinoma, variable in lung adenocarcinoma and ovarian carcinoma, and upregulated in prostate and thyroid cancers.[76] The expression of CD26 is also associated with more aggressive T-cell lymphomas.[76] With respect to colon cancer, one study found that CD26 mRNA and protein activity were increased within the colorectal cancer tissue of CRC patients compared to healthy subjects.[98]

The specific roles of both transmembrane and soluble CD26 in colorectal carcinomas are disputed; the molecular functions of CD26 suggest anticancer properties on the cellular level but its tissue distribution hints that it may help rather than hinder metastasis. It has been observed that CRC patients whose tumor tissues expressed high quantities of the CD26 protein had a significantly worse rate of survival than those whose tumors had a low expression of CD26.[99] The same study also found CD26 expression to be positively associated with late TNM stage and poorer differentiation, indicating its relevance to the metastatic state.[99] With regards to cancer metastasis, it was found that the presence of CD26+ cells predicted distant metastasis on follow up in CRC patients.[100] Likewise, CD26+ cells but not CD26- cells led to the development of distant metastases in a mouse model.[100] Lastly, tumor cell migration, invasion, and adhesion to ECM of CD26+ cells were higher than CD26- cells.[100]

In contrast to tissue CD26, there does not seem to be a correlation between plasma CD26 levels and tumor metastasis or patient survival. For instance, one study found that the amount of

plasma CD26 was increased in CRC patients compared to healthy subjects and that it was further increased in patients whose tumors had metastasized.[101] Conversely, another study found that serum CD26 concentrations were significantly lower in CRC patients than in healthy plasma donors.[102] Some studies show that plasma CD26 levels may indicate the occurrence of a metastatic event. Unstable plasma CD26 levels were found in postoperative CRC patients prior to local tumor recurrence; these increased and then decreased.[103] Patients with metastatic disease had elevated plasma CD26 levels compared to patients whose tumors had gone into remission.[103] However, the levels of CD26 protein in the plasma may not correlate with the CD26 enzymatic activity. It was found that CRC patients had lower plasma CD26 activity than did healthy subjects.[98]

It was found that the expression of CD26 protein and its enzyme activity were both correlated with the presence of a differentiated phenotype in HT-29 and Caco-2 cells.[104] In Caco-2 cells, this was found to be the period when the cells were no longer growing, but instead confluent.[104] In HT-29 cells, differentiation was found to occur when they were grown in a glucose-deprived, inosine-supplemented medium.[104]

In HCT116 and HCT-15 colon cancer cell lines, more CD26 mRNA and protein was produced as these cell lines became more confluent.[105] The presence of c-Myc was found to repress CD26 expression whereas the presence of Cdx2 was found to increase it.[105] Furthermore, serum depletion increased CD26 expression.[105]

1.4.2 CD38

The CD38 protein functions as both an enzyme and a receptor.[75] Initially discovered to be a lymphocyte antigen, it is highly expressed within the immune system and is also expressed within the prostatic epithelium, pancreatic islet cells, kidney tubule cells, Purkinje cells, and

astrocytes.[75] CD38 is believed to function when situated in the plasma membrane as well as when it is found in the intracellular and extracellular environments.[75] As an enzyme, CD38 can either mobilize intracellular Ca^{2+} by converting NAD+ into cADPR or induce an influx of extracellular Ca^{2+} by potentiating the activity of the transient receptor potential cation channel M2 (TRPM2).[106] With respect to colorectal carcinomas, it has only been observed that CD38 expression is heterogeneous and that it declines in the later stages of the tumor.[107]

1.4.3 CD73

CD73 is an enzyme involved in the metabolism of extracellular adenosine- it converts adenosine monophosphate (AMP) into adenosine.[73] CD73 is overexpressed in many cancer cell lines, including cancers of the colon, breast, ovaries, stomach, and gallbladder.[108] It is said that the upregulation of CD73 within tumors is an adaptation that helps cancer cells evade immune system surveillance.[109] The production of adenosine by CD73 helps promote tumor growth by providing an immunosuppressive extracellular environment.[110] CD73 levels are also upregulated during hypoxia and it has been hypothesized that CD73 decreases the permeability of the intestinal epithelium.[111] Mice lacking CD73 are more susceptible to colitis and have altered gastrointestinal inflammatory responses.[112] With respect to colon cancer, CD73 is of particular interest because it has been found in significantly higher quantities within metastatic colon tumors than in primary colon tumors or in normal colonic mucosa.[109]

1.5 Culture Cell Density

The population density of cultured cancer cells is an important characteristic which can significantly affect experimental outcomes.[113] Shifts in culture cell density can generate changes in protein and miRNA expression, cell invasiveness, and molecular uptake.

1.5.1 Cell Density and Protein Expression

As cancer cells approach confluence, they encounter more intercellular interactions and junctions and correspondingly less space into which to grow, which has an effect on their transcriptome. In MDA-MB-231 breast cancer cells, it was found that over 2,000 genes had at least a 2-fold difference in expression between 50% and 90% confluent cultures.[114] An increase in cell density has been shown to affect cellular lipid composition and endocytosis as well as specific signalling pathways.[113] These include a decrease in MTOR signalling, a decreased expression of the mature lysosomal protein cathepsin D, and a decrease in the levels of the autophagic proteins p62 and pS6 in a number of cancer cell lines.[113] Of note is that the quantity of abundant proteins like GAPDH and β-actin remains unchanged as cell density increases.[113] This demonstrates that only a subset of cell proteins are affected by cell density, and in particular suggests that these two specific proteins are appropriate choices for loading controls in PCR and immunoblotting experiments.

1.5.2 Cell Density and miRNA Expression

The Hippo pathway is said to mediate cell-density dependent signals that determine the metastatic ability of cancer cells.[115] The proteins YAP and TAZ act as effectors which bind the TEAD transcription factor target genes, translocate into the nucleus and alter gene transcription upon activation by upstream extracellular signalling and cell-cell contact.[115] When cells are subconfluent, nuclear YAP binds p72 which in turn suppresses microRNA expression.[116] Indeed, many human cancers have been found to have decreased miRNA in comparison to their normal tissue of origin.[116]

1.5.3 Cell Density and Invasiveness

Cell growth density has been shown to be an important factor in the invasiveness of cells. Human breast cancer MDA-MB-231 cells at low density were shown to easily invade an endothelial cell monolayer.[117] In contrast, the same cells cultured to confluence had a significantly less invasive phenotype.[117] This effect was found to not be caused by altered cell proliferation or apoptosis rates which could have been induced by the difference in culture confluence.[117] As well, this effect was reversed once the cells were reverted to the low-confluence culture state.[117] The invasiveness of Colo357 pancreatic cancer cells was not affected by cell density, however.[117] In MDA-MB-231 cells, the same effect was seen *in vivo*: cells grown at low density were more readily able to invade to vasculature of zebrafish yolk sacs and also extravasate from the blood vessels of the adult fish into surrounding tissues.[117]

1.5.4 Cell Density and Molecular Uptake

Cell growth density may also be relevant to the uptake of molecules into the cells. In both HT-29 and Caco-2 cell lines, it has been shown that excitatory amino acid transporters (EAATs) localize from the nuclei to the plasma membrane when the cells became confluent.[118] This in turn significantly increased their glutamate uptake and decreased their glutamate secretion, respectively.[118] Curiously, confluent HT-29 cells also saw a reduced passive diffusion of anthracycline drugs across their membranes in comparison to subconfluent cells.[119] This suggests that changes occur within the plasma membrane itself as cells approach the confluent state.

Confluence dependent resistance is an observed phenomenon in cell lines including colon cancers. Confluent HT-29 cells were found to be more resistant to and have a lower intracellular accumulation of doxorubicin, vincristine, etoposide, cisplatin, melphalan, and 5-fluorouracil than subconfluent cells.[120] The levels of p27 have also been found to be increased in confluent HT-29

cells.[121] Furthermore, the transfection of p27 into subconfluent HT-29 cells increases their resistance to cisplatin, doxorubicin, etoposide, and 5-fluorouracil.[121]

1.6 Rationale of the Project

The cell-surface proteins CD26, CD38, and CD73 are present in colon cancers although their specific contributions to the development of these cancers are as yet incompletely known. It may though be possible to modulate their presence and function within colon cancer cells using substances which alter their production, metabolism, or enzymatic activity. This may have an impact on development of the cancer.

One such substance is the dietary flavonoid apigenin, which has been found to increase the levels of CD26 protein on the surface of HT-29 colorectal cancer cells.[15] However, the specific mechanism by which this phenomenon occurs has not been identified. The flavonoids genistein, kaempferol, and luteolin are similar in structure to apigenin and are similarly bioactive in many models of other cancer cell functions. It is therefore likely that they exert the same effects on CD26 as does apigenin. It is possible that apigenin, genistein, kaempferol, and luteolin also affect the levels of CD38 and CD73 in HT-29 cells.

Cancer cell behaviour *in vitro* and *in vivo* is modulated by the extracellular environment. Cancer cell population density in particular is a crucial factor which can give rise to numerous behavioural adaptations. CD26 expression has been shown to be dependent upon cell culture density.

Therefore the purpose of this project was to examine the effects of apigenin, genistein, kaempferol, and luteolin on the levels of CD26, CD38, and CD73 within both confluent and subconfluent cultures of HT-29 colorectal cancer cells.

1.7 Hypothesis

Apigenin and the related flavonoids genistein and luteolin will increase the levels of CD26, CD38 and CD73 in HT-29 colorectal carcinoma cells, and this will be dependent on cell population density (culture confluency).

1.8 Specific Objectives

The objectives of this project were to:

1. Compare changes in CD26, CD38, and CD73 mRNA in HT-29 cells in response to apigenin treatment at different confluence levels, and to decide which of these three components to investigate further.

2. Quantify changes in HT-29 cell viability in response to treatment with increasing concentrations of apigenin, genistein, kaempferol, or luteolin, to exclude changes that may be dependent on toxicity.

… and then having identified CD26 as the preferred candidate and accounted for the possibility of toxicity due to high doses of these flavonoids:

3. Quantify changes in the mRNA and protein expression of CD26 in HT-29 cells treated with apigenin, genistein, kaempferol, or luteolin in both confluent and subconfluent states.

4. Observe changes in the abundance and localization of CD26 protein in HT-29 cells treated with apigenin, genistein, kaempferol, or luteolin.

2 Methods

2.1 Cell Culture

HT-29 colorectal cancer cells were cultured in T-75 flasks (Thermo Scientific) kept at 37ºC and 10% CO_2. HyClone Dulbecco's Modified Eagle Medium (GE Life Sciences) containing 4mM L-glutamine, 4500mg/L glucose, 1mM sodium pyruvate (ThermoFisher Scientific), and 10% newborn calf serum v/v (Fisher Scientific) was used. Cells nearing confluence were passaged using 1mL 0.25% Trypsin-EDTA (Fisher Scientific) for 2min at room temperature to release them from the flasks.

Apigenin (Sigma-Aldrich), genistein (Sigma-Aldrich), kaempferol (Sigma-Aldrich), and luteolin (Sigma-Aldrich) dissolved in DMSO were used to treat the cells. Flavonoid aliquots dissolved in DMSO were stored at -80ºC until the time of treatment, in which culture media was replaced with serum-free medium containing the required dilution of flavonoid. The concentration of DMSO was no greater than 0.4% v/v for every treatment.

2.2 Cell Counting

A Neubauer chamber hemacytometer (Fisher Scientific; 0267110) was used to count cells prior to their being seeded in 6-well plates (Thermo Scientific; 140675). Cells suspended in media were taken into a glass pipette using capillary action and introduced to the hemacytometer to fill it. A Nikon Eclipse TE200 inverted microscope with a 20x objective lens was used to view the cells. The central big square of the Neubauer chamber was used to count the number of cells. Cells on the upper and left-hand boundaries of this square were included into the count whereas cells on the lower and right-hand boundaries were not. Six measurements were made with the hemacytometer being cleaned with water and dried off between each count. The six

measurements were averaged into one value, representative of the number of cells per 0.1µL of cell suspension.

The number of cells seeded per unit area was kept constant at 62,500/cm^2 throughout all experiments conducted. Therefore the number of cells seeded into one well of a 6-well plate, 8-well chamber slide, or 96-well plate was 600,000, 61,250, and 18,263, respectively.

2.3 MTT Assay

A 96-well plate was loaded with a 200µL suspension of cells in media. After 48 hours, the media was replaced with cell media treated with the reagents of interest. Each treatment was used 6 times. After 48 hours of treatment, 20µL of 5mg/mL MTT (Sigma-Aldrich) in phosphate-buffered saline (PBS; see Appendix) was added to each well. The plates were manually shaken and then incubated at 37ºC for 1h. The plates were then inverted such that the media and MTT solution was drained off and 100µL DMSO was added to each well. The plates were then shaken using a Hoefer Red Rotor PR70 platform shaker for 30min at room temperature. The absorbance of the wells at 492nm was measured on a M5 Spectramax spectrophotometer (Molecular Devices).

2.4 RNA Extraction

Upon reaching the desired confluence level, the 6-well plates were placed on ice. The media was aspirated and the flasks rinsed twice with 2mL ice-cold phosphate-buffered saline with calcium and magnesium (PBS Ca^{2+}/ Mg^{2+}; see Appendix). The PBS Ca^{2+}/ Mg^{2+} was discarded and 1.25mL TRI Reagent (Invitrogen) was added to each well. The cells were lysed by pipetting this solution several times within the flasks. The flasks were then left for 5min at room temperature before the contents were transferred to 1.5mL microcentrifuge tubes and 0.25mL chloroform (Sigma-Aldrich) was added to each tube. The tubes were vortexed for 3-5s to mix the

solutions together and then incubated for 5min at room temperature. The tubes were then centrifuged at 12,000 x *g* for 10min at 4ºC using an Eppendorf 5424R microcentrifuge. The aqueous phases containing the RNA were then transferred to a new set of 15mL tubes. 3M potassium acetate (Sigma-Aldrich) consisting of 10% of the volume of the aqueous RNA was added to each tube as well as 625µL of isopropanol. The tubes were inverted several times to mix them before being incubated for 10min at room temperature and subsequently centrifuged at 12,000 x *g* for 15min at 4ºC. The supernatant was removed after which 1.25mL of 75% ethanol was added to the pellet and mixed in by inverting the tubes. The tubes were then centrifuged at 12,000 x *g* for 5min at 4ºC. The supernatant was removed and discarded and the pellet dissolved in 50µL autoclaved Milli-Q® water. The RNA concentration and purity values were then measured using a nanodrop (Thermo Scientific 200c).

2.5 Reverse Transcription

Master mix 1 was composed of volumes of 0.5µg/µL oligo dT (Jena Bioscience), 10mM dNTP (Thermo Scientific), and with diethyl pyrocarbonate (DEPC)-treated water mixed in a 2:5:3 ratio. Each reaction tube was filled with 5µL master mix 1 and 2.5µg of RNA before being diluted with DEPC-treated water until the total volume reached 25µL. The tubes were then placed into the thermocycler (Fisher® Techne Genius #FGENO2TP) and heated to 65ºC for 5min. Afterwards, they were chilled on ice for 15s before 20µL of master mix 2 was added to each tube. Master mix 2 was comprised of a 4:2:1:1 ratio, by volume, of 5x first strand (FS) buffer (Invitrogen), dithiothreitol (DTT) (Invitrogen), RNAse Out™ ribonuclease inhibitor (Invitrogen), and 200 U/µL Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen). The tubes were then put back into the thermocycler, heated to 37ºC for 50min, and

then heated to 70ºC for 15min, after which the product was stored at -80ºC. Negative controls were made using autoclaved Milli-Q® water instead of M-MLV RT.

2.6 Primer Design

DNA primer sets for qPCR were purchased from Invitrogen Canada. The sequences were designed as follows:

1. CD26
    a. Forward: 5'-CGCTCCTTCTCTGAACGCTC-3'
    b. Reverse: 5'-AGCATCATCTGTGCCTTTGTTC-3'
2. CD38
    a. Forward: 5'-ATTCCAGAGACTTATGCCAGA-3'
    b. Reverse: 5'-GCTAAAACAACCACAGCGACT-3'
3. CD73
    a. Forward: 5'-GATGAACGCAACAATGGCAC-3'
    b. Reverse: 5'-GCACGCTATGCTCAAAGGC-3'
4. GAPDH
    a. Forward: 5'-CGACCACTTTGTCAAGCTCA-3'
    b. Reverse: 5'-AGGGGTCTACATGGCAACTG-3'

The primer sets were designed using the National Center for Biotechnology Information (NCBI) PrimerBLAST program after referencing the sequences for human CD26 and GAPDH mRNA using the NCBI Nucleotide database. Primers were selected such that they met as many of the following criteria as possible: the primer pair was separated by at least one intron on the corresponding genomic DNA, each primer spanned 18-25 base pairs long, had a product size

between 80-250 base pairs, a melting temperature (T_m) between 58-61ºC, not more than a 2ºC difference in T_m between the pair, and not more than 3 G or C bases out of the last 5 bases on the 3' end of the primer.

2.7 End-Point PCR

Each end point PCR reaction contained 2.5µg cDNA, 12.5µL GoTaq Green, 0.5µL forward primer, 0.5µL reverse primer, and 9.5µL MilliQ H_2O. A Techne Genius FGEN02TP thermal cycler was used to heat the reactions to 36 cycles of the following: 94ºC for 45s (denaturing), 60ºC for 45s (annealing), and 72ºC for 60s (extension), before a final extension period of 72ºC for 10 min. Next, 5µL of each sample was loaded into a 1% agarose gel. Electrophoresis ran at 95V for 30min after which an Alpha Imager HP machine was used to image the gels. Intermediary troubleshooting steps, including primer and reagent verification, can be seen in Figures 2.1 and 2.2.

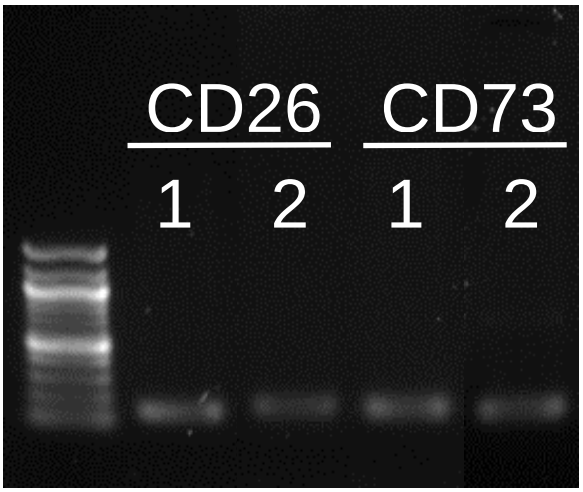


Figure 2.1. Testing different primer sets on a collection of HT-29 RNA using gel PCR.

