Characterizing Changes in Dipeptidyl Peptidase IV/CD26 on Colon Carcinoma Cells in Response to Prostaglandin 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 the thesis, including any required final revisions as accepted by my examiners.

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Abstract

Dipeptidyl peptidase IV (DPPIV) – also known as CD26 – is a multifunctional transmembrane protease found on the surface of most human cells, and shows variable expression between different types of cancer. DPPIV activity may play a role in inhibiting cancer progression by interacting with components of the tumour microenvironment. DPPIV degrades several signalling molecules including the chemokine CXCL12. CXCL12 has been shown to facilitate tumour development when associating with its cell-surface receptor CXCR4. The cell-surface expressions of CXCR4 and DPPIV/CD26 exhibit an inverse relationship; an increase in DPPIV/CD26 is accompanied by a decrease in CXCR4, and vice versa. J-series prostaglandins (i.e. PGD_2, PGJ_2 and 15d-PGJ_2) have been shown to downregulate CXCR4 expression on colorectal carcinoma cells, which in turn may upregulate the expression and activity of DPPIV/CD26. Therefore, I investigated whether J-series prostaglandins altered DPPIV/CD26 expression and activity on colorectal cancer cells. Results showed that these prostaglandins did not upregulate DPPIV/CD26 mRNA expression, nor did they increase its whole-cell or cell-surface protein expression. J-series prostaglandins did increase the mean DPPIV/CD26 dipeptidase activity, however this increase was not statistically significant due to substantial inter-experimental variability. 15d-PGJ_2 treatment also significantly decreased the rate of cell migration on both CRC cells and normal fibroblasts. These findings suggest that J-series prostaglandins may promote DPPIV/CD26 specific activity in colorectal carcinoma. However, 15d-PGJ_2 was found to be significantly unstable at high concentrations in serum-supplemented culture media and this instability in culture medium may explain the inconsistent cell response *in vitro*.

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List of Abbreviations

Δ^{12}-PGJ_2	9-deoxy- Δ-^{9,12}-prostaglandin J_2
15d-PGJ_2	15-deoxy-Δ-^{12,14}-prostaglandin J_2
ADA	Adenosine deaminase
ADAbp	Adenosine deaminase binding protein
ANOVA	Analysis of variance
APS	Ammonium persulfate
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
CA-CRC	Colitis-associated colorectal cancer
CD26	Cluster of differentiation antigen 26
cDNA	Complementary DNA
COX	Cyclooxygenase
Cq	Quantification cycle
CRC	Colorectal cancer
CXCR4	CXC receptor 4
DAPI	2-(4-amidinophenyl)-1H-indole-6-carboxamidine
DEPC	Diethyl pyrocarbonate

DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
DPPIV	Dipeptidyl peptidase IV
dT	Deoxythymine
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FS	First-strand
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase

IgG	Immunoglobin G
M-MLV	Moloney murine leukemia virus
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCS	Newborn calf serum
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Nonsteroidal anti-inflammatory drug
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2

$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGJ_2	Prostaglandin J_2
PMSF	Phenylmethylsulfonyl fluoride
*p*NA	*para*-nitroanilide
PPARγ	Peroxisome proliferator-activated receptor gamma
PPRE	Peroxisome proliferator-activated receptor element
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RXR	Retinoid X receptor
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline-Tween 20
TFA	Trifluoroacetic acid
TEMED	Tetramethylethylenediamine
TME	Tumour microenvironment
TRIS	Tris(hydroxymethyl)aminomethane

TZD	Thiazolidinedione

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1. Introduction

1.1 Colorectal Cancer

1.1.1 Colorectal Cancer: A Perspective

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide (Siegel, DeSantis, & Jemal, 2014). Over 1 million people are diagnosed with the disease each year (Siegel et al., 2014). Fortunately, early-stage CRC mortality rates have decreased due to significant improvements in diagnostic techniques, patient care, and therapeutic treatments (Arnold et al., 2017). However, most deaths from CRC occur in Stage IV when the cancer has spread to distant organs or *metastasized*. The 5-year survival rate of CRC patients dramatically drops from 80-90% to 12.5% when the cancer metastasizes (Siegel et al., 2014). We must uncover the biological processes behind metastasis in order to develop new therapies and prevention strategies. In this project I have examined the relationship between three constituents of the tumour microenvironment (TME) – prostaglandins (PGs), CXCR4 and dipeptidyl peptidase IV/CD26 – that influence CRC progression and metastasis (Figure 1.1).

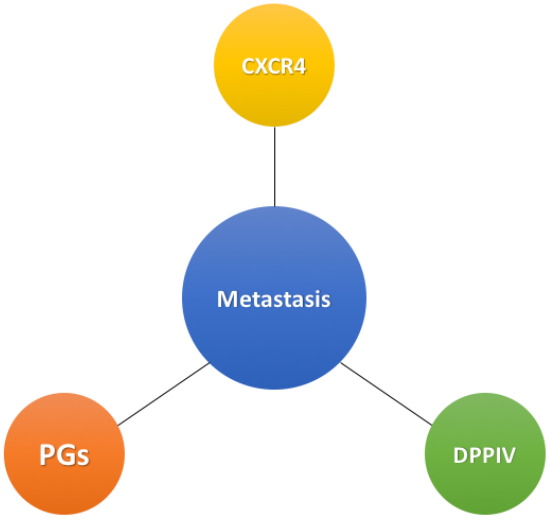

Figure 1.1: Metastasis is influenced by the activities of prostaglandins (PGs), CXCR4 and dipeptidyl peptidase IV (DPPIV) in the tumour microenvironment.

1.1.2 Colorectal Risk Factors and Tumourigenesis

Colorectal cancer can arise from lifestyle or hereditary factors. Most cases of CRC are sporadic; they occur spontaneously without genetic predisposition (Aaltonen et al., 1998). Risk factors for sporadic CRC are obesity, lack of physical activity, alcohol consumption, smoking, and excessive red meat intake (Itzkowitz & Yio, 2004; Johnson et al., 2013). Nevertheless, a small minority (2-5%) of CRC cases are hereditary (Jasperson, Tuohy, Neklason, & Burt, 2010). Individuals with hereditary CRC have inherited a condition that increases the risk of developing CRC such as familial adenomatous polyposis (FAP) and Lynch syndrome (Johnson et al., 2013). Individuals with FAP have a mutated adenomatous polyposis coli (*APC*) gene that causes tens to thousands of polyps to form in the colorectal mucosa, and these polyps can become cancerous (Half,

Bercovich, & Rozen, 2009). Individuals with Lynch syndrome have faulty DNA mismatch repair mechanisms, which increase the risk of developing cancerous mutations during DNA replication (Poulogiannis, Frayling, & Arends, 2010). Another more recently defined CRC subtype is colitis-associated colorectal cancer (CA-CRC). CA-CRC develops from chronic gastrointestinal (GI) inflammatory conditions such as ulcerative colitis and Crohn's disease (Munkholm, 2003).

Cancer arises from the gradual accumulation of mutations in a somatic cell. Somatic mutations can occur at the single nucleotide level (e.g. point mutations) or at the chromosomal level (e.g. translocation, deletion, amplification and aneuploidy) (Loeb & Loeb, 2000). Mutations can result from a) physical DNA damage by environmental mutagens and free radicals, or b) errors in DNA replication by DNA polymerase (Loeb & Loeb, 2000). Mutations that activate proto-oncogenes (e.g. Ras genes HRas, KRas and NRas) and inhibit tumour suppressor genes (e.g. TP53 gene and APC gene) induce the malignant transformation of the cell (Downward, 2003; Muller & Vousden, 2013). The cell thereby acquires hallmark capabilities such as uncontrolled cell growth and division, and evasion of *apoptosis* or programmed cell death (Hanahan & Weinberg, 2000).

Colorectal cancer occurs in the epithelia adjacent to the GI lumen. Accumulated genetic mutations result in uncontrolled proliferation of epithelial cells (Hanahan & Weinberg, 2000). Hyperproliferative cells form a circumferential tumour that invades the mucosa and submucosa of the GI tract (Gutman & Fidler, 1995). The growing tumour eventually protrudes into the lumen and expands through the successive layers of the colon – the muscularis propria and serosa (Zeng et al., 1992). A new capillary network forms around the tumour via *angiogenesis* – the formation of new blood vessels from

existing vasculature – providing the tumour with sufficient oxygen and nutrients to grow beyond 2 mm^3 (Hillen & Griffioen, 2007). Tumour cells detach from the primary mass and enter the circulation by penetrating surrounding blood vessels (Carmeliet & Jain, 2000). The few surviving malignant cells eventually exit the capillaries and enter the parenchyma of distant organs, where they further multiply and form a secondary tumour known as a *metastasis* (Fidler, 2002). CRC most often metastasizes to the liver since it directly connects to the GI tract via the hepatic portal vein (Figure 1.2) (Leporrier et al., 2006).

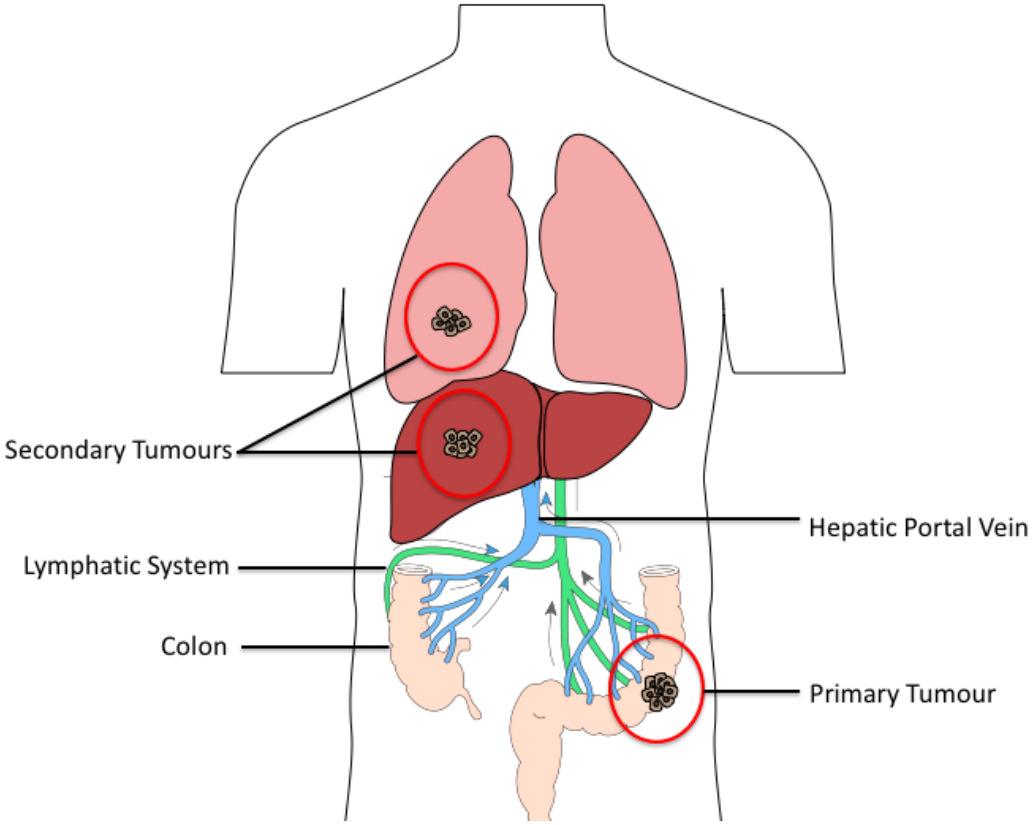

Figure 1.2: Colorectal cancer metastasis.

Cancer cells detach from the colonic tumour and enter the circulation through surrounding capillaries. Circulating tumour cells can travel through the hepatic portal system to reach the liver, and then from the liver to the lung via the inferior vena cava and pulmonary circulation. Tumour cells exit the circulation and embed in the parenchyma of these distant organs where they hyperproliferate to form a secondary tumour. The liver is the most common site for CRC metastasis followed by the lung; however, a secondary tumour can also form in other organs.

1.1.3 Colorectal Carcinogenesis and Inflammation

Chronic colorectal inflammation can lead to CA-CRC. Cancer often arises at the site of inflammation, and the risk of developing cancer increases with the duration and extent of inflammation (Rutter et al., 2004). During inflammation, signalling molecules recruit leukocytes from the venous system to destroy pathogenic substances, digest necrotic cells and repair damaged tissue (Ryan & Majno, 1977). Inflammation becomes chronic when it persists for several months to years without resolution (Shacter & Weitzman, 2002). Chronic inflammation involves repeated tissue damage and regeneration, coupled with the release of mutagenic free radicals and pro-tumorigenic signalling molecules, which creates an ideal environment for the onset of carcinogenesis (Franceschi & Campisi, 2014). Approximately 20% of patients with inflammatory bowel disease develop CA-CRC, and 50% of CA-CRC patients will die from the cancer (Ekbom, Helmick, Zack, & Adami, 1990a, 1990b; Lakatos & Lakatos, 2008).

1.1.4 Colorectal Carcinogenesis and Cyclooxygenase Enzymes

Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are two isozymes that likely promote CA-CRC development by sustaining inflammation in the tissue microenvironment. COX-1 and COX-2 catalyze the biosynthesis of signalling molecules called *prostanoids* including pro-inflammatory prostaglandins (Marnett, Rowlinson, Goodwin, Kalgutkar, & Lanzo, 1999). COX-1 is constitutively expressed, whereas COX-2 expression is induced by inflammatory and growth stimuli (e.g. cytokines, tumour promoters and growth factors) (Vane, Bakhle, & Botting, 1998). COX-2 expression is significantly upregulated in patient colorectal tumours which is associated with worse patient prognosis through increased tumour size, invasiveness and metastasis (Chen et al.,

2001; Eberhart & Dubois, 1995; Masunaga et al., 2000). Inhibiting COX-2 activity may significantly reduce the risk of CRC (Meade, Smith & DeWitt, 1993; Flossman & Rothwell, 2007; Rostom, Dube & Lewin, 2007). Nonsteroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin and ibuprofen) are over-the-counter COX inhibitors that have shown to hinder both the onset and progression of CRC (Meade, Smith, & DeWitt, 1993). Regular NSAID use over 10 to 15 years has shown to reduce the relative risk of CRC development by 40 to 50% (Flossmann & Rothwell, 2007; Rostom, Dube, & Lewin, 2007). NSAID intake has also been associated with improved survival of patients with CRC, and reduced risk of cancer recurrence (Sandler et al., 2003). Targeting the COX pathway may prevent the onset of CRC, or help treat CRC after its onset.

1.2 Prostaglandins

1.2.1 Prostaglandin Structure

Prostaglandins are small lipid molecules that mediate diverse physiological responses (Smith, 1989). They are a subclass of *eicosanoids* which derive from 20-carbon polyunsaturated fatty acids (Smith, 1989). Eicosanoids are synthesized by nucleated cells in most tissues and organs (Smith, 1989). Prostaglandins differ from other eicosanoids by their unique structure which has two side chains attached to a five-carbon ring at carbon-8 and carbon-12 (Silver & Smith, 1975). Individual prostaglandins differ by the presence and positioning of double bonds, carbonyl groups and hydroxyl groups (Silver & Smith, 1975). Each prostaglandin is named A through J, followed by a subscript denoting the number of double bonds within the side chains (Goldyne, 1975). The presence of "α" or "β" after the subscript indicates the spatial orientation of the substitutions on the 5-carbon

ring (Goldyne, 1975). The structural differences between prostaglandins (Figure 1.3) distinguish their biological functions.

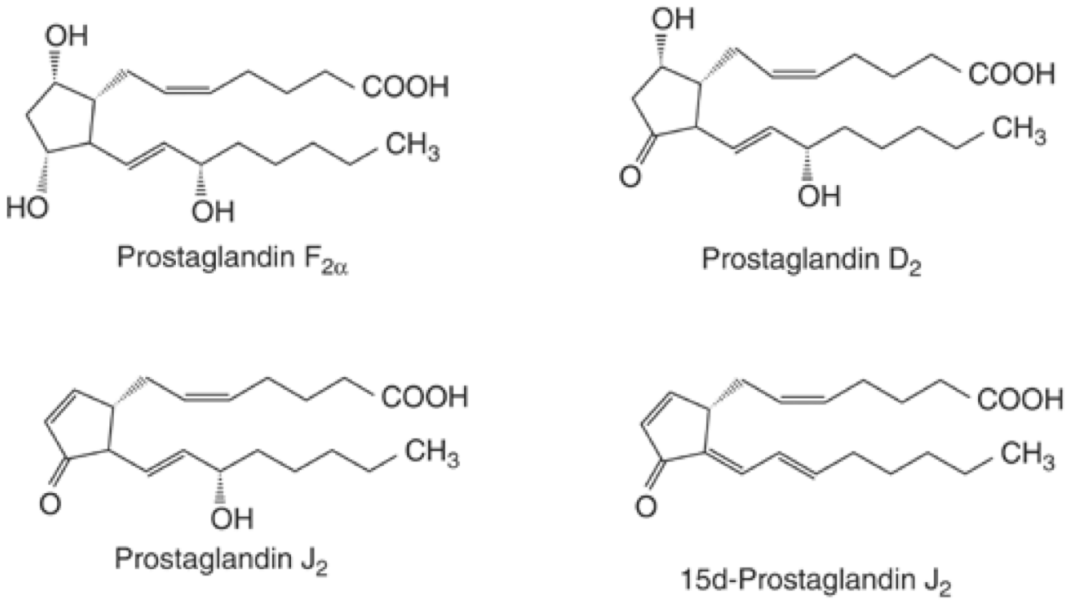

Figure 1.3: The molecular structures of $PGF_{2\alpha}$, PGD_2, PGJ_2 and 15d-PGJ_2 (Adapted from Figarella et al., 2006).

1.2.2 Prostaglandin Biosynthesis

Prostaglandins derive from arachidonic acid (AA) – an omega-6 polyunsaturated fatty acid that is stored in glycophospholipids of the plasma membrane (Anggard & Samuelsson, 1965). Arachidonic acid is either directly obtained from dietary sources (e.g. meat, eggs, and fish) or synthesized from linoleic acid (Meyer et al., 2003). Prostaglandin synthesis commences when phospholipase A_2 catalyzes the release of AA from

membrane glycerophospholipids (Figure 1.4) (T. Shimizu, Ohto, & Kita, 2006). COX-1 and COX-2 add two oxygen molecules to AA to yield prostaglandin H_2 (PGH_2) (Marnett et al., 1999). Since PGH_2 is highly unstable, it is immediately converted by inducible prostaglandin synthases and isomerases into bioactive prostaglandins (Vane et al., 1998). Inflammatory stimuli induce prostaglandin E synthase and prostaglandin F synthase to convert PGH_2 into PGE_2 and $PGF_{2\alpha}$, respectively (Herschman, 1996). PGH_2 can also enter the J-series prostaglandin pathway; prostaglandin D synthase first converts PGH_2 to PGD_2 (Fitzpatrick & Wynalda, 1983). PGD_2 is unstable in aqueous solution and spontaneously undergoes several dehydration reactions to form PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2 (Fitzpatrick & Wynalda, 1983). These PGD_2 derivatives are collectively known as the J-series prostaglandins.

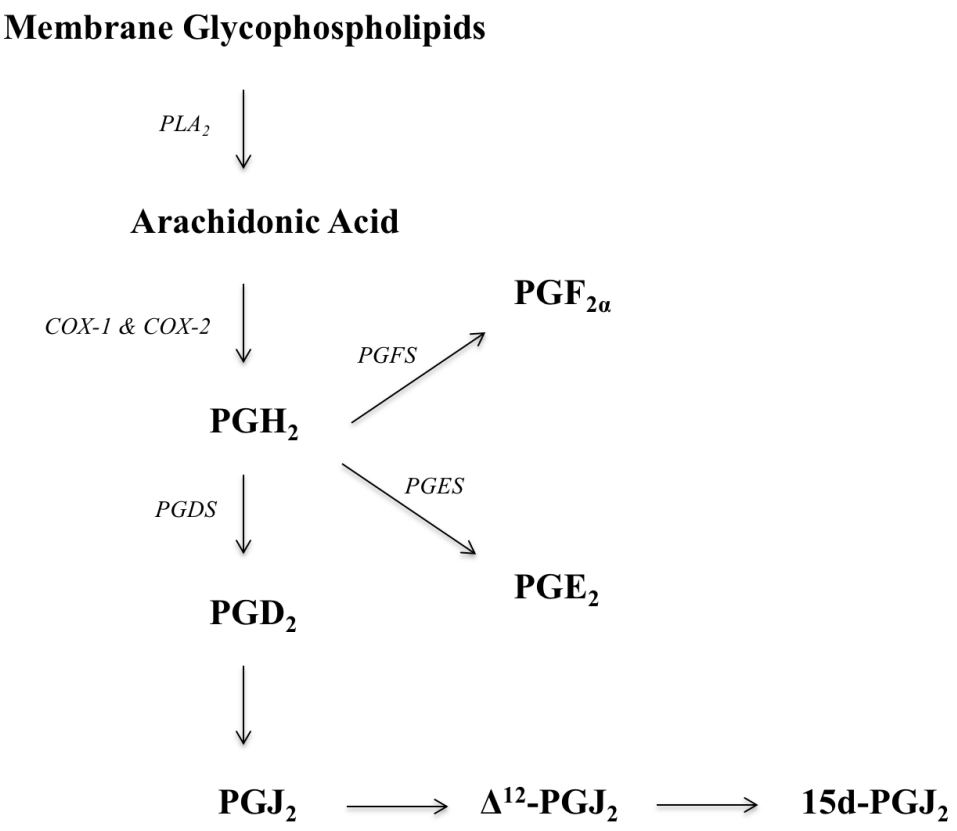

Figure 1.4: Prostaglandin biosynthesis pathway.

Arachidonic acid is released from glycophospholipids of the plasma membrane via phospholipase A_2 (PLA_2). Arachidonic acid is converted to PGH_2 by cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). PGH_2 is converted to either a) $PGF_{2\alpha}$ by prostaglandin F synthase (PGFS), b) PGE_2 by prostaglandin E synthase (PGES), or c) PGD_2 by prostaglandin D synthase (PGDS). PGD_2 is subsequently converted to PGJ_2, which is then transformed to Δ^{12}-PGJ_2 and then 15d-PGJ_2 (Adapted from Scher & Pillinger, 2004).

1.2.3 Prostaglandin Activity

Prostaglandins are called "autocoids" or "local hormones" because they exhibit autocrine or paracrine signalling, respectively (Shimizu & Nakamure, 1985). Unlike regular hormones, prostaglandins do not exhibit endocrine signalling; their short half-lives render them biologically inactive in circulation (Folco & Murphy, 2006; Funk, 2001). Prostaglandins are not usually stored in cells but are rather synthesized and secreted within 5 to 60 seconds of extracellular stimulation (Athari, Hanecke, & Jungermann, 1994; Camacho, Lopez-Belmonte, & Vila, 1998). Prostaglandins exit the cell by passive diffusion or through the multidrug resistance protein MRP4 transporter (Reid et al., 2003). Prostaglandins then activate specific G protein-coupled receptors (GPCRs) on the cell surface (Williams & Higgs, 1988). PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 preferentially bind to DP, EP, FP, and IP receptors, respectively (Table 1.1) (Williams & Higgs, 1988). The J-series prostaglandins PGJ_2, Δ^{12}-PGJ_2 and 15d-PGJ_2 bind to DP_2 receptors with high affinity (Monneret, Li, Vasilescu, Rokach, & Powell, 2002). 15d-PGJ_2 also activates the intranuclear receptor peroxisome proliferator-activated receptor-gamma (PPARγ) in addition to DP_2 (Forman et al., 1995). Prostaglandins are catabolized by 15-hydroxyprostaglandin dehydrogenase which oxidizes the hydroxyl group on carbon-15 (Ensor & Tai, 1995).

Prostaglandins modulate a broad range of homeostatic and immunologic functions. They primarily regulate inflammation, blood pressure and platelet aggregation amongst other physiological activities (Table 1.1). Several prostaglandins can also alter the motility and absorption of the GI tract – namely PGE_2 and $PGF_{2\alpha}$, which are the most abundant prostaglandins in the human colon (Bennett, Hensby, Sanger, & Stamford,

1981; Boughton-Smith, Hawkey, & Whittle, 1983; Matuchansky & Bernier, 1973; Milton-Thompson, Cummings, Newman, Billings, & Misiewicz, 1975). Depending on the type of prostaglandin they can either promote or inhibit a particular response. For example, PGE_2 and PGD_2 promote inflammatory responses, whereas 15d-PGJ_2 is anti-inflammatory (Ricciotti & FitzGerald, 2011; Rossi et al., 2000).

Prostaglandin	Receptor(s)	Main Functions
PGD_2	DP_1 DP_2	Promotes sleep Promotes allergic responses (i.e. sneezing, bronchoconstriction) Promotes allergic inflammation (i.e. airway hyperreactivity, mucus production) Suppresses other types of inflammation (i.e. inhibits production of some immune cells and cytokines)
PGE_2	EP_1 EP_2 EP_3 EP_4	Promotes inflammation (i.e. inducing pain, heat and fever) Induces vasodilation Inhibits PGD_2-induced allergic responses
$PGF_{2\alpha}$	FP	Generates uterine contractions & parturition Promotes acute inflammation Promotes renal function Induces vasoconstriction
15d-PGJ_2	DP_1 DP_2 PPARγ	Promotes anti-inflammatory responses Promotes adipocyte differentiation
PGI_2	IP	Inhibits platelet aggregation Induces vasodilation Inhibits allergic inflammation Promotes embryo implantation

Table 1.1: The receptors and functions of major prostaglandins.

(Ricciotti & FitzGerald, 2011; Rossi et al., 2000).

1.2.4 Prostaglandins in Cancer

Prostaglandins can either inhibit or promote cancer progression depending on the molecule. PGE_2 is upregulated in many different cancers, and has shown pro-tumorigenic effects in numerous *in vitro* and *in vivo* studies (Pugh & Thomas, 1994). For example, treating Caco-2 CRC cells with PGE_2 has increased their survival by inhibiting apoptosis (Leone et al., 2007). Wang et al. (2004) also showed that PGE_2 treatment promoted the survival of CRC cells, and increased intestinal polyp formation in Apc^{min} mice (i.e. mice with a mutated *APC* gene). Blocking PGE_2 receptors EP3 and EP4 has reduced the number and size of intestinal polyps in *APC* knockout mice (Sonoshita et al., 2001). PGE_2 may also facilitate metastasis by stimulating angiogenesis and suppressing immunosurveillance in the tumour microenvironment (Leahy, Koki, & Masferrer, 2000; Namkoong et al., 2005; Pai et al., 2001; Y. Sakai, Fujita, Sakai, & Mizuno, 2001).

It has been suggested that COX inhibitors (e.g. NSAIDs) may prevent the onset and progression of CRC by inhibiting PGE_2 production (Ghosh, Chaki, Mandal, & Mandal, 2010). However, other COX-derived prostaglandins can actually exert anticancer effects – particularly those synthesized through the J-series pathway. Yoshida et al. (1998) found that PGD_2 treatment inhibited the proliferation of multiple CRC cell lines. The authors also measured the level of PGD_2 in human patients' resected colon tumours and found it to be negatively correlated with the incidence of liver metastasis. PGD_2 metabolites have also shown anticancer activity *in vitro*, primarily by inhibiting cell growth and inducing apoptosis (Kim et al., 1993; Narumiya & Fukushima, 1985; Clay et al., 1999). Δ^{12}-PGJ_2 treatment has inhibited the growth of leukemia cells and caused them to undergo apoptosis in a dose-dependent manner (Kim et al., 1993;

Narumiya & Fukushima, 1985). Narumiya and Fukushima (1985) also found that Δ^{12}-PGJ_2 was more potent than PGD_2 in inhibiting cell growth, and suggested that the effects of PGD_2 was actually exerted by the presence of Δ^{12}-PGJ_2, since PGD_2 is rapidly converted to Δ^{12}-PGJ_2 in culture media. Furthermore, Δ^{12}-PGJ_2 and 15d-PGJ_2 treatments have shown to induce apoptosis in breast cancer cells (Clay et al., 1999). 15d-PGJ_2 has also caused apoptosis in gastric, pancreatic, lung and colorectal cancer cells, as well as leukemia cells (Chen, Zhong, Qin, Bing, & He, 2003; Hashimoto, Farrow, & Evers, 2004; Shin et al., 2009).

15d-PGJ_2 may also interfere with angiogenesis. In one study by Xin, Yang, Kowalski & Gerritsen (1999), 15d-PGJ_2 treatment showed to inhibit a) the tubular organization of human umbilical endothelial cells (HUVECs) in three-dimensional collagen gels, and b) vascular formation in the rat cornea (Xin, Yang, Kowalski & Gerritsen, 1999). 15d-PGJ_2 treatment greatly reduced the area of new blood vessels that had resulted from exposure to the angiogenic protein vascular endothelial growth factor (VEGF) (Xin, Yang, Kowalski, & Gerritsen, 1999). Furthermore, 15d-PGJ_2 has suppressed pancreatic xenograft tumour growth in nude mice by reducing the expression of VEGF, thereby hindering angiogenesis (Dong, Wang & Wu, 2009).

1.2.5 15d-PGJ_2 and Peroxisome Proliferator-Activated Receptor Gamma

15d-PGJ_2 may exert its anticancer effects through PPARγ. PPARγ is a transcription factor that is mainly expressed in adipocytes and colorectal epithelial cells (Fajas et al., 1997). It regulates the expression of numerous genes including those that code for proteins involved in cell differentiation, proliferation, apoptosis and migration as well as angiogenesis (Hsu & Chi, 2014; Lea, Sura, & Desbordes, 2004; Sarraf et al.,

1998). 15d-PGJ_2 is a natural ligand for PPARγ, whereas thiazolinediones (TZDs) (which are used to treat type II diabetes) are synthetic PPARγ ligands (Forman et al., 1995).

PPARγ activation commences when 15d-PGJ_2 binds to inactive PPARγ in the cytoplasm (Scher & Pillinger, 2005). PPARγ then dissociates from a co-repressor protein and the 15d-PGJ_2/PPARγ complex translocates to the nucleus (Scher & Pillinger, 2005). The complex forms a heterodimer with the nuclear retinoid X receptor (RXR), which then binds to PPAR-response elements (PPRE) (Scher & Pillinger, 2005). PPREs are short DNA segments within the promoter region of a gene that bind peroxisome-proliferator receptors (PPARs) (Abott, 2009). PPARγ thereby regulates the expression of genes that contain certain PPREs such as those that regulate apoptosis, cell growth, migration and angiogenesis (Hsu & Chi, 2014).

1.3 The CXCL12/CXCR4 Axis

Chemokines are small (8-10 kiloDalton) cytokines that induce chemotaxis in nearby cells (Luster, 1998). Chemotaxis is the directional movement of a cell in response to a chemokine concentration gradient (Keller et al., 1977). Over 40 different chemokines have been discovered, which are categorized into families according to the position of the first two N-terminal cysteine (C) amino acid residues (Luster, 1998). “CC” chemokines exhibit adjacent cysteine residues, whereas one amino acid separates two cysteine residues in “CXC” chemokines (Luster, 1998). Chemokines are produced and secreted by immune cells, fibroblasts, endothelial cells and stromal cells throughout the body (Coondoo, 2011; Landskron, De la Fuente, Thuwajit, Thuwajit, & Hermoso, 2014). Chemokines bind to GPCRs on the cell surface; they may activate multiple receptors, and

each receptor may have more than one ligand (Teicher & Fricker, 2010). Chemokines exhibit autocrine, paracrine, or endocrine signalling mechanisms to induce chemotaxis (Coondoo, 2011; Schrader et al., 2002). Although the mechanism behind chemotaxis is not completely understood, intracellular signalling mechanisms likely cause chemotactic motility by promoting actin polymerization on one end of the cell, and myosin contraction on the opposite end (Levine & Rappel, 2013). This phenomenon creates a "pseudopod" which elongates the cell towards a chemoattractant or away from a chemorepellent (Levine & Rappel, 2013). Chemotaxis is especially crucial for regulating inflammation, tissue homeostasis, and embryonic development (Landskron et al., 2014).

CXCL12 – also known as stromal-derived factor 1 (SDF-1) – is an 8-kilodalton chemokine that is widely distributed throughout the body (Shirozu et al., 1995). There exist two primary isoforms of CXCL12 – CXCL12a (i.e. SFD1-α), and CXCL12b (i.e. SDF1-β) – that result from alternative splicing of the same gene. CXCL12 is a potent chemoattractant, drawing responsive cells towards its highest concentration. CXCL12 induces chemotaxis through binding to its receptor CXC receptor 4 (CXCR4) and is the only known ligand for this receptor (Jiang, Wu, Shi, Wu, & Yin, 2006). Although a second receptor for CXCL12 has been identified as CXC receptor 7 (CXCR7), it does not induce typical migratory responses upon activation (Burns et al., 2006; Naumann et al., 2010). CXCR4 is mainly expressed on epithelial and endothelial cells, lymphocytes, hematopoietic stem cells and stromal fibroblasts, but can also be found on neurons, astrocytes, and glial cells (Guyon, 2014).

CXCL12 guides cell migration in embryonic development, inflammation and wound healing. During embryogenesis, CXCL12 directs $CXCR4^{+}$ progenitor cells

towards their final destination where they undergo differentiation (Cheng et al., 2014). This controls the development of the nervous, cardiovascular and hematopoietic systems (Barrientos et al., 2013; Li & Ransohoff, 2009; Tachibana et al., 1998; Zou, Kottmann, Kuroda, Taniuchi, & Littman, 1998). The CXCL12/CXCR4 axis also regulates inflammation and wound healing. CXCL12 recruits leukocytes and endothelium-committed stem cells to the site of tissue damage for reparation (Kucia et al., 2004).

1.3.1 CXCL12/CXCR4 Signalling in Cancer

CXCR4 is the most commonly expressed chemokine receptor on cancer cells (Balkwill, 2004). Many types of cancers exhibit upregulated CXCR4 including breast, prostate, ovarian, pancreatic, esophageal, cervical, gastric, and head and neck squamous cell cancer, as well as leukemia and melanoma (Akashi et al., 2008; Muller et al., 2001; Richard & Blay, 2008; Scala et al., 2005). CXCR4 expression is particularly abundant in CRC. The receptor is highly expressed on human CRC cell lines (e.g. HT-29 and SW480 cells) and moderately expressed on others (e.g. Caco-2 and LoVo cells) (Ottaiano et al., 2005). CXCR4 is also overexpressed in human colorectal tumours compared to healthy colorectal tissues (Ottaiano et al., 2005).

The overexpression of CXCR4 marks the progression of many different cancers. CXCR4 overexpression in colorectal tumours has predicted poor patient survival and risk of recurrence (Kim et al., 2005). It has also been positively correlated with the occurrence of metastasis to lymph nodes and distant organs (Schimanski et al., 2005). In cervical cancer CXCR4 overexpression in primary tumours has been associated with increased tumour size, lymph node metastasis, and decreased survival (Kodama et al., 2007). CXCR4 overexpression also correlates with reduced survival and increased metastasis in

patients with melanoma, head and neck squamous cell cancer, hepatocellular carcinoma, esophageal cancer, and prostate cancer (Darash-Yahana et al., 2004; Kaifi et al., 2005; Scala et al., 2005; Schimanski et al., 2006; Taki et al., 2008). CXCR4 may serve as a reliable prognostic marker for cancer patients.

CXCL12 signalling may foster tumour growth and metastasis by a) promoting angiogenesis and b) directing primary tumour cell migration. One major function of the CXCL12/CXCR4 axis is blood vessel development and repair, thus it can also help vascularize a malignant tumour (Heidemann et al., 2004). CXCL12 stimulates the release of VEGF which causes the proliferation, migration and assembly of endothelial cells during angiogenesis (Guleng et al., 2005). CXCL12 also attracts endothelial cells to the site of angiogenesis (Orimo et al., 2005). Furthermore, CXCL12 may also promote metastatic spread by stimulating the directional migration of circulating tumour cells (Frick et al., 2011; Singh, Singh, Grizzle, & Lillard, 2004). It has been postulated that these $CXCR4^+$ cells migrate towards organs rich in CXCL12 where they can form a secondary tumour (Chambers, Groom, & MacDonald, 2002). The most common sites for metastasis – the lung, liver, lymph nodes and bone marrow – express the highest levels of CXCL12, whereas the least common organs for metastasis express the lowest CXCL12 levels (Muller et al., 2001).

Decreasing CXCR4 expression and activity may impede metastasis. Silencing CXCR4 mRNA expression via small interfering RNA (siRNA) has inhibited CXCL12-induced migration of colon cancer cells and breast cancer cells (Chen, Stamatoyannopoulos & Song, 2003; Frick et al., 2011; Liang et al., 2005). Silencing CXCR4 also decreased the number of lung metastases in mice inoculated with breast

cancer cells (Liang et al., 2004). Blocking CXCR4 activity with either CXCR4 antagonists or anti-CXCR4 antibodies has inhibited breast cancer cell migration toward CXCL12 (Liang et al., 2004). CXCR4 antagonists have also reduced CXCL12-mediated migration of CRC cells (Li et al., 2008). Decreasing CXCR4 expression may impede metastasis by preventing cancer cell migration to distant organs.

1.3.2 The Relationship Between Prostaglandins and CXCR4

Prostaglandins may downregulate CXCR4 expression in CRC. An important study by Richard, Lowthers and Blay (2007) showed that J-series prostaglandins reduced CXCR4 mRNA and cell-surface protein expression on HT-29 CRC cells, and 15d-PGJ_2 exerted the most pronounced effect (Richard, Lowthers, & Blay, 2007). The effects of these prostaglandins on CXCR4 expression were likely achieved by altering the activity of transcription factors PPARγ and nuclear factor-κB (NFκB) (Richard, Lowthers & Blay, 2007). 15d-PGJ_2 and synthetic PPARγ agonists were able to downregulate CXCR4 expression to a similar degree, however the addition of PPARγ antagonists could not completely reverse this effect. The authors then showed that the cyclopentenone structure of J-series prostaglandins permitted the inhibition of NFκB, thereby suppressing the expression of the *CXCR4* gene. Therefore, 15d-PGJ_2 can downregulate CXCR4 expression by activating PPARγ and inhibiting NFκB (Richard, Lowthers & Blay, 2007).

1.4 Dipeptidyl Peptidase IV/CD26

1.4.1 DPPIV/CD26 Structure and Function

Dipeptidyl peptidase IV (DPPIV), or CD26, is a transmembrane protease that has several important functions in the tissue microenvironment. The enzyme is primarily anchored to the outer membrane of most mammalian cells, but also resides in the cytosol and nucleus to a lesser extent (Mccaughan, Wickson, Creswick, & Gorrell, 1990; Yamada et al., 2009). It contains a short cytoplasmic domain, a transmembrane domain, and a relatively large extracellular domain (Figure 1.5). The extracellular domain is the site of enzymatic activity and contains a) a glycosylation site, b) a cysteine-rich site that binds collagen and fibronectin, as well as adenosine deaminase (ADA), and c) a catalytic site that cleaves certain polypeptides (Huhn, Ehrlich, Fleischer, & von Bonin, 2000; Kameoka, Tanaka, Nojima, Schlossman, & Morimoto, 1993; Piazza, Callanan, Mowery, & Hixson, 1989). DPPIV is also referred to as cluster of differentiation antigen 26 (CD26) and adenosine deaminase binding protein (ADAbp) since these proteins were discovered to be structurally homologous to DPPIV (Darmoul et al., 1991; Morrison, Vijayasaradhi, Engelstein, Albino, & Houghton, 1993). The designation "DPPIV" typically refers to its enzymatic activity. DPPIV/CD26 has been investigated for its potential role in cancer due to its multifunctional nature and its differential expression on cancer cells versus healthy cells, as discussed below.

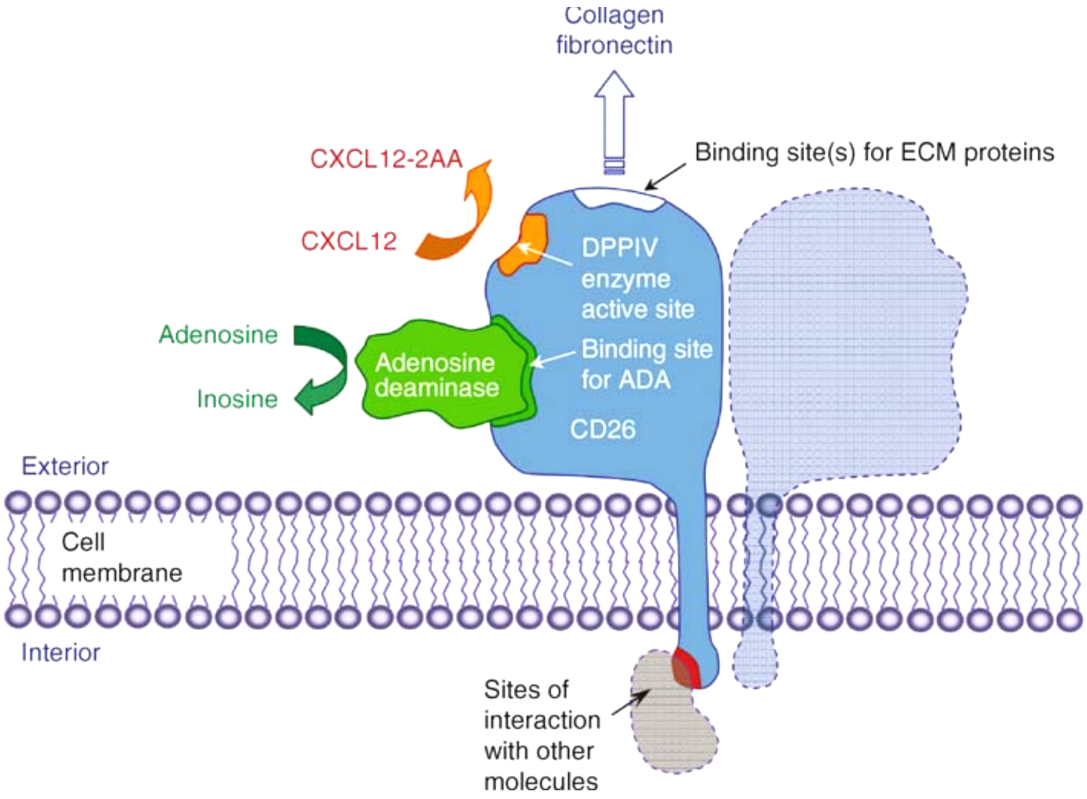

Figure 1.5: Dipeptidyl peptidase IV/CD26 within the plasma membrane. (Reproduced from Blay, 2008 with permission).

1.4.2 DPPIV/CD26 Expression and Distribution

Dipeptidyl peptidase IV/CD26 is ubiquitously expressed in normal tissues. Epithelial cells primarily express DPPIV/CD26, yet the enzyme is also found on T- and B-lymphocytes, fibroblasts and endothelial cells (Mccaughan et al., 1990). DPPIV/CD26 is distributed within most mammalian tissues and organs, and the level of expression depends on the type of tissue and the location within that tissue (Abott, Baker, Sutherland & McCaughan, 1995; Hartel, Gossrau, Hanski & Reutter, 1988). The enzyme is constitutively expressed in the kidney, small intestine and liver (Kettmann, Humbel, & Holzhausen, 1992). The brush border membranes of epithelial cells in the kidney cortex and small intestine strongly express DPPIV/CD26 (Stange, Kettmann, & Holzhausen,

1996). In the small intestine, the ileum and jejunum exhibit the highest DPPIV/CD26 expression and DPPIV activity (Darmoul et al., 1994). In the liver, DPPIV/CD26 is found on the bile duct epithelia, and on hepatocytes around the bile caniculi (Fukasawa et al., 1981; Hartel et al., 1988). The enzyme is also expressed in small amounts on colon epithelial cells (Dinjens et al., 1989). Furthermore, DPPIV/CD26 is also situated on epithelial cells lining the sweat, adrenal, prostate and salivary glands (Sahara & Suzuki, 1984). In the cardiovascular system, DPPIV/CD26 is expressed on endothelial cells that comprise the capillary beds of the lung, spleen, pancreas, myocardium and blood-brain barrier (Hartel et al., 1988; Lojda, 1979). Soluble DPPIV/CD26 (sDPPIV), which only consists of its extracellular domain, is dissolved in blood plasma, urine, amniotic fluid, semen and synovial fluid (Durinx et al., 2000). sDPPIV retains its binding capacity and peptidase activity (Durinx et al., 2000).

1.4.3 DPPIV Dipeptidase Activity of DPPIV/CD26

Dipeptidyl peptidase IV selectively removes an N-terminal dipeptide from many natural polypeptides (Figure 1.6) (Mentlein, Gallwitz, & Schmidt, 1993). Substrates with proline or alanine at the penultimate position (P_1) – the second residue from the N-terminus – are cleaved with the highest catalytic efficiency (Mentlein et al., 1993). However, proline-containing substrates are preferred. Other substrates that are cleaved less efficiently may contain hydroxyproline, dehydroproline, serine, glycine, valine, threonine or leucine at P_1 (de Meester, Lambeir, Proost, & Scharpe, 2003). The dipeptidase activity of DPPIV/CD26 is primarily studied through its cleavage of synthetic chromogenic or fluorogenic substrates such as Gly-Pro-*p*-nitroanilide and Ala-Pro-cresyl violet, respectively.

Dipeptidyl peptidase IV/CD26 can either suppress or enhance the activity of its substrates upon cleavage, thereby adjusting the bioactivity of many regulatory polypeptides in the body (de Meester et al., 2003). DPPIV/CD26 cleaves a diverse range of bioactive substrates including cytokines, chemokines, growth factors, peptide hormones and neuropeptides (Table 1.2). Consequently, the enzyme modulates a broad range of physiological processes including development, immune and inflammatory responses, blood vessel dilation and constriction, hunger, energy homeostasis, and digestion (Blay, 2008). Targeting the dipeptidase activity of DPPIV/CD26 may confer diverse therapeutic advantages.

Dipeptidyl peptidase IV/CD26 degrades numerous chemokines with different levels of efficiency. The chemokine that is degraded with the highest catalytic efficiency is CXCL12, which contains a proline penultimate residue (Lambeir et al., 2001). DPPIV/CD26 truncates CXCL12 and terminates its bioactivity (De La Luz Sierra et al., 2004; Shioda et al., 1998). As a result, CXCL12-mediated chemotaxis is impeded (Shioda et al., 1998). CXCL12 inactivation by DPPIV/CD26 has reduced the migration of hematopoietic and progenitor cells *in vitro*, and inhibiting DPPIV dipeptidase activity on these cells restored their ability to migrate (Christopherson, Hangoc, & Broxmeyer, 2002). Cleavage of CXCL12 by DPPIV/CD26 may contribute to the regulation of cell migration in the body.

Dipeptidyl peptidase IV/CD26 may impede metastasis by decreasing CXCL12-mediated cell migration. CXCL12 activity is upregulated in various types of cancer (Liekens, Schols, & Hatse, 2010). The CXCL12/CXCR4 axis facilitates metastasis by directing primary tumour cells to secondary organs and tissues (Vandercappellen, Van

Damme, & Struyf, 2008). Reducing bioavailable CXCL12 via DPPIV/CD26 has been shown to downregulate cancer metastasis *in vivo* (Sun et al., 2008). In one study by Sun et al. (2008) the authors modelled metastasis by establishing primary tumours in immunodeficient mice and subsequently measuring the number of primary tumour cells that migrated into different tissues. Mice that were treated with the DPPIV/CD26 inhibitor diprotin A exhibited significantly more primary tumour cells in the brain, lymph nodes, mandible, humerus and tibia than mice that were treated with the vehicle control. The authors concluded that DPPIV/CD26 inhibited cancer cell migration likely through its degradation of CXCL12 (Sun et al., 2008).

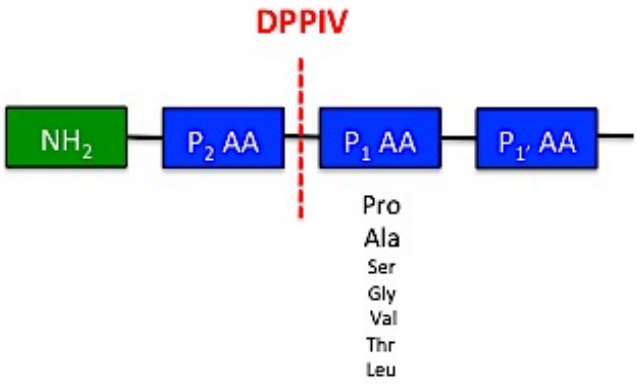

Figure 1.6: DPPIV/CD26 dipeptidase activity.

DPPIV/CD26 cleaves the peptide bond after the penultimate (P_1) residue. Possible P_1 amino acid (AA) residues include proline (Pro), alanine (Ala), serine (Ser), glycine (Gly), valine (Val), threonine (Thr) and leucine (Leu). Catalytic efficiency of the substrate decreases with decreasing font. (Adapted from Klemann, Wagner, Stephan & von Horsten, 2016).

Molecule	Full name and main function
CXCL12	SDF-1α (stromal cell-derived factor-1α); *Involved in the development of the nervous system, bone marrow and intestine, and in the homing of stem cells.*
CCL22	Macrophage-derived chemokine; *An attractant for various types of white cells and functions in immune and inflammatory responses.*
GRP	Gastrin releasing peptide; *Released by nerves in the stomach to cause the production of gastrin from G cells in the mucosa.*
NPY	Neuropeptide Y; *Peptide neurotransmitter found in the brain that regulates normal physiological processes such as hunger, energy homeostasis and blood pressure.*
GLP-1	Glucagon-like peptide-1; *Gut hormone secreted by L cells in the intestine, has a role in control of insulin levels.*
CCL11	The chemokine Eotaxin-1; *Causes the recruitment of eosinophils into tissues and plays a role in allergic responses.*
CCL5	The chemokine RANTES ('Regulated on Activation, Normal T Expressed and Secreted'); *Selective attractant for memory T lymphocytes and monocytes.*
VIP	Vasoactive intestinal peptide; *Peptide hormone produced by various tissues, with effects on blood vessels and secretory processes.*

Table 1.2: Some of the major substrates for dipeptidyl peptidase IV and their activities in human tissues.

(Reproduced from Blay, 2008 with permission).

1.4.4 DPPIV/CD26 Dipeptidase Activity in Diabetes

The dipeptidase activity of DPPIV/CD26 is also implicated in type II diabetes. Common DPPIV/CD26 substrates are *incretins* (e.g. glucagon-like peptide-1 (GLP-1),

glucose-dependent insulinotropic polypeptide (GIP)) which are intestinal polypeptides that promote the secretion of insulin, and thus lower blood glucose levels (Green, Flatt & Bailey, 2006; Nielsen, 2005). DPPIV/CD26 rapidly degrades incretins, inhibiting their effect on insulin release (Green, Flatt & Bailey, 2006; Nielsen, 2005). DPPIV inhibitors called *gliptins* prevent the degradation of incretins, thereby increasing insulin levels in the blood and thus lowering blood glucose levels in diabetic patients (Green, Flatt & Bailey, 2006; Nielsen, 2005).

1.4.5 DPPIV/CD26 and Cell Adhesion

Another major function of DPPIV/CD26 is promoting cell adhesion. Cell adhesion is the process by which cells associate with each other and with the ECM (Zhu, Bao, & Wang, 2000). The ECM is a dynamic network of macromolecules outside the cell that provides structural and biochemical support (Bosman & Stamenkovic, 2003). Two abundant fibrous proteins within the ECM are collagen and fibronectin (Bosman & Stamenkovic, 2003). Fibronectin anchors cells to collagen to promote adhesion to the ECM (Klebe, 1974). Cell-surface DPPIV/CD26 binds to both collagen and fibronectin, serving as an additional anchor to the ECM (Hanski, Huhle, Gossrau, & Reutter, 1988). In fact, the cysteine-rich region of DPPIV/CD26 has specific binding sites for collagen and fibronectin that are distinct from the catalytic domain (H. C. Cheng, Abdel-Ghany, & Pauli, 2003; Loster, Zeilinger, Schuppan, & Reutter, 1995).