The lanes labelled "1" and "2" denote two different sets of primers used for CD26 and CD73, respectively. CD26 primer set 1 and CD73 primer set 1 resulted in better band resolution than CD26 primer set 2 and CD73 primer set 2, respectively.

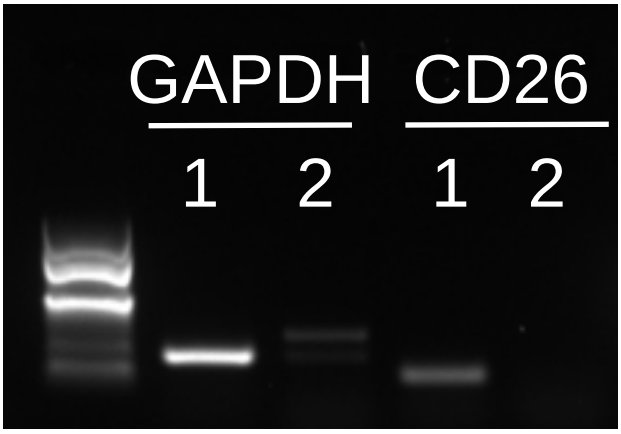


Figure 2.2. Testing different sources of water to be used in the reverse transcription reaction.

The water used to reverse transcribe the cDNA in the lanes labelled "1" was DEPC-treated H_2O and gave clear banding at the appropriate molecular weights according to the ladder. This indicates that the reverse transcription was successfully completed. The water used to reverse transcribe the cDNA in the lanes labelled "2" was MilliQ. It was likely contaminated with RNAses and as such the reverse transcription was not successfully completed. This is evident from the absence of banding seen in the lanes labelled "2".

2.8 Quantitative PCR

First, cDNA was diluted to 5ng/µL in DEPC-treated water. Next, the qPCR reactions were made such that each 20µL reaction contained 5ng cDNA, 10µL SSO Advanced SYBR Green qPCR Mix (Bio-Rad Laboratories), 200nm forward primer, and 200nm reverse primer. A StepOnePlusTM Real-Time PCR System was used to heat the qPCR reactions to 95ºC for 30s to activate the polymerase and then 40 cycles of 15s at 95ºC for denaturation and 60s at 60ºC for annealing and extension. The $\Delta\Delta C_T$ relative quantification method was used to determine changes in the expression of the CD26 gene. LinRegPCR® was used for data analysis and to produce a quantification cycle (C_T) value for each sample, the number of cycles needed to reach the fluorescence threshold. This was then used to determine the N0 value which is relative to the

starting concentration of CD26 cDNA. The N0 values of CD26 in each sample were normalized to N0 values of GAPDH.

2.9 Protein Extraction

The media was aspirated from each well before the cells were washed with ice-cold PBS Ca^{2+}/ Mg^{2+}. Next, the PBS Ca^{2+}/ Mg^{2+} was aspirated and 0.4mL ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (see Appendix) was added to each well. The cells were then incubated for 30min at 4ºC (on ice) and agitated using a Hoefer Scientific Red Rotor #PR70-115V. The lysates were pipetted, collected into 1.5mL microcentrifuge tubes, and vortexed for 10s (Fischer Scientific; 02215370). An Eppendorf 5424R microcentrifuge was used to centrifuge the tubes at 14,000 x *g* for 20min at 4ºC to pellet the membranes. The supernatant was collected and transferred to a fresh microcentrifuge tube before protein concentration was determined using the Bradford assay.

2.10 Bradford Assay

Protein standards of 0, 50, 125, 250, 375, 500, 750, and 100µg/mL were made using bovine serum albumin (Alfa Aesar) in 20% RIPA buffer. A 96-well plate was loaded with 5µL of each standard in triplicate. Protein samples were diluted 5-fold in Milli-Q water and 5µL of each sample was loaded into the plate using a total of 6 replicates per sample. Next, 200µL of Bradford reagent (Bio-Rad Laboratories) were added to each well and mixed by pipetting. The absorbance of the wells at 595nm was measured on a M5 Spectramax spectrophotometer (Molecular Devices). The protein concentrations were calculated using the standard curve given by the absorbance values of the protein standards.

2.11 Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein detection. The protein samples, dissolved in RIPA buffer, were further dissolved into 2X Laemmli sample buffer (see Appendix) such that there were equal proportions of RIPA buffer and 2X Laemmli sample buffer. They were heated at 90ºC for 10min after which 30µL of each sample as well as 5-10µL of protein ladder (FroggaBio) was loaded into the wells. The electrophoresis was then completed using polyacrylamide gels (see Appendix) in running buffer (see Appendix) at 30mA for 3h (Bio-Rad ® PowerPac™ HC 250v/3.0A/300w western blot SDS-PAGE). The proteins were then transferred from the gel onto an Immun-Blot® PVDF membrane (Bio-Rad Laboratories) for 16h at 10V (Bio-Rad Laboratories PowerPac™ HC 250v/3.0A/300w western blot SDS-PAGE) at 4ºC in transfer buffer (see Appendix). The membrane was then blocked by being incubated with 3% BSA in TBS-T (see Appendix) for 1h at room temperature. After the blocking solution was discarded, 10mL of rabbit IgG anti-human CD26 primary antibody (Abcam; ab129060) diluted 1:50,000 in TBS-T with 1% BSA was added to the membrane and incubated for 2h at room temperature. For use as a loading control, 10mL solution of HRP-conjugated mouse IgG anti-β-actin antibody (Abcam) diluted 1:1,000 in TBS-T with 1% BSA was added to the membrane and left to incubate for 2h at room temperature. The membrane was then washed 5 times for 5min each with 20mL TBS-T at room temperature. A 10mL solution of horseradish peroxidase (HRP)-conjugated goat IgG anti-rabbit IgG secondary antibody (ThermoFisher Scientific; 31460) diluted between 1:1,000-1:5,000 in TBS-T with 1% BSA was added to the membrane and left to incubate for 1h at room temperature. The membrane was again washed 5 times for 5min each with 20mL TBS-T at room temperature. Then, 3mL chemiluminescent solution (see Appendix) was added to the membrane for 5min at room temp.

Afterwards, the Kodak Image Station 4000 MM Pro was used to image the proteins on the membrane. Intermediary troubleshooting steps, including antibody verification and protocol optimization, can be seen in Figures 2.3, 2.4, and 2.5.

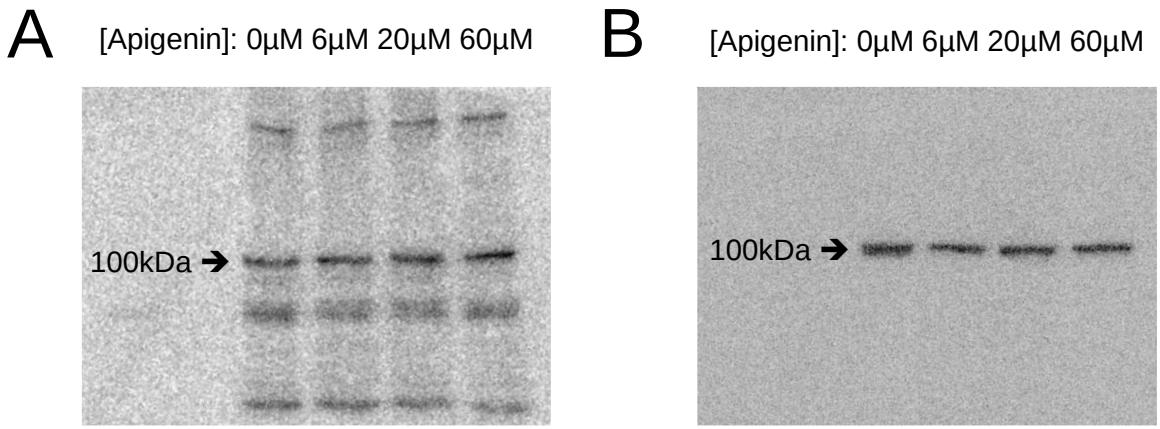


Figure 2.3. Immunoblots comparing two CD26 antibodies.

Protein collected from HT-29 cells treated with vehicle control (0μM), 6μM, 20μM, or 60μM apigenin was transferred onto PVDF membranes and immunoblotted with two different rabbit IgG anti-human CD26 primary antibodies. The antibody used in panel A (Abcam; ab28340) shows nonspecific binding whereas the antibody used in panel B (Abcam; ab129060) does not. The 100kDa protein marker has been noted on the image to indicate the expected location of CD26 banding according to its molecular weight.

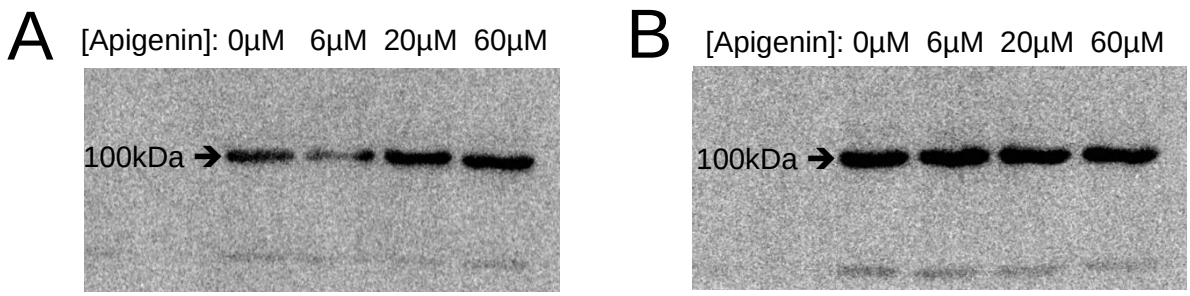


Figure 2.4. Comparing two different wet transfer protocols.

Protein collected from HT-29 cells treated with vehicle control (0μM), 6μM, 20μM, or 60μM apigenin was transferred onto PVDF membranes using two different protocols. The proteins in panel A were transferred at 100V for 1h whereas the proteins in panel B were transferred at 10V for 16h. The membranes were immunoblotted with the same rabbit IgG anti-human CD26

primary antibody (Abcam; ab129060). The transferring protocol used in panel B displayed better protein resolution. The 100kDa protein marker has been noted on the image to indicate the expected location of CD26 banding according to its molecular weight.

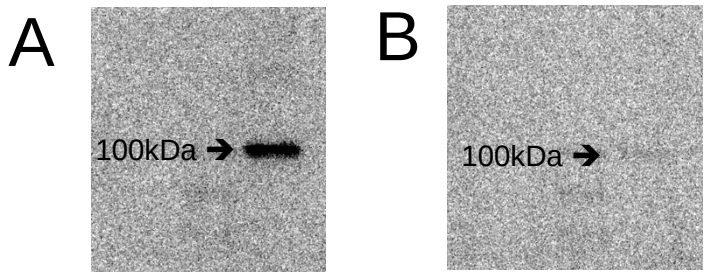


Figure 2.5. Confirming that the antibody selected for experimental work binds the CD26 protein.

Purified CD26 protein (Abcam) was transferred onto PVDF membranes. The membrane in panel A was immunoblotted with a rabbit IgG anti-human CD26 primary antibody (Abcam; ab129060), whereas the membrane in panel B was immunoblotted with the same antibody in combination with a CD26 peptide (Abcam). The antibody in panel A bound the purified CD26 protein in the membrane. However, the antibody in panel B bound the CD26 peptide but not the CD26 protein in the membrane, confirming that this antibody specifically binds CD26. The 100kDa protein marker has been noted on the image to indicate the expected location of CD26 banding according to its molecular weight.

2.12 Immunofluorescent Staining

Cells were grown in 8-chamber slides (SPL Life Sciences). The plates were placed on ice and the media was aspirated. Each well was washed with 200µL cold PBS Ca^{2+}/ Mg^{2+}. The wash was aspirated and the cells fixed with freshly prepared 1% paraformaldehyde (EMD Millipore; see Appendix) in PBS Ca^{2+}/ Mg^{2+} for 10min at room temperature. The cells were then washed three times with PBS Ca^{2+}/ Mg^{2+}. The cells were blocked using 3% BSA (Alfa Aesar) v/v in 200µL PBS Ca^{2+}/ Mg^{2+} per well for 30min at room temperature. This was aspirated and 200µL rabbit IgG anti-human CD26 primary antibody (Abcam; ab28340) in PBS Ca^{2+}/ Mg^{2+} with 1mg/mL BSA was added to each well. The chamber slides were incubated in a humidified

chamber for 1.5h at room temperature. The primary antibody solution was aspirated and the wells washed three times with 200µL PBS Ca^{2+}/ Mg^{2+} containing 1mg/mL BSA. Each wash was performed on a rotary platform mixer (Hoefer Red Rotor PR70) at low speed for 5min at room temperature. Next, 200µL goat IgG anti-rabbit IgG secondary antibody conjugated to green AlexaFluor 488 (Life Technologies) in PBS Ca^{2+}/ Mg^{2+} with 1mg/mL BSA was added to each well. The chamber slides were again incubated in a humidified chamber for 1h at room temperature. The secondary antibody solution was aspirated and the wells washed three times with 200µL PBS Ca^{2+}/ Mg^{2+} containing 1mg/mL BSA. Each wash was performed on a rotary platform mixer at low speed for 10min at room temperature. This was aspirated and the wells washed three times with 200µL cold PBS Ca^{2+}/ Mg^{2+} per well. After aspirating this wash, cells were treated with either CellMask Deep Red (ThermoFisher Scientific) to stain the plasma membrane or Fluoroshield aqueous gel mountant containing 1 µg/mL 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (Sigma-Aldrich) before the coverslip was added. With the assistance of Dr. Marianna Foldvari and Dr. Roger Chen, fluorescence was visualized using a Zeiss LSM710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) using Ar-laser (488nm) and HeNe-laser (633nm) lines and either the Plan-Apochromat 20x/0.80 dry objective or the 63x/1.40 oil immersion objective. A 'no treatment' sample was used to confirm gain and pinhole settings to exclude noise and autofluorescence background for the subsequent treatment samples. Images were captured and processed using the Zen 2009 software. A Leica DM2000 microscope with a 100x oil immersion objective was also used to visualize cells before capturing and processing images with the QCapture Pro 7 software.

2.13 Statistical Analysis

GraphPad Prism 5 software was used to analyze the data collected. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test were used to analyze the data obtained from the MTT experiments. Two-way ANOVA followed by Bonferroni's multiple comparisons test were used to analyze the data obtained from the qPCR and western immunoblotting experiments. A value of $p<0.05$ was chosen as the threshold for statistical significance.

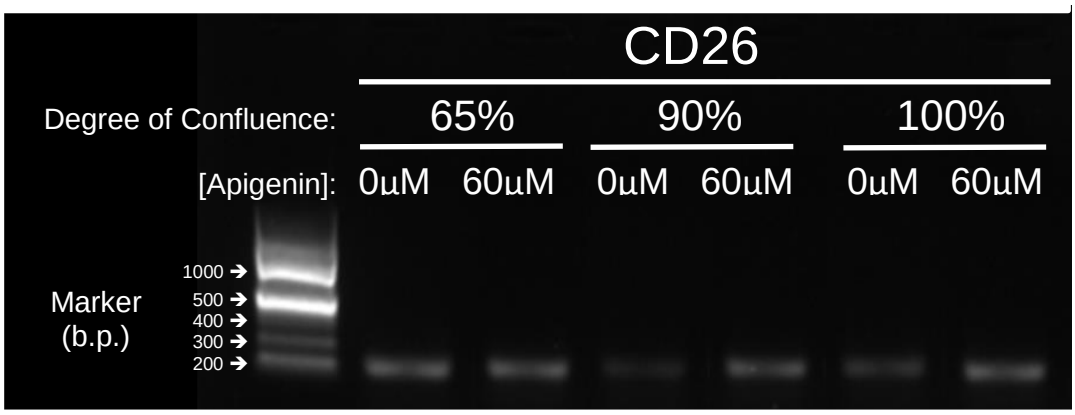
3 Results

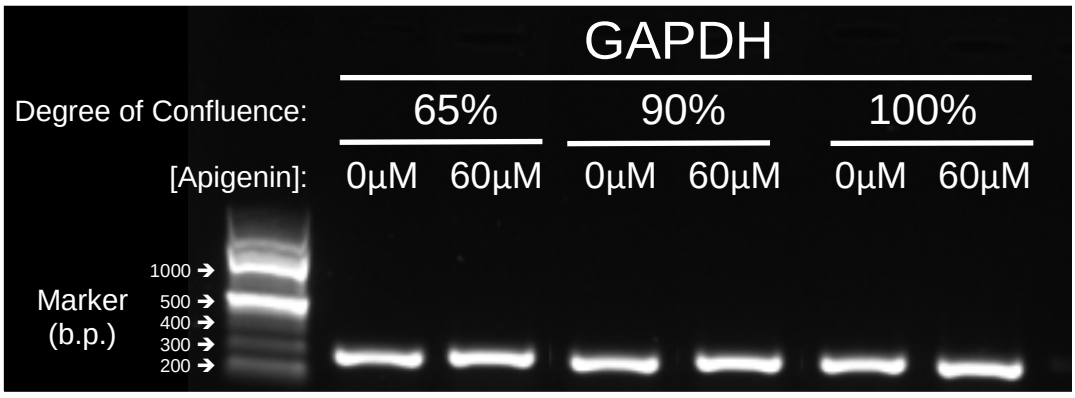
3.1 Evaluating Changes in the Levels of CD26, CD38, and CD73 mRNA in Response to Apigenin Treatment at Different Cell Confluence Levels

The potential effects of the flavonoid apigenin on the mRNA expression for the enzymes CD26, CD38, and CD73 were investigated in HT-29 cells. To account for the possibility that cell confluence level might influence the transcription of these genes and their response to flavonoid, HT-29 cells were treated with apigenin for 24h such that the mRNA could be collected from cells when the population was at 65%, 90%, or 100% confluence. End-point PCR was used in conjunction with agarose gel electrophoresis as a method by which differences in gene transcription could be rapidly identified.