Dipeptidyl peptidase IV/CD26 may prevent metastasis by suppressing the migration of primary tumour cells. DPPIV/CD26 overexpression has been associated with increased cell-cell adhesion, and reduced migration and invasive potential of ovarian carcinoma cells (Inamoto et al., 2007; Kikkawa et al., 2005). Furthermore, inducing

DPPIV/CD26 expression in non-small cell lung cancer cell lines has been shown to reduce their migration on both fibronectin- and collagen-coated chambers by 50 to 75% (Wesley, Tiwari, & Houghton, 2004).

1.4.6 DPPIV/CD26 and Extracellular Adenosine

Dipeptidyl peptidase IV/CD26, or ADAbp, also promotes the breakdown of extracellular adenosine. DPPIV/CD26 has a distinct binding site for adenosine deaminase (ADA) – an enzyme that catalyzes the irreversible metabolism of the nucleosides adenosine and 2'deoxyadenosine (Brady, 1942; Spencer, Hopkinson, & Harris, 1968). DPPIV/CD26 and ADA co-localize on the cell surface to augment the deamination of adenosine (Dong et al., 1996). Deamination inactivates these nucleosides and prevents their accumulation in the extracellular fluid (Spencer et al., 1968).

Metabolizing adenosine may impede cancer progression. Extracellular adenosine can promote tumour survival and growth by suppressing cell-mediated immune responses. The nucleoside prevents the activation and proliferation of lymphocytes, and can also be cytotoxic to lymphocytes (Hovi, Smyth, Allison, & Williams, 1976). Furthermore, adenosine can promote metastasis by stimulating cancer cell motility and proliferation as well as angiogenesis (Hasko & Cronstein, 2004; Mujoomdar, Bennett, Hoskin, & Blay, 2004; Mujoomdar, Hoskin, & Blay, 2003; Woodhouse et al., 1998) (Barcz, Sommer, Janik, Marianowski, & Skopinska-Rozewska, 2000; Olah & Caldwell, 2003). The extracellular fluid of solid tumours in mice has exhibited high levels of extracellular adenosine (Blay, White, & Hoskin, 1997). Interestingly, high levels of adenosine can decrease DPPIV/CD26 expression and ADA binding in CRC cells (Tan et

al., 2006). The relationship between adenosine and DPPIV/CD26 may provide insight into the metastatic progression of cancer.

1.4.7 DPPIV/CD26 and the Immune Response

DPPIV/CD26 also regulates the immune response through T lymphocyte activation (Gorrell, Gysbers, & McCaughan, 2001). T lymphocytes are a subpopulation of white blood cells that become activated in response to an antigen, and subsequently trigger immunologic processes (Reinherz & Schlossman, 1980). T cell-surface expression of DPPIV/CD26 increases after antigenic stimulation, and these activated $CD26^{+}$ lymphocytes rapidly proliferate to induce a robust immune response (Ulmer, Mattern, & Flad, 1992). Upregulated DPPIV/CD26 expression on the cell surface is therefore considered a marker of T cell activation. This effect may influence immunosurveillance in the TME.

1.4.8 DPPIV/CD26 Expression in Cancer

Dipeptidyl peptidase IV/CD26 expression is highly variable in cancer. The enzyme is upregulated in some cancers and downregulated in others. Malignant mesothelioma and prostate cancer tissues have both shown significantly higher DPPIV/CD26 expression than their respective benign tissues (Inamoto et al., 2007; Wilson et al., 2000). Renal carcinoma cells have also exhibited upregulated DPPIV/CD26 expression compared to healthy cells (Inamoto et al., 2006). Meanwhile, a downregulation in DPPIV/CD26 expression has been reported in melanoma, endometrial cancer, liver cancer, and non-small cell lung cancer (Houghton, Albino, Cordon-Cardo, David & Eisinger, 1988; Khin et al., 2003; Stecca et al., 1997; Wesley et al., 2004).

Dipeptidyl peptidase IV/CD26 can also exhibit variable expression within one cancer type. Findings on DPPIV/CD26 expression in colorectal, prostate and ovarian cancer are inconsistent across the literature (Havre et al., 2008). Several studies have shown considerable variation in DPPIV/CD26 abundance and localization between different colorectal tumours, and between cells within one colorectal tumour (Ten Kate, Vandeningh, Khan & Bosman, 1986; Ten Kate et al., 1985). Furthermore, the amount of soluble DPPIV/CD26 (sCD26) dissolved in the blood plasma of CRC patients also varied between studies. Cordero et al. (2000) found reduced sCD26 levels in CRC patients, whereas de la Haba-Rodriguez et al. (2002) reported upregulated sCD26, with the highest levels present in patients with metastatic CRC. Although the differential expression of DPPIV/CD26 in CRC is still being elucidated, it might be explained by the level of differentiation of the carcinoma, as well as the heterogeneity of cell subpopulations in the tumour (Kate, Vandeningh, Khan & Bosman, 1986).

The prognostic significance of DPPIV/CD26 expression also varies between different cancers. In some cancers, DPPIV/CD26 expression is positively correlated with improved patient prognosis. For instance, overexpressed DPPIV/CD26 in malignant pleural mesothelioma tumours has been associated with prolonged patient survival (Aoe et al., 2012). DPPIV/CD26 overexpression has also been correlated with reduced prostate cancer metastasis (Bogenrieder et al., 1997). In contrast, DPPIV/CD26 overexpression is associated with metastasis in several types of cancer. Lam et al. (2014) found that DPPIV/CD26 expression was higher in primary tumours of patients with metastatic CRC than patients with local CRC, and DPPIV/CD26 overexpression indicated worse patient

survival. Worse patient survival has also been correlated with increased levels of DPPIV/CD26 in ovarian tumours (M. Z. Zhang, Qiao, & Suo, 2008).

Increasing DPPIV/CD26 expression *in vitro* has been shown to reduce the metastatic activity of some cancers. For example, a study by Kajiyama et al. (2002) reported that $CD26^+$ ovarian carcinoma cells showed less migration and invasion than $CD26^-$ ovarian carcinoma cells. The authors also found a correlation between DPPIV/CD26 overexpression and a prolonged survival time of nude mice inoculated with CD26-positive ovarian carcinoma cells. Furthermore, transfecting CD26 into melanoma cells has reduced their invasiveness by 75% (Pethiyagoda, Welch, & Fleming, 2001).

Dipeptidyl peptidase IV/CD26 expression and activity in cancer probably depends on multiple parameters. Since DPPIV/CD26 exhibits pleiotropic effects between different tissues, its effect on cancer progression likely depends on the location of the tumour (Blay, 2008). In fact, a study on differential gene expression in the colon reported significantly higher DPPIV/CD26 mRNA levels in right-sided than left-sided colorectal tumours (Fric et al., 2000). Second, the level of differentiation of the cancer ought to be considered. Darmoul et al. (1992) found that DPPIV/CD26 expression on HT-29 and Caco-2 CRC cells positively correlated with their degree of differentiation. Sakamoto et al. (1993) also found high DPPIV/CD26 expression in well-differentiated cancers, whereas its expression was virtually absent in poorly differentiated cancers. Finally, studies on DPPIV/CD26 expression vary greatly in the methods by which they detected the enzyme, with some methods being more sensitive than others.

1.4.9 The Relationship Between DPPIV/CD26 and CXCR4

Dipeptidyl peptidase IV/CD26 and the chemokine receptor CXCR4 are related in several ways. First, DPPIV/CD26 degrades and inactivates the CXCR4 ligand CXCL12 with high affinity (Lambeir et al., 2001). DPPIV/CD26 and CXCR4 also exhibit an inverse relationship; the cell-surface expression of CXCR4 has shown to decrease with increased DPPIV/CD26 expression, and vice versa (Cutler et al., 2015; Tan et al., 2006; Richard, Tan & Blay, 2006; Kajiyama et al., 2007). The administration of chemotherapeutic agents to CRC cells increases cell-surface levels of DPPIV/CD26 while simultaneously downregulating CXCR4 (Cutler et al., 2015). The maximum decrease in CXCR4 averaged to 78.6% between different anticancer drug treatments, while the maximum increase in DPPIV/CD26 ranged from 22.4% to 72.1%. Furthermore, treating HT-29 CRC cells with adenosine has both suppressed CXCR4 expression and upregulated DPPIV expression and activity in separate studies (Tan et al., 2006; Richard, Tan & Blay, 2006). On the contrary, a concomitant decrease in DPPIV/CD26 and increase in CXCR4 expression has been observed in human peritoneal mesothelial cells in response to transforming growth factor-β1 treatment (Kajiyama et al., 2007).

1.5 Rationale of the Project

Dipeptidyl peptidase IV/CD26 regulates several anti-metastatic activities in the tumour microenvironment. Although DPPIV/CD26 has shown variable expression in CRC, upregulation of the enzyme may suppress metastasis by (i) decreasing extracellular adenosine, (ii) promoting cellular adhesion to the extracellular matrix, and (iii) degrading the pro-tumorigenic chemokine CXCL12. It is unclear why DPPIV/CD26 expression varies in CRC, although tissue factors likely play a role. However, DPPIV/CD26 expression and activity have shown to increase with a concurrent decrease in CXCR4 in response to various treatments. CXCR4 expression on colorectal cancer cells can be downregulated by several J-series prostaglandins (particularly 15d-PGJ_2). Therefore, we investigated whether such prostaglandin treatment conversely upregulates DPPIV/CD26 levels on CRC cells (Figure 1.7). Upregulated DPPIV/CD26 would likely suppress the metastatic potential of CRC cells.

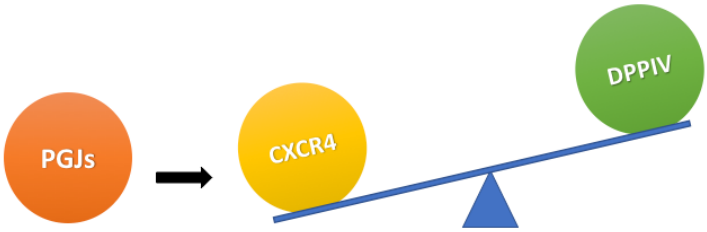

Figure 1.7: The relationship between prostaglandins, CXCR4 and DPPIV/CD26 in the tumour microenvironment.

J-series prostaglandins (PGJs) have shown to downregulate CXCR4 expression on CRC cells. Downregulated CXCR4 expression has been previously associated with an increase in DPPIV expression and activity. Increased DPPIV may also occur with J-series prostaglandin (PGJ) treatment.

2. Objectives and Hypothesis

2.1. Objectives

The specific objectives of my project were to:

1. Quantify changes in DPPIV/CD26 dipeptidase activity on colon carcinoma cells in response to prostaglandin treatment.
2. Identify changes in DPPIV/CD26 mRNA and protein expression in colon carcinoma cells in response to prostaglandin treatment.
3. Determine changes in cell-ECM interactions due to prostaglandin treatment on colon cancer cells.

2.2. Hypothesis

Prostaglandin treatment will induce an upregulation in DPPIV/CD26 expression and activity on colon carcinoma cells, and this will increase the DPPIV/CD26-mediated cellular interactions with the extracellular matrix.

3. Materials and Methods

3.1 Materials

HT-29 and Caco-2 human colon carcinoma cells, and NIH3T3 mouse fibroblasts, were obtained from the American Type Culture Collection (Manassas, VA). Culture flasks and 96- and 24-well plates (Nunc™), as well as media (HyClone Laboratories Inc.) and trypsin-EDTA (0.25%) (Gibco™) were from ThermoFisher Scientific (Mississauga, ON, Canada). Fetal bovine serum (FBS) was from VWR International (Mississauga, ON, Canada) and newborn calf serum (NCS) was from ThermoFisher Scientific (Mississauga, ON, Canada). 15d-PGJ_2, PGJ_2, PGD_2 and $PGF_{2\alpha}$ were from Cayman Chemical (Ann Arbor, MI, USA). Dimethyl sulfoxide (DMSO) (Fisher Bioreagents™) was from Fisher Scientific (Mississauga, ON, Canada). Gly-Pro-*p*-nitroanilide (Gly-Pro-*p*NA) DPPIV substrate and bovine serum albumin (BSA) were from Sigma-Aldrich (Oakville, ON, Canada), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Fisher BioReagents™) was from Fisher Scientific (Mississauga, ON, Canada). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Alfa Aesar™) was obtained from Fisher Scientific (Mississauga, ON, Canada). Tris(hydroxymethyl)aminomethane (TRIS) buffer (AMRESCO Inc.) was from VWR International (Mississauga, ON, Canada). Bio-Rad Protein Assay Dye Reagent was from BioRad Laboratories (Mississauga, ON, Canada). Protein inhibitors phenylmethylsulfonyl fluoride (PMSF) and leupeptin (Tocris Bioscience), as well as aprotinin (Cayman Chemical) were from Cedarlane Laboratories (Burlington, ON, Canada). Ethylenediaminetetraacetic acid (EDTA), glycine, glycerol and Tween 20 were purchased from Fisher Scientific (Mississauga, ON, Canada). Triton X-11, p-coumaric

acid and luminol were purchased from Sigma-Aldrich (Oakville, ON, Canada). 96-well polypropylene plates and the C18 high performance liquid chromatography (HPLC) column were from Agilent Technologies (Mississauga, ON, Canada). Chloroform and HPLC-grade acetonitrile were purchased from Sigma Aldrich (Oakville, ON, Canada). LCMS-grade trifluoroacetic acid was from CovaChem LLC (Loves Pak, IL, USA). TRI Reagent® and potassium acetate were purchased from Sigma-Aldrich (Oakvilla, ON, Canada). First strand (FS) buffer, dithiothreitol (DTT), RNAse Out® and Moloney Murine Leukemia Virus (M-MLV) were from Invitrogen Canada (Burlington, ON, Canada). Oligo deoxythymine (dT) and CD26 and GAPDH polymerase chain reaction (PCR) primers (Jena Bioscience) were from Cedarlane Labs, and deoxynucleotide triphosphate (dNTP) mix was from ThermoScientific (Mississauga, ON, Canada). SSO Advanced SYBR Green quantitative PCR (qPCR) Mix was from BioRad Laboratories (Mississauga, ON, Canada).

3.2 Antibodies and Primers

3.2.1 Antibodies

For Western blot experiments, rabbit monoclonal antibodies (mAb) against human CD26 (clone EPR5883), and mouse mAb (HRP conjugated) against human ß-actin (clone mAbcam 8226) were from Abcam Inc. (Toronto, ON, Canada). Goat anti-rabbit IgG (HRP-conjugated) was purchased from Invitrogen Canada (Burlington, ON, Canada). For immunofluorescence experiments, rabbit polyclonal antibodies (pAb) against human CD26 were purchased from Abcam Inc. (Toronto, ON, Canada). Alexa Fluor® 488 conjugated goat anti-rabbit IgG was obtained from Invitrogen Canada (Burlington, ON, Canada).

3.2.2 Primers

DNA primer sets for PCR and qPCR were obtained from Invitrogen Canada (Burlington, ON, Canada) and had the following sequences:

1. CD26:	Forward: 5'-CTCACTTCCGAGGAGACGCC-3'
	Reverse: 5'-TCATCTGTGCCTTTGTTCAGCAGA-3'
2. GAPDH:	Forward: 5'-CGACCACTTTGTCAAGCTCA-3'
	Reverse: 5'-AGGGGTCTACATGGCAACTG-3'

3.2.3 Primer Selection

Short DNA primers were used in PCR and qPCR experiments to identify DNA fragments of CD26. Primers were designed using the National Center for Biotechnology Information (NCBI) PrimerBLAST program. Criteria for primer selection were based on recommendations from the StepOnePlus PCR manual (Applied Biosystems). 2000 primers were screened for a size between 18-25 base pairs, an amplicon length between 80-250 base pairs, a melting temperature (T_m) between 58-61°C, and a 30-80% G/C content with a maximum of 3 G and/or C bases within the five nucleotides on the 3' end. The maximum mononucleotide repeat (max poly-X) score, self-complementarity score, and template mispriming score were minimized (5 or lower). Selected primers fulfilled most, if not all, of the above criteria.

3.3 Cell Culture

HT-29, Caco-2 and NIH3T3 cell lines were maintained as stocks in 20-cm^2 flasks and incubated at 37°C in a humidified atmosphere of 90% air/10% CO_2. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (without antibiotics) with 1%

sodium pyruvate, supplemented with either 5% or 10% $^{v}/_{v}$ heat-inactivated fetal bovine serum (FBS) (HT-29 and Caco-2 cells), or 10% $^{v}/_{v}$ heat-inactivated newborn calf serum (NCS) (HT-29 cells and NIH3T3 cells). Cells were subcultured when approximately 80% confluent and split at a 1:5 ratio.

Cells were treated with specific concentrations of one prostaglandin when approximately 70% confluent (although in some experiments cells were treated when grown to confluence). The vehicle for all prostaglandins was dimethyl sulfoxide (DMSO) at a final concentration at or below 0.2% v/v. Prostaglandins 15d-PGJ_2, PGJ_2, PGD_2 and $PGF_{2\alpha}$ were stored as 20 mM stocks at -80˚C. Concentrations of prostaglandins were prepared so that each treatment contained the same amount of DMSO.

3.4 DPPIV/CD26 Enzyme Activity Assay

A chromogenic assay was used to measure DPPIV/CD26 dipeptidase activity. HT-29 cells were seeded in a 24-well flat-bottomed plate at a density of 5 x 10^4 cells. The cells were then treated with different concentrations of one prostaglandin and incubated at 37˚C for 48 h. After incubation, the plate was placed on ice, the media was aspirated, and the wells were washed 3 x with 500 µL ice-cold PBS (0.8% NaCl, 0.02% KCl, 0.12% Na_2HPO_4, 0.02% KH_2PO_4 $^{m}/_{v}$ in distilled water; pH 7.4) with Ca^{2+} and Mg^{2+}. Cells were then subjected to the chromogenic DPPIV substrate Gly-Pro-*p*-nitroanilide (Gly-Pro-*p*NA). 2 mM of Gly-Pro-*p*NA was prepared in 100 mM HEPES buffer (pH 7.6) with 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose and 10 mg/mL bovine serum albumin (BSA). 250 µL of this Gly-Pro-*p*NA solution was added to each well, and the plate was incubated at 37˚C for 2 h. Cleavage of Gly-Pro-*p*NA by DPPIV yielded the product *p*NA, which transforms the clear solution into a yellow colour. 100 µL of yellow

supernatant was transferred into a 96-well flat-bottomed plate, and the absorbance was measured by a SpectraMax® microplate reader at 405 nm. The absorbance indicates the amount of yellow *p*NA product. The amount of product is proportional to the amount of DPPIV dipeptidase activity.

3.5 MTT Assay

An MTT assay was performed to measure cell viability in response to prostaglandin treatment. Cells were seeded in a flat-bottomed 96-well plate at a density of 5 x 10^4 cells. Cells were treated with different concentrations of one prostaglandin for 48 h, after which they were subjected to the tetrazolium dye precursor MTT. MTT is reduced to a purple formazan crystal by mitochondrial enzymes within viable cells. 5 mg/mL MTT was prepared in PBS, which was then vortexed and passed through Whatman® filter paper to remove particulates. 20 µL MTT reagent was pipetted into each well, and the plate was mixed for 5 min on a plate rotator and then incubated at 37°C for 2 h. The plate was then carefully inverted over an absorbent pad to remove the media without losing cells. The formazan crystal that resulted from MTT reduction was solubilized in 100 µL DMSO, and the plate was incubated at RT on a platform shaker for 30 min. During this time the plate was wrapped in aluminum foil to avoid exposure to light. Absorbance of the formazan product was measured on a SpectraMax® microplate reader at 492 nm. Absorbance was correlated with the relative amount of viable cells.

3.6 Cell Counting Assay

Cells were counted to measure cell viability in response to prostaglandin treatment. Cells were seeded in a flat-bottomed 24-well plate at a density of 5 x 10^4 cells and treated with various concentrations of a prostaglandin for 48 h. Then, the 24-well

plate was washed 3 x with 500 µL PBS. 500 µL trypsin-EDTA was added to each well and the plate was incubated at 37˚C for 10 min. 500 µL of the trypsin-EDTA was then thoroughly pipetted up and down to ensure complete dissociation of cells, and then transferred into a cuvette containing 9.5 mL PBS. The cuvette was placed in the digital multisizer (Beckman Multisizer™ 4) for cell counting. Three cell count values were taken for each sample.

3.7 Western Blot

3.7.1 Protein Collection

HT-29 cells were seeded in 25 cm^2 flasks at a density of 7 x 10^5 cells. Cells grew until approximately 70% of confluence, after which they were treated with a prostaglandin at different concentrations for either 24 h or 48 h depending on the experiment. After treatment, the flasks were placed on ice and washed twice with 5 mL PBS containing Ca^{2+} and Mg^{2+} to remove dead cells and debris without disrupting the monolayer. 500 µL RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in 1 mM EDTA; pH 8.0) with protease inhibitors (1 mM PMSF freshly added, 5 mM EDTA, 1 µg/mL aprotinin and 5 µg/mL leupeptin) was added to each flask, and the flasks were incubated at 4˚C on a plate rotator for 30 min. The lysate was pipetted 10 x to detach proteins from the monolayer, and collected into separate 2 mL tubes. The tubes were centrifuged at 14,000 x *g* and 4˚C for 20 min to pellet the membranes, nuclear material and cytoskeletal proteins. 400 µL of the supernatant was then carefully transferred into fresh 2 mL tubes and stored at -80˚C overnight.

3.7.2 Bradford Assay

The Bradford assay was used to measure the relative concentration of protein within the collected protein samples. 5 µL of bovine serum albumin (BSA) protein standards (0, 50, 125, 250, 375, 500, 750 and 1000 µL/mg) were pipetted in triplicate into a flat-bottomed 96-well plate to generate a standard concentration curve. 0.5 µL of collected protein sample was pipetted in triplicate and diluted 1/10 with 10% RIPA buffer and 80% distilled water. Using a multichannel pipette, 200 µL of Bradford Dye Reagent was dispensed into each well, which turns blue upon protein binding. The absorbance of blue product was read spectrophotometrically on a SpectraMax® microplate reader at 595 nm. The absorbance value was correlated with the amount of protein in the sample. Sample concentrations were equalized by diluting them in RIPA buffer accordingly.

3.7.3 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to separate proteins by molecular weight. 100 µL of protein sample was transferred into a fresh tube and combined with 100 µL 2X Laemmli sample buffer [0.5 M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-beta-mercaptoethanol, 0.01% bromophenol blue]. Samples were vortexed for 20 s, and then boiled for 5 min to denature proteins. Equal volumes of sample protein were loaded into each lane of the 4% stacking/7.5% resolving polyacrylamide gel. The gel was run in running buffer (5.1 mM SDS, 25 mM Tris base, 0.192 M glycine, 2% methanol in distilled water; pH ~8.3) and samples were separated by electrophoresis at 10 mA per gel for approximately 4 h.

3.7.4 Transfer and Immunoblotting

After the proteins were separated by SDS-PAGE, the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane using the wet transfer method. For the wet transfer method, the gel was equilibrated in transfer buffer (192 mM glycine, 25 mM Tris base in methanol, pH 8.3) for 15 min and the PVDF membrane was equilibrated in methanol. The protein samples were electroblotted in transfer buffer at 10 V for 16 h at 4˚C.

Blotted membranes were blocked with 3% BSA m/v in 20 mL Tris-buffered saline (TBS) (50 mM Tris base, 150 mM NaCl in distilled water; pH 7.6) with 0.1% Tween 20 (TBS-T) for 1 h at RT. Membranes were washed with 20 mL TBS-T for 5 min on a plate rotator, and then probed with 10 mL primary antibody for 2 h at RT. Antibodies were diluted in TBS-T with 1% BSA $^{m}/_{v}$; primary antibody dilutions were 1:50,000 for anti-CD26 and 1:1,000 for anti-ß-actin. The blotted membranes were then washed 3 x 5 min with 20 mL TBS-T, and then probed with 10 mL secondary antibody for 1 h at RT. Secondary antibodies were horseradish peroxidase (HRP)-conjugated, and the dilutions for anti-mouse and anti-rabbit IgG secondary antibodies were 1:2000. The membranes were then washed 4 x 5 min with 20 mL TBS-T. Protein bands were visualized using enhanced chemiluminescence; each membrane was exposed to 1.5 mL of chemiluminescence solution [100 mM Tris-HCl (pH 8.5), 200 µM p-coumaric acid, 1.25 mM luminol, 0.01% H_2O_2] for 5 min. Images of the membrane were captured using a Kodak™ Image Station 4000 mm Pro at exposure times ranging from 30 s to 3 min.

3.8 Immunofluorescence

The localization of DPPIV/CD26 on HT-29 cells was visualized using immunofluorescence. Cells were seeded into an 8-well chamber slide in 500 µL DMEM supplemented with 10% NCS, and grown to 30% of confluence. Each well was then exposed to a prostaglandin for 48 h. The media was then carefully aspirated using a capillary pipette, and the wells were washed with 200 µL PBS with Ca^{2+} and Mg^{2+}. The cell monolayer was then fixed onto the slide using freshly prepared 1% paraformaldehyde (PFA). The monolayer was exposed to 200 µL 1% PFA for 10 min at RT, then washed three times with 200 µL PBS with Ca^{2+} and Mg^{2+}. To prevent nonspecific antibody binding, the monolayer was then exposed to 200 µL 3% BSA for 30 min at RT. The BSA solution was then aspirated, and 200 µL of CD26 primary antibody in 1% BSA in PBS with Ca^{2+} and Mg^{2+} (1:150 dilution) was added to each well. The wells were incubated for 90 min at RT. The wells were then washed three times for 5 min with 1% BSA in PBS with Ca^{2+} and Mg^{2+} on a rotary platform mixer. 200 µL 488-Alexafluor fluorophore-conjugated anti-rabbit secondary antibody in 1% BSA in PBS with Ca^{2+} and Mg^{2+} was then added to each well, and incubated for 30 min at RT in an aluminum foil-covered box to prevent exposure to light. Wells were then washed once with 1% BSA in PBS with Ca^{2+} and Mg^{2+}. The chambers were then removed from the slide, and one drop of Fluoroshield aqueous gel mountant containing 1 µg/mL 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) was added to each well to stain the nuclei and mount the cover slip. DAPI was allowed to dry for 1 h at RT. Green fluorescence indicated CD26 expression, which was visualized under a Zeiss LSM710 laser scanning confocal microscope, and images on this microscope were captured with Zen 2009 software.

3.9 High Performance Liquid Chromatography

3.9.1 15d-PGJ_2 Extraction

High performance liquid chromatography (HPLC) was used to determine the amount of remaining 15d-PGJ_2 in culture medium at different time points after cell treatment. HT-29 cells were seeded in 6-well flat-bottomed plates at a density of 3 x 10^5 cells and grown in DMEM with 10% NCS until approximately 70% confluent. 15d-PGJ_2 was added to each well for a defined amount of time (depending on the time course of the experiment), after which 500 µL of the media was withdrawn and collected in separate 2 mL tubes. 500 µL of ice-cold methanol was added to each sample, and the samples were incubated in -20˚C for 10 min. The samples were then centrifuged at maximum speed for at 4˚C for 10 min. 1 mL chloroform/500 µL media was then added to each sample. The samples were vortexed for 30 s, and then centrifuged again at maximum speed for 10 min at 4˚C. ~1 mL of the bottom organic layer was carefully pipetted into a fresh 15 mL tube, and the chloroform was completely evaporated under a gentle nitrogen stream for approximately 3 h. The samples were then stored in -80˚C until further use.

3.9.2 HPLC Method

Chromatography separations were performed using a 1200 series HPLC system (Agilent Technologies®) with a C-18 reverse phase column. The extracted 15d-PGJ_2 samples were dissolved in 20% methanol, and 25 µL of each sample was pipetted into a round-bottom 96-well polyethylene plate. The temperatures of the sample chamber and the column were 4˚C and 30˚C, respectively. The mobile phase consisted of 60% acetonitrile and 0.01% trifluoroacetic acid (TFA) (pH 3.5) with a flow rate of 1 mL/min. Each sample was passed through the column for 10 min, and UV absorption was detected

at 230 nm. The quantity of prostaglandin was based on electronic integration of the distinct 15d-PGJ_2 peaks (i.e. calculating the area under the curve). A standard curve was generated to plot known concentrations against the prostaglandin peak area for determining unknown prostaglandin concentrations. The average retention time for 15d-PGJ_2 was 7.85 min. Three separate injections were taken per sample.

3.10 Polymerase Chain Reaction

3.10.1 mRNA Extraction

HT-29 cells were seeded in a flat-bottomed 6-well plate in DMEM containing 10% NCS at a density of 3 x 10^5 cells. Once grown to 70% of confluence, cells were treated with a particular prostaglandin for 24 h. Then, total mRNA was extracted from the cells using TRIzol® reagent. TRIzol dissolves DNA, RNA and protein. It contains guanidinium isothiocyanate that deactivates RNA-degrading enzymes, as well as acidic phenol that separates RNA into an aqueous supernatant. 1.25 mL of TRIzol® reagent was added to the cell monolayer to lyse the cells and incubated at RT for 5 min. 250 µL chloroform was added to each sample for phase separation and vortexed for 5 s, then incubated at RT for 5 min. The samples were then centrifuged at 12,000 x *g* at 4˚C for 15 min which separated the contents into three phases: the bottom red organic phase containing protein, a middle white phase containing DNA, and the top colourless aqueous phase containing RNA. Approximately 600 µL of the colourless aqueous phase was carefully pipetted into a 2 mL tube. 3M potassium acetate (pH 5.2) and isopropanol were added to the sample at 10% and 70% of the volume of the aqueous phase, respectively. The samples were then incubated at RT for 10 min, and then centrifuged at 12,000 x g at 4˚C for 20 min. The supernatant was discarded and 1 mL of 75% ethanol per 1 mL of

TRI® reagent was added to the samples to wash salts out of the RNA. The samples were centrifuged at 12,000 x *g* at 4˚C for 15 min. The supernatant was discarded and the resultant RNA pellet was air dried for 10 min. The pellet was dissolved in 50 µL of nuclease-free water, and the concentration of RNA was measured using a Nanodrop® 2000c. The purity of the samples was discerned by the ratio of absorbance at 260 nm (wavelength of nucleic acid absorbance) to absorbance at 280 nm (wavelength of protein absorbance). A purity ratio of approximately 2 was acceptable. The mRNA samples were stored at -80˚C until further use.

3.10.2 Reverse Transcription

RNA samples were reverse transcribed into complementary DNA (cDNA) for PCR. Each reverse transcription reaction started with 2.5 µg RNA and Master Mix 1. Master Mix 1 contained 0.5 µg/µL oligo dT and 10 mM dNTP in diethyl pyrocarbonate (DEPC) H_2O. Oligo dTs served as primers for reverse transcriptase, dNTPs were the building blocks for cDNA synthesis, and DEPC H_2O prevented RNA degradation by inactivating RNase enzymes. The samples were loaded into a Techne Genius Thermal Cycler and exposed to 65˚C for 5 min (oligo dT primer annealing stage), then were removed from the thermal cycler and chilled on ice for 15 s. 20 µL Master Mix 2 was added to each sample. Master Mix 2 contained a) 0.1 M DTT which loosened RNA structure and facilitated reverse transcriptase activity, b) RNase Out™ which prevented RNA degradation by ribonucleases, c) 200 U/µL M-MLV reverse transcriptase, and d) FS buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCL_2$; pH 8.3). The samples were loaded back into the thermal cycler and underwent the following program: 37˚C for 50

min (DNA polymerization), 70˚C for 15 min (reverse transcriptase deactivation), and 4˚C indefinitely (holding stage).

3.10.3 End Point PCR and Gel Electrophoresis

End point PCR was performed to amplify cDNA samples for visualization. Each reaction contained 2.5 µg cDNA, 12.5 µL GoTaq® Green, 0.5 µL forward primer, 0.5 µL reverse primer, and 9.5 µL Milli Q H_2O. PCR was performed using a thermal cycler, which underwent 36 cycles of the following program: 94ºC for 110 s (denaturing), 60ºC for 45 s (annealing), 72ºC for 60s (extension), and 72ºC for 10 min (final extension). 5 µL of PCR samples were loaded into the wells of a 1% agarose gel. Electrophoresis was run at 95 V for approximately 30 min. Images of the gel were captured under UV light using an Alpha Imager® HP machine (Alpha Innotech - MultiImage®II).

3.10.4 Quantitative Real-Time PCR

Quantitative PCR was used to quantify mRNA expression of DPPIV/CD26. First, cDNA concentration was diluted to 5 ng/µL in DEPC distilled water. qPCR reactions were performed in a low profile semi-skirted 96-well PCR plate. Each qPCR reaction contained 25 ng cDNA, 200 nM forward primer, 200 nM reverse primer, and 10 µL SSO Advanced SYBR Green qPCR Mix. qPCR was performed using a StepOnePlus™ Real-Time PCR System under the following thermal conditions: 95˚C for 30 s (polymerase activation), 95˚C for 15 s (denaturation), and 60˚C for 60 s (annealing and extension) for 40 cycles. The ΔΔCq relative quantification method was used to determine the fold change in gene expression. The software LinRegPCR® was used to analyze the fluorescence data and produce a quantification cycle (Cq) value for each sample. The Cq value represents the number of cycles needed to reach the fluorescence threshold. Cq

values were used to calculate the N0 value of each sample. The N0 value indicates the starting concentration of the target gene and is expressed in arbitrary fluorescence units. N0 values of CD26 were normalized to GAPDH N0 values.

3.11 Cell Migration Assay

A cell migration assay was used to investigate changes in cellular interactions with the ECM. General cancer cell migration was measured using HT-29 cells, and normal stromal tissue wound healing/remodeling was measured using NIH3T3 cells. HT-29 and NIH3T3 cells were separately seeded in flat-bottomed 6-well plates in DMEM containing 10% NCS at a density of 3 x 10^5 cells. At approximately 80% confluence, the cells were treated with the vehicle control, or different concentrations of 15d-PGJ_2, for 48 h. Then, two vertical parallel scratches were etched into the monolayer using a P1000 pipette tip. The media was aspirated, and the wells were washed once with DMEM containing 20% FBS. 3 mL of DMEM containing 20% FBS was then added to each well to stimulate cell migration. Reference markings on the bottom of each well were etched with a thin sharp needle. The scratches, or wounds, were observed under a Leica DM2000 light microscope, and images on this microscope were captured using a Micropublisher 5.0 RTV camera with QCapture Pro 5 software. Images of each scratch wound were captured immediately after the wounds were created, and afterwards at 24 h intervals or less according to the pace of wound healing. ImageJ software was used to calculate the area of each scratch wound at different time intervals.

3.12 Analysis and Statistics

GraphPad Prism 7 software was used for data analysis. ImageJ® was used for quantitation of intensity of images in end-point PCR and Western blotting. Analysis of variance (ANOVA) was performed to analyze statistically significant differences in cell response. A one-way ANOVA was followed by Dunnett's multiple comparisons test, and a 2-way ANOVA was followed by Bonferroni's multiple comparisons test. $P<0.05$ was chosen as the threshold for statistically significant differences. Analyzed data were either triplicate wells of one experiment, or means from at least three independent experimental replicates. For non-quantitative results, figures that were most representative of all experimental findings were selected for inclusion.

4. Results

In this project I measured the expression of DPPIV/CD26 and its dipeptidase activity on HT-29 colon carcinoma cells after prostaglandin treatment. I then explored the effect of prostaglandins on nondirectional cell migration. I investigated PGD_2 and its J-series derivatives PGJ_2 and 15d-PGJ_2, and used $PGF_{2\alpha}$ as a non-J-series prostaglandin for comparison.

4.1 Measuring changes in DPPIV/CD26 dipeptidase activity after prostaglandin treatment

I first investigated whether each of these prostaglandins altered the level of DPPIV/CD26 dipeptidase activity on colon carcinoma cells. Cultured cells were treated with different concentrations of one prostaglandin for 48 h. DPPIV/CD26 enzyme activity was quantified by spectrophotometrically measuring *p*NA release from the DPPIV substrate Gly-Pro-*p*NA. Results showed that PGD_2 decreased DPPIV/CD26 activity by 26% on HT-29 cells (Figure 4.1 A), but had no significant effect on Caco-2 cells (Figure 4.1 B). The J-series prostaglandins showed to significantly decrease DPPIV/CD26 dipeptidase activity at the highest concentrations on both HT-29 cells and Caco-2 cells. PGJ_2 decreased DPPIV/CD26 activity by 73% on HT-29 cells (Figure 4.2 A), and by 77% on Caco-2 cells (Figure 4.2 B). The highest concentration of 15d-PGJ_2 treatment showed to reduce DPPIV/CD26 activity by 86% on HT-29 cells (Figure 4.3 A) and by 80% on Caco-2 cells (Figure 4.3 B). $PGF_{2\alpha}$ slightly increased DPPIV/CD26 enzyme activity by up to 9% in a concentration-dependent manner beginning at 10 μM (Figure 4.4).

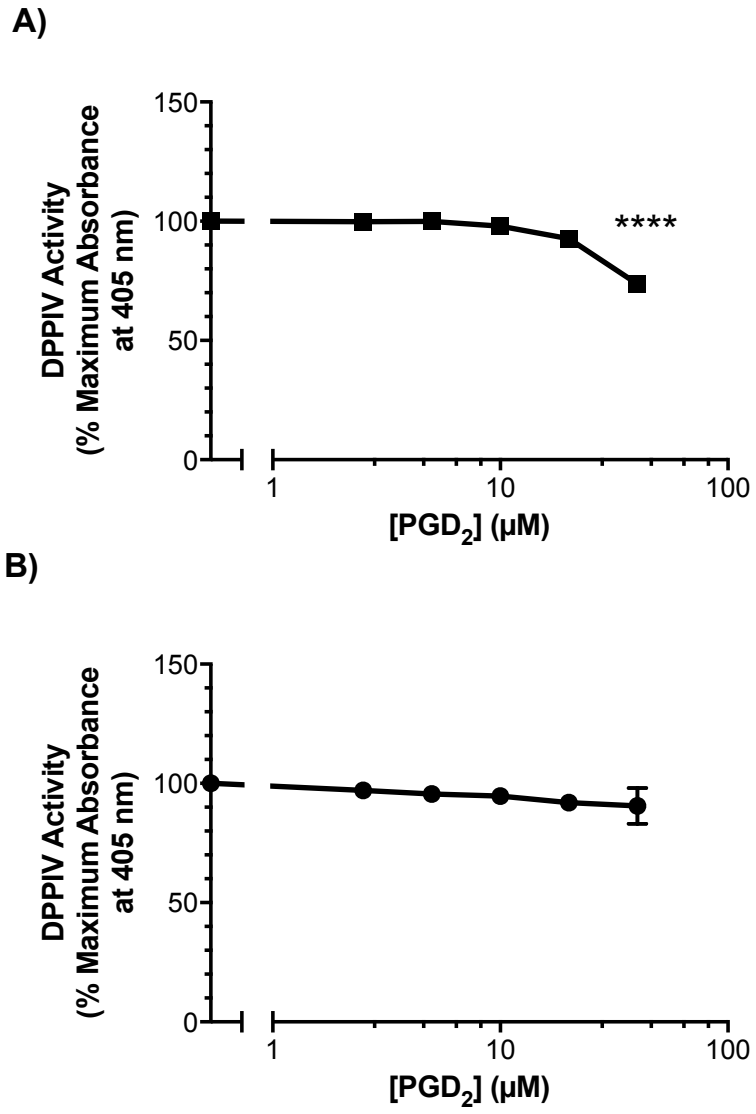

Figure 4.1: Concentration-dependent effect of PGD_2 on DPPIV dipeptidase activity

HT-29 cells (A) and Caco-2 cells (B) were treated with PGD_2 at the indicated concentrations for 48 h. DPPIV/CD26 peptidase activity was quantified by the absorbance of *p*-nitroanilide at 405 nm. Percent Maximum Absorbance was calculated by subtracting the absorbance of blank control samples from the absorbance of experimental samples, divided by the average absorbance of control-treated experimental samples. Data of each graph are means ± standard error of the mean (SEM) from triplicate wells of one independent experiment. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. ****$P<0.0001$ shows a significant difference in DPPIV activity between the control treatment and PGD_2 treatment.

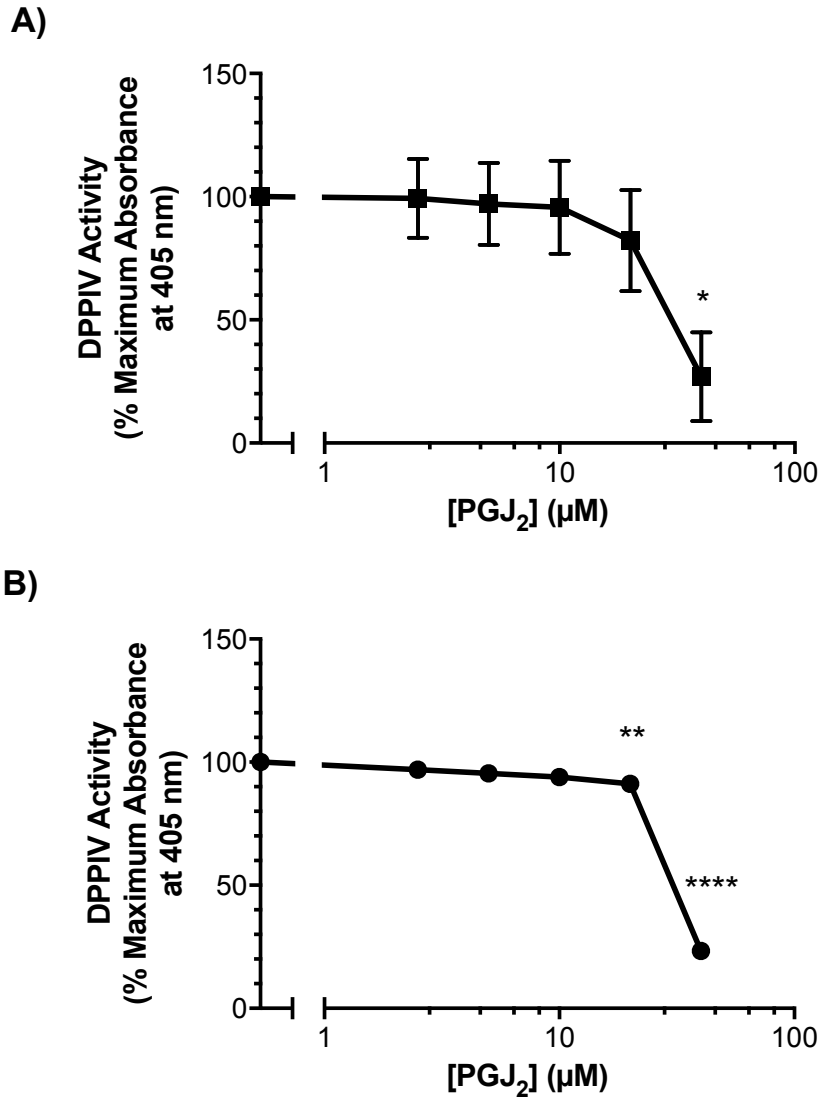

Figure 4.2: Concentration-dependent effect of PGJ_2 on DPPIV dipeptidase activity

HT-29 cells (A) and Caco-2 cells (B) were treated with PGJ_2 at the indicated concentrations for 48 h. DPPIV/CD26 peptidase activity was quantified by the absorbance of *p*-nitroanilide at 405 nm. Percent Maximum Absorbance was calculated by subtracting the absorbance of blank control samples from the absorbance of experimental samples, divided by the average absorbance of control-treated experimental samples. The data are means ± SEM. Graph A shows data from three independent experiments; Graph B shows data from one independent experiment. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. *P<0.05, **P<0.01 and ****P<0.0001 show a significant decrease in DPPIV activity between the control treatment and PGJ_2 treatment.

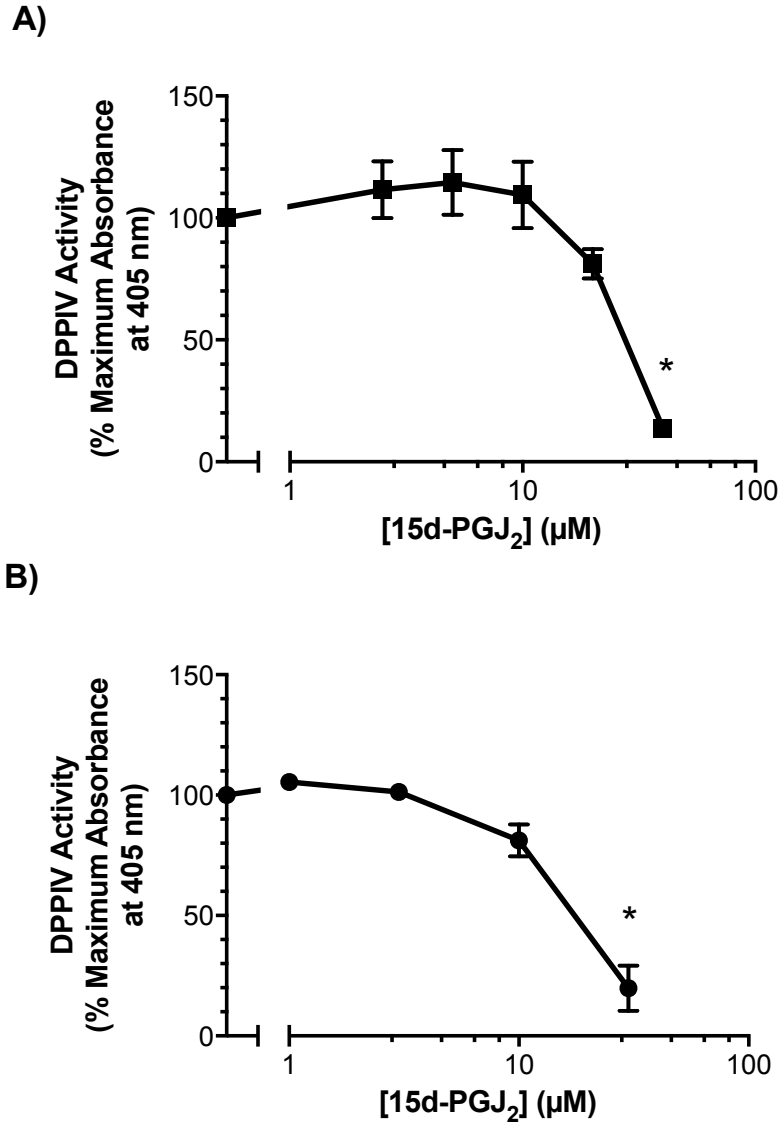

Figure 4.3: Concentration-dependent effect of 15d-PGJ$_2$ on DPPIV activity

HT-29 cells (A) and Caco-2 cells (B) were treated with 15d-PGJ$_2$ for 48 h at the indicated concentrations. DPPIV/CD26 peptidase activity was quantified as the absorbance of *p*-nitroanilide at 405 nm. Percent Maximum Absorbance was calculated by subtracting the absorbance of blank control samples from the absorbance of experimental samples, divided by the average absorbance of control-treated experimental samples. The data are means ± SEM. Graph A represents three independent experiments; Graph B represents four independent experiments. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. *P<0.05 shows a significant difference in DPPIV activity between the control treatment and 15d-PGJ$_2$ treatment.

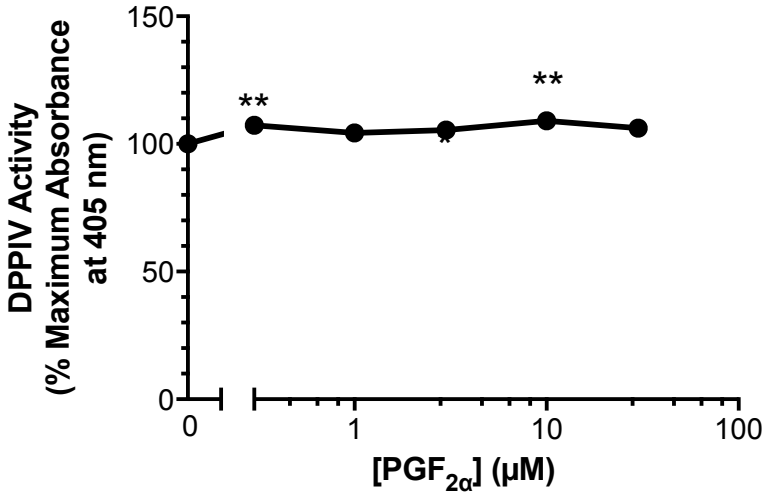

Figure 4.4: Concentration-dependent effect of $PGF_{2\alpha}$ on DPPIV dipeptidase activity

Caco-2 cells were treated with $PGF_{2\alpha}$ at the indicated concentrations for 48 h. DPPIV/CD26 peptidase activity was quantified by the absorbance of *p*-nitroanilide at 405 nm. Percent Maximum Absorbance was calculated by subtracting the absorbance of blank control samples from the absorbance of experimental samples, divided by the average absorbance of control-treated experimental samples. Data are means ± SEM from three independent experiments. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. **P<0.01 shows a significant increase in DPPIV activity between the control treatment and $PGF_{2\alpha}$ treatment.

Through the course of performing DPPIV/CD26 enzymatic activity assays I noticed that the absolute magnitude of the response sometimes varied between independent experiments. Realizing that cultures might be at different stages of growth, I decided to investigate whether *cell confluency* at the time of prostaglandin treatment affected cell response. Cell confluency is the proportion of a culture flask that is covered by adherent cells. HT-29 cells were grown to either 60% or 90% of confluence, and then treated with different concentrations of 15d-PGJ_2 or $PGF_{2\alpha}$ for 48 h. Results showed that cell confluency indeed influenced the effect of both prostaglandins on DPPIV/CD26 enzyme activity. At the highest concentration of 15d-PGJ_2, cells that were 60% confluent at the time of treatment exhibited a more substantial decrease (91%) in DPPIV/CD26 activity than 90% confluent cells (41%) (Figure 4.5 A). $PGF_{2\alpha}$ did not appear to change DPPIV/CD26 activity on 60 percent confluent cells, yet induced a slight concentration-dependent increase (3%) in DPPIV/CD26 activity on 90% confluent cells starting at 10 μM (Figure 4.5 B). Since the magnitude of cell response changed with cell confluency, I controlled for cell confluency at the time of prostaglandin treatment for all future experiments.