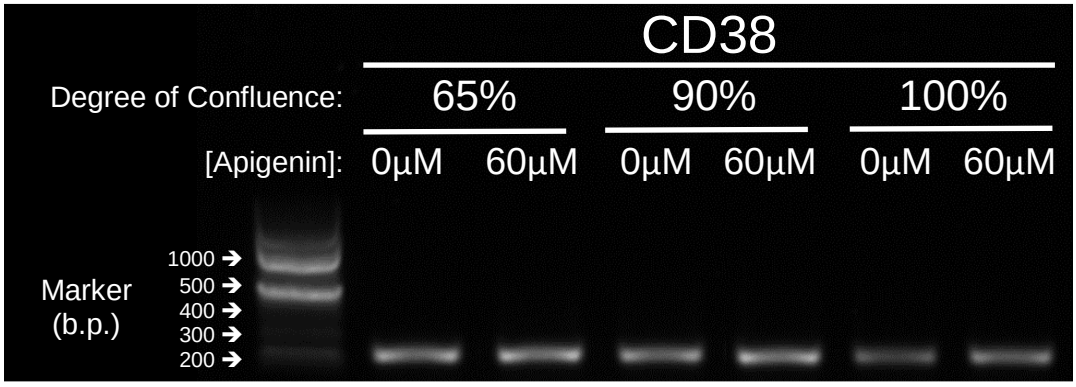
The results are shown in Figure 3.1. Based on indications from the gel band intensities, there was strong suggestion of an upregulation of CD26 mRNA levels in apigenin-treated cells harvested at 90% and 100% confluence but not in those harvested at 60% confluence (Figure 3.1A). There might also be a slight upregulation of CD38 mRNA in apigenin-treated cells collected only at 100% confluence (Figure 3.1B), however this change if present was too minor to be likely to be biologically relevant and was therefore not investigated further. There was no sign of a change in CD73 mRNA due to apigenin treatment at any confluence level or time of mRNA collection (Figure 3.1C). It was therefore decided not to further investigate potential changes in CD38 or CD73 in response to flavonoids, of which apigenin is the main example. CD26 was chosen as the focus of subsequent experimental work.

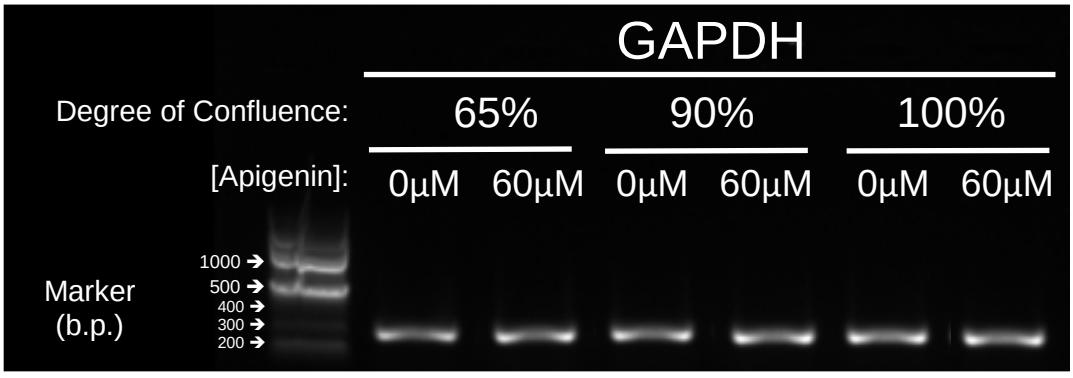
A


CD26
Degree of Confluence:
65%
90%
100%
[Apigenin]:
0μM 60μM 0μM 60μM 0μM 60μM
Marker (b.p.)
1000
500
400
300
200


GAPDH
Degree of Confluence:
65%
90%
100%
[Apigenin]:
0μM 60μM 0μM 60μM 0μM 60μM
Marker (b.p.)
1000
500
400
300
200

B


CD38
Degree of Confluence:
65%
90%
100%
[Apigenin]:
0μM 60μM 0μM 60μM 0μM 60μM
Marker (b.p.)
1000
500
400
300
200


GAPDH
Degree of Confluence:
65%
90%
100%
[Apigenin]:
0μM 60μM 0μM 60μM 0μM 60μM
Marker (b.p.)
1000
500
400
300
200

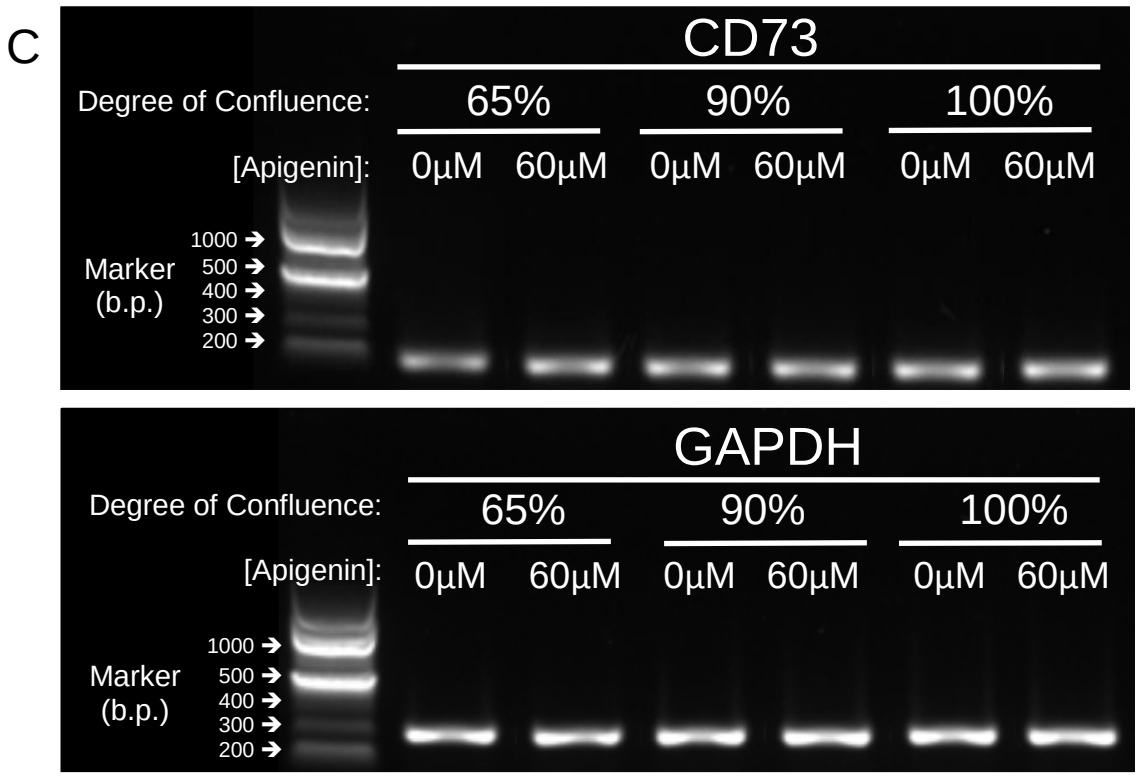


Figure 3.1. The effects of apigenin treatment on the amounts of CD26, CD38, and CD73 mRNA in HT-29 cells collected at different confluence levels.

HT-29 cells were treated with vehicle control (0μM) or 60μM apigenin for 24h. Three groups of cells were treated such that mRNA was collected from cells at approximately 65%, 90%, or 100% confluent densities, respectively. mRNA reverse transcribed to cDNA was used for these gel electrophoresis experiments. Then the amounts of (A) CD26, (B) CD38, and (C) CD73 cDNA in each sample were visualized. The first lane in each image shows a DNA ladder, and the number of base pairs (b.p.) in each band has been marked with arrows. CD26 and CD73 amplicon lengths were approximately 150 b.p. long, and as such the bands can be seen slightly below the 200 b.p. band on the DNA ladder. CD38 amplicon lengths were approximately 200 b.p. long, and as such the bands can be seen adjacent to the 200 b.p. band on the DNA ladder. GAPDH loading control amplicon lengths were approximately 230 b.p. long, and as such the bands can be seen slightly above the 200 b.p. band on the DNA ladder. Images are representative of three experimental replicates.

3.2 Assessing Flavonoid Cytotoxicity Using a MTT Assay

I now added three flavonoids structurally and functionally related to apigenin, namely genistein, kaempferol, and luteolin, to this study to investigate the potential effects of all four related flavonoids on the abundance, expression, and localization of CD26 as a single marker.

The potential cytotoxicity of each flavonoid within HT-29 cells was investigated using MTT assays so as to determine the maximal flavonoid concentrations would not induce cytotoxicity. HT-29 cells were treated with each of these flavonoids for 24h. Based on data from the MTT assay, only luteolin treatments significantly changed cell viability (Figure 3.2). Apigenin, genistein and kaempferol were without any effect at concentrations up to 100μM (Figure 3.2 panels A-C).

The effect of luteolin was unexpectedly biphasic (Figure 3.2D). Treatments of 3μM and 10μM luteolin increased cell growth, by approximately 11% and 13%, respectively, whereas 100μM luteolin decreased cell viability by approximately 20% (Figure 3.2).

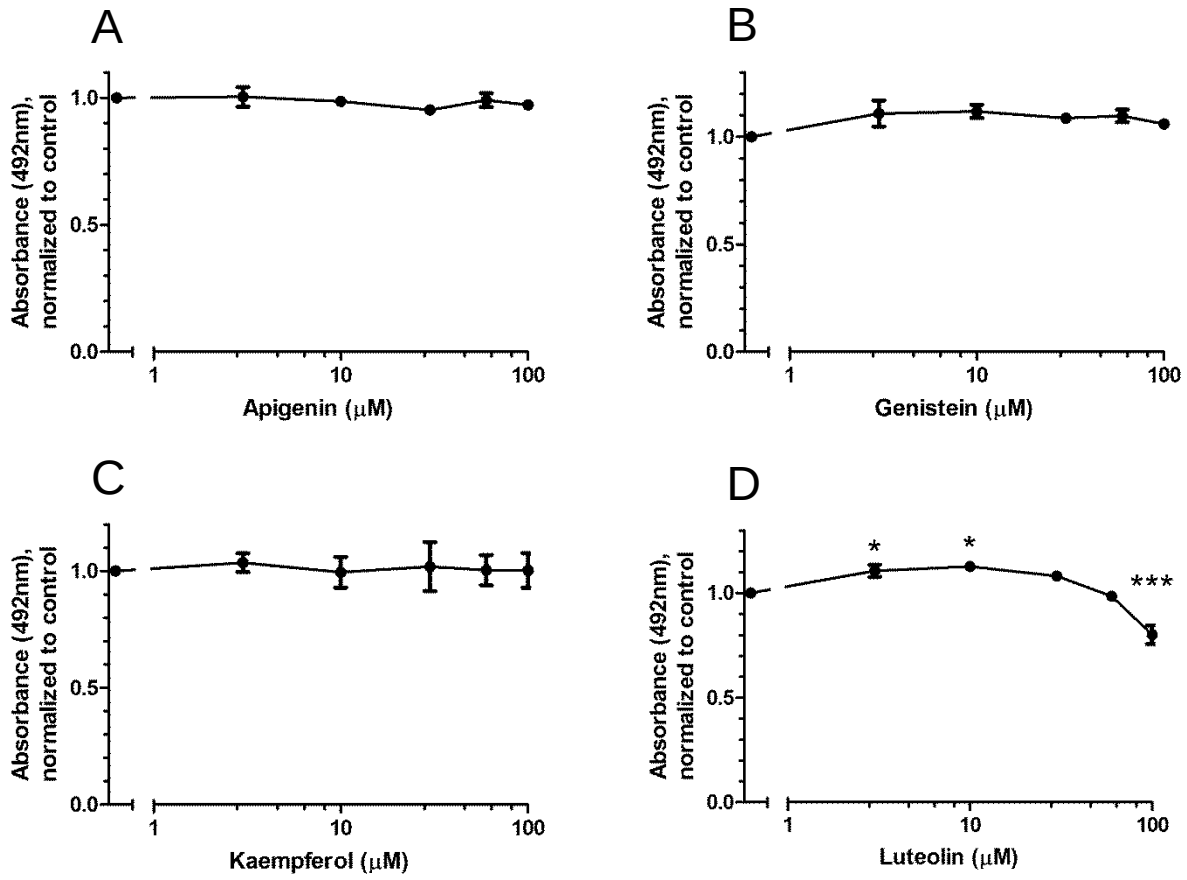


Figure 3.2: Cell viability after flavonoid treatment as measured by an MTT assay.

HT-29 cells were treated with (A) apigenin, (B) genistein, (C) kaempferol, or (D) luteolin for 24h at the indicated concentrations. Cell viability was measured using an MTT assay in which each reaction was performed in six replicates. Percent maximal absorbance was calculated by dividing the absorbance of each experimental sample by the average absorbance of the control samples. The data are means ± SEM. Each graph represents three independent experiments. Statistical analysis was done using one-way ANOVA with Dunnett's multiple comparisons test. 3μM and 10μM luteolin significantly increased cell growth, whereas 100μM luteolin significantly decreased cell viability. * $p < 0.05$, *** $p < 0.001$

3.3 Measurements of the Transcription of CD26 mRNA in Response to Flavonoids

Possible changes in the transcription of CD26 mRNA were further explored and quantified using qPCR. Based upon the prior findings with end-point PCR (Figure 3.1A) I allowed for the possibility of dependence on cell population density in these experiments.

HT-29 cells were grown to less than 60% (low) confluent density (no response expected, Figure 3.1A) or to 90-100% (high) confluence (response expected, Figure 3.1A) before being treated with vehicle control (0μM), 30μM or 60μM of apigenin, genistein, kaempferol or luteolin for 24h.

The effects of each flavonoid on the mRNA of CD26 in HT-29 cells treated at these low or high confluence levels are shown in Figures 3.3, 3.4, 3.5, and 3.6. Despite significant differences in average values (e.g. 60μM apigenin at high confluence, +55%, 60μM luteolin at low confluence, +110%) there were no statistically significant changes with respect to the transcription of CD26 mRNA following treatment with either concentration of any of the four related flavonoids at either confluence level. It may be that statistical significance was difficult to attain due to the potential variation from environmental factors such as population density (see further comments in Discussion).

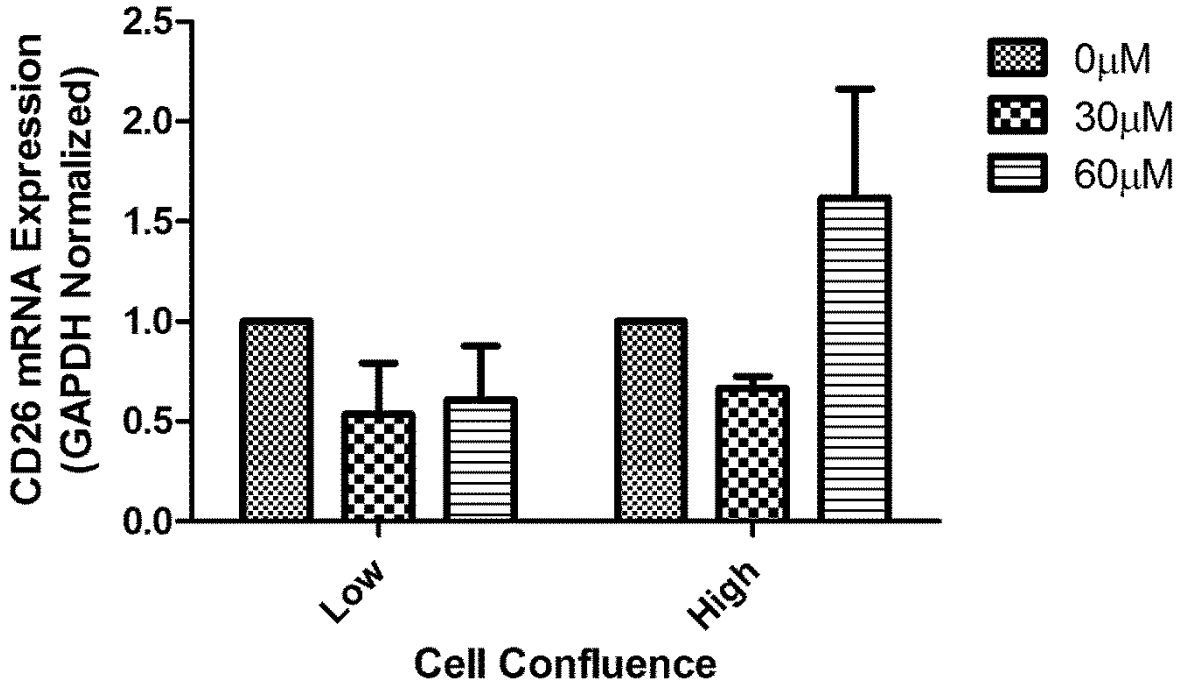


Figure 3.3 CD26 mRNA expression in HT-29 cells treated with apigenin at different confluence levels.

HT-29 cells were treated at low or high confluence with apigenin for 24h at the indicated concentrations. mRNA reverse transcribed to cDNA was used in triplicate reactions for each independent experiment. CD26 mRNA data was first normalized to the levels of GAPDH mRNA found in each sample before the ΔΔCt method was used to quantify the fold difference in CD26 mRNA expression in each sample relative to the untreated control. Data is presented as mean ± SEM from three independent experiments. Statistical analysis was done using two-way ANOVA with Bonferroni's post-test used to compare the replicate means.