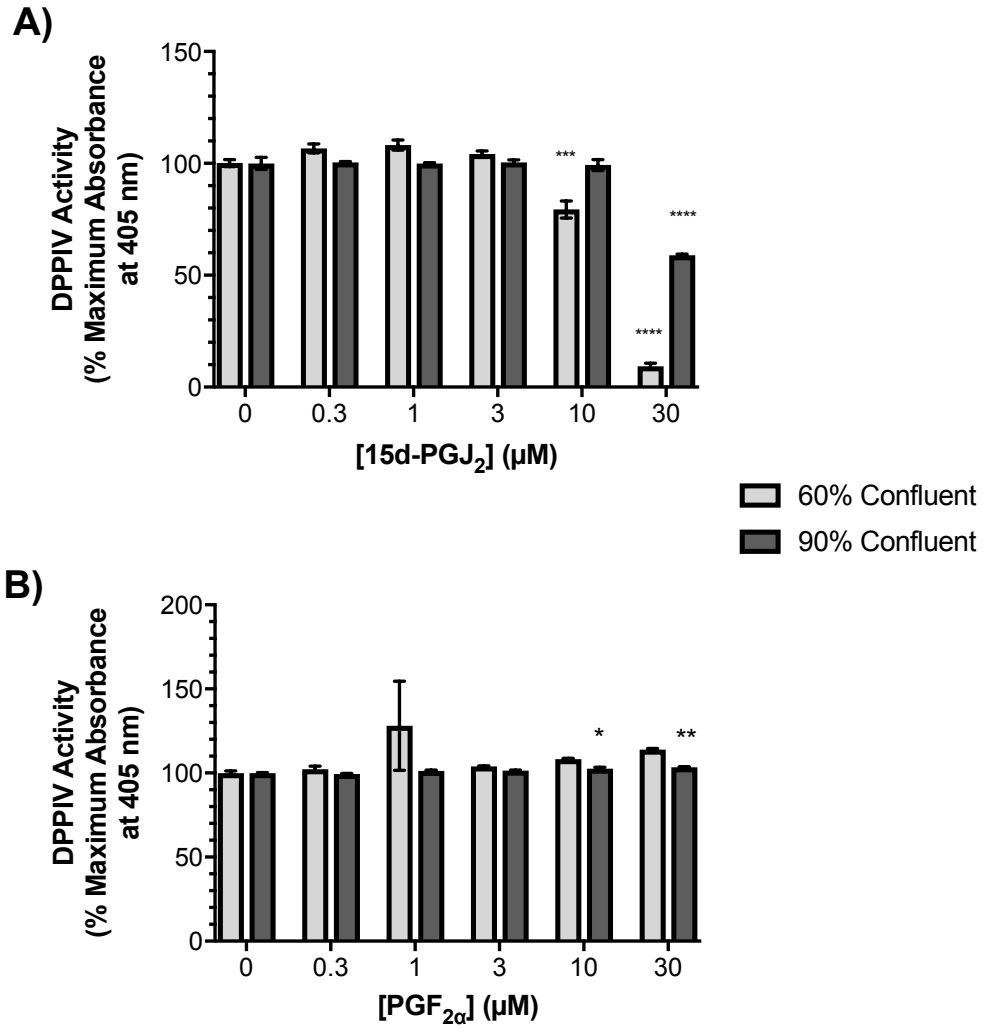

Figure 4.5: The effects of cell confluence on prostaglandin-induced changes in DPPIV/CD26 activity

Caco-2 cells were grown to 60 percent confluence or 90 percent confluence, and treated with 15d-PGJ_2 (A) or $PGF_{2\alpha}$ (B) for 48 h at the indicated concentrations. DPPIV peptidase activity was quantified by the absorbance of *p*-nitroanilide at 405 nm. Percent Maximum Absorbance was calculated by subtracting the absorbance of blank control samples from the absorbance of experimental samples, divided by the average absorbance of control-treated experimental samples. The data in each graph are means ± SEM from one independent experiment. Data were analyzed by a two-way ANOVA – which indicated a significant interaction between the two levels of cell confluence – followed by a Bonferroni multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 indicate significant differences between control treatment and prostaglandin treatment at one confluence percentage.

4.2 Measuring the potential toxicity of prostaglandins on HT-29 cells

4.2.1 Measuring prostaglandin cytotoxicity using an MTT assay

In the investigations shown above, PGJ_2 and 15d-PGJ_2 both appeared to reduce DPPIV/CD26 dipeptidase activity at high concentrations. Previous studies have shown that similar or lower concentrations of J-series prostaglandins can induce cancer cell death (Clay et al., 1999; Yen et al., 2014). Thus, the apparent decrease in DPPIV/CD26 enzyme activity may have actually resulted from a decrease in the number of viable cells, reducing the amount of functional DPPIV/CD26 to be measured. To investigate the potential cytotoxicity of each prostaglandin, I first employed an MTT assay. The MTT assay estimates the viability of cells by quantifying their mitochondrial activity. Caco-2 and HT-29 cells were exposed to a prostaglandin at the same concentrations used in the DPPIV activity assays for the same amount of time (48 h). The prostaglandins did not significantly decrease cell viability according to the MTT assay at any concentration over the course of my experiments (Figure 4.6), despite some individual experimental replicates suggesting a decrease that was not statistically significant.

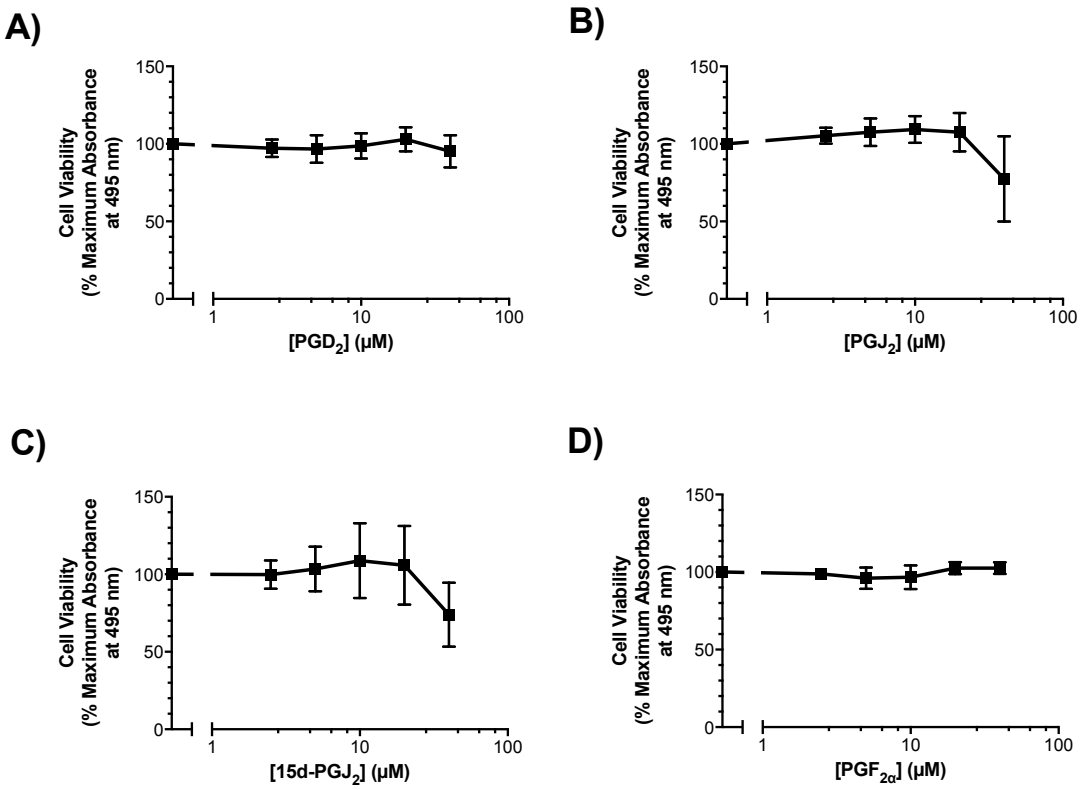

Figure 4.6: Cell viability after prostaglandin treatment measured by an MTT assay

HT-29 cells were treated with PGD_2 (A), PGJ_2 (B), 15d-PGJ_2 (C) or $PGF_{2\alpha}$ (D) for 48 h at the indicated concentrations. Cell viability was measured using an MTT assay. Percent Maximum Absorbance was calculated by subtracting the absorbance of blank control samples from the absorbance of experimental samples, divided by the average absorbance of control-treated experimental samples. The data are means ± SEM. Graphs A represents four independent experiments, Graphs B and D each represent three independent experiments, and Graph C represents five independent experiments. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. No significant difference was found between the control treatment and any prostaglandin treatment.

4.2.2 Measuring prostaglandin cytotoxicity by counting viable cells

To confirm the MTT results that reflect an indirect approach, I directly counted the number of viable cells after prostaglandin treatment. HT-29 cells were exposed to different concentrations of the prostaglandin for 48 h. Intact, full-sized (and therefore presumed viable) cells were then counted using a digital multisizer. Results showed that PGD_2 and PGJ_2 both significantly reduced the number of viable cells at high concentrations by up to 38% ($P<0.001$) and 99% ($P<0.0001$), respectively (Figure 4.7 A; Figure 4.7 B). Furthermore, 15d-PGJ_2 significantly decreased cell viability at the highest concentration by 88% ($P<0.05$) (Figure 4.7 C). $PGF_{2\alpha}$ had a very little effect on cell viability, producing a small (11%) reduction at the highest concentration (Figure 4.7 D). These cell count results contradicted the MTT results which showed that PGD_2, PGJ_2 and 15d-PGJ_2 did not significantly alter HT-29 cell viability. This casts doubt on the reliability of the MTT assay. Since counting viable cells is a more direct measure of prostaglandin cytotoxicity than using an MTT assay, I concluded that the J-series prostaglandins and their parent compound PGD_2 were indeed cytotoxic to HT-29 cells at the highest concentrations.

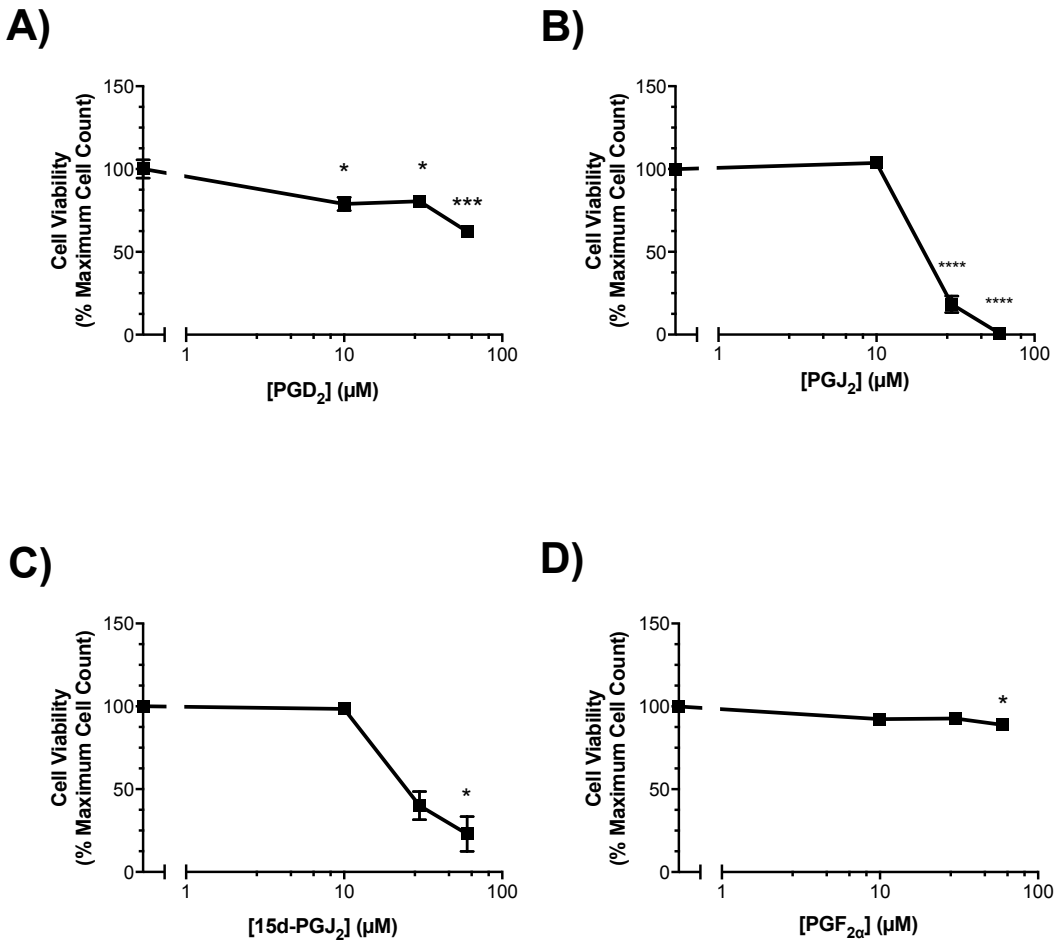

Figure 4.7: Mean percent viable cell count after prostaglandin treatment

HT-29 cells were treated with PGD_2 (A), PGJ_2 (B), 15d-PGJ_2 (C) or $PGF_{2\alpha}$ (D) for 48 h at the indicated concentrations. Viable cell count was measured using a digital multisizer. The data for Graphs A, B and D are means ± SEM of triplicate wells, each from one independent experiment. The data for Graph C are means ± SEM from seven independent experiments. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. *P<0.05, ***P<0.001, and ****P<0.0001 indicate a significant difference between the vehicle control and prostaglandin treatment.

4.3 Measuring DPPIV/CD26 dipeptidase activity after prostaglandin treatment while controlling for cytotoxic effects

Since the cell counting results showed that PGJ_2 and 15d-PGJ_2 both decreased the number of viable HT-29 cells, I decided to measure DPPIV/CD26 dipeptidase activity while controlling for prostaglandin cytotoxicity; i.e. to study the activity normalized to per viable cell. HT-29 cells were exposed to different concentrations of a prostaglandin for 48 h as before. For each experiment, half of the cultures were measured for DPPIV/CD26 dipeptidase activity, while cells in the parallel cultures were counted. DPPIV/CD26 enzyme activity was then normalized to the number of viable cells. As expected, PGD_2 did not alter the level of DPPIV/CD26 dipeptidase activity on viable cells (Figure 4.8 A). The increases in mean DPPIV/CD26 activity after PGJ_2 (150%) and 15d-PGJ_2 (93%) treatments were not statistically significant; these results exhibited much higher variability between experimental replicates (Figure 4.8 B; Figure 4.8 C). Interestingly, $PGF_{2\alpha}$ slightly increased DPPIV/CD26 activity at the lowest concentration by 21% and this increase was statistically significant (Figure 4.8 D). These findings contradicted my initial results on DPPIV/CD26 activity, which showed a decline after PGJ_2 and 15d-PGJ_2 treatments without controlling for cell death (Figure 4.2; Figure 4.3).

In this series of experiments I noticed that the magnitude of responses differed between experimental replicates. It seemed possible that culture growth parameters contributed to variations in cell response, in addition to the confluence level studied earlier. For practical reasons to do with cell culture, cells used in some replicates of the DPPIV/CD26 enzymatic assay had been grown in DMEM supplemented with 10% NCS, whereas others were grown in DMEM supplemented with 5% FBS. NCS differs from

FBS in that it contains less growth factors and higher total protein levels. The type of serum has shown to affect cancer cell morphology and the rate of cell proliferation, which in return can lead to alterations in cell response (Fang, Wu, Fang, Chen, & Chen, 2017). However, when comparing DPPIV/CD26 dipeptidase activity on HT-29 cells grown in NCS vs. FBS, there was no significant interaction between serum type and 15d-PGJ_2 concentration (Figure 4.9). Therefore, the type of culture serum did not affect DPPIV/CD26 enzyme activity in response to 15d-PGJ_2. Another difference between experimental replicates was the rate of cell growth. After seeding HT-29 cells in 24-well plates for DPPIV/CD26 enzyme activity assays at the same density, they grew slowly during some experiments (i.e. seven days to reach 60 percent confluence), and during others they grew quickly (i.e. three days to reach 60 percent confluence). I decided to investigate whether the rate of HT-29 cell growth indicated changes in cell response to 15d-PGJ_2 by comparing DPPIV/CD26 enzyme activity between slow-growing cells and fast-growing cells. There was no statistically significant interaction between the pace of cell growth and 15d-PGJ_2 concentration (Figure 4.10). It is likely that there are many factors related to cell culture that contribute to experimental variability.

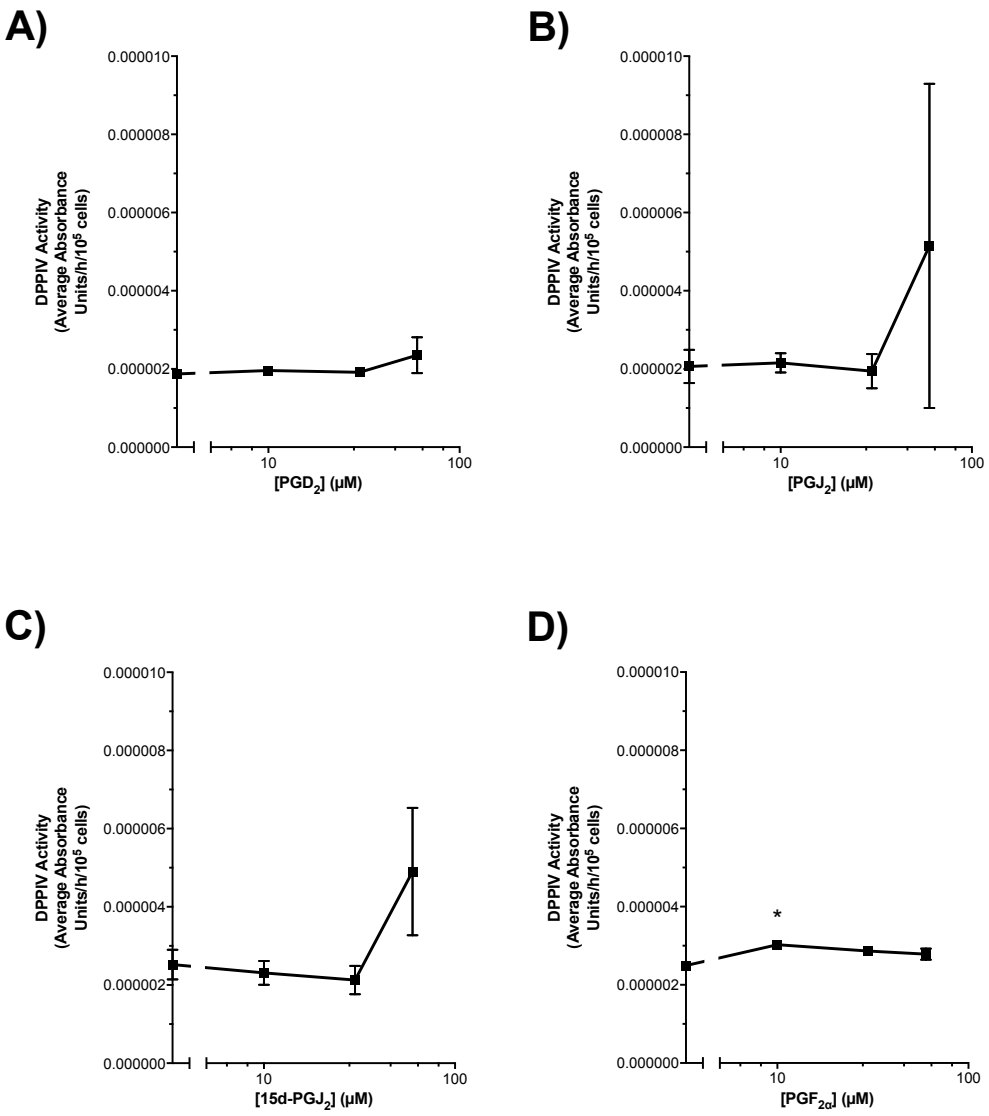

Figure 4.8: DPPIV/CD26 dipeptidase activity normalized to viable cell count after prostaglandin treatment

HT-29 were treated with PGD_2 (A), PGJ_2 (B), 15d-PGJ_2 (C), or $PGF_{2\alpha}$ (D) for 48 h at the indicated concentrations. DPPIV peptidase activity was quantified as the absorbance of *p*-nitroanilide at 405 nm. Viable cells were counted using a digital multisizer. DPPIV Activity was calculated by taking the absorbance over time, divided by the average number of cells counted. The data for Graphs A and D are means ± SEM of triplicate wells, each from one independent experiment. The data for Graph C are means ± SEM from three independent experiments, and the data for Graph B are means ± SEM from eight independent experiments. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. *$P<0.05$ shows a significant difference between the control treatment and $PGF_{2\alpha}$ treatment.

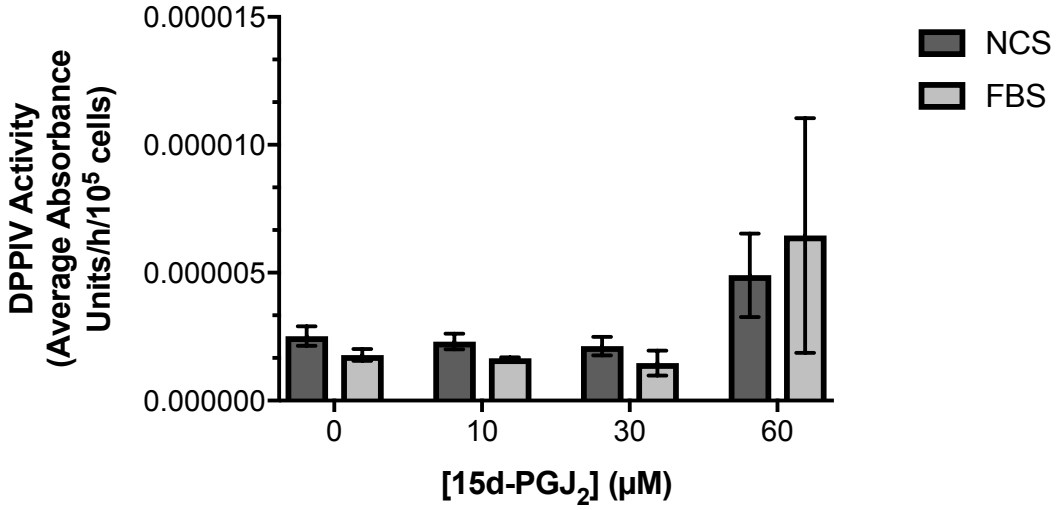

Figure 4.9: Lack of effect of cell culture serum on 15d-PGJ_2-induced changes in DPPIV/CD26 dipeptidase activity

HT-29 cells were grown in DMEM supplemented with either 10% NCS or 5% FBS, and then treated with 15d-PGJ_2 for 48 h at the indicated concentrations. DPPIV/CD26 dipeptidase activity was quantified as the absorbance of *p*-nitroanilide at 405 nm. Viable cells were counted using a digital multisizer. DPPIV Activity was calculated by taking the absorbance over time, divided by the average number of cells counted. The 10% NCS data are means ± SEM from five independent experiments. The 5% FBS data are means ± SEM from three independent experiments. Data were analyzed by a two-way ANOVA with a Bonferroni multiple comparisons test. No significant interaction was found between the type of serum and 15d-PGJ_2 concentration.

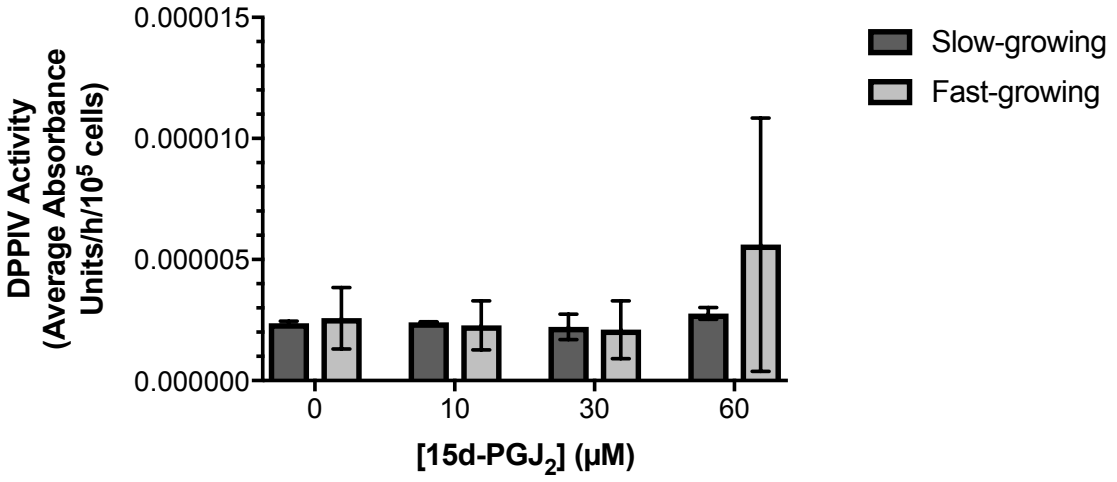

Figure 4.10: Lack of effect of pace of cell growth on 15d-PGJ_2-induced changes in DPPIV/CD26 dipeptidase activity

HT-29 cells were seeded and grown to 60 percent confluence, which either took approximately seven days (slow-growing) or three days (fast-growing). They were then treated with 15d-PGJ_2 for 48 h at the indicated concentrations. DPPIV/CD26 dipeptidase activity was quantified as the absorbance of *p*-nitroanilide at 405 nm. Viable cells were counted using a digital multisizer. DPPIV Activity was calculated by taking the absorbance over time, divided by the average number of cells counted. The slow-growing data are means ± SEM from two independent experiments. The fast-growing data are means ± SEM from six independent experiments. Data were analyzed by a two-way ANOVA with a Bonferroni multiple comparisons test. No significant interaction was found between the pace of cell growth and 15d-PGJ_2 concentration.

4.4 Characterizing changes in DPPIV/CD26 mRNA expression in response to prostaglandin treatment

Given the uncertainty of my findings about the level of DPPIV/CD26 enzyme activity after exposure to different prostaglandins, I decided to quantify the expression and abundance of DPPIV/CD26 at the mRNA and protein level. I first investigated DPPIV/CD26 mRNA expression in HT-29 cells after 24 to 36 h prostaglandin treatment via end-point PCR with agarose gel analysis (Figure 4.11). The J-series prostaglandins PGD_2 (Figure 4.11 A) and PGJ_2 (Figure 4.11 B), and the structurally distinct $PGF_{2\alpha}$ (Figure 4.11 D), showed no evidence for a dose-dependent increase in DPPIV/CD26 mRNA expression either visually or on ImageJ® analysis of band intensity (not shown). However, 15d-PGJ_2 showed evidence of a slight increase in DPPIV/CD26 mRNA expression at the highest treatment dose of 40 μM when independent experiments were analyzed using Image J® (mean increase 12.0%, n=3 experiments, $P<0.05$).

I sought to extend these results using DPPIV/CD26 expression after using qPCR, which is much more sensitive to changes in expression than end point PCR. Interestingly, these qPCR results – normalized to GAPDH to control for cytotoxicity – revealed a substantial and significant concentration-dependent decline in DPPIV/CD26 mRNA abundance by up to 85% at higher concentrations of 15d-PGJ_2 (Figure 4.12).

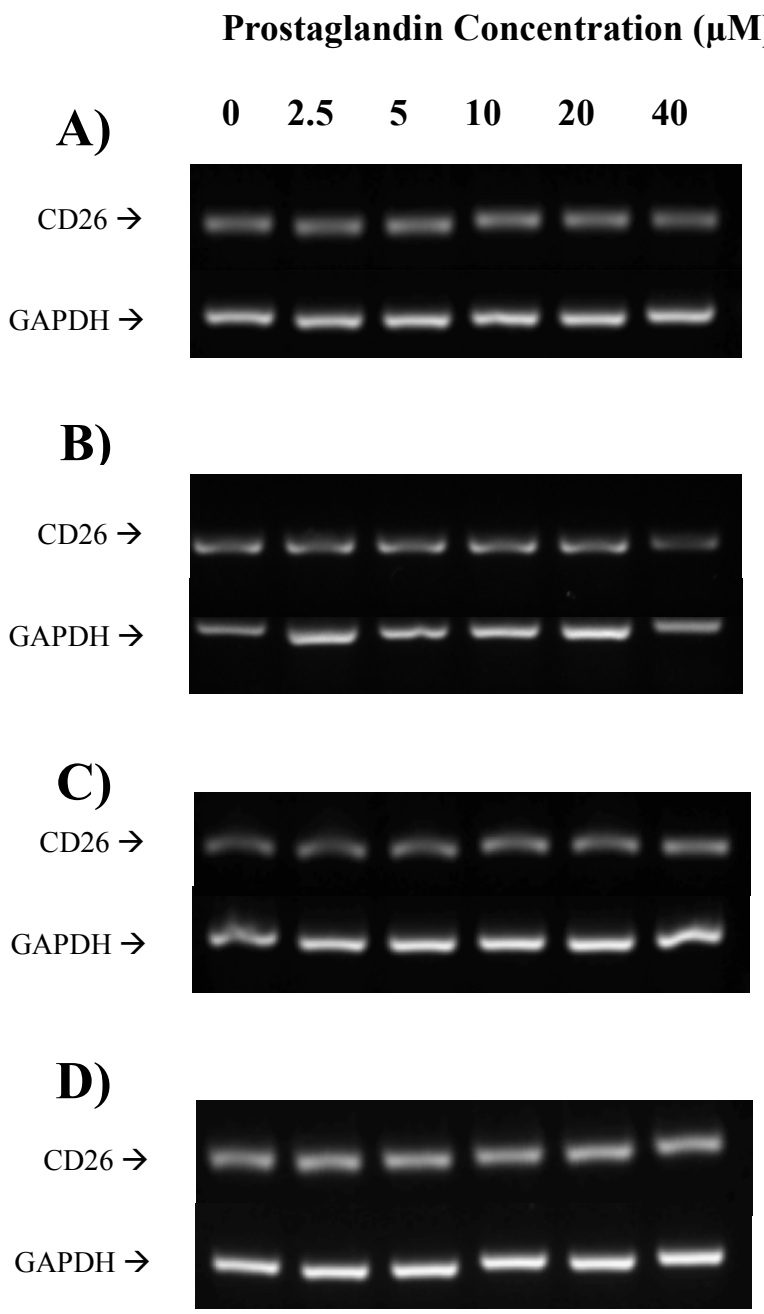

Figure 4.11: DPPIV/CD26 mRNA expression after prostaglandin treatment

HT-29 cells were treated with either PGD_2 (A), PGJ_2 (B), 15d-PGJ_2 (C) or $PGF_{2\alpha}$ (D) at the indicated concentrations for 24 to 36 h. mRNA was extracted, reverse transcribed to cDNA, and amplified via PCR. Images were taken on an Alpha Imager® HP machine. GAPDH expression was used as a positive control compared to CD26 expression. Images A and B show single independent experiments; Image C is representative of three independent experiments; Image D is representative of two independent experiments.

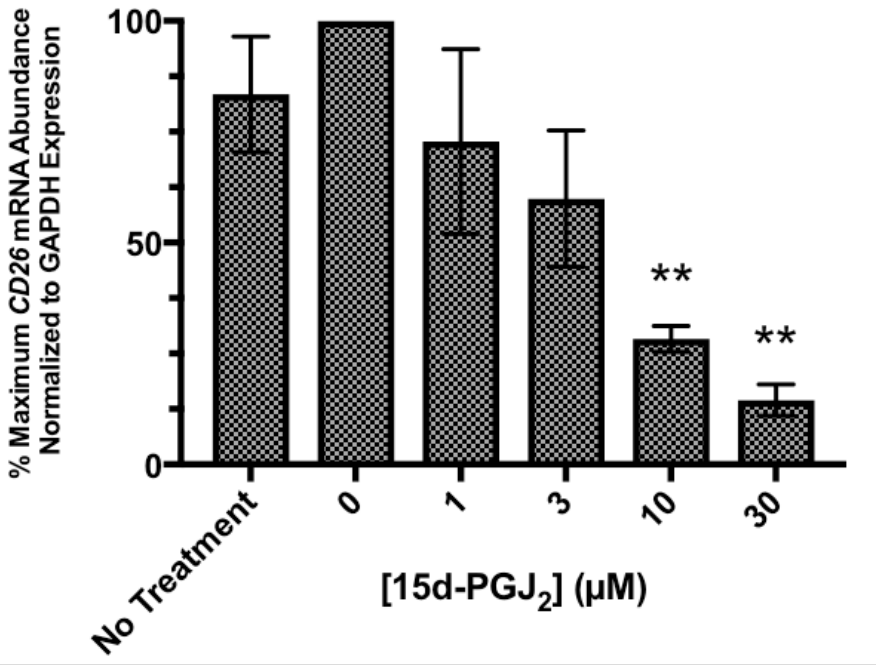

Figure 4.12: DPPIV/CD26 mRNA expression in HT-29 cells after 15d-PGJ_2 treatment

HT-29 cells were treated with 15d-PGJ_2 at the indicated concentrations for 24 h. mRNA was extracted, reverse transcribed to cDNA, and amplified and quantified via qPCR. GAPDH expression was used as a positive control. mRNA expression was expressed in arbitrary fluorescence units. Percent Maximum DPPIV/CD26 mRNA Abundance was calculated as the ratio of CD26 mRNA fluorescence to the mean GAPDH mRNA fluorescence, and this ratio was then normalized to that at 0 μM 15d-PGJ_2. The data represent means ± SEM from three independent experiments. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. **$P<0.01$ shows a significant decrease in CD26 mRNA expression between the vehicle control and 15d-PGJ_2 treatment.

4.5 Characterizing changes in DPPIV/CD26 whole cell protein expression in response to prostaglandin treatment

The qPCR results showed that 15d-PGJ_2 induced a concentration-dependent decrease in DPPIV/CD26 mRNA expression. Oftentimes changes in the transcription of a gene are not reflected in alterations emerging after protein translation (Vogel & Marcotte, 2012). I investigated whether the decrease in DPPIV/CD26 mRNA expression was reflected at the protein level. HT-29 cells were dosed with either the vehicle control or different concentrations of one prostaglandin, and whole-cell DPPIV/CD26 protein expression was visualized by Western blotting. Results showed that there was no *consistent* shift in whole-cell DPPIV/CD26 protein expression after PGD_2, PGJ_2 or 15d-PGJ_2 treatments (Figure 4.13). Analysis of band intensity using ImageJ® software confirmed that in spite of variations in measured intensity of up to 20% from the mean in certain lanes, there was no consistent change in protein expression (*P* value for test of change in DPPIV/CD26 protein expression at the highest treatment dose of 40 μM of 15d-PGJ_2 > 0.35, n=3 independent experiments). Therefore, the apparent decline in DPPIV/CD26 mRNA expression after 15d-PGJ_2 exposure (as measured by qPCR) was not paralleled by a decrease in DPPIV/CD26 whole-cell protein abundance. Furthermore, the possible increase in DPPIV dipeptidase activity at high concentrations of PGJ_2 and 15d-PGJ_2 suggested by the averaged data could not be related to an increase in DPPIV/CD26 protein. The possible changes in both mRNA and DPPIV enzyme activity are therefore not supported by evidence for a change in whole-cell protein.

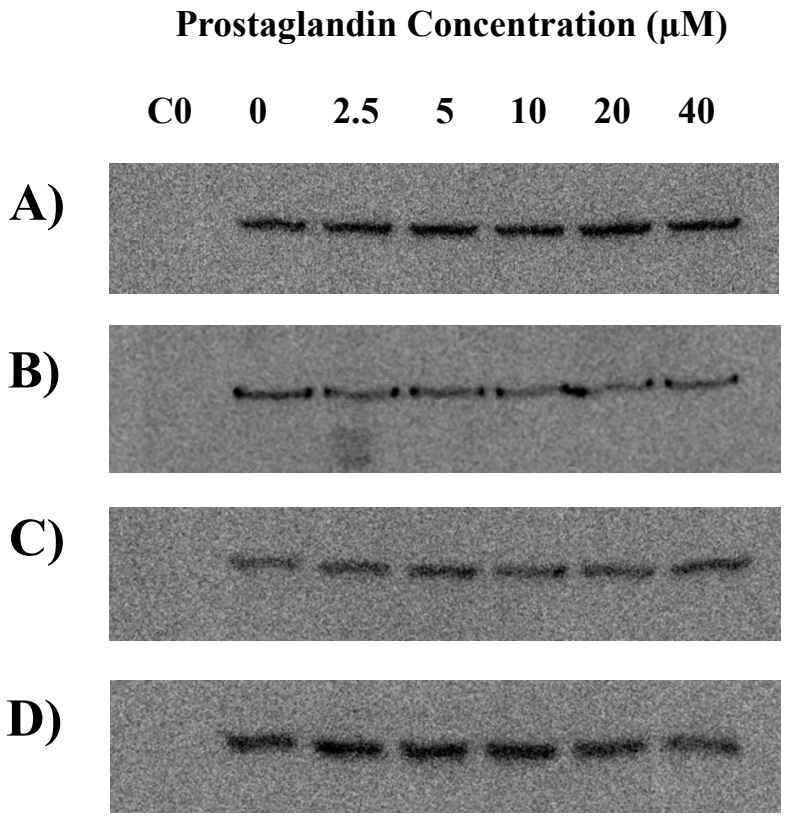

Figure 4.13: Whole-cell DPPIV/CD26 protein expression after 24 h prostaglandin treatment

HT-29 cells were treated with either PGD_2 (A), PGJ_2 (B), or 15d-PGJ_2 (C, D) at the indicated concentrations for 24 h. Protein samples were extracted, separated by SDS-PAGE, blotted to a PVDF membrane, and probed for CD26. Images were captured using a Kodak™ Image Station 4000 mm Pro. Image A is representative of two independent experiments; Image B shows the outcome of one independent experiment; Image C is representative of three independent experiments. Image D depicts 15d-PGJ_2-treated protein samples probed for β-actin as a positive control to confirm uniform protein loading.

4.6 Characterizing the localization of DPPIV/CD26 protein expression on HT-29 cells in response to prostaglandin treatment

Since the prostaglandins did not change the abundance of DPPIV/CD26 protein within the whole cell as shown by the Western blot results, I decided to investigate whether they instead altered the display of functional DPPIV/CD26 protein at the cell surface. Increased cell-surface DPPIV/CD26 protein expression may have resulted in a potential increase in DPPIV/CD26 dipeptidase activity that was suggested in some of my experiments. I used immunofluorescence staining to visualize cell-surface DPPIV/CD26 expression after 48 h treatment with the prostaglandins at different concentrations. DPPIV/CD26 was abundantly expressed on the cell surface as indicated by the high level of fluorescence intensity, however the surface localization of immunoreactive DPPIV/CD26 did not change with treatment of $PGF_{2\alpha}$, PGD_2, PGJ_2 or 15d-PGJ_2 at any concentration (Figure 4.14). Therefore, J-series prostaglandins did not change cell-surface DPPIV/CD26 protein expression.

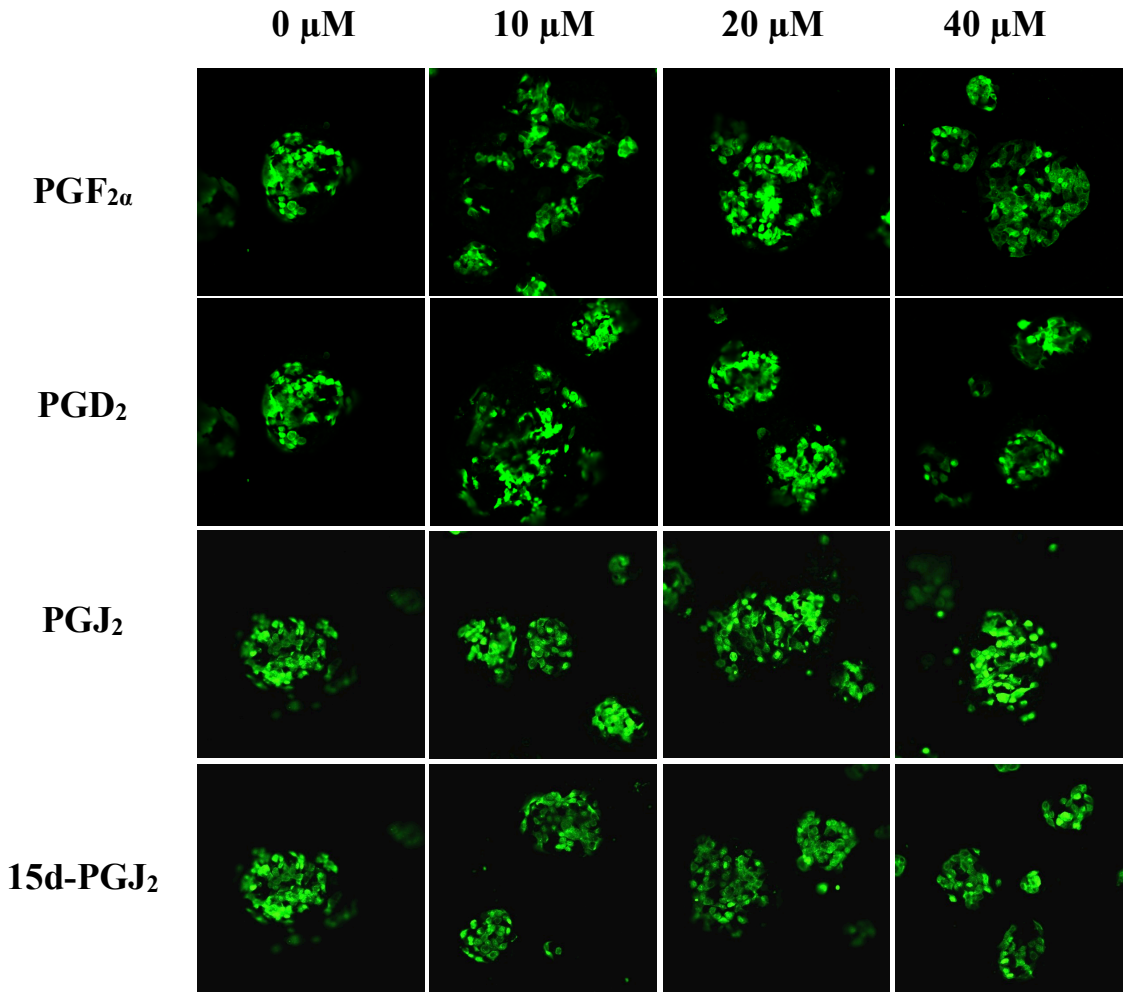

Figure 4.14: Cell-surface DPPIV/CD26 expression after prostaglandin treatment

HT-29 cells were treated with either $PGF_{2\alpha}$, PGD_2, PGJ_2 or 15d-PGJ_2 at the indicated concentrations for 48 h. Cells were then fixed onto a chamber slide with 1% paraformaldehyde, and fluorescently probed for cell-surface CD26. Images were taken on a Zeiss® LSM710 laser scanning confocal microscope by Roger Chen from the laboratory of Dr. Marianna Foldvari. This figure represents the results of two independent experiments.

4.7 Measuring the effect of 15d-PGJ_2 on cell migration on the extracellular matrix

Results thus far suggested that prostaglandin treatment did not significantly impact DPPIV/CD26 expression and dipeptidase activity on HT-29 cells. Nevertheless, to be sure there was no consistent response to 15d-PGJ_2 I decided to measure another aspect of DPPIV/CD26 activity, which was cell migration over the ECM. I characterized cell migration by measuring the rate of non-directed cell movement across an area that had been first cleared of cells but has ECM that was already produced by those cells; this technique is usually known as a wound-healing assay. I used HT-29 cells to mimic cancer cell migration at the tumour invasive front, and NIH3T3 cells to mimic the healing of wounds by normal cells, or fibrous remodelling. Results appeared to show that the highest concentration of 15d-PGJ_2 significantly decreased the rate of both HT-29 and NIH3T3 cell migration by 79% and 17%, respectively (Figure 4.15). However, it is important to note that when observed under a light microscope at different time points, HT-29 cells were proliferating more quickly than usual, and some cells appeared to be dead due to their morphology. This may have indicated that the area of the wound decreased due to accelerated HT-29 cell proliferation, rather than gradual migration across the wound (Figure 4.16). On the other hand, NIH3T3 cells appeared to be migrating toward the wound, due to their gradual dispersion and lack of cell death (Figure 4.17).

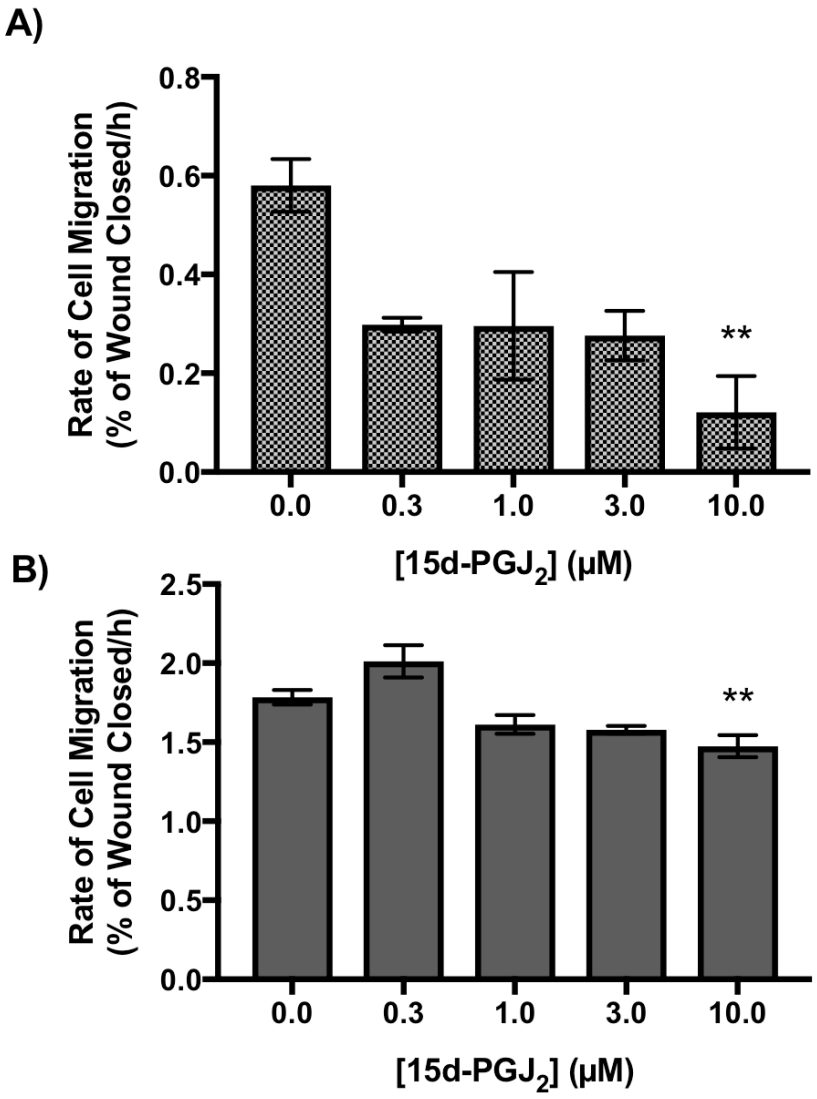

Figure 4.15: The rate of HT-29 and NIH3T3 cell migration in response to 15d-PGJ_2 treatment

HT-29 (A) and NIH3T3 (B) cells were treated with 15d-PGJ_2 at the indicated concentrations for 48 h, after which a wound was made in which cells were removed from the monolayer in a vertical line. The area of the wound was measured at 0 h and 36 h. The rate of cell migration was calculated as the percent of the wound covered by cells over the time after the wound was made. The data represent means ± SEM from one independent experiment performed in triplicate. Data were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test. **P<0.01 shows a significant decrease between the vehicle control and prostaglandin treatment.

0 h 36 h

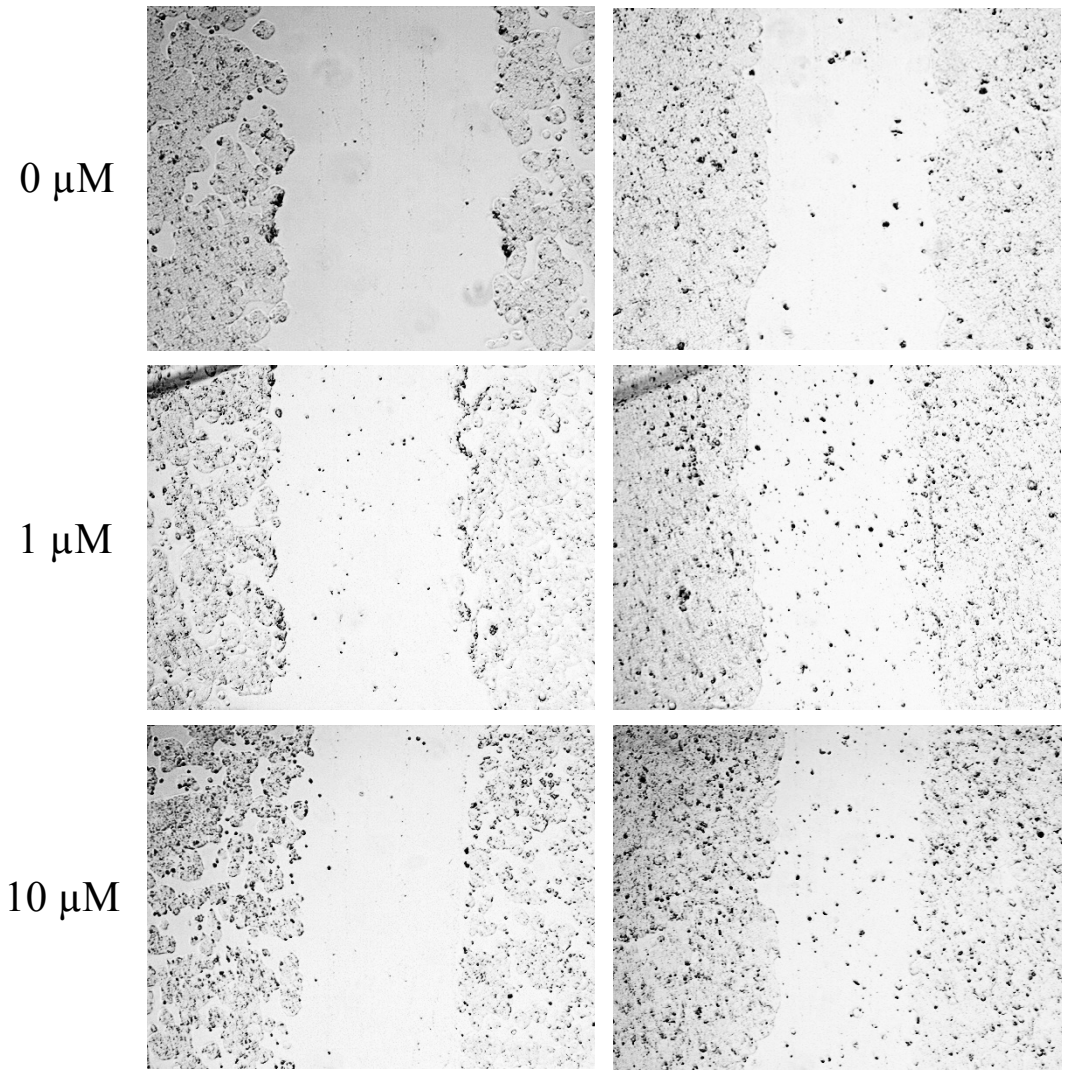

Figure 4.16: Wound healing by HT-29 cells at different 15d-PGJ_2 concentrations over time

HT-29 cells were treated with 15d-PGJ_2 at the indicated concentrations for 48 h, after which a wound was made where cells were removed from the monolayer in a vertical line. The area of the wound was measured at 0 h and 36 h. Cells were observed under a Leica DM2000 light microscope, and images on this microscope were captured using a Micropublisher 5.0 RTV camera with QCapture Pro 5 software by Judah Glogauer (0 h) and myself (36 h). These images are representative of one experiment.