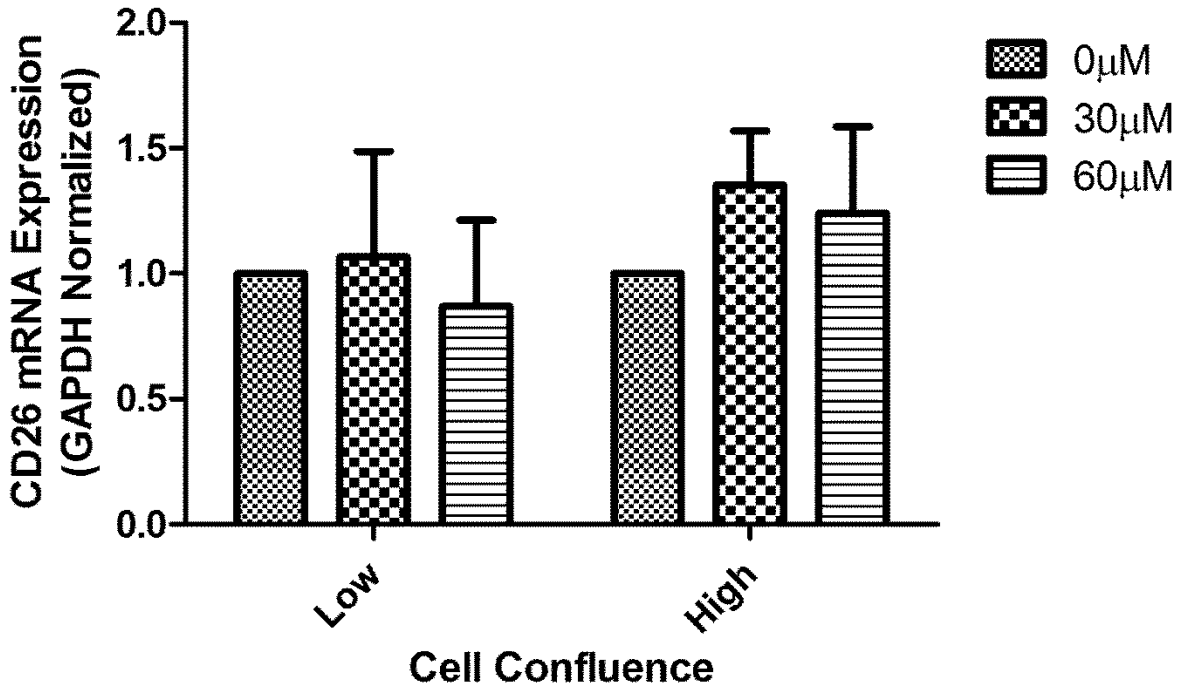


Figure 3.4 CD26 mRNA expression in HT-29 cells treated with genistein at different confluence levels.

HT-29 cells were treated at low or high confluence with genistein for 24h at the indicated concentrations. mRNA reverse transcribed to cDNA was used in triplicate reactions for each independent experiment. CD26 mRNA data was first normalized to the levels of GAPDH mRNA found in each sample before the ΔΔCt method was used to quantify the fold difference in CD26 mRNA expression in each sample relative to the untreated control. Data is presented as mean ± SEM from three independent experiments. Statistical analysis was done using two-way ANOVA with Bonferroni's post-test used to compare the replicate means.

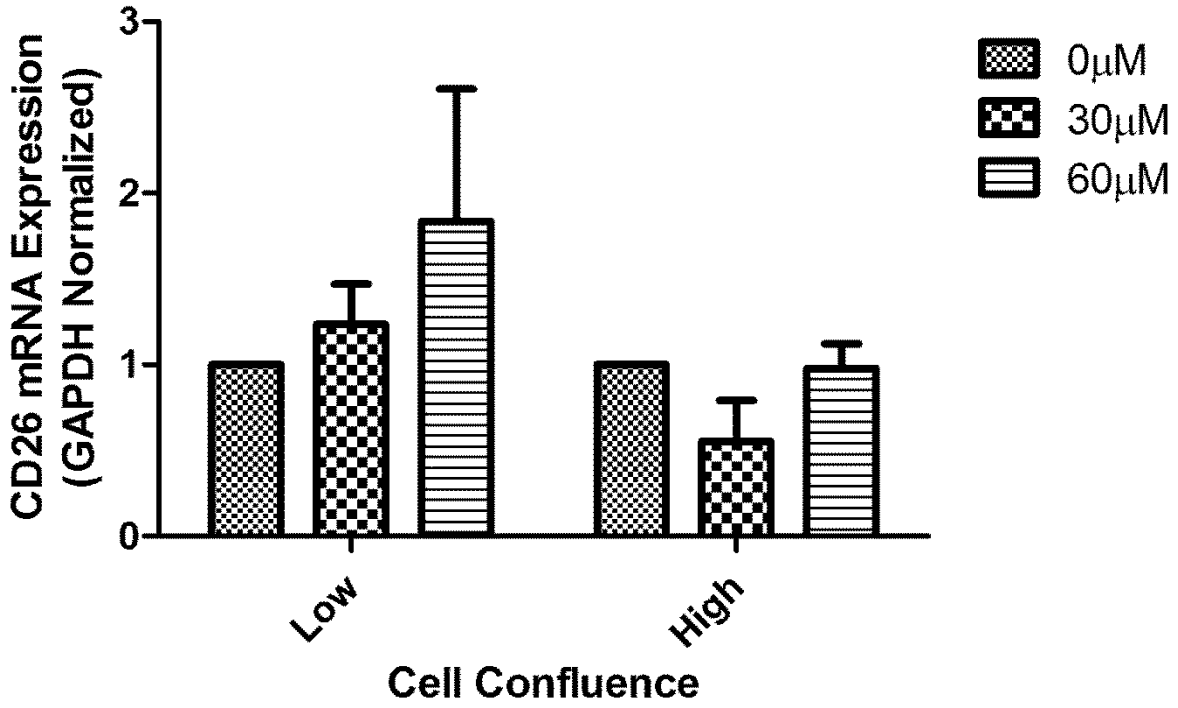


Figure 3.5 CD26 mRNA expression in HT-29 cells treated with kaempferol at different confluence levels.

HT-29 cells were treated at low or high confluence with kaempferol for 24h at the indicated concentrations. mRNA reverse transcribed to cDNA was used in triplicate reactions for each independent experiment. CD26 mRNA data was first normalized to the levels of GAPDH mRNA found in each sample before the ΔΔCt method was used to quantify the fold difference in CD26 mRNA expression in each sample relative to the untreated control. Data is presented as mean ± SEM from three independent experiments. Statistical analysis was done using two-way ANOVA with Bonferroni's post-test used to compare the replicate means.

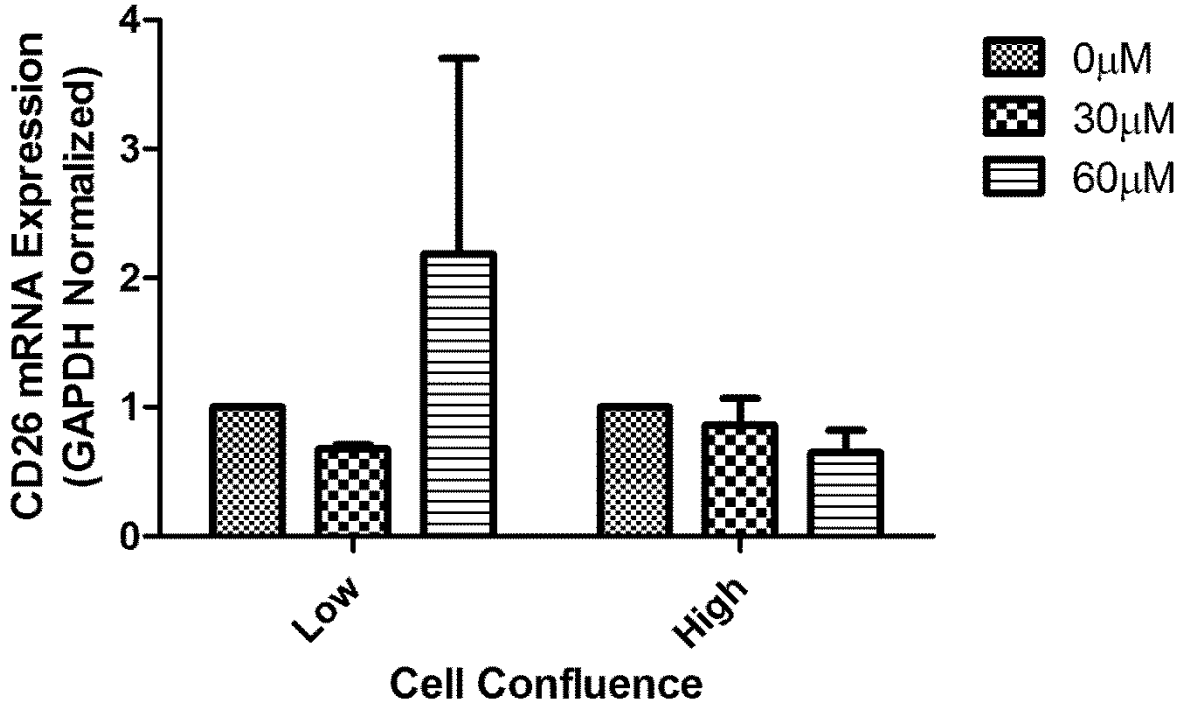


Figure 3.6 CD26 mRNA expression in HT-29 cells treated with luteolin at different confluence levels.

HT-29 cells were treated at low or high confluence with luteolin for 24h at the indicated concentrations. mRNA reverse transcribed to cDNA was used in triplicate reactions for each independent experiment. CD26 mRNA data was first normalized to the levels of GAPDH mRNA found in each sample before the ΔΔCt method was used to quantify the fold difference in CD26 mRNA expression in each sample relative to the untreated control. Data is presented as mean ± SEM from three independent experiments. Statistical analysis was done using two-way ANOVA with Bonferroni's post-test used to compare the replicate means.

3.4 Measurement of CD26 Protein Levels Using Western Immunoblotting

Whole cell levels of CD26 protein were quantified using western immunoblotting, followed by densitometry of the images and normalization to levels of β-actin. As before, HT-29 cells were grown to grown to less than 60% (low) confluent density or to 90-100% (high) confluence before being treated with vehicle control (0μm), 30μM or 60μM of apigenin, genistein, kaempferol, or luteolin for 24h. This is consistent with the timecourse for initial changes in protein appearance in response to apigenin as measured in terms of immunoreactive CD26 at the surface of viable cells.[15] It was chosen rather than a later timepoint of e.g. 48h to remain close to the desired confluence levels (cultures from the lower confluence level may have then been approaching the higher cell density).

The results of examining the potential effects of each flavonoid on the total protein levels of CD26 in HT-29 cells treated at low or high confluence levels are shown in Figures 3.7, 3.8, 3.9, and 3.10. Despite consistent upward trends in averaged data at increasing doses of apigenin (Figure 3.7) and numerical increases of mean data of as high as 40%, there were no statistically significant findings with respect to the amount of CD26 protein following treatment with any concentration of any flavonoid at any confluence level. However, it is apparent that averaged data show less elevation from control for the three other flavonoids (Figures 3.8, 3.9, and 3.10) than for apigenin (Figure 3.7).

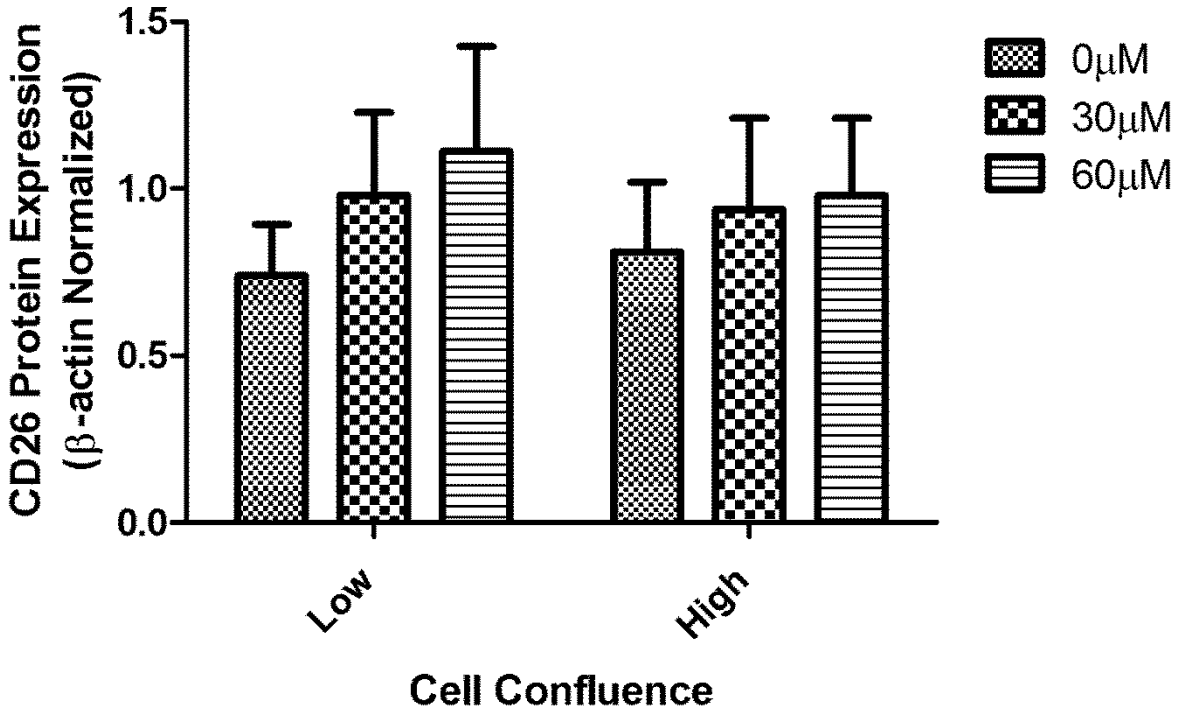


Figure 3.7 CD26 protein expression in HT-29 cells treated with apigenin at different confluence levels.

HT-29 cells were treated at low or high confluence with apigenin for 24h at the indicated concentrations. Protein fractions were immunoblotted for expression of CD26. β-actin was used as a loading control to which the levels of CD26 protein in each sample were normalized. Data is presented as mean ± SEM from three independent experiments. Statistical analysis was done using two-way ANOVA with Bonferroni's post-test used to compare the replicate means.

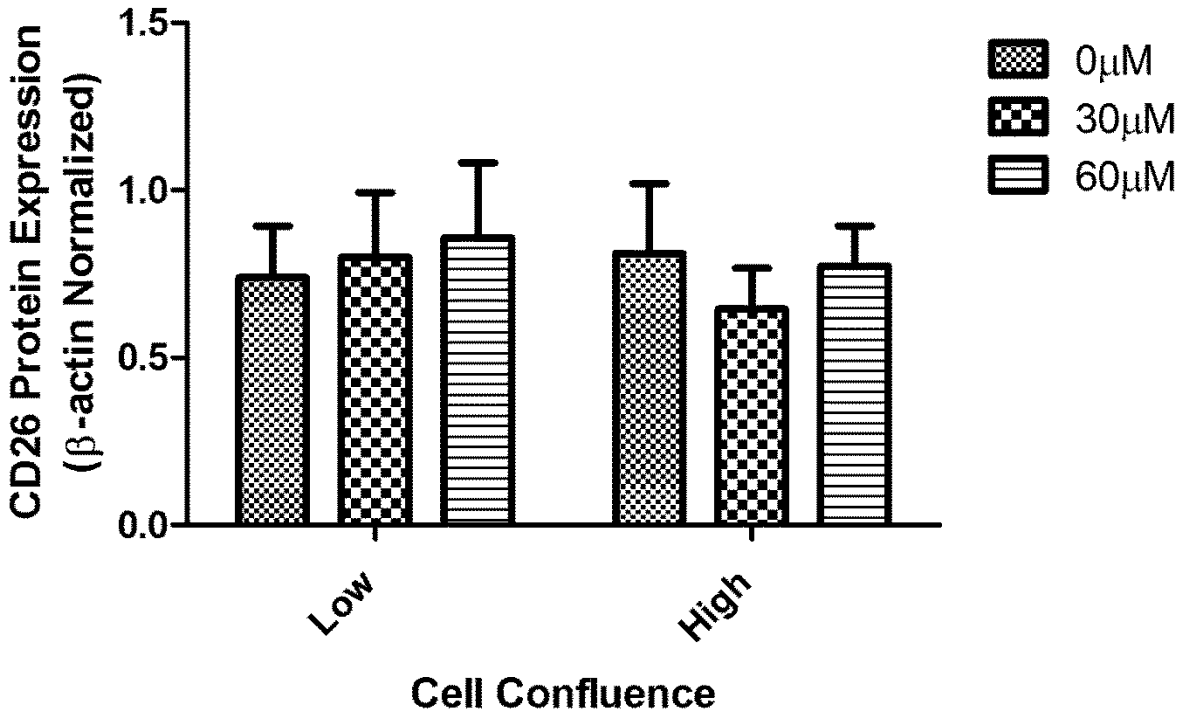


Figure 3.8 CD26 protein expression in HT-29 cells treated with genistein at different confluence levels.

HT-29 cells were treated at low or high confluence with genistein for 24h at the indicated concentrations. Protein fractions were immunoblotted for expression of CD26. β-actin was used as a loading control to which the levels of CD26 protein in each sample were normalized. Data is presented as mean ± SEM from three independent experiments. Statistical analysis was done using two-way ANOVA with Bonferroni's post-test used to compare the replicate means.

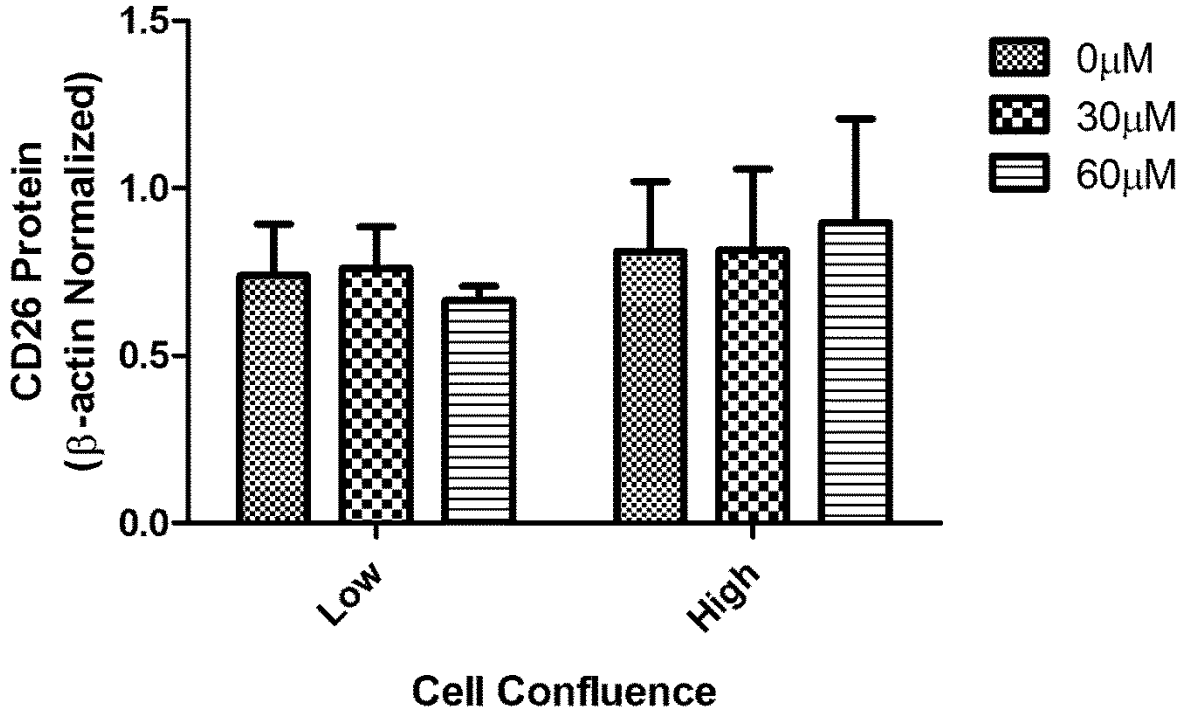


Figure 3.9 CD26 protein expression in HT-29 cells treated with kaempferol at different confluence levels.

HT-29 cells were treated at low or high confluence with kaempferol for 24h at the indicated concentrations. Protein fractions were immunoblotted for expression of CD26. β-actin was used as a loading control to which the levels of CD26 protein in each sample were normalized. Data is presented as mean ± SEM from three independent experiments. Statistical analysis was done using two-way ANOVA with Bonferroni's post-test used to compare the replicate means.

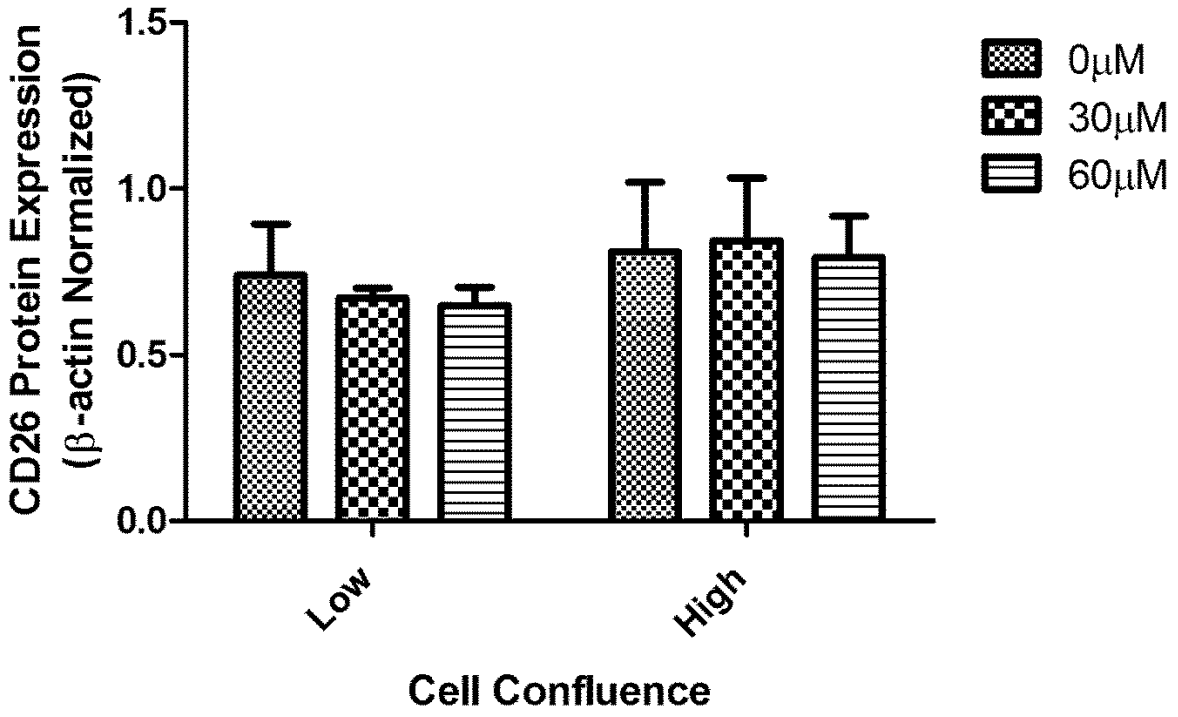


Figure 3.10 CD26 protein expression in HT-29 cells treated with luteolin at different confluence levels.

HT-29 cells were treated at low or high confluence with luteolin for 24h at the indicated concentrations. Protein fractions were immunoblotted for expression of CD26. β-actin was used as a loading control to which the levels of CD26 protein in each sample were normalized. Data is presented as mean ± SEM from three independent experiments. Statistical analysis was done using two-way ANOVA with Bonferroni's post-test used to compare the replicate means.

3.5 Changes in the Cellular Localization of CD26 in Response to Apigenin

Given that I found no statistically significant elevations in either CD26 mRNA expression or whole cell CD26 protein despite previous laboratory findings of a substantial increase in cell-surface CD26 in HT-29 cells after apigenin treatment at these concentrations, it was important to consider whether there might be a shift in the cellular localization of CD26 after treatment with this flavonoid.[15]

The localization of CD26 protein in HT-29 cells following flavonoid treatment was investigated using immunofluorescence microscopy. Based upon previous data the CD26 was expected to be predominantly located at the cell surface.[15] Surprisingly, substantial CD26 was found to be located intracellularly (Figure 3.11). This aside, there were substantial changes based upon exposure of HT-29 cells to apigenin (100μM) or the degree of confluence (Figure 3.11).

Apigenin-treated cells at low confluence displayed a greater amount of CD26 immunoreactivity than vehicle-treated cells, and strong reactivity was found in a significant proportion of apigenin-treated cells in circular bodies in the middle of the cells (Figure 3.11, panels A, B). In contrast, apigenin-treated cells in high confluence monolayer displayed slightly less CD26 protein compared to their correspondent vehicle control and did not show the dramatic response seen at low confluence (Figure 3.11, panels C, D). In neither situation of cell density was there substantial staining in the area of the plasma membrane as identified with the CellMask Deep Red plasma membrane stain.

The circular bodies in the middle of the cells (e.g. Figure 3.11B) appeared to resemble nuclei. It has previously been shown that CD26 may localize in nuclear regions.[95, 122, 123] I therefore wished to examine this further, and to see whether this localization effect could also be induced by the flavonoids genistein, kaempferol, and luteolin.

The most dramatic appearance in this context (Figure 3.11) was evident with cells at low confluence. Therefore HT-29 cells were treated with 60μM apigenin, genistein, kaempferol, or luteolin at low confluence and a DAPI stain was used to identify cell nuclei (Figure 3.12). Both apigenin- and genistein-treated cells contain greater amounts of CD26 protein in areas which coincide with their nuclei as stained with DAPI, compared to vehicle-treated cells (Figure 3.12, panels A, B). These effects were not observed in kaempferol- and luteolin-treated cells (Figure 3.12, panels C,D). Cells treated with kaempferol or luteolin instead showed a slight decrease in CD26 staining compared to vehicle-treated cells (Figure 3.12, panel E).

Although seeming to be nuclei, there is a possibility that the CD26-rich areas are instead structures which overlay or surround the nucleus, such as the endoplasmic reticulum or the Golgi apparatus. Interestingly in this approach the nuclei of apigenin- and genistein-treated cells were also smaller and the nuclear material was more condensed (Figure 3.12, panels A, B). However, this had not been the case with apigenin treatment in Figure 3.11 (panel B).

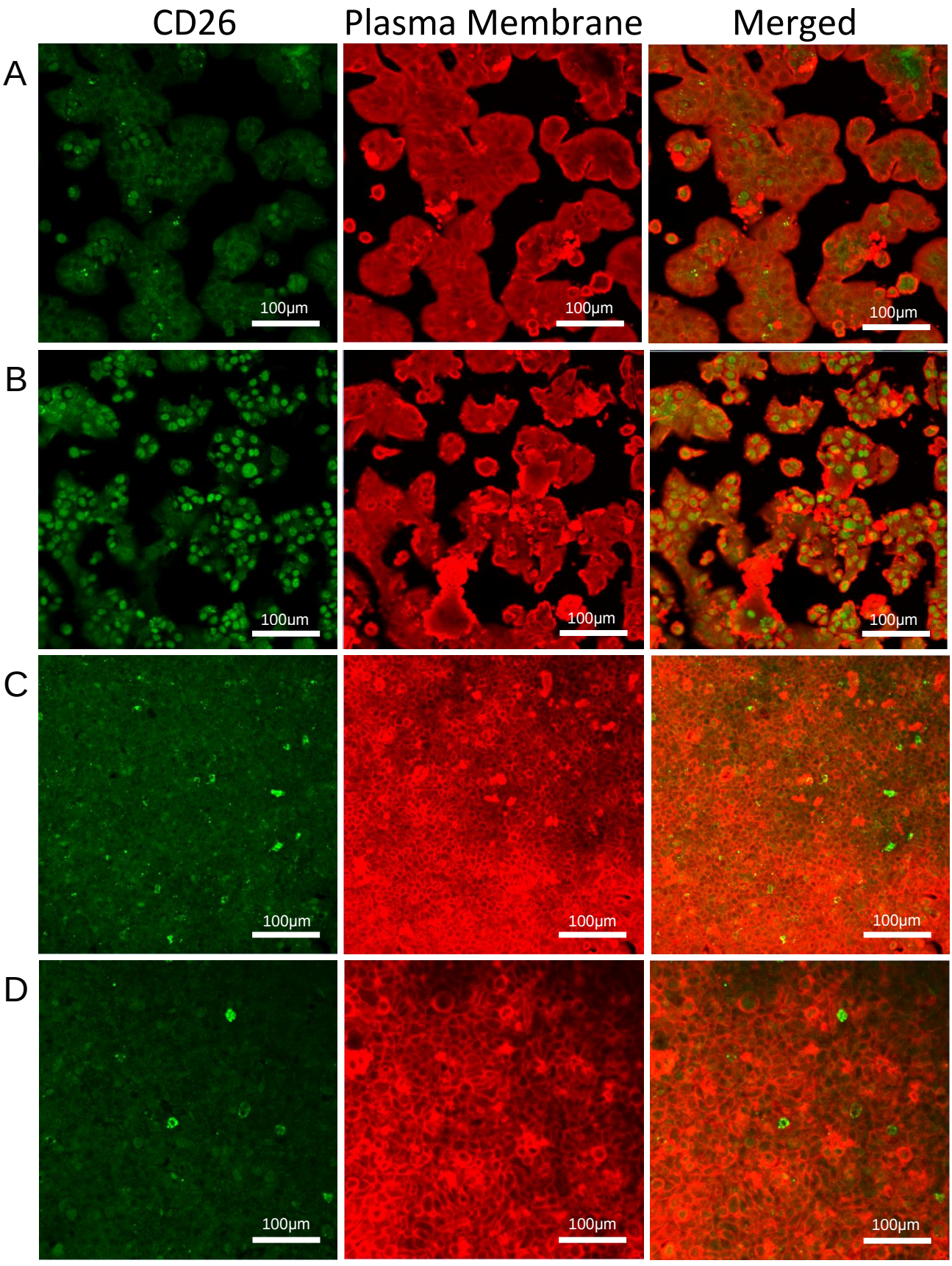

CD26
Plasma Membrane
Merged
A
B
C
D
100μm

Figure 3.11. CD26 visualized in apigenin-treated HT-29 cells at different confluence levels by confocal microscopy.

HT-29 cells were treated with (A, C) vehicle control or (B, D) 100μM apigenin for 24h. They were stained with CD26 primary antibody, anti-rabbit AlexaFluor 488 secondary antibody, and a CellMask Deep Red plasma membrane stain. Images of areas at low (A, B) or high (C, D) confluence are shown. Images shown are representative of two independent experiments.

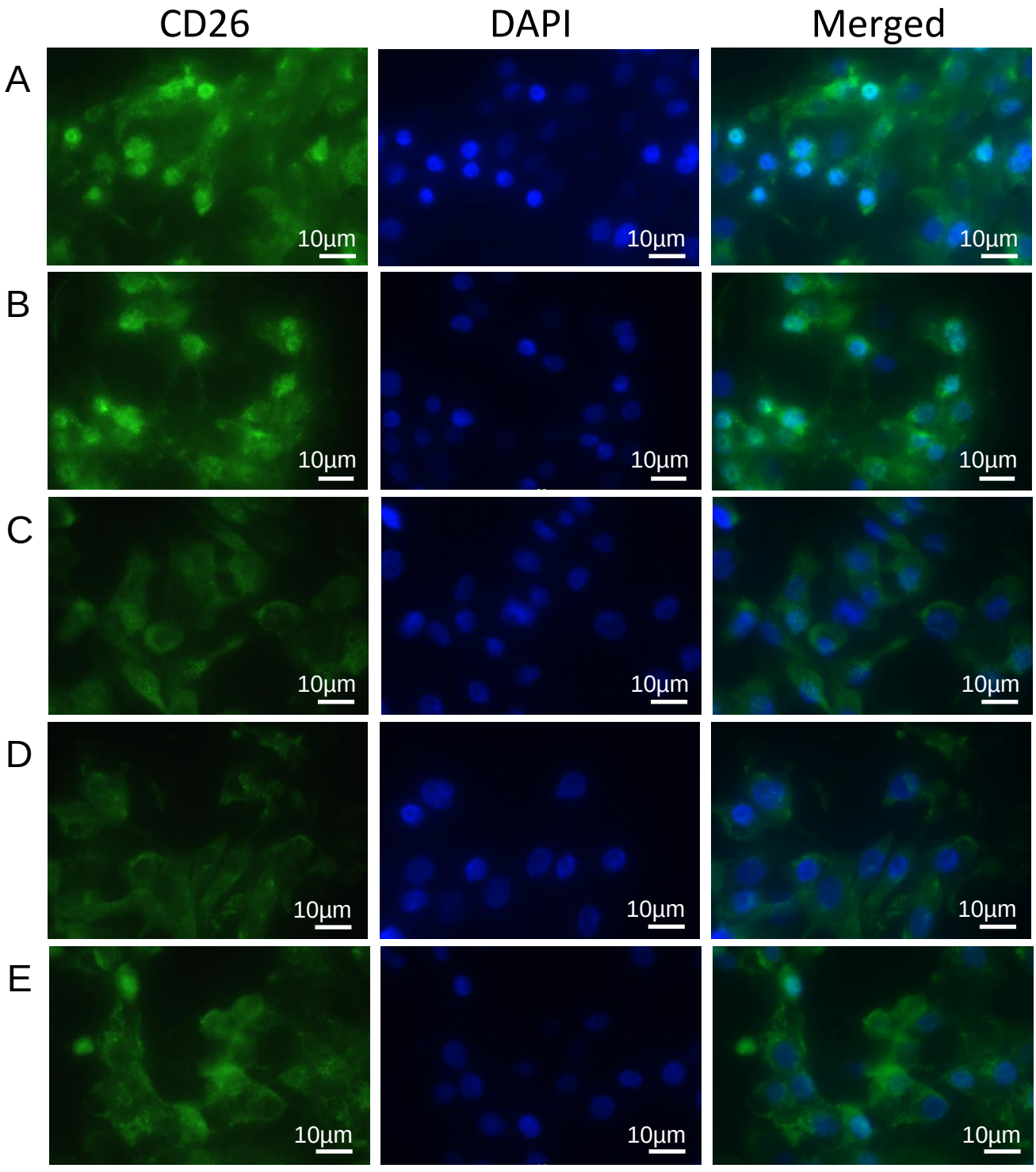

CD26
DAPI
Merged
A
B
C
D
E
10μm

Figure 3.12. CD26 visualized in HT-29 cells treated with apigenin, genistein, kaempferol, or luteolin at a low level of confluence by fluorescence microscopy.

HT-29 cells were treated with 60μM (A) apigenin, (B) genistein, (C) kaempferol, or (D) luteolin, relative to (E) vehicle control for 24h .They were stained with CD26 primary antibody and anti-rabbit AlexaFluor 488 (green) secondary antibody. Afterwards they were stained using DAPI (blue) to visualize nuclei. Images shown are representative of three independent experiments.

4 Discussion

4.1 Overview

The data collected during this project demonstrate the effects - or lack thereof - of flavonoids on three key cell-surface enzymes associated with the development of colon cancer.

First, HT-29 colorectal carcinoma cells cultured to different levels of confluence were treated with apigenin to determine whether this would affect the mRNA transcription of CD26, CD38, and CD73. Finding that the levels of mRNA for CD38 and CD73 showed no observable change in response to this key flavonoid, CD26 was selected as the focus of subsequent experimental work.

Focusing then on CD26, three flavonoids similar to apigenin in both chemical structure and biological function were also used in the further experiments: genistein, kaempferol, and luteolin. The mRNA transcription, protein quantity, and protein localization of CD26 were investigated in HT-29 cells treated with the four flavonoids at different confluence levels.

Although there was no significant change in mRNA transcription or total whole cell protein, I found clear evidence for a shift in the cellular localization of CD26 in response to two of the flavonoids. There appeared to be an increase in the nuclear localization of CD26 protein in cells treated at low confluence with 60μM apigenin or genistein. This data both complements and contrasts with that of Lefort and Blay, whose work showed that a 24h 30μM apigenin treatment significantly upregulated the cell-surface CD26 levels in HT-29 cells.[15]

4.2 Target Identification

The enzymes CD26, CD38 and CD73 are all relevant to the development and progression of colorectal cancer although their precise roles within this context remain to be elucidated.[70-72, 108, 109, 124] Using end-point PCR in conjunction with agarose gel electrophoresis, the presence of

CD26, CD38 and CD73 mRNA was detected in HT-29 colon cancer cells, suggesting that the corresponding protein is also produced in this cell line.[125] I focused on whether the mRNA quantities of these enzymes could be modulated by apigenin treatment, and, if so, whether this effect was dependent on cell confluence. In comparison with vehicle-treated control cells, the levels of CD26 mRNA appeared to increase only in apigenin-treated cells harvested at 90% and 100% confluence. CD38 mRNA levels appeared to increase somewhat but only in apigenin-treated cells collected at 100% confluence. The amount of CD73 mRNA did not change following apigenin treatment regardless of the confluence level of the cells at the time of RNA collection. CD26 was chosen as the most promising target on which to focus. Moreover the putative shifts in CD26 mRNA levels appeared to depend on cell confluence. Therefore I predicted that the abundance of CD26 protein would be modulated by both flavonoid treatment and cell density.