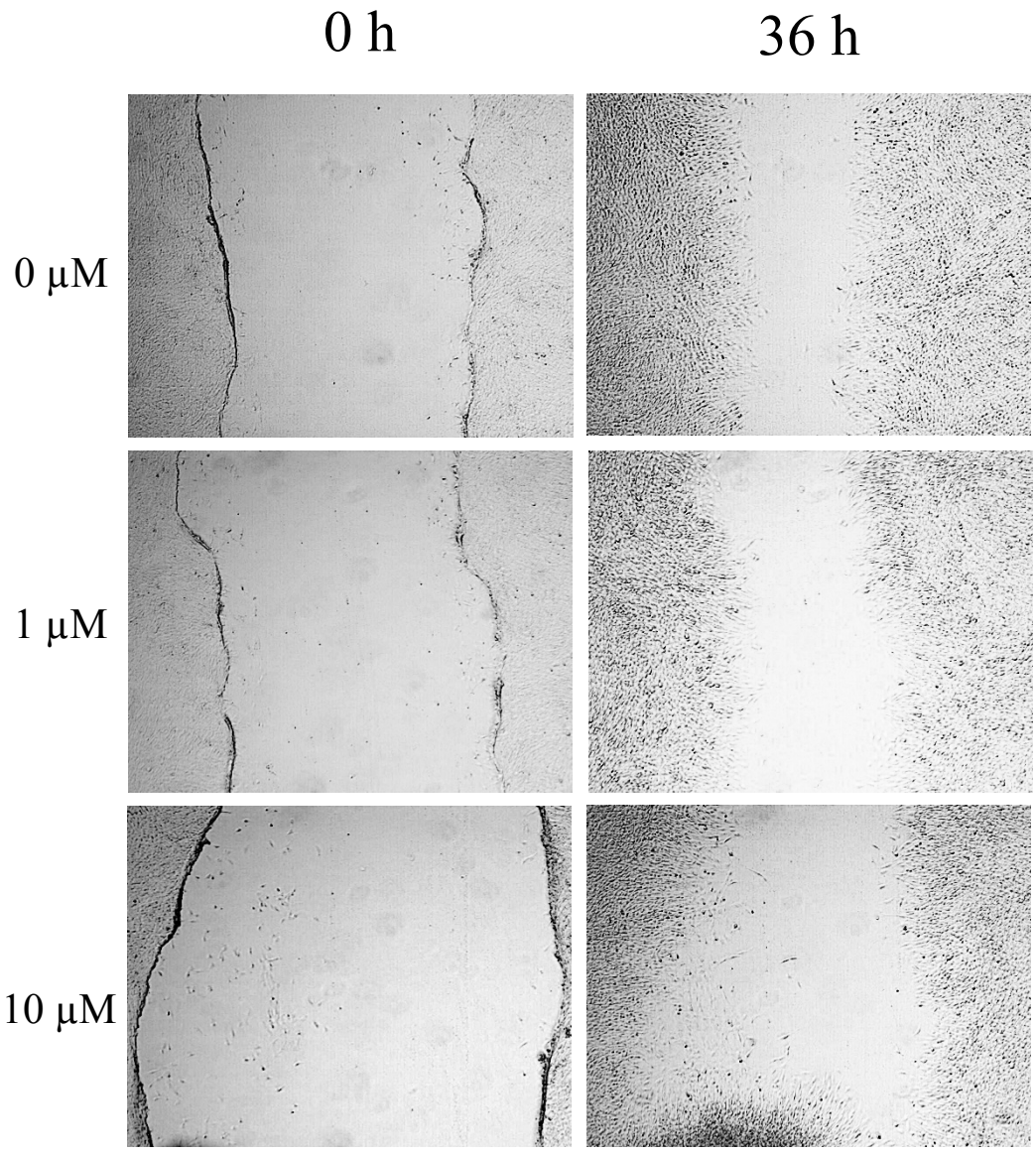

Figure 4.17: Wound healing by NIH3T3 cells at different 15d-PGJ_2 concentrations over time

NIH3T3 cells were treated with 15d-PGJ_2 at the indicated concentrations for 48 h, after which a wound was made where cells were removed from the monolayer in a vertical line. The area of the wound was measured at 0 h and 36 h. Cells were observed under a Leica DM2000 light microscope, and images on this microscope were captured using a Micropublisher 5.0 RTV camera with QCapture Pro 5 software by Judah Glogauer (0 h) and myself (36 h). These images are representative of one experiment.

4.7 Quantifying the abundance of 15d-PGJ_2 treatment in cell culture medium over time using high-performance liquid chromatography

Results thus far showed substantial conflicts and variability in my results for (i) DPPIV/CD26 dipeptidase activity, (ii) DPPIV/CD26 mRNA expression, and (iii) DPPIV/CD26 expression at the protein level. This was not obviously due to technical issues, and was seen both within experiments and between experimental replicates. I therefore sought to confirm whether the J-series prostaglandins were consistently available during treatment to produce a cell response in a predictable and concentration-dependent manner. Prostaglandins, and eicosanoids in general, have short half-lives ranging from seconds to minutes, and thus may break down before they can induce a consistent cell response (Folco & Murphy, 2006; Funk, 2001). I used HPLC to identify the amount of 15d-PGJ_2 in cell culture media from HT-29 cell cultures at different time points after treatment. Results showed that most of the added 15d-PGJ_2 was still present in the media 30 min after treatment at the lower concentrations that were used in my studies (Figure 4.18; Figure 4.19). However, intriguingly at the highest concentration, there was an enhanced breakdown of 15d-PGJ_2 (Figure 4.18; Figure 4.19). The level of 15d-PGJ_2 substantially declined by 38% 5 min after treatment, and significantly declined by 82% 30 min after treatment. This dramatic breakdown of 15d-PGJ_2 at only the highest concentration may indicate a shift in cellular metabolism that affects cell regulation due to this eicosanoid, and may be a further complicating factor in defining the cellular responses that may occur in response to J-series prostaglandins.

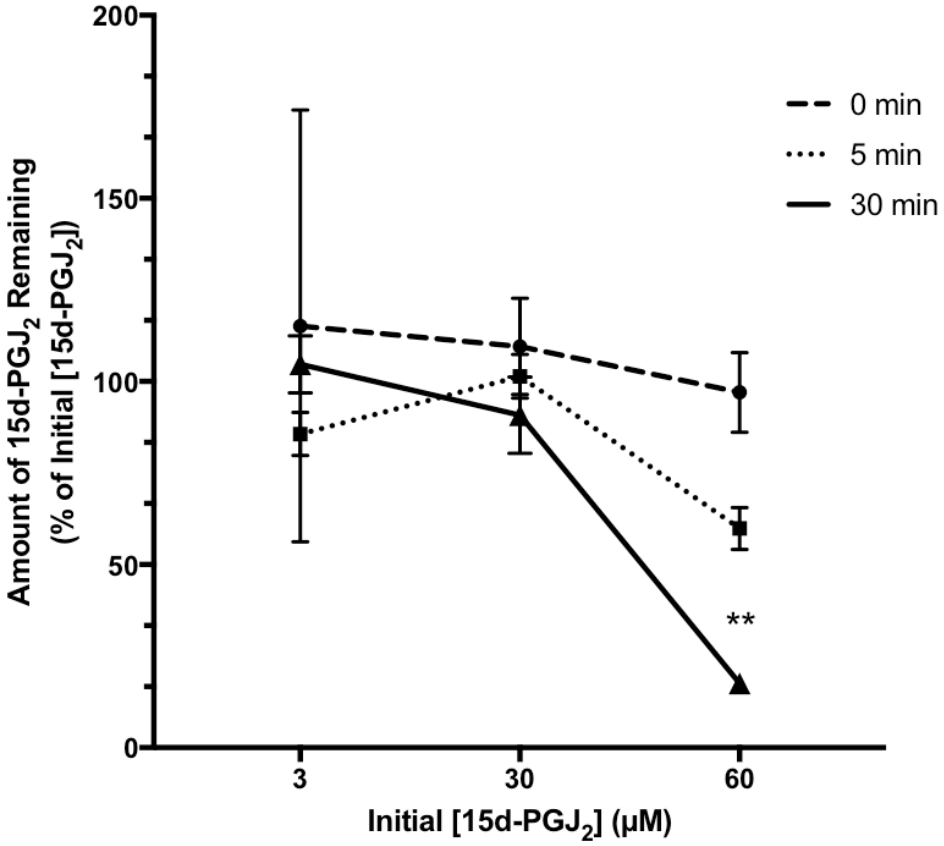

Figure 4.18: The amount of 15d-PGJ$_2$ treatment remaining in cell culture media over time at different initial concentrations

HT-29 cells were treated with 15d-PGJ$_2$ at the indicated concentrations. 15d-PGJ$_2$ was then extracted from the media at the indicated time points. The amount of extracted 15d-PGJ$_2$ was quantified using HPLC. The data are means ± SEM of triplicate wells, from one independent experiment. Data were analyzed by a two-way ANOVA with a Bonferroni multiple comparisons test. **P<0.01 indicates a significant decrease in 15d-PGJ$_2$ between 0 min and another time point.

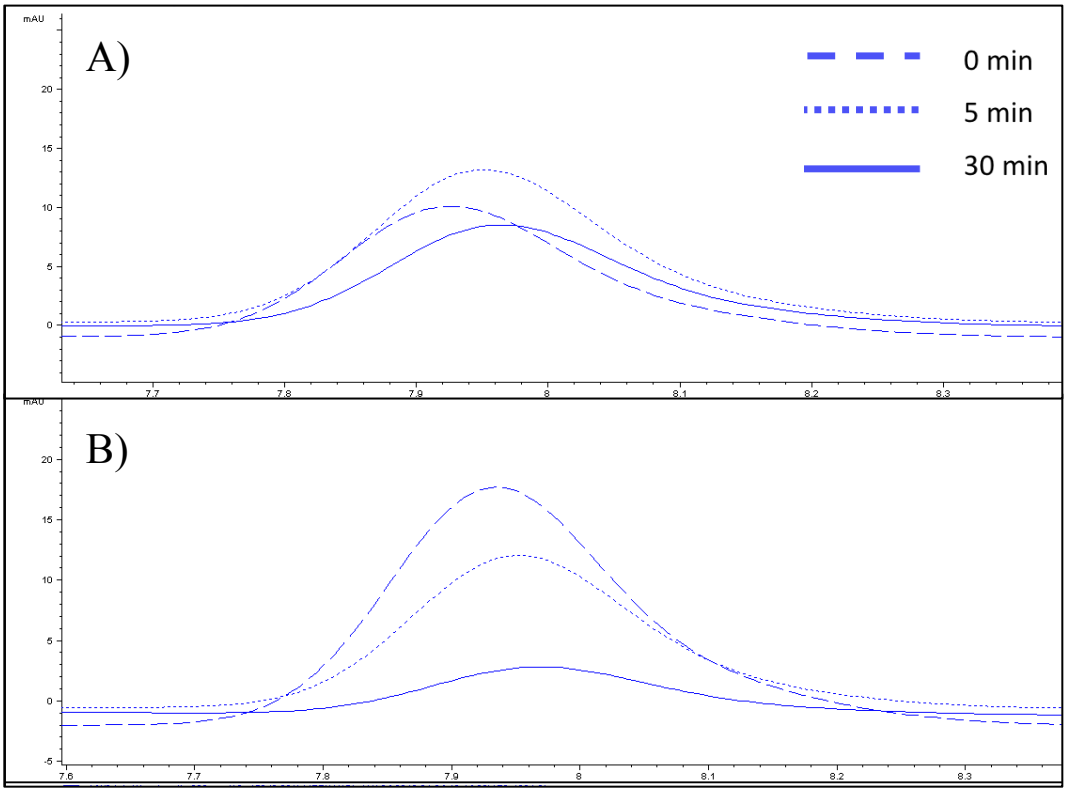

Figure 4.19: Chromatograms of 15d-PGJ$_2$ detection by HPLC over time

HT-29 cells were treated with 15d-PGJ$_2$ at 30 µM (A) and 60 µM (B), which was then extracted from the media 0 min, 5 min and 30 min after treatment. The amount of extracted 15d-PGJ$_2$ was quantified using HPLC. The X-axis represents the retention time in minutes, and the Y-axis represents the intensity of absorbance at 230 nm in milli-Absorbance Units (mAU). The area under each curve is proportional to the amount of 15d-PGJ$_2$ remaining at that time. The image is representative of three independent experiments.

5. Discussion

Dipeptidyl peptidase IV/CD26 on the surface of cancer cells exerts several anti-metastatic functions, and shows variable expression in cancer that may relate to the tendency to metastasize. In this project I studied the effect of prostaglandins, primarily of the J-series, on DPPIV/CD26 expression and activity on human colon adenocarcinoma cells. I mainly examined HT-29 cells, which sufficiently express DPPIV/CD26, and reproduced some findings using Caco-2 cells. Both cell lines can form differentiated epithelia, and therefore can be used to model the intestinal epithelium (Darmoul et al., 1991; Howell, Kenny, & Turner, 1992).

I investigated four prostaglandins – three that are sequential products in the J-structural series (PGD_2, PGJ_2 and 15d-PGJ_2), and another ($PGF_{2\alpha}$) that provides a different structural example. The J-series prostaglandins are present in the colonic tumour microenvironment: PGD_2 has been studied by other groups (Boughton-Smith et al., 1983; Fajas et al., 1997) and 15d-PGJ_2 has been measured in tumour xenografts by microdialysis (Blay et al., unpublished; Blay, White & Hoskin, 1997). Previous work in the Blay laboratory has shown that J-series prostaglandins decrease CXCR4 expression on HT-29 cells (Richard, Lowthers & Blay, 2007). As discussed earlier, CXCR4 and DPPIV/CD26 are likely regulated in parallel and usually in opposite directions. Thus, I investigated whether the J-series prostaglandins would induce an increase in DPPIV/CD26 expression and activity on HT-29 colon carcinoma cells.

5.1 Prostaglandins initially appeared to downregulate DPPIV/CD26 dipeptidase activity

Dipeptidyl peptidase IV/CD26 may suppress metastasis through its dipeptidase activity. DPPIV cleaves and thus inactivates the pro-tumorigenic chemokine CXCL12 (Sun et al., 2008). I first measured DPPIV dipeptidase activity in response to prostaglandin treatment. Initially PGD_2, PGJ_2 and 15d-PGJ_2 appeared to decrease DPPIV activity; this contradicted my hypothesis that J-series prostaglandins would instead increase DPPIV activity. However, these data showed high variability, and suggested the occurrence of cell death at high prostaglandin concentrations.

5.2 The apparent decrease in DPPIV/CD26 dipeptidase activity reflected cell death after prostaglandin treatment

To investigate possible cell death, an MTT assay was first employed. In this assay, the tetrazolium salt MTT penetrates viable cells, and is reduced to formazan by NAD(P)H-dependent enzymes in the mitochondria (Riss et al., 2004). Cell viability is quantified by the amount of formazan produced. The MTT assay showed no significant decrease in cell viability after prostaglandin treatment. However, a subsequent cell counting assay revealed a significant decrease in cell viability at high concentrations of PGD_2, PGJ_2, and 15d-PGJ_2.

There was a remarkable discrepancy between the cytotoxicity shown by the actual number of viable cells and that estimated by the MTT assay. This is likely due to differences in the way that each assay measures cell viability. While cell counting directly quantifies viable cells, an MTT assay estimates those cells indirectly by measuring their mitochondrial activity. But if the compound of interest alters

mitochondrial activity in any way, cell viability can be misjudged. For example, Pagliacci et al. (1993) found that the MTT assay consistently overestimated the number of viable breast cancer and leukemia cells remaining after treatment with the plant flavonoid genistein. The authors discovered that genistein increased mitochondrial activity in these cells, thereby increasing formazan production and overestimating cell viability. A direct count of viable cells revealed a dramatic decrease in cell number. Counting viable cells was a more valid measure of genistein cytotoxicity than the MTT assay for these authors and in my own study.

PGD_2, PGJ_2 and 15d-PGJ_2 significantly decreased HT-29 colon cancer cell survival at high concentrations. These results agree with previous findings by others on prostaglandin cytotoxicity. J-series prostaglandins have been shown to induce cytotoxic effects in various cancer cell lines. In leukemia cells, PGJ_2 and its metabolite, Δ^{12}-PGJ_2, induced apoptosis (Y. C. Chen, Shen, & Tsai, 2005; I. K. Kim et al., 1993). Δ^{12}-PGJ_2 is also cytotoxic to osteosarcoma, melanoma, ovarian, prostate, colon, pancreas, small cell lung cancer, and breast cancer cell lines (McClay, Winski, Jones, Jennerette, & Gattoni-Celli, 1996; T. Sakai et al., 1989). The final PGJ_2 metabolite, 15d-PGJ_2, has been extensively studied for its ability to induce apoptosis, especially in colorectal cancer cells (Clay et al., 1999; Dionne, Levy, Levesque, & Seidman, 2010; S. J. Lee, Kim, Park, Woo, & Kim, 2008; Shimada, Kojima, Yoshiura, Hiraishi, & Terano, 2002; Shin et al., 2009).

The cytotoxic effects of J-series prostaglandins likely resulted in part from PPARγ activation. PPARγ is a lipid-activated transcription factor that regulates cell proliferation, differentiation and apoptosis (Gupta et al., 2003; Sarraf et al., 1998). It is

particularly abundant in the colon mucosa, and exhibits upregulated expression in colonic tumours (DuBois et al., 1998; Jain et al., 1998). 15d-PGJ_2 is an endogenous agonist for PPARγ (Soares et al., 2005). PPARγ activation can suppress tumour progression by upregulating the expression of genes that promote apoptosis, and downregulating genes that inhibit apoptosis. For example, a study by Shimada et al. (2002) found that PPARγ activation by 15d-PGJ_2 in CRC cells resulted in upregulated expression of the pro-apoptotic gene *gadd153,* and downregulated expression of *c-myc* – a critical gene whose protein product suppresses apoptosis. Furthermore, Chen et al. (2002) found that PPARγ activation by 15d-PGJ_2 inhibited the expression of two major pro-tumorigenic molecules, NFκB and Bcl-2, in HT-29 cells. NFκB and Bcl-2 are normally involved in protecting cells against apoptosis; therefore, inhibiting their expression induces apoptosis (Chaves et al., 2009; Tsujimoto, 1998).

In this study PGJ_2 and 15d-PGJ_2 induced a greater decrease in cell viability than PGD_2. This might have resulted from the metabolism of PGD_2 into 15d-PGJ_2. PGD_2 readily undergoes dehydration to form PGJ_2 and then 15d-PGJ_2 over time (Fitzpatrick & Wynalda, 1983; Shibata et al., 2002). In fact, it has been shown that 80% of PGD_2 in culture medium is converted into J-series prostaglandins over 24 h (Narumiya & Fukushima, 1985). In this study, perhaps PGD_2 treatment did not induce cell death via its cell-surface DP receptors. Instead, it is likely that a significant amount of PGD_2 was converted into 15d-PGJ_2, which then induced apoptosis via PPARγ activation (Richard, Lowthers & Blay, 2007). Furthermore, the rate of PGJ_2 conversion to 15d-PGJ_2 is likely faster than that of PGD_2 since it undergoes one less step in the biosynthesis pathway. As a result, more 15d-PGJ_2 would be produced during PGJ_2 metabolism over unit time,

producing a greater decrease in cell viability than PGD_2. $PGF_{2\alpha}$ had little effect on cell viability likely because it is not produced in the J-series prostaglandin biosynthesis pathway.

5.3 Prostaglandins did not significantly upregulate DPPIV/CD26 dipeptidase activity after controlling for cell death

In order to control for cell death, DPPIV/CD26 dipeptidase activity was normalized to viable cell number. This method revealed that prostaglandins did not significantly upregulate DPPIV enzyme activity on HT-29 cells. There was however a much higher mean enzyme activity at high J-series prostaglandin concentrations (which did not reach statistical significance as a definite change). Interestingly, these data showed substantial variability between experimental replications. The magnitude of heightened average DPPIV enzyme activity varied greatly between experiments; some showed a dramatic increase in DPPIV activity, while others only showed a slight increase. This led me to consider whether the variability in results may have resulted from different aspects of cell culture in each experiment. Cell confluency affected the magnitude of cell response to prostaglandin treatment. Initial results had suggested that 15d-PGJ_2 had a greater effect on DPPIV activity on 60% confluent cells than on 90% confluent cells, although cytotoxicity assays ultimately showed that these results were skewed by cell death.

Increasing cell confluency tends to decrease cell sensitivity to a range of cell treatments, shifting the dose-response curve to the right. This has for example been shown on HT-29 cells, where increasing cell number resulted in a steady decrease in the maximum cell growth response to adenosine treatment (Mujoomdar, Hoskin & Blay,

2003). Although the reason for this confluence-dependent decrease in sensitivity is likely complex, it may have affected DPPIV enzyme activity data differently in different experiments, thereby preventing a statistically significant outcome.

Although no statistically significant elevation in DPPIV/CD26 dipeptidase activity was found, there was a subjective sense that the enzyme activity may still have been upregulated. This was because some replicates of the DPPIV enzyme assay revealed dramatic increases in dipeptidase activity at high concentrations of PGJ_2 and 15d-PGJ_2. If the J-series prostaglandins did indeed increase DPPIV/CD26 dipeptidase activity, this may inactivate the pro-tumorigenic chemokine CXCL12 to a higher degree. As a result, CXCL12-mediated tumour cell migration would decrease, which has been shown by a previous study on prostate cancer cells (Sun et al., 2008). Furthermore, DPPIV-induced inactivation of CXCL12 has also been shown to inhibit CXCL12-mediated endometrial cancer cell proliferation (Mizokami et al., 2004). Therefore, upregulated DPPIV dipeptidase activity could impede tumour growth and metastasis by further inactivating CXCL12. The mechanism by which J-series prostaglandins could have upregulated DPPIV/CD26 dipeptidase activity remains to be elucidated.

5.4 Prostaglandins did not upregulate DPPIV/CD26 expression

If in fact DPPIV dipeptidase was upregulated by J-series prostaglandins, this apparently did not result from a higher abundance of DPPIV/CD26. End-point PCR showed no apparent change in DPPIV/CD26 mRNA expression after prostaglandin treatment. However, qPCR – a more sensitive technique than end-point PCR – showed a significant decrease in DPPIV/CD26 mRNA at high concentrations of 15d-PGJ_2. This finding contradicted my prediction that 15d-PGJ_2 would increase DPPIV/CD26

expression. Although the reason behind decreased DPPIV/CD26 mRNA expression is unclear, there was no apparent functional significance of decreased DPPIV/CD26 mRNA, since its whole-cell protein expression remained unchanged after 15d-PGJ_2 treatment.

The reason for the contradictory results between end-point PCR and qPCR is unclear. The same CD26 and GAPDH primers were used in both techniques, a melt curve analysis revealed no evidence for the formation of primer dimers, and the amplification efficiency of each primer was 1.95 or higher (with 2 being the maximum efficiency). Furthermore, negative control samples did not indicate genomic DNA contamination of the distilled water used for primer dilution, nor of the RNA samples that were not reverse transcribed. Although qPCR is more sensitive to changes in mRNA expression than end-point PCR (and is thus the most common method for quantifying mRNA expression) the technique is still somewhat limited (Bustin, 2002). For instance, mRNA is unstable, and therefore the concentration of corresponding cDNA may not be equal to the starting concentration of mRNA. Measuring GAPDH expression controls for these potential differences in cDNA loading between samples; however, using GAPDH as a housekeeping gene may actually be problematic, as its levels have shown to be heterogenous in cell subpopulations of the same origin (Goidin et al., 2001).

Despite these inconsistent changes in DPPIV/CD26 mRNA expression, the abundance of whole-cell DPPIV/CD26 protein did not change with 15d-PGJ_2 treatment, as assessed with Western blotting. It was not particularly unusual that DPPIV/CD26 protein expression remained constant despite my qPCR findings, since mRNA expression does not always predict protein expression (de Sousa Abreu, Penalva, Marcotte, & Vogel,

2009). This discrepancy is mostly attributed to processes downstream of transcription, such as post transcriptional modifications (Maier, Guell, & Serrano, 2009). Post-transcriptional modifications control gene expression at the RNA level, and include RNA processing (i.e. capping and poly-A tail addition), alternative splicing, and the functions of small interfering RNA (siRNA) and microRNA (miRNA) (Zhao, Roundtree, & He, 2017). RNA processing modifies the primary mRNA transcript to eventually promote its expression, whereas alternative splicing results in different proteins being expressed from the same gene (Y. Lee & Rio, 2015; Licatalosi & Darnell, 2010). Furthermore, mRNA can be degraded by miRNA and siRNA, thereby suppressing protein translation (Iwakawa & Tomari, 2015; Y. Zeng, Yi, & Cullen, 2003). There are many complex processes that can occur between transcription and translation that may have prevented the translation of DPPIV/CD26 mRNA directly after it was downregulated by 15d-PGJ_2.

Prostaglandin treatment also did not change cell-surface DPPIV/CD26 protein expression or localization. The high intensity of immunofluorescence remained constant at each prostaglandin concentration, as did the cellular pattern. Any increase in DPPIV enzyme activity after J-series prostaglandin treatment (which was suggested by some of my data but needs to be confirmed for possible statistical significance) would therefore not be explained by an increase in DPPIV/CD26 cell-surface protein expression. However, this observation did confirm that a substantial amount of DPPIV/CD26 was present on the surface of HT-29 cells (Tan, Mujoomdar & Blay, 2004).

5.5 15d-PGJ_2 may have decreased DPPIV/CD26-mediated cell migration across the extracellular matrix

Thus far, prostaglandin treatment did not appear to significantly upregulate DPPIV dipeptidase activity or protein expression. My last experiments explored another function of DPPIV/CD26, which is promoting cell adhesion to and migration across the ECM (Piazza et al., 1989). A wound-healing assay was performed to measure the rate of non-directed cell migration across the ECM. Two scenarios with different cell lines mimicked i) general cancer cell migration at the tumour invasive front, and ii) normal cell migration during wound healing. The highest concentration of 15d-PGJ_2 significantly decreased the rate of wound closure by both HT-29 and NIH3T3 cells. However, it is unclear whether wound closure was the result of cell migration into the wound, or cell proliferation around the wound which resulted in a greater number of cells contributing to would closure. NIH3T3 cells appeared to be migrating into the wound due to their increased dispersion over time, whereas HT-29 cells exhibited behaviours and morphologies indicative of accelerated proliferation and subsequent cell death. The accelerated proliferation of HT-29 cells was likely due to the addition of culture medium containing 20% FBS intended to stimulate cell migration, but which also contains more growth factors than the 10% NCS that the cells were growing in before the assay. It therefore seems unlikely that 15d-PGJ_2 did in fact increase HT-29 cell migration across the ECM, as would be expected if there was a change in functional DPPIV/CD26 protein, although the capacity of 15d-PGJ_2 to alter cell migration was confirmed by the NIH3T3 normal fibroblast model.