4.3 Flavonoid Cytotoxicity

The flavonoids genistein, kaempferol, and luteolin were chosen for this study as they are structurally and functionally very similar to apigenin. They may have comparable effects but in the case of changes in CD26 may function differently.[11, 35-49] Lefort and Blay found that treatment with genistein increased cell-surface CD26 in HT-29 cells similarly to apigenin albeit to a lesser degree.[15] They also found that kaempferol treatment had no effect on cell-surface CD26.[15] Kaempferol was included in this project for this reason - essentially as a control - but also because it shares the cytosolic effects induced by apigenin, genistein, and luteolin (see Tables 1.1, 1.2, and 1.3). Luteolin is a bioactive flavonoid in its own right and also the primary metabolite of apigenin.

The cytotoxicity of these flavonoids to HT-29 cells needed to be determined such that only subtoxic concentrations would be used throughout the remainder of this project. In this way, any results obtained would not be confounded by any cytotoxic cell processes these substances may have induced. Although the cytotoxicity of apigenin, genistein, kaempferol, and luteolin has been previously studied in other laboratories, changes to cell culture conditions such as the media type or oxygen concentration can change the chemosensitivity of cancer cells.[126, 127] Therefore MTT assays were used to determine the cytotoxicity of the four flavonoids in HT-29 cells under the conditions used in this study.

It was found that neither apigenin, genistein, nor kaempferol induced significant cytotoxicity at any of the concentrations used over the 24h treatment period. However, 100μM luteolin decreased cell viability by 20% and this was statistically significant. The second highest treatment, 60μM, was chosen as the usual maximal flavonoid concentration to be used in this project because it did not induce any cytotoxicity for any of the flavonoids tested.

Curiously, cells treated with 3μM and 10μM luteolin appeared to experience an increase in mitochondrial activity - up to 10% - and this effect was found to be statistically significant for the luteolin treatments. This phenomenon may also be biologically significant because it may indicate an increase in cell growth. Several studies have shown that genistein stimulates cell growth at concentrations under 10μM in breast and cervical cancer cell lines.[128-131] Genistein, a phytoestrogen, can induce cell proliferation at low concentrations by acting as an estrogen receptor agonist.[128-131] Indeed, this effect has led some to suggest that dietary genistein may not be safe for postmenopausal women with estrogen dependent breast cancers.[132] Estrogen receptors are present within the colonic epithelium and several studies show that their expression is decreased in colorectal cancer tissues compared to normal epithelium.[133-135] The mRNA of

estrogen receptor β (ERβ) has been found in the HT-29 cell line although it is as yet unknown whether these cells express ERβ protein.[136] Being that genistein is the most estrogenic flavonoid and ubiquitous in the diet, it is the focus of most flavonoid estrogenicity studies.[137] Luteolin and apigenin are estimated to have 58% and 16% of the estrogenic activity of genistein, respectively.[137] The estrogenic potential of kaempferol is estimated to be equal to or slightly less than that of apigenin.[138] If a biologically significant proliferative effect occurred, it is likely that it was mediated by estrogen receptors; this would explain why it was observed in cells treated with luteolin but not in those treated with apigenin or kaempferol. However, it remains curious that the same effect was not observed in genistein-treated cells.

Because the 30μM treatments neither inhibited nor stimulated cell viability, this concentration was chosen as the second subtoxic flavonoid treatment to be used in this project.

4.4 Quantifying CD26 mRNA Transcription

Next, changes in the transcription of CD26 mRNA were quantified using qPCR. Using 24h treatments of either 30μM or 60μM flavonoids, cells were treated at 50% (low) or 100% (high) confluence. There were no statistically significant findings or relationships between the flavonoid used, flavonoid concentrations, and cell confluence levels.

In contrast to the results observed in the end-point PCR experiments, the amount of CD26 mRNA appeared to decrease following the treatment of low confluence cells with any concentration of apigenin.

The lack of statistically significant findings from the mRNA data could be attributed to the high degree of variability often found with cancer cells. A study of human breast, liver, lung, and colon cancer tissues found that roughly 75% of genes had increased data variances compared to adjacent normal tissues.[139] These variances in mRNA expression, dubbed “noise”, have been

observed and their potential origins discussed in a number of studies.[140-144] Even within a population of identical cells, such noise occurs and can be attributed to the inherent "stochasticity", or randomness, of the chemical reactions involved within biological processes.[140, 141] Other relevant factors include the location of the gene in question, the rate of mRNA degradation, and cell doubling time.[142] Indeed, yeast cells exhibit more gene expression noise at lower growth rates and within environmentally-regulated genes which are not otherwise constitutively expressed.[143] Because CD26 is not constitutively expressed or explicitly required for a cell's survival, one could expect its transcription to exhibit a greater degree of noise. However, it is possible that the actions of the flavonoids themselves within the HT-29 cells contributed to the high degree of variance in the mRNA expression levels. As previously discussed, flavonoids exert myriad epigenetic effects including histone acetylation. Histone modifications have been hypothesized to be a source of genetic noise.[144] In the future, absolute quantification of CD26 mRNA within HT-29 cells could determine whether the transcription of CD26 has an inherently high degree of noise and whether this could be further modulated following flavonoid treatment.

4.5 Quantifying CD26 Protein

The amount of whole-cell CD26 protein in HT-29 cells was then quantified to observe whether the flavonoid-induced increases in cell-surface CD26 protein discovered by Lefort and Blay originated as a result of an increase in the production of functional CD26 protein, irrespective of the magnitude of any change in CD26 mRNA.[15] There were no significant differences in the amount of CD26 protein between cells treated with any concentration of the four selected flavonoids, at any level of confluence.

While protein quantities are primarily determined by the amount of mRNA present when cells are in a "steady state", post-transcriptional processes play a significant role in short-term adaptations to changes in the environment.[145] Whether an increase in translation or targeted breakdown via ubiquitination, these processes can quickly regulate the amount of protein in the cell and may also determine the levels of CD26 protein after cellular exposure to flavonoids.[145] Post-translational modifications are also involved, including those that may target the protein for transport.[145] Although the protein levels of CD26 were not significantly altered following flavonoid treatment, it is possible that the CD26 protein was modified post-translationally such that it was targeted to the apical surface of the plasma membrane in the HT-29 cells. This may explain the results found by Lefort and Blay.

Although not statistically significant, apigenin treatment appears to suggest a slight upregulation in the levels of CD26 protein in both low and high confluence cells. This compares with the trend observed by Lefort and Blay in which apigenin treatment produced a significant upregulation of cell-surface CD26 in HT-29 cells.[15] It is possible that the effect observed by Lefort and Blay is produced by the upregulation of whole cell CD26 protein in conjunction with an increased transport of CD26 to the surface of the cell. Of note is the observation that low confluence cells appeared to experience a greater upregulation of CD26 upon treatment with apigenin than high confluence cells. This suggests that cell confluence may affect the degree of the cellular response to apigenin treatment.

Cells treated with genistein at low confluence also appeared to experience an upregulation of CD26 protein, although this upregulation appeared to occur to a lesser degree than it did in apigenin-treated cells. This also follows the trends observed by Lefort and Blay, in which genistein treatment increased the levels of cell-surface CD26 to a lesser degree than did

apigenin treatment. However, high confluence cells treated with genistein appeared to experience a downregulation of CD26 protein compared to vehicle-treated control, with the 30μM concentration appearing to produce this effect to a greater degree.

The levels of CD26 protein found in cells treated with either kaempferol or luteolin appear to be very similar to those found in control cells. With respect to kaempferol, this was expected; Lefort and Blay found that it had no effect on the levels of cell-surface CD26.

4.6 Visualizing CD26 Protein

Finally, the localization of intracellular CD26 protein was visualized using immunofluorescence microscopy.

In the first such experiment, HT-29 cells were treated with 100μM apigenin, a concentration which was not found to be cytotoxic in this cell line. Populations of cells throughout each well were found at different levels of confluence. Vehicle-treated cells displayed a small amount of CD26 evenly distributed throughout each cell, regardless of confluence level. Cells treated with apigenin at high confluence displayed CD26 in a similar fashion. However, cells treated at low confluence were markedly different. Round structures in the middle of the cells became apparent and were significantly brighter than the CD26 staining observed in the control cells. These results follow the pattern observed in the western immunoblots: CD26 protein levels were more susceptible to apigenin treatment in low confluence cells than in high confluence cells. The bright and circular CD26 staining patterns were enclosed within the confines delineated by the CellMask Deep Red plasma membrane stain, suggesting that these were cytosolic structures. It was believed that these were the cell nuclei although this could not yet be confirmed without using a nuclear stain. Both the confluent and subconfluent cell populations imaged in this experiment had been cultured together within the

same well, meaning that the environmental conditions to which these cells had been exposed were identical. Having observed markedly different patterns in CD26 staining in these two cell subpopulations, this reinforced the idea that confluence was a determining factor in the cellular response to apigenin treatment.

In order to confirm whether the round structures previously seen were indeed nuclei, the experiment was repeated using a DAPI nuclear stain. This time, 60μM treatments - the highest subtoxic concentration - of each of the four flavonoids were compared. Furthermore, only those subpopulations of cells which had reached a low level of confluence were imaged so as to more clearly investigate the results produced in the previous immunofluorescence experiment.

In this experiment, apigenin- and genistein-treated cells displayed markedly brighter areas which overlapped their nuclei compared to control cells. This was attributed to increased nuclear CD26 staining. In contrast to the results found by Lefort and Blay, however, there did not appear to be an increase in plasma membrane CD26 staining. Kaempferol- and luteolin-treated cells did not exhibit such nuclear CD26 staining, and even appeared to have slightly less CD26 than did the vehicle-treated control cells.

The role of nuclear CD26 has not been well characterized in the literature although its translocation to the nucleus has been found to occur in response to treatment with anti-CD26 antibodies.[95, 122] If this phenomenon had occurred in these experiments, nuclear CD26 would have been found in every treatment group and the vehicle-treated control as well. This was not the case, however, so it can be inferred that treatment with apigenin and genistein must have caused this localization. Indeed, even Yamada *et al*. found that only one of the two antibodies they used induced the nuclear translocation of CD26.[122] This suggests that this effect requires that a ligand binds a specific epitope on the CD26 protein.[122] Yamada *et al*. also found that CD26

is translocated into the nucleus using caveolin-dependent endocytosis.[95] It is likely that this phenomenon occurred in the apigenin- and genistein-treated HT-29 cells as well. Given that the levels of CD26 mRNA and protein levels were not significantly altered, a translocation of CD26 is the most probable explanation for the increase in CD26 protein observed in the area of the nucleus. Yamada *et al*. observed that nuclear CD26 interacted with the POLR2A gene and repressed the transcription of its gene product, a subunit of RNA polymerase II.[95] In turn this reduced the cellular proliferation of the cancer cells used.[95] Although there is nothing to suggest that this is what occurred in the HT-29 cells, it remains a possibility considering that apigenin and genistein are both known to hinder cellular proliferation in this cell line.

Apigenin and genistein are isomers as are kaempferol and luteolin. The latter two have an additional hydroxyl group compared to the former two and this makes them more hydrophilic. It is possible that the molecular structure of apigenin and genistein allowed these substances to bind CD26 in such a way that it translocated to the nucleus. In addition, it is also possible that, being more lipophilic than kaempferol or luteolin, they penetrated the plasma membrane more easily and could therefore bind the epitope of CD26 and induce its nuclear translocation.

It is important to note that not every cell nucleus displayed CD26. Generally, the nuclei in which CD26 was abundant were the ones which had shrunk the most. This may indicate that there is a subpopulation of cells which are more susceptible to the effects of apigenin and genistein treatment on the localization of CD26 protein. Since cancer cells are, by definition, genomically unstable, genetic and phenotypic cell heterogeneity has been identified in numerous cancer cell lines including the colorectal carcinoma lines Caco-2 and HT-29.[146-150]

One might argue that the nuclear shrinking observed is actually evidence of an apoptotic process in which chromatin is condensed, fragmented, and subsequently destroyed.[151] This

nuclear shrinking is comprised of three stages, each of which are morphologically distinct and which altogether last 30-45min.[151] In the first stage, a continuous ring of condensed chromatin appears within the nucleus, and this ring becomes progressively more beaded and discontinuous in appearance as the second stage begins and the nucleus begins to shrink.[151] This step only lasts for 15-30min before the nucleus has become entirely fragmented.[151] Indeed, the annulus which characterizes the second stage can be seen in the form of circles within the nuclei of the apigenin- and genistein-treated cells. However, these banding patterns come from the green CD26 antibody and not from the blue DAPI stain. Because DAPI binds DNA directly, one would expect the banding patterns to come from the blue DAPI stain binding the condensed chromatin ring. Furthermore, the banding patterns observed in the HT-29 cells are different from those observed by Toné *et al*. because they occupy a greater proportion of the nucleus.[151] Lastly, similar banding patterns can be observed in the low-confluence HT-29 cells which had been treated with 100μM apigenin. These cells were not treated with any nuclear stain and yet these banding patterns are visible in regions bound by CD26 antibody. As previously mentioned, it is possible that CD26 is in the nucleus to repress (or perhaps induce) a transcriptional event. Any CD26-independent transcriptional or epigenetic effects induced by flavonoid treatment would have also been observed in kaempferol- and luteolin-treated cells.

4.7 Limitations and Future Directions

Although all colorectal cancers produce similar and predictable symptoms, colorectal cancers are actually a heterogeneous group of diseases with multiple genetically distinct subtypes.[152, 153] Therefore the results obtained using a single colorectal cancer cell line cannot be generalized to other cell lines. Neither can they be generalized to colorectal cancer *in situ*. This is further evidenced by the fact that experiments using cultured cells, as with any other *in vitro*

tumor model, observe the behaviour of these cells in isolation and not necessarily as they would exist *in vivo* under physiological conditions.[154]

In terms of further elucidation of events observed here, first it must be confirmed whether a caveolin-dependent endocytotic translocation of CD26 is what occurred following treatment with apigenin and genistein; this can be done by simultaneously treating the cells with an endocytosis inhibitor. Next, the activity of CD26 in the cell nucleus and its significance with respect to cell activity must be elucidated. This work must then be continued using other colorectal cancer cell lines to establish the presence of CD26 and determine whether its translocation to the nucleus is similarly induced following treatment with apigenin or genistein.

If any sufficiently significant result were to be obtained following such more thorough explanation of the treatment of cancer cells with flavonoids, it would be difficult to make any conclusions regarding the normal dietary consumption of these substances. As previously mentioned, most epidemiological data gathered on flavonoids is with regard to genistein simply uses information based on their ubiquity in the human diet. Given the structural similarity of genistein to the other flavonoids, one could expect that they are metabolized in similar ways. The quantity of flavonoids consumed can vary greatly depending on one's location and diet. Even within raw soybeans, known to be rich in flavonoids, the flavonoid content ranges between 18 to 562 mg/100g due to myriad environmental factors.[155] Ethnic origin of the subject, age, gut transit time, individual flavonoid metabolism phenotypes, and the plant source of the flavonoids also greatly affect their bioavailability in the blood plasma.[156-159] Furthermore, the flavonoid concentrations used in many studies, including this one, are much greater than what is naturally found in human blood plasma. Therefore new delivery methods, such as liposome encapsulation, are under development to enhance flavonoid bioavailability.[160]

5 References

[1] Brenner H, Kloor M, Pox CP: Colorectal cancer. The Lancet 2014, 383:1490-502.
[2] Kuipers EJ, Grady WM, Lieberman D, Seufferlein T, Sung JJ, Boelens PG, van de Velde CJ, Watanabe T: Colorectal cancer. Nat Rev Dis Primers 2015, 1:15065.
[3] Erdrich J, Zhang X, Giovannucci E, Willett W: Proportion of colon cancer attributable to lifestyle in a cohort of US women. Cancer Causes Control 2015, 26:1271-9.
[4] Platz EA, Willett W, Colditz GA, Rimm EB, Spiegelman D, Giovannucci E: Proportion of colon cancer risk that might be preventable in a cohort of middle-aged US men. Cancer Causes Control 2000, 11:579-88.
[5] Aune D, Lau R, Chan DS, Vieira R, Greenwood DC, Kampman E, Norat T: Nonlinear reduction in risk for colorectal cancer by fruit and vegetable intake based on meta-analysis of prospective studies. Gastroenterology 2011, 141:106-18.
[6] Ravishankar D, Rajora AK, Greco F, Osborn HM: Flavonoids as prospective compounds for anti-cancer therapy. Int J Biochem Cell Biol 2013, 45:2821-31.
[7] Kuo S-M: Dietary flavonoid and cancer prevention: evidence and potential mechanism. Critical Reviews™ in Oncogenesis 1997, 8:47-69.
[8] Lefort EC, Blay J: Apigenin and its impact on gastrointestinal cancers. Mol Nutr Food Res 2013, 57:126-44.
[9] Wikipedia Commons. Chemical structure of apigenin. Retrieved November 24, 2016, from https://commons.wikimedia.org/wiki/File:Apigenin.png
[10] Walle T, Ta N, Kawamori T, Wen X, Tsuji PA, Walle UK: Cancer chemopreventive properties of orally bioavailable flavonoids--methylated versus unmethylated flavones. Biochem Pharmacol 2007, 73:1288-96.
[11] Zhang Q, Zhao XH, Wang ZJ: Cytotoxicity of flavones and flavonols to a human esophageal squamous cell carcinoma cell line (KYSE-510) by induction of G2/M arrest and apoptosis. Toxicol In Vitro 2009, 23:797-807.
[12] Wu K, Lin-Hong Y, Wei X: Inhibitory effects of apigenin on the growth of gastric carcinoma SGC-7901 cells. World Journal of Gastroenterology 2005, 11:4461-4.
[13] Chung CS, Jiang Y, Cheng D, Birt DF: Impact of adenomatous polyposis coli (APC) tumor supressor gene in human colon cancer cell lines on cell cycle arrest by apigenin. Mol Carcinog 2007, 46:773-82.
[14] Ujiki MB, Ding XZ, Salabat MR, Bentrem DJ, Golkar L, Milam B, Talamonti MS, Bell RH, Jr., Iwamura T, Adrian TE: Apigenin inhibits pancreatic cancer cell proliferation through G2/M cell cycle arrest. Mol Cancer 2006, 5:76.
[15] Lefort EC, Blay J: The dietary flavonoid apigenin enhances the activities of the anti-metastatic protein CD26 on human colon carcinoma cells. Clin Exp Metastasis 2011, 28:337-49.
[16] Russo M, Russo GL, Daglia M, Kasi PD, Ravi S, Nabavi SF, Nabavi SM: Understanding genistein in cancer: The "good" and the "bad" effects: A review. Food Chem 2016, 196:589-600.
[17] Wikipedia Commons. Genistein. Retrieved July 17, 2018 from https://commons.wikimedia.org/wiki/File:Genistein.svg.
[18] Mukund V, Mukund D, Sharma V, Mannarapu M, Alam A: Genistein: Its role in metabolic diseases and cancer. Crit Rev Oncol Hematol 2017, 119:13-22.
[19] Spagnuolo C, Russo GL, Orhan IE, Habtemariam S, Daglia M, Sureda A, Nabavi SF, Devi KP, Loizzo MR, Tundis R, Nabavi SM: Genistein and cancer: current status, challenges, and future directions. Adv Nutr 2015, 6:408-19.

[20] Tse G, Eslick GD: Soy and isoflavone consumption and risk of gastrointestinal cancer: a systematic review and meta-analysis. Eur J Nutr 2016, 55:63-73.
[21] Yan L, Spitznagel EL, Bosland MC: Soy consumption and colorectal cancer risk in humans: a meta-analysis. Cancer Epidemiol Biomarkers Prev 2010, 19:148-58.
[22] Lu D, Pan C, Ye C, Duan H, Xu F, Yin L, Tian W, Zhang S: Meta-analysis of Soy Consumption and Gastrointestinal Cancer Risk. Sci Rep 2017, 7:4048.
[23] Yu Y, Jing X, Li H, Zhao X, Wang D: Soy isoflavone consumption and colorectal cancer risk: a systematic review and meta-analysis. Sci Rep 2016, 6:25939.
[24] Calderon-Montano JM, Burgos-Moron E, Perez-Guerrero C, Lopez-Lazaro M: A Review on the Dietary Flavonoid Kaempferol. Mini-Reviews in Medicinal Chemistry 2011, 11:298-344.
[25] Wikipedia Commons. Kaempferol. Retrieved November 24, 2016, from https://commons.wikimedia.org/wiki/File:Kaempferol.svg.
[26] Chen AY, Chen YC: A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention. Food Chem 2013, 138:2099-107.
[27] Shimoi K, Okada H, Furugori M, Goda T, Takase S, Suzuki M, Hara Y, Yamamoto H, Kinae N: Intestinal absorption of luteolin and luteolin '7-O-Beta-glucoside in rats and humans. FEBS Letters 1998, 438:220-4.
[28] Wikipedia Commons. Luteolin. Retrieved November 24, 2016, from https://commons.wikimedia.org/wiki/File:Luteolin.svg.
[29] Tarahovsky YS, Kim YA, Yagolnik EA, Muzafarov EN: Flavonoid-membrane interactions: involvement of flavonoid-metal complexes in raft signaling. Biochim Biophys Acta 2014, 1838:1235-46.
[30] Walle T: Absorption and metabolism of flavonoids. Free Radic Biol Med 2004, 36:829-37.
[31] Kosinova P, Berka K, Wykes M, Otyepka M, Trouillas P: Positioning of antioxidant quercetin and its metabolites in lipid bilayer membranes: implication for their lipid-peroxidation inhibition. J Phys Chem B 2012, 116:1309-18.
[32] Ajdzanovic V, Mojic M, Maksimovic-Ivanic D, Bulatovic M, Mijatovic S, Milosevic V, Spasojevic I: Membrane fluidity, invasiveness and dynamic phenotype of metastatic prostate cancer cells after treatment with soy isoflavones. J Membr Biol 2013, 246:307-14.
[33] Pawlikowska-Pawlega B, Misiak LE, Zarzyka B, Paduch R, Gawron A, Gruszecki WI: FTIR, (1)H NMR and EPR spectroscopy studies on the interaction of flavone apigenin with dipalmitoylphosphatidylcholine liposomes. Biochim Biophys Acta 2013, 1828:518-27.
[34] Selvaraj S, Krishnaswamy S, Devashya V, Sethuraman S, Krishnan UM: Influence of membrane lipid composition on flavonoid-membrane interactions: Implications on their biological activity. Prog Lipid Res 2015, 58:1-13.
[35] Zhu Y, Mai Y, Chen H, Lin Y, Hu Z, Wu J, Xu X, Xu X, Qin J, Xie L: Apigenin promotes apoptosis, inhibits invasion and induces cell cycle arrest of T24 human bladder cancer cells. Cancer Cell International 2013, 13.
[36] Yang J, Pi C, Wang G: Inhibition of PI3K/Akt/mTOR pathway by apigenin induces apoptosis and autophagy in hepatocellular carcinoma cells. Biomed Pharmacother 2018, 103:699-707.
[37] Lee Y, Sung B, Kang YJ, Kim DH, Jang JY, Hwang SY, Kim M, Lim HS, Yoon JH, Chung HY, Kim ND: Apigenin-induced apoptosis is enhanced by inhibition of autophagy formation in HCT116 human colon cancer cells. Int J Oncol 2014, 44:1599-606.
[38] Tseng TH, Chien MH, Lin WL, Wen YC, Chow JM, Chen CK, Kuo TC, Lee WJ: Inhibition of MDA-MB-231 breast cancer cell proliferation and tumor growth by apigenin through

induction of G2/M arrest and histone H3 acetylation-mediated p21(WAF1/CIP1) expression. Environ Toxicol 2017, 32:434-44.
[39] Zhou P, Wang C, Hu Z, Chen W, Qi W, Li A: Genistein induces apoptosis of colon cancer cells by reversal of epithelial-to-mesenchymal via a Notch1/NF-kappaB/slug/E-cadherin pathway. BMC Cancer 2017, 17:813.
[40] Gao Y, Yin J, Rankin GO, Chen YC: Kaempferol Induces G2/M Cell Cycle Arrest via Checkpoint Kinase 2 and Promotes Apoptosis via Death Receptors in Human Ovarian Carcinoma A2780/CP70 Cells. Molecules 2018, 23.
[41] Cho HJ, Park JHY: Kaempferol induces cell cycle arrest in HT-29 human colon cancer cells. Journal of Cancer Prevention 2013, 18:257-63.
[42] Lin CM, Chen HH, Lin CA, Wu HC, Sheu JJ, Chen HJ: Apigenin-induced lysosomal degradation of beta-catenin in Wnt/beta-catenin signaling. Sci Rep 2017, 7:372.
[43] Park S, Choi J: Inhibition of beta-catenin/Tcf signaling by flavonoids. J Cell Biochem 2010, 110:1376-85.
[44] Sahin K, Tuzcu M, Basak N, Caglayan B, Kilic U, Sahin F, Kucuk O: Sensitization of Cervical Cancer Cells to Cisplatin by Genistein: The Role of NFkappaB and Akt/mTOR Signaling Pathways. J Oncol 2012, 2012:461562.
[45] Huang WW, Tsai SC, Peng SF, Lin MW, Chiang JH, Chiu YJ, Fushiya S, Tseng MT, Yang JS: Kaempferol induces autophagy through AMPK and AKT signaling molecules and causes G2/M arrest via downregulation of CDK1/cyclin B in SK-HEP-1 human hepatic cancer cells. Int J Oncol 2013, 42:2069-77.
[46] Wang Q, Wang H, Jia Y, Ding H, Zhang L, Pan H: Luteolin reduces migration of human glioblastoma cell lines via inhibition of the p-IGF-1R/PI3K/AKT/mTOR signaling pathway. Oncol Lett 2017, 14:3545-51.
[47] Lim W, Yang C, Bazer FW, Song G: Luteolin Inhibits Proliferation and Induces Apoptosis of Human Placental Choriocarcinoma Cells by Blocking the PI3K/AKT Pathway and Regulating Sterol Regulatory Element Binding Protein Activity. Biol Reprod 2016, 95:82.
[48] Xu M, Wang S, Song YU, Yao J, Huang K, Zhu X: Apigenin suppresses colorectal cancer cell proliferation, migration and invasion via inhibition of the Wnt/beta-catenin signaling pathway. Oncol Lett 2016, 11:3075-80.
[49] Johnson JL, Rupasinghe SG, Stefani F, Schuler MA, Gonzalez de Mejia E: Citrus flavonoids luteolin, apigenin, and quercetin inhibit glycogen synthase kinase-3beta enzymatic activity by lowering the interaction energy within the binding cavity. J Med Food 2011, 14:325-33.
[50] Lu L, Wang Y, Ou R, Feng Q, Ji L, Zheng H, Guo Y, Qi X, Kong AN, Liu Z: DACT2 Epigenetic Stimulator Exerts Dual Efficacy for Colorectal Cancer Prevention and Treatment. Pharmacol Res 2018, 129:318-28.
[51] Iizumi Y, Oishi M, Taniguchi T, Goi W, Sowa Y, Sakai T: The flavonoid apigenin downregulates CDK1 by directly targeting ribosomal protein S9. PLoS One 2013, 8:e73219.
[52] Malumbres M, Barbacid M: Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer 2009, 9:153-66.
[53] Bendris N, Lemmers B, Blanchard JM: Cell cycle, cytoskeleton dynamics and beyond: the many functions of cyclins and CDK inhibitors. Cell Cycle 2015, 14:1786-98.
[54] Taylor RC, Cullen SP, Martin SJ: Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 2008, 9:231-41.

[55] Ersahin T, Tuncbag N, Cetin-Atalay R: The PI3K/AKT/mTOR interactive pathway. Mol Biosyst 2015, 11:1946-54.
[56] Johnson SM, Gulhati P, Rampy BA, Han Y, Rychahou PG, Doan HQ, Weiss HL, Evers BM: Novel expression patterns of PI3K/Akt/mTOR signaling pathway components in colorectal cancer. J Am Coll Surg 2010, 210:767-76, 76-8.
[57] Klaus A, Birchmeier W: Wnt signalling and its impact on development and cancer. Nature Reviews Cancer 2008, 8:387-98.
[58] Polakis P: Wnt signaling in cancer. Cold Spring Harb Perspect Biol 2012, 4.
[59] Giles RH, van Es JH, Clevers H: Caught up in a Wnt storm: Wnt signaling in cancer. Biochimica et Biophysica Acta 2003, 1653:1-24.
[60] Busch C, Burkard M, Leischner C, Lauer UM, Frank J, Venturelli S: Epigenetic activities of flavonoids in the prevention and treatment of cancer. Clin Epigenetics 2015, 7:64.
[61] Pandey M, Kaur P, Shukla S, Abbas A, Fu P, Gupta S: Plant flavone apigenin inhibits HDAC and remodels chromatin to induce growth arrest and apoptosis in human prostate cancer cells: in vitro and in vivo study. Mol Carcinog 2012, 51:952-62.
[62] Berger A, Venturelli S, Kallnischkies M, Bocker A, Busch C, Weiland T, Noor S, Leischner C, Weiss TS, Lauer UM, Bischoff SC, Bitzer M: Kaempferol, a new nutrition-derived pan-inhibitor of human histone deacetylases. J Nutr Biochem 2013, 24:977-85.
[63] Sundaram MK, Ansari MZ, Al Mutery A, Ashraf M, Nasab R, Rai S, Rais N, Hussain A: Genistein induces alterations of epigenetic modulatory signatures in human cervical cancer cells. Anti-Cancer Agents in Medicinal Chemistry 2018, 18:412-21.
[64] Xie Q, Bai Q, Zou LY, Zhang QY, Zhou Y, Chang H, Yi L, Zhu JD, Mi MT: Genistein inhibits DNA methylation and increases expression of tumor suppressor genes in human breast cancer cells. Genes Chromosomes Cancer 2014, 53:422-31.
[65] Fang M, Chen D, Yang CS: Dietary Polyphenols May Affect DNA Methylation. The Journal of Nutrition 2007, 137:223S-8S.
[66] Paredes-Gonzalez X, Fuentes F, Su ZY, Kong AN: Apigenin reactivates Nrf2 anti-oxidative stress signaling in mouse skin epidermal JB6 P + cells through epigenetics modifications. AAPS J 2014, 16:727-35.
[67] Zuo Q, Wu R, Xiao X, Yang C, Yang Y, Wang C, Lin L, Kong AN: The dietary flavone luteolin epigenetically activates the Nrf2 pathway and blocks cell transformation in human colorectal cancer HCT116 cells. J Cell Biochem 2018, 119:9573-82.
[68] Zhang Y, Li Q, Chen H: DNA methylation and histone modifications of Wnt genes by genistein during colon cancer development. Carcinogenesis 2013, 34:1756-63.
[69] Vanhees K, Coort S, Ruijters EJ, Godschalk RW, van Schooten FJ, Barjesteh van Waalwijk van Doorn-Khosrovani S: Epigenetics: prenatal exposure to genistein leaves a permanent signature on the hematopoietic lineage. FASEB J 2011, 25:797-807.
[70] Lam CS, Cheung AH, Wong SK, Wan TM, Ng L, Chow AK, Cheng NS, Pak RC, Li HS, Man JH, Yau TC, Lo OS, Poon JT, Pang RW, Law WL: Prognostic significance of CD26 in patients with colorectal cancer. PLoS One 2014, 9.
[71] Pang R, Law WL, Chu AC, Poon JT, Lam CS, Chow AK, Ng L, Cheung LW, Lan XR, Lan HY, Tan VP, Yau TC, Poon RT, Wong BC: A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell 2010, 6:603-15.
[72] Perenkov AD, Novikov DV, Sakharnov NA, Alyasova AV, Utkin OV, Baryshnikov AY, Novikov VV: Heterogeneous CD38 expression in tumor tissues of patients with colorectal cancer. Molecular Biology 2012, 46:705-9.

[73] Faas MM, Saez T, de Vos P: Extracellular ATP and adenosine: The Yin and Yang in immune responses? Mol Aspects Med 2017, 55:9-19.
[74] Meester ID, Korom S, Damme JV, Scharpe S: CD26, let it cut or cut it down. Immunology Today 1999, 20:367-75.
[75] Deaglio S, Mehta K, Malavasi F: Human CD38: a (r)evolutionary story of enzymes and receptors Leukemia Research 2001, 25:1-12.
[76] Pro B, Dang NH: CD26/dipeptidyl peptidase IV and its role in cancer. Histology and Histopathology 2004:1345-51.
[77] Beckenkamp A, Davies S, Willig JB, Buffon A: DPPIV/CD26: a tumor suppressor or a marker of malignancy? Tumour Biol 2016, 37:7059-73.
[78] Mujoomdar M, Hoskin D, Blay J: Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism. Biochem Pharmacol 2003, 66:1737-47.
[79] Richard CL, Tan EY, Blay J: Adenosine upregulates CXCR4 and enhances the proliferative and migratory responses of human carcinoma cells to CXCL12/SDF-1alpha. Int J Cancer 2006, 119:2044-53.
[80] Young A, Mittal D, Stagg J, Smyth MJ: Targeting cancer-derived adenosine: new therapeutic approaches. Cancer Discov 2014, 4:879-88.
[81] Blay J: Adenosine and Tumor Microenvironment. Encyclopedia of Cancer Edited by Schwab M. Springer-Verlag: Heidelberg, 2012. pp. 49-52.
[82] Chung KM, Huang CH, Cheng JH, Tsai CH, Suen CS, Hwang MJ, Chen X: Proline in transmembrane domain of type II protein DPP-IV governs its translocation behavior through endoplasmic reticulum. Biochemistry 2011, 50:7909-18.
[83] David F, Baricault L, Sapin C, Gallet X, Marguet D, Thomas-Soumarmon A, Trugnan G: Reduced cell surface expression of a mutated dipeptidyl peptidase IV (DPP IV/ CD26) correlates with the generation of a beta strand in its C-terminal domain. Biochemical and Biophysical Research Communications 1996, 222:833-8.
[84] Jascur T, Matter K, Hauri HP: Oligomerization and intracellular protein transport: dimerization of intestinal dipeptidylpeptidase IV occurs in the golgi apparatus. Biochemistry 1991, 30:1908-15.
[85] Fan H, Meng W, Kilian C, Grams S, Reutter W: Domain specific N-glycoslylation of the membrane glycoprotein dipeptidylpeptidase IV (CD26) influences its subcellular trafficking, biological stability, enzyme activity, and protein folding. European Journal of Biochemistry 1997, 246:243-51.
[86] Alfalah M, Jacob R, Naim HY: Intestinal dipeptidyl peptidase IV is efficiently sorted to the apical membrane through the concerted action of N- and O-glycans as well as association with lipid microdomains. J Biol Chem 2002, 277:10683-90.
[87] Aït Slimane T, Lenoir C, Sapin C, Maurice M, Trugnan G: Apical Secretion and Sialylation of Soluble Dipeptidyl Peptidase IV Are Two Related Events. Experimental Cell Research 2000, 258:184-94.
[88] Weisz OA, Machamer CE, Hubbard AL: Rat liver dipeptidylpeptidase IV contains competing apical and basolateral targeting information. The Journal of Biological Chemistry 1992, 267:22282-8.
[89] Baricault L, Garcia M, Cibert C, Sapin C, Geraud G, Codogno P, Trugnan G: Forskolin blocks the apical expression of dipeptidyl peptidase IV in Caco-2 cells and induces its retention in Lamp-1 containing vesicles. Experimental Cell Research 1993, 209:277-87.

[90] Baricault L, Fransen JAM, Garcia M, Sapin C, Codogno P, Ginsel LA, Trugnan G: Rapid sequestration of DPP IV/ CD26 and other cell surface proteins in an autophagic-like compartment in Cac-2 cells treated with forskolin. Journal of Cell Science 1995, 108:2109-21.
[91] Theard D, Steiner M, Kalicharan D, Hoekstra D, van Ijzendoorn SCD: Cell polarity development and protein trafficking in hepatocytes lacking E-cadherin/beta-catenin based adherens junctions. Molecular Biology of the Cell 2007, 18:2313-21.
[92] Ishii T, Ohnuma K, Murakami A, Takasawa N, Kobayashi S, Dang NH, Schlossman SF, Morimoto C: CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO. Proc Natl Acad Sci U S A 2001, 98:12138-43.
[93] Pereira DA, Gomes L, El-Cheikh MC, Borojevic R: Dipeptidyl peptidase IV (CD26) activity in the hematopoietic system: differences between the membrane-anchored and the released enzyme activity. Brazilian Journal of Medical and Biological Research 2003, 36:567-78.
[94] Ohnuma K, Uchiyama M, Yamochi T, Nishibashi K, Hosono O, Takahashi N, Kina S, Tanaka H, Lin X, Dang NH, Morimoto C: Caveolin-1 triggers T-cell activation via CD26 in association with CARMA1. J Biol Chem 2007, 282:10117-31.
[95] Yamada K, Hayashi M, Madokoro H, Nishida H, Du W, Ohnuma K, Sakamoto M, Morimoto C, Yamada T: Nuclear localization of CD26 induced by a humanized monoclonal antibody inhibits tumor cell growth by modulating of POLR2A transcription. PLoS One 2013, 8:e62304.
[96] Patlolla JMR, Swamy MV, Raju J, Rao CV: Overexpression of caveolin-1 in experimental colon adenocarcinomas and human colon cancer cell lines. Oncology Reports 2004, 11:957-63.
[97] Bender FC, Reymond MA, Bron C, Quest AFG: Caveleolin-1 levels are downregulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. Cancer Research 2000, 60:5870-8.
[98] Larrinaga G, Perez I, Sanz B, Beitia M, Errarte P, Fernandez A, Blanco L, Etxezarraga MC, Gil J, Lopez JI: Dipeptidyl-peptidase IV activity is correlated with colorectal cancer prognosis. PLoS One 2015, 10:e0119436.
[99] Lam CS, Cheung AH, Wong SK, Wan TM, Ng L, Chow AK, Cheng NS, Pak RC, Li HS, Man JH, Yau TC, Lo OS, Poon JT, Pang RW, Law WL: Prognostic significance of CD26 in patients with colorectal cancer. PLoS One 2014, 9:e98582.
[100] Pang R, Law WL, Chu AC, Poon JT, Lam CS, Chow AK, Ng L, Cheung LW, Lan XR, Lan HY, Tan VP, Yau TC, Poon RT, Wong BC: A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell 2010, 6:603-15.
[101] de la Haba-Rodriguez J, Macho A, Calzado MA, Blazquez MV, Gomez MA, Munoz EE, Aranda E: Soluble dipeptidyl peptidase IV (CD-26) in serum of patients with colorectal carcinoma. Neoplasma 2002, 49:307-11.
[102] Cordero OJ, Ayude D, Nogueira M, Rodriguez-Berrocal FJ, de la Cadena MP: Preoperative serum CD26 levels: diagnostic efficiency and predictive value for colorectal cancer. British Journal of Cancer 2000, 83:1139-46.
[103] De Chiara L, Rodriguez-Pineiro AM, Cordero OJ, Vazquez-Tunas L, Ayude D, Rodriguez-Berrocal FJ, de la Cadena MP: Postoperative serum levels of sCD26 for surveillance in colorectal cancer patients. PLoS One 2014, 9:e107470.
[104] Darmoul D, Lacasa M, Baricault L, Marguet D, Sapin C, Trotot P, Barbat A, Trugnan G: Dipeptidyl peptidase IV (CD26) gene expression in enterocyte-like colon cancer cell lines HT-29 and Caco-2. The Journal of Biological Chemistry 1992, 267:4824-2833.

[105] Abe M, Havre PA, Urasaki Y, Ohnuma K, Morimoto C, Dang LH, Dang NH: Mechanisms of confluence-dependent expression of CD26 in colon cancer cell lines. BMC Cancer 2011, 11:51.
[106] Wei W, Graeff R, Yue J: Roles and mechanisms of the CD38/cyclic adenosine diphosphate ribose/Ca(2+) signaling pathway. World J Biol Chem 2014, 5:58-67.
[107] Perenkov AD, Novikov DV, Sakharnov NA, Alyasova AV, Utkin OV, Baryshnikov AY, Novikov VV: Heterogeneous CD38 expression in tumor tissues of patients with colorectal cancer. Molecular Biology 2012, 46:705-9.
[108] Gao ZW, Dong K, Zhang HZ: The roles of CD73 in cancer. Biomed Res Int 2014, 2014:460654.
[109] Pancione M, Giordano G, Remo A, Febbraro A, Sabatino L, Manfrin E, Ceccarelli M, Colantuoni V: Immune escape mechanisms in colorectal cancer pathogenesis and liver metastasis. J Immunol Res 2014, 2014:686879.
[110] Di Virgilio F, Adinolfi E: Extracellular purines, purinergic receptors and tumor growth. Oncogene 2017, 36:293-303.
[111] Synnestvedt K, Furuta GT, Comerford KM, Louis N, Karhausen J, Eltzschig HK, Hansen KR, Thompson LF, Colgan SP: Ecto-5′-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. Journal of Clinical Investigation 2002, 110:993-1002.
[112] Bynoe MS, Waickman AT, Mahamed DA, Mueller C, Mills JH, Czopik A: CD73 is critical for the resolution of murine colonic inflammation. J Biomed Biotechnol 2012, 2012:260983.
[113] Trajkovic K, Valdez C, Ysselstein D, Krainc D: Fluctuations in cell density alter protein markers of multiple cellular compartments, confounding experimental outcomes. PLoS One 2019, 14:e0211727.
[114] Kim SW, Kim SJ, Langley RR, Fidler IJ: Modulation of the cancer cell transcriptome by culture media formulations and cell density. Int J Oncol 2015, 46:2067-75.
[115] Sharif GM, Wellstein A: Cell density regulates cancer metastasis via the Hippo pathway. Future Oncology 2015, 11:3253-60.
[116] Mori M, Triboulet R, Mohseni M, Schlegelmilch K, Shrestha K, Camargo FD, Gregory RI: Hippo signaling regulates microprocessor and links cell-density-dependent miRNA biogenesis to cancer. Cell 2014, 156:893-906.
[117] Sharif GM, Schmidt MO, Yi C, Hu Z, Haddad BR, Glasgow E, Riegel AT, Wellstein A: Cell growth density modulates cancer cell vascular invasion via Hippo pathway activity and CXCR2 signaling. Oncogene 2015, 34:5879-89.
[118] Varini K, Benzaria A, Taieb N, Di Scala C, Azmi A, Graoudi S, Maresca M: Mislocalization of the exitatory amino-acid transporters (EAATs) in human astrocytoma and non-astrocytoma cancer cells: effect of the cell confluence. J Biomed Sci 2012, 19:10.
[119] Pelletier H, Millot JM, Chauffert B, Manfait M, Genne P, Martin F: Mechanisms of resistance of confluent human and rat colon cancer cells to anthracyclines: alteration of drug passive diffusion. Cancer Research 1990, 50:6626-31.
[120] Dimanche-Boitrel MT, Pelletier H, Genne P, Petit JM, Le Grimellec C, Canal P, Ardiet C, Bastian G, Chauffert B: Confluence-dependent resistance in human colon cancer cells: role of reduced drug accumulation and low intrinsic chemosensitivity of resting cells. International Journal of Cancer 1992, 50:677-82.

[121] Dimanche-Boitrel MT, Micheau O, Hammann A, Haugg M, Eymin B, Chauffert B, Solary E: Contribution of the cyclin-dependent kinase inhibitor p27KIP1 to the confluence-dependent resistance of HT29 human colon carcinoma cells. International Journal of Cancer 1998, 77:796-802.
[122] Yamada K, Hayashi M, Du W, Ohnuma K, Sakamoto M, Morimoto C, Yamada T: Localization of CD26/DPPIV in nucleus and its nuclear translocation enhanced by anti-CD26 monoclonal antibody with anti-tumor effect. Cancer Cell Int 2009, 9:17.
[123] Xie Y, Zhu S, Song X, Sun X, Fan Y, Liu J, Zhong M, Yuan H, Zhang L, Billiar TR, Lotze MT, Zeh HJ, 3rd, Kang R, Kroemer G, Tang D: The Tumor Suppressor p53 Limits Ferroptosis by Blocking DPP4 Activity. Cell Rep 2017, 20:1692-704.
[124] Larrinaga G, Perez I, Sanz B, Beitia M, Errarte P, Fernandez A, Blanco L, Etxezarraga MC, Gil J, Lopez JI: Dipeptidyl-peptidase IV activity is correlated with colorectal cancer prognosis. PLoS One 2015, 10.
[125] Wilhelm M, Schlegl J, Hahne H, Gholami AM, Lieberenz M, Savitski MM, Ziegler E, Butzmann L, Gessulat S, Marx H, Mathieson T, Lemeer S, Schnatbaum K, Reimer U, Wenschuh H, Mollenhauer M, Slotta-Huspenina J, Boese JH, Bantscheff M, Gerstmair A, Faerber F, Kuster B: Mass-spectrometry-based draft of the human proteome. Nature 2014, 509:582-7.
[126] Strese S, Fryknas M, Larsson R, Gullbo J: Effects of hypoxia on human cancer cell line chemosensitivity. BMC Cancer 2013, 13.
[127] Tsai CM, Perng RP, Chang KT, Venzon D, Gazdar AF: Evaluation of the relative cytotoxic effects of anticancer agents in serum-supplemented versus serum-free media using a tetrazolium colorimetric assay. Japanese Journal of Cancer Research 1996, 87:91-7.
[128] Uifalean A, Schneider S, Ionescu C, Lalk M, Iuga CA: Soy Isoflavones and Breast Cancer Cell Lines: Molecular Mechanisms and Future Perspectives. Molecules 2015, 21:E13.
[129] Lucki NC, Sewer MB: Genistein stimulates MCF-7 breast cancer cell growth by inducing acid ceramidase (ASAH1) gene expression. J Biol Chem 2011, 286:19399-409.
[130] Chen HH, Chen SP, Zheng QL, Nie SP, Li WJ, Hu XJ, Xie MY: Genistein Promotes Proliferation of Human Cervical Cancer Cells Through Estrogen Receptor-Mediated PI3K/Akt-NF-kappaB Pathway. J Cancer 2018, 9:288-95.
[131] Poschner S, Maier-Salamon A, Zehl M, Wackerlig J, Dobusch D, Pachmann B, Sterlini KL, Jager W: The Impacts of Genistein and Daidzein on Estrogen Conjugations in Human Breast Cancer Cells: A Targeted Metabolomics Approach. Frontiers in Pharmacology 2017, 8.
[132] Ju YH, Allred KF, Allred CD, Helferich WG: Genistein stimulates growth of human breast cancer cells in a novel, postmenopausal animal model, with low plasma estradiol concentrations. Carcinogenesis 2006, 27:1292-9.
[133] Campbell-Thompson M, Lynch IJ, Bhardwaj B: Expression of Estrogen Receptor (ER) Subtypes and ER Beta Isoforms in Colon Cancer. Cancer Research 2001, 61:632-40.
[134] Castiglione F, Taddei A, Rossi Degl'Innocenti D, Buccoliero AM, Bechi P, Garbini F, Chiara FG, Moncini D, Cavallina G, Marascio L, Freschi G, Gian LT: Expression of estrogen receptor beta in colon cancer progression. Diagn Mol Pathol 2008, 17:231-6.
[135] Elbanna HG, Ebrahim MA, Abbas AM, Zalata K, Hashim MA: Potential value of estrogen receptor beta expression in colorectal carcinoma: interaction with apoptotic index. J Gastrointest Cancer 2012, 43:56-62.
[136] Arai N, Strom A, Rafter JJ, Gustafsson JA: Estrogen receptor beta mRNA in colon cancer cells: growth effects of estrogen and genistein. Biochem Biophys Res Commun 2000, 270:425-31.

[137] Zand RSS, Jenkins DJA, Diamandis EP: Steroid hormone activity of flavonoids and related compounds. Breast Cancer Research and Treatment 2000, 62:35-49.
[138] Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, Van Der Saag PT, Van Der Burg B, Gustafsson JA: Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998, 139:4252-63.
[139] Han R, Huang G, Wang Y, Xu Y, Hu Y, Jiang W, Wang T, Xiao T, Zheng D: Increased gene expression noise in human cancers is correlated with low p53 and immune activities as well as late stage cancer. Oncotarget 2016, 7:72011-20.
[140] Raser JM, O'Shea EK: Noise in Gene Expression: Origins, Consequences, and Control. Science 2005, 309:2010-3.
[141] Munsky B, Neuert G, van Oudenaarden A: Using Gene Expression Noise to Understand Gene Regulation. Science 2012, 336:183-7.
[142] Peterson JR, Cole JA, Fei J, Ha T, Luthey-Schulten ZA: Effects of DNA Replication on mRNA Noise. PNAS 2015, 112:15886-91.
[143] Keren L, van Dijk D, Weingarten-Gabbay S, Davidi D, Jona G, Weinberger A, Milo R, Segal E: Noise in gene expression is coupled to growth rate. Genome Res 2015, 25:1893-902.
[144] Wu S, Li K, Li Y, Zhao T, Li T, Yang YF, Qian W: Independent regulation of gene expression level and noise by histone modifications. PLoS Comput Biol 2017, 13:e1005585.
[145] Liu Y, Beyer A, Aebersold R: On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell 2016, 165:535-50.
[146] Altschuler SJ, Wu LF: Cellular heterogeneity: when do differences make a difference? Cell 2010, 141:559-63.
[147] Abdallah BY, Horne SD, Stevens JB, Liu G, Ying AY, Vanderhyden B, Krawetz SA, Gorelick R, Heng HH: Single cell heterogeneity: why unstable genomes are incompatible with average profiles. Cell Cycle 2013, 12:3640-9.
[148] Walter E, Kissel T: Heterogeneity in the human intestinal cell line Caco-2 leads to differences in transepithelial transport. European Journal of Pharmaceutical Sciences 1995, 3:215-30.
[149] Lesuffleur T, Kornowski A, Luccioni C, Muleris M, Barbat A, Beaumatin J, Dussaulx E, Dutrillaux B, Zweibaum A: Adaptation to 5-fluorouracil of the heterogeneous human colon tumor cell line HT-29 results in the selection of cells committed to differentiation. International Journal of Cancer 1991, 49:721-30.
[150] Lesuffleur T, Porchet N, Aubert JP, Swallow D, Gum JR, Kim YS, Real FX, Zweibaum A: Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. Journal of Cell Sciences 1993, 106:771-83.
[151] Tone S, Sugimoto K, Tanda K, Suda T, Uehira K, Kanouchi H, Samejima K, Minatogawa Y, Earnshaw WC: Three distinct stages of apoptotic nuclear condensation revealed by time-lapse imaging, biochemical and electron microscopy analysis of cell-free apoptosis. Exp Cell Res 2007, 313:3635-44.
[152] Sztupinszki Z, Gyorffy B: Colon cancer subtypes: concordance, effect on survival and selection of the most representative preclinical models. Sci Rep 2016, 6:37169.
[153] Yamauchi M, Lochhead P, Morikawa T, Huttenhower C, Chan AT, Giovannucci E, Fuchs C, Ogino S: Colorectal cancer: a tale of two sides or a continuum? Gut 2012, 61:794-7.

[154] Katt ME, Placone AL, Wong AD, Xu ZS, Searson PC: In Vitro Tumor Models: Advantages, Disadvantages, Variables, and Selecting the Right Platform. Front Bioeng Biotechnol 2016, 4:12.
[155] Mortensen A, Kulling SE, Schwartz H, Rowland I, Ruefer CE, Rimbach G, Cassidy A, Magee P, Millar J, Hall WL, Kramer Birkved F, Sorensen IK, Sontag G: Analytical and compositional aspects of isoflavones in food and their biological effects. Mol Nutr Food Res 2009, 53 Suppl 2:S266-309.
[156] Zheng Y, Hu J, Murphy PA, Alekel DL, Franke WD, Hendrich S: Rapid gut transit time and slow fecal isoflavone disappearance phenotype are associated with greater genistein bioabailability in women. Journal of Nutrition 2003, 133:3110-6.
[157] Vergne S, Sauvant P, Lamothe V, Chantre P, Asselineau J, Perez P, Durand M, Moore N, Bennetau-Pelissero C: Influence of ethnic origin (Asian v. Caucasian) and background diet on the bioavailability of dietary isoflavones. Br J Nutr 2009, 102:1642-53.
[158] Halm BM, Ashburn LA, Franke AA: Isoflavones from soya foods are more bioavailable in children than adults. Br J Nutr 2007, 98:998-1005.
[159] Hollman PCH: Absorption, Bioavailability, and Metabolism of Flavonoids. Pharmaceutical Biology 2009, 42:74-83.
[160] Huang M, Su E, Zheng F, Tan C: Encapsulation of flavonoids in liposomal delivery systems: the case of quercetin, kaempferol and luteolin. Food Funct 2017, 8:3198-208.

6 Appendix

RIPA Buffer

NaCl	150mM
Tris-HCl	50mM
Triton X-100	1%
Na-Deoxycholate	0.5%
SDS	0.1%
EDTA	1mM
Aprotinin	1µg/mL
Leupeptin	5µg/mL
Phenylmethylsulfonylfluoride	1mM
Ethylenediaminetetraacetic acid	5mM
MilliQ H_2O	

pH 7.4	

Polyacrylamde Resolving Gel

Tris-HCl, pH 8.8	0.4M
Acrylamide/bisacrylamide	8%
SDS	0.1%
Ammonium Persulfate	0.05%
TEMED	7%
MilliQ H_2O	

Polyacrylamide Stacking Gel

Tris-HCl, pH 6.8	0.13M
Acrylamide/bisacrylamide	4%
SDS	0.1%

Ammonium Persulfate	0.05%
TEMED	0.1%
MilliQ H_2O	

Running Buffer

SDS	0.1%
Glycine	192mM
Tris Base	25mM
Methanol	20%
MilliQ H_2O	

Transfer Buffer

Glycine	192mM
Tris Base	25mM

Methanol	20%
MilliQ H_2O	

TBS-T

Tris Base	50mM
NaCl	150mM
Tween-20	0.1%
MilliQ H_2O	
pH 7.6	

1% PFA

PFA	1%
MilliQ H_2O	

PBS

NaCl	0.8%
KCl	0.02%
Na_2HPO_4	0.115%
KH_2PO_4	0.02%
MilliQ H_2O	

PBS Ca^{2+}/ Mg^{2+}

NaCl	0.8%
KCl	0.02%
Na_2HPO_4	0.115%
KH_2PO_4	0.02%
$CaCl_2 \cdot 2H_2O$	0.0132%
$MgCl_2 \cdot 6H_2O$	0.01%

MilliQ H_2O	

2x Laemmli Sample Buffer

Tris-HCl pH 6.8	0.5M
Glycerol	10%
SDS	2%
2-β-Mercaptoethanol	5%
Bromophenol Blue	0.01%
MilliQ H_2O	

Chemiluminescent Solution

Hydrogen Peroxide	0.01%
Luminol	1.25mM
p-Coumaric Acid	0.2mM
Tris-HCl pH 8.5	