15d-PGJ_2 may have decreased cell migration across the ECM via DPPIV-dependent or DPPIV-independent mechanisms. The potential increase in DPPIV/CD26 dipeptidase activity may have resulted in an increased inactivation of CXCL12, thereby decreasing CXCL12-mediated cell migration. CXCL12-mediated cell migration could have also decreased independently of DPPIV/CD26 activity, as 15d-PGJ_2 downregulates its receptor CXCR4 likely through activating PPARγ and inhibiting NFκB (Richard, Lowthers & Blay, 2007). PPARγ activation has also shown to reduce hepatocarcinoma cell migration by downregulating the expression of matrix metalloproteinases (MMPs) MMP9 and MMP13 (Shen et al., 2012). MMPs facilitate cell migration by degrading components of the ECM; MMP9 primarily degrades type IV and V collagen, whereas MMP13 degrades type I, II and III collagen (Howes et al., 2014; Z. S. Zeng, Cohen, & Guillem, 1999). Degradation of ECM collagen creates cavities through which cells can freely move (Howes et al., 2014).

5.6 15d-PGJ_2 treatment degraded at high concentrations in cell culture medium

In the different approaches of my project, prostaglandin treatment did not significantly upregulate DPPIV expression and activity as hypothesized. The lack of cell response might have occurred due to an insufficient amount of bioactive prostaglandins available during treatment. After HPLC analysis, 15d-PGJ_2 was found to significantly degrade in culture medium after 30 min, intriguingly only at the highest concentration. Therefore, during treatment, a large portion of the prostaglandin at the normally effective concentrations may have degraded before it could affect DPPIV/CD26 expression and activity. Eicosanoids are quite unstable and have short half-lives ranging from seconds to

minutes, rendering them difficult to study in dose-response relationships (Folco & Murphy, 2006; Funk, 2001).

The instability of prostaglandins in serum-supplemented culture medium may be due to the presence of serum albumin. Prostaglandins have been shown to bind to the same site of albumin whose microenvironment is significantly alkaline (with a pH equal to or greater than 10.0) (Fitzpatrick & Waynalda, 1981). Albumin subsequently catalyzes dehydration and isomerization reactions to break down bound prostaglandins (Fitzpatrick & Waynalda, 1981). 15d-PGJ_2 has shown to bind to bovine serum albumin (BSA) when added to cardiomyocytes cultured in 10% fetal calf serum (FCS) (Rajakariar et al., 2007). As a result, less than 4% of the initial 15d-PGJ_2 treatment was detectable by liquid chromatography tandem mass spectrometry after 24 h. Furthermore, prostaglandins containing a β-hydroxy ketone have been shown to readily bind to albumin, and this greatly reduces their stability (Fitzpatrick & Waynalda, 1981; Fitzpatrick & Wynalda, 1983). The use of serum-free medium may decrease extracellular breakdown of prostaglandins *in vitro*. *In vivo*, 15d-PGJ_2 can be loaded into solid lipid nanoparticles to prevent its degradation (de Melo et al., 2016).

5.7 Perspectives

In this study I examined the relationships between three components of the colonic tumour microenvironment – prostaglandins, CXCR4 and DPPIV/CD26. Contrary to my hypothesis, prostaglandin treatment did not significantly upregulate DPPIV/CD26 expression and activity on HT-29 cells. However, there were suggestions of increased DPPIV/CD26 dipeptidase activity after J-series prostaglandin treatment. Although reasons for this possible upregulation are unclear, perhaps it suggests that DPPIV/CD26

and CXCR4 are only related through the DPPIV substrate and CXCR4 ligand CXCL12, rather than through more complex mechanisms.

If DPPIV/CD26 and CXCR4 do exhibit a complex relationship, it may involve the extracellular signal-regulated kinase (ERK) 1/2 signal- mitogen-activated protein kinase (MAPK) pathway. Increased DPPIV/CD26 expression in prostate cancer cells has been associated with the inactivation of ERK 1/2 MAP kinase (Wesley, McGroarty, & Homoyouni, 2005). Inactivating ERK 1/2 MAP kinase inhibits the transcription factor NFκB, which has been shown to promote the expression of CXCR4 in breast cancer cells (Helbig et al., 2003). Inhibiting NFκB would thus reduce the expression of CXCR4 on the cell-surface. Therefore, increasing DPPIV/CD26 may subsequently decrease CXCR4 expression via inactivating ERK 1/2 MAP kinase.

The relationship between DPPIV/CD26 and relationship may be unidirectional. Manipulating DPPIV/CD26 may cause the opposite change in CXCR4 expression; however, targeting CXCR4 expression might not induce the opposite change in DPPIV/CD26. In fact, a study by Miyanishi et al. (2010) found that knocking down CXCR4 expression via siRNA actually decreased DPPIV/CD26 protein expression on ovarian carcinoma cells. Therefore, DPPIV/CD26 expression and activity may not have increased in my own study through targeting CXCR4 via prostaglandin treatment.

The possibility remains that DPPIV/CD26 and CXCR4 are not always inversely related. Interestingly, the two proteins have been shown to physically associate on the membranes of human T-lymphocytes (Herrera et al., 2001). In a study by Herrera et al. (2001), DPPIV/CD26 and CXCR4 were shown to colocalize on the cell surface, as fluorescent micrographs revealed the physical overlap of their structures. Furthermore,

these two proteins coimmunoprecipitated from the plasma membrane, and cointernalized upon exposure to CXCL12 (Herrera et al., 2001). Thus, DPPIV/CD26 and CXCR4 may either directly associate on the plasma membrane, or associate through a larger protein complex (Herrera et al., 2001).

5.8 Limitations

Cancer is a highly heterogeneous disease, and its characteristics substantially differ between individual patients. This project primarily focused on one human colon carcinoma cell line, HT-29, which originated from a 44-year old Caucasian female patient. Even when derived from the same type of tumour, cell lines can differ in morphology, degree of differentiation, and DPPIV/CD26 expression as well as other traits. Different CRC cell lines may show different responses to prostaglandin treatment. Therefore, multiple cell lines derived from colonic tumours should be compared.

Tumour-derived cell lines help us understand the mechanisms of cancer, and serve as preclinical models for assessing the toxicity and efficacy of novel therapies. However, these *in vitro* studies come with several limitations. First, cell lines can be misidentified due to changes in morphology when cultured over time, or cross contamination with another cell line. Second, *in vitro* concentrations of a compound are difficult to extrapolate to *in vivo* doses. The chosen prostaglandin concentrations in this project cannot be directly translated into corresponding doses for animal models. Finally, we cannot exactly replicate the colonic tumour microenvironment *in vitro*. Cells will inevitably behave differently when isolated from organisms and grown in culture medium.

In vitro studies on DPPIV/CD26 are limited by the existence of several DPPIV homologues. These homologues exhibit structural and/or functional analogies to DPPIV (H. Zhang, Chen, Keane, & Gorrell, 2013). For example, DPP8 and DPP9 exhibit similar dipeptidase activity to DPPIV/CD26 in that they also cleave Xaa-Pro polypeptides at the penultimate position (C. A. Abbott et al., 2000). Therefore, when intending to exclusively measure DPPIV activity through its cleavage of Gly-Pro-pNA, DPP8 and DPP9 might interfere with these results by cleaving the same substrate. However, this would only be a minor interference, since DPP8 and DPP9 cleave substrates with a weaker catalytic efficiency than DPPIV, and are located in the cytoplasm rather than on the cell surface (C. A. Abbott et al., 2000; Ajami et al., 2008; Bjelke et al., 2006). Nevertheless, one should be wary of the functional and structural similarities between DPPIV and its homologues when exclusively studying one of these enzymes.

Studies on DPPIV/CD26 in colorectal cancer are also limited by the discordant literature on its levels of expression and activity. Some studies have reported upregulated DPPIV/CD26 expression in CRC, while others have reported a downregulation (Havra et al., 2008). Furthermore, despite exerting several anti-metastatic functions, the net effect of DPPIV/CD26 on cancer progression varies in the literature (Havre et al., 2008). The location of the tumour in the colon may determine the level and significance of DPPIV activity (Kate, Vandeningh, Khan & Bosman, 1986; Kate et al., 1985). If so, there are likely individual differences in DPPIV/CD26 between CRC patients that ought to be considered if DPPIV eventually serves as a prognostic biomarker, or is targeted by a novel therapy.

5.9 Future Directions

In this project, the unstable prostaglandin 15d-PGJ_2 may have degraded in the culture medium before it could induce a consistent cell response at high concentrations. Further studies would need to confirm whether 15d-PGJ_2 treatment is sufficiently available to cultured cells to affect DPPIV/CD26 levels. This may include lysing cells and measuring intracellular 15d-PGJ_2 after treatment by HPLC, and using serum-free culture medium to prevent albumin from degrading 15d-PGJ_2. If optimal concentrations of stable 15d-PGJ_2 are established, then the effect of J-series prostaglandins on DPPIV/CD26 dipeptidase activity can be further investigated with perhaps less variability between results. For instance, the effect of 15d-PGJ_2 on CXCL12 degradation by DPPIV/CD26 could be investigated, followed by its effect on CXCL12-mediated directional CRC cell migration *in vitro* and *in vivo*. Furthermore, the directionality of the inverse relationship between DPPIV/CD26 and CXCR4 may be further investigated by targeting DPPIV/CD26 instead of CXCR4. This can be accomplished by using DPPIV/CD26 inhibitors such as diprotin A, or DPPIV/CD26 knockdown via siRNA.

6. References

Aaltonen, L. A., Salovaara, R., Kristo, P., Canzian, F., Hemminki, A., Peltomaki, P., . . . ValKamo, E. (1998). Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. *New England Journal of Medicine, 338*(21), 1481-1487.

Abbott, B. D. (2009). Review of the expression of peroxisome proliferator-activated receptors alpha (PPAR alpha), beta (PPAR beta), and gamma (PPAR gamma) in rodent and human development. *Reproductive Toxicology, 27*(3-4), 246-257.

Abbott, C. A., Baker, E., Sutherland, G. R., & Mccaughan, G. W. (1995). Genomic Organization, Exact Localization, and Tissue Expression of the Human Cd26 (Dipeptidyl Peptidase-Iv) Gene (Vol 40, Pg 331, 1994). *Immunogenetics, 42*(1), 76-76.

Abbott, C. A., Yu, D. M., Woollatt, E., Sutherland, G. R., McCaughan, G. W., & Gorrell, M. D. (2000). Cloning, expression and chromosomal localization of a novel human dipeptidyl peptidase (DPP) IV homolog, DPP8. *European Journal of Biochemistry, 267*(20), 6140-6150.

Ajami, K., Pitman, M. R., Wilson, C. H., Park, J., Menz, R. I., Starr, A. E., . . . Gorrell, M. D. (2008). Stromal cell-derived factors 1alpha and 1beta, inflammatory protein-10 and interferon-inducible T cell chemo-attractant are novel substrates of dipeptidyl peptidase 8. *FEBS Lett, 582*(5), 819-825.

Akashi, T., Koizumi, K., Tsuneyama, K., Saiki, I., Takano, Y., & Fuse, H. (2008). Chemokine receptor CXCR4 expression and prognosis in patients with metastatic prostate cancer. *Cancer Science, 99*(3), 539-542.

Anggard, E., & Samuelsson, B. (1965). Biosynthesis of prostaglandins from arachidonic acid in guinea pig lung. Prostaglandins and related factors. 38. *J Biol Chem, 240*(9), 3518-3521.

Aoe, K., Amatya, V. J., Fujimoto, N., Ohnuma, K., Hosono, O., Hiraki, A., . . . Morimoto, C. (2012). CD26 Overexpression Is Associated with Prolonged Survival and Enhanced Chemosensitivity in Malignant Pleural Mesothelioma. *Clinical Cancer Research, 18*(5), 1447-1456.

Arnold, M., Sierra, M. S., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2017). Global patterns and trends in colorectal cancer incidence and mortality. *Gut, 66*(4), 683-691.

Athari, A., Hanecke, K., & Jungermann, K. (1994). Prostaglandin F2 alpha and D2 release from primary Ito cell cultures after stimulation with noradrenaline and ATP but not adenosine. *Hepatology, 20*(1), 142-148.

Balkwill, F. (2004). The significance of cancer cell expression of the chemokine receptor CXCR4. *Seminars in Cancer Biology, 14*(3), 171-179.

Barcz, E., Sommer, E., Janik, P., Marianowski, L., & Skopinska-Rozewska, E. (2000). Adenosine receptor antagonism causes inhibition of angiogenic activity of human ovarian cancer cells. *Oncology Reports, 7*(6), 1285-1291.

Barrientos, G., Tirado-Gonzalez, I., Freitag, N., Kobelt, P., Moschansky, P., Klapp, B. F., . . . Blois, S. M. (2013). CXCR4(+) dendritic cells promote angiogenesis during embryo implantation in mice. *Angiogenesis, 16*(2), 417-427.

Bennett, A., Hensby, C. N., Sanger, G. J., & Stamford, I. F. (1981). Metabolites of arachidonic acid formed by human gastrointestinal tissues and their actions on the muscle layers. *Br J Pharmacol, 74*(2), 435-444.

Bjelke, J. R., Christensen, J., Nielsen, P. F., Branner, S., Kanstrup, A. B., Wagtmann, N., & Rasmussen, H. B. (2006). Dipeptidyl peptidases 8 and 9: specificity and molecular characterization compared with dipeptidyl peptidase IV. *Biochem J, 396*(2), 391-399.

Blay, J. (2008). CD26/DPPIV in cancer progression and spread. In *Encyclopedia of Cancer*: Springer, Berlin, Heidelberg.

Blay, J., White, T. D., & Hoskin, D. W. (1997). The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. *Cancer Research, 57*(13), 2602-2605.

Bogenrieder, T., Finstad, C. L., Freeman, R. H., Papandreou, C. N., Scher, H. I., Albino, A. P., . . . Nanus, D. M. (1997). Expression and localization of aminopeptidase A, aminopeptidase N, and dipeptidyl peptidase IV in benign and malignant human prostate tissue. *Prostate, 33*(4), 225-232.

Bosman, F. T., & Stamenkovic, I. (2003). Functional structure and composition of the extracellular matrix. *J Pathol, 200*(4), 423-428.

Boughton-Smith, N. K., Hawkey, C. J., & Whittle, B. J. (1983). Biosynthesis of lipoxygenase and cyclo-oxygenase products from [14C]-arachidonic acid by human colonic mucosa. *Gut, 24*(12), 1176-1182.

Brady, T. (1942). Adenosine deaminase. *Biochemical Journal, 36*, 478-484.

Burns, J. M., Summers, B. C., Wang, Y., Melikian, A., Berahovich, R., Miao, Z. H., . . . Schall, T. J. (2006). A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *Journal of Experimental Medicine, 203*(9), 2201-2213.

Bustin, S. A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of molecular endocrinology*, *29*(1), 23-39.

Camacho, M., Lopez-Belmonte, J., & Vila, L. (1998). Rate of vasoconstrictor prostanoids released by endothelial cells depends on cyclooxygenase-2 expression and prostaglandin I synthase activity. *Circ Res, 83*(4), 353-365.

Carmeliet, P., & Jain, R. K. (2000). Angiogenesis in cancer and other diseases. *Nature, 407*(6801), 249-257.

Chambers, A. F., Groom, A. C., & MacDonald, I. C. (2002). Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer, 2*(8), 563-572.

Chaves, J. C., Fagundes, D. J., Simoes Mde, J., Bertoletto, P. R., Oshima, C. T., Taha, M. O., . . . Fagundes, A. L. (2009). Hyperbaric oxygen therapy protects the liver from apoptosis caused by ischemia-reperfusion injury in rats. *Microsurgery, 29*(7), 578-583.

Chen, Zhong, X. Y., Qin, Y. F., Bing, W., & He, L. Z. (2003). 15d-PGJ2 inhibits cell growth and induces apoptosis of MCG-803 human gastric cancer cell line. *World J Gastroenterol, 9*(10), 2149-2153.

Chen, G. G., Lee, J. F., Wang, S. H., Chan, U. P., Ip, P. C., & Lau, W. Y. (2002). Apoptosis induced by activation of peroxisome-proliferator activated receptor-gamma is associated with Bcl-2 and NF-kappaB in human colon cancer. *Life Sci, 70*(22), 2631-2646.

Chen, W. S., Wei, S. J., Liu, J. M., Hsiao, M., Kou-Lin, J., & Yang, W. K. (2001). Tumor invasiveness and liver metastasis of colon cancer cells correlated with cyclooxygenase-2 (COX-2) expression and inhibited by a COX-2-selective inhibitor, etodolac. *Int J Cancer, 91*(6), 894-899.

Chen, Y. C., Shen, S. C., & Tsai, S. H. (2005). Prostaglandin D(2) and J(2) induce apoptosis in human leukemia cells via activation of the caspase 3 cascade and production of reactive oxygen species. *Biochim Biophys Acta, 1743*(3), 291-304.

Chen, Y. C., Stamatoyannopoulos, G., & Song, C. Z. (2003). Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion in vitro. *Cancer Research, 63*(16), 4801-4804.

Cheng, H. C., Abdel-Ghany, M., & Pauli, B. U. (2003). A novel consensus motif in fibronectin mediates dipeptidyl peptidase IV adhesion and metastasis. *J Biol Chem, 278*(27), 24600-24607.

Cheng, J. W., Sadeghi, Z., Levine, A. D., Penn, M. S., von Recum, H. A., Caplan, A. I., & Hijaz, A. (2014). The role of CXCL12 and CCL7 chemokines in immune regulation, embryonic development, and tissue regeneration. *Cytokine, 69*(2), 277-283.

Christopherson, K. W., 2nd, Hangoc, G., & Broxmeyer, H. E. (2002). Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34+ progenitor cells. *J Immunol, 169*(12), 7000-7008.

Clay, C. E., Namen, A. M., Atsumi, G., Willingham, M. C., High, K. P., Kute, T. E., . . . Chilton, F. H. (1999). Influence of J series prostaglandins on apoptosis and tumorigenesis of breast cancer cells. *Carcinogenesis, 20*(10), 1905-1911.

Coondoo, A. (2011). Cytokines in dermatology - a basic overview. *Indian J Dermatol, 56*(4), 368-374.

Cordero, O. J., Ayude, D., Nogueira, M., Rodriguez-Berrocal, F. J., & de la Cadena, M. P. (2000). Preoperative serum CD26 levels: diagnostic efficiency and predictive value for colorectal cancer. *British Journal of Cancer, 83*(9), 1139-1146.

Cutler, M. J., Lowthers, E. L., Richard, C. L., Hajducek, D. M., Spagnuolol, P. A., & Blay, J. (2015). Chemotherapeutic agents attenuate CXCL12-mediated migration of colon cancer cells by selecting for CXCR4-negative cells and increasing peptidase CD26. *Bmc Cancer, 15*.

Darash-Yahana, M., Pikarsky, E., Abramovitch, R., Zeira, E., Pal, B., Karplus, R., . . . Peled, A. (2004). Role of high expression levels of CXCR4 in tumor growth, vascularization, and metastasis. *Faseb Journal, 18*(9), 1240-1242.

Darmoul, D., Lacasa, M., Baricault, L., Marguet, D., Sapin, C., Trotot, P., . . . Trugnan, G. (1992). Dipeptidyl peptidase IV (CD 26) gene expression in enterocyte-like colon cancer cell lines HT-29 and Caco-2. Cloning of the complete human coding sequence and changes of dipeptidyl peptidase IV mRNA levels during cell differentiation. *J Biol Chem, 267*(7), 4824-4833.

Darmoul, D., Rouyerfessard, C., Blais, A., Voisin, T., Sapin, C., Baricault, L., . . . Trugnan, G. (1991). Dipeptidyl Peptidase Iv Expression in Rat Jejunal Crypt-Villus Axis Is Controlled at Messenger-Rna Level. *American Journal of Physiology, 261*(5), 763-769.

Darmoul, D., Voisin, T., Couvineau, A., Rouyerfessard, C., Salomon, R., Wang, Y. X., . . . Laburthe, M. (1994). Regional Expression of Epithelial Dipeptidyl Peptidase-Iv in the Human Intestines. *Biochemical and Biophysical Research Communications, 203*(2), 1224-1229.

De La Haba-Rodriguez, J., Macho, A., Calzado, M. A., Blazquez, M. V., Gomez, M. A., Munoz, E. E., & Aranda, E. (2002). Soluble dipeptidyl peptidase IV (CD-26) in serum of patients with colorectal carcinoma. *Neoplasma, 49*(5), 307-311.

De La Luz Sierra, M., Yang, F., Narazaki, M., Salvucci, O., Davis, D., Yarchoan, R., . . . Tosato, G. (2004). Differential processing of stromal-derived factor-1alpha and stromal-derived factor-1beta explains functional diversity. *Blood, 103*(7), 2452-2459.

de Meester, I., Lambeir, A. M., Proost, P., & Scharpe, S. (2003). Dipeptidyl peptidase IV substrates. An update on in vitro peptide hydrolysis by human DPPIV. *Adv Exp Med Biol, 524*, 3-17.

de Melo, N. F., de Macedo, C. G., Bonfante, R., Abdalla, H. B., da Silva, C. M., Pasquoto, T., . . . Napimoga, M. H. (2016). 15d-PGJ2-Loaded Solid Lipid Nanoparticles: Physicochemical Characterization and Evaluation of Pharmacological Effects on Inflammation. *PLoS One, 11*(8), e0161796.

de Sousa Abreu, R., Penalva, L. O., Marcotte, E. M., & Vogel, C. (2009). Global signatures of protein and mRNA expression levels. *Mol Biosyst, 5*(12), 1512-1526.

Dinjens, W. N., ten Kate, J., van der Linden, E. P., Wijnen, J. T., Khan, P. M., & Bosman, F. T. (1989). Distribution of adenosine deaminase complexing protein (ADCP) in human tissues. *J Histochem Cytochem, 37*(12), 1869-1875.

Dionne, S., Levy, E., Levesque, D., & Seidman, E. G. (2010). PPARgamma ligand 15-deoxy-delta 12,14-prostaglandin J2 sensitizes human colon carcinoma cells to TWEAK-induced apoptosis. *Anticancer Res, 30*(1), 157-166.

Dong, R. P., Kameoka, J., Hegen, M., Tanaka, T., Xu, Y., Schlossman, S. F., & Morimoto, C. (1996). Characterization of adenosine deaminase binding to human CD26 on T cells and its biologic role in immune response. *J Immunol, 156*(4), 1349-1355.

Dong, Y. W., Wang, X. P., & Wu, K. (2009). Suppression of pancreatic carcinoma growth by activating peroxisome proliferator-activated receptor gamma involves angiogenesis inhibition. *World J Gastroenterol, 15*(4), 441-448.

Downward, J. (2003). Targeting ras signalling pathways in cancer therapy. *Nature Reviews Cancer, 3*(1), 11-22.

DuBois, R. N., Gupta, R., Brockman, J., Reddy, B. S., Krakow, S. L., & Lazar, M. A. (1998). The nuclear eicosanoid receptor, PPARgamma, is aberrantly expressed in colonic cancers. *Carcinogenesis, 19*(1), 49-53.

Durinx, C., Lambeir, A. M., Bosmans, E., Falmagne, J. B., Berghmans, R., Haemers, A., . . . De Meester, I. (2000). Molecular characterization of dipeptidyl peptidase activity in serum - Soluble CD26/dipeptidyl peptidase IV is responsible for the release of X-Pro dipeptides. *European Journal of Biochemistry, 267*(17), 5608-5613.

Eberhart, C. E., & Dubois, R. N. (1995). Eicosanoids and the gastrointestinal tract. *Gastroenterology, 109*(1), 285-301.

Ekbom, A., Helmick, C., Zack, M., & Adami, H. O. (1990a). Increased risk of large-bowel cancer in Crohn's disease with colonic involvement. *Lancet, 336*(8711), 357-359.

Ekbom, A., Helmick, C., Zack, M., & Adami, H. O. (1990b). Ulcerative-Colitis and Colorectal-Cancer - a Population-Based Study. *New England Journal of Medicine, 323*(18), 1228-1233.

Ensor, C. M., & Tai, H. H. (1995). 15-Hydroxyprostaglandin dehydrogenase. *J Lipid Mediat Cell Signal, 12*(2-3), 313-319.

Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebvre, A. M., Saladin, R., . . . Auwerx, J. (1997). The organization, promoter analysis, and expression of the human PPARgamma gene. *J Biol Chem, 272*(30), 18779-18789.

Fang, C. Y., Wu, C. C., Fang, C. L., Chen, W. Y., & Chen, C. L. (2017). Long-term growth comparison studies of FBS and FBS alternatives in six head and neck cell lines. *PLoS One, 12*(6), e0178960.

Fidler, I. J. (2002). The organ microenvironment and cancer metastasis. *Differentiation, 70*(9-10), 498-505.

Fitzpatrick, F. A., & Waynalda, M. A. (1981). Albumin-lipid interactions: prostaglandin stability as a probe for characterizing binding sites on vertebrate albumins. *Biochemistry, 20*(21), 6129-6134.

Fitzpatrick, F. A., & Wynalda, M. A. (1983). Albumin-catalyzed metabolism of prostaglandin D2. Identification of products formed in vitro. *J Biol Chem, 258*(19), 11713-11718.

Flossmann, E., & Rothwell, P. M. (2007). Commentary: aspirin and colorectal cancer an epidemiological success story. *Int J Epidemiol, 36*(5), 962-965.

Folco, G., & Murphy, R. C. (2006). Eicosanoid transcellular biosynthesis: from cell-cell interactions to in vivo tissue responses. *Pharmacol Rev, 58*(3), 375-388.

Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., & Evans, R. M. (1995). 15-Deoxy-Delta(12,14)-Prostaglandin J(2) Is a Ligand for the Adipocyte Determination Factor Ppar-Gamma. *Cell, 83*(5), 803-812.

Franceschi, C., & Campisi, J. (2014). Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. *Journals of Gerontology Series a-Biological Sciences and Medical Sciences, 69*, S4-S9.

Fric, P., Sovova, V., Sloncova, E., Lojda, Z., Jirasek, A., & Cermak, J. (2000). Different expression of some molecular markers in sporadic cancer of the left and right colon. *European Journal of Cancer Prevention, 9*(4), 265-268.

Frick, V. O., Rubie, C., Ghadjar, P., Faust, S. K., Wagner, M., Graber, S., & Schilling, M. K. (2011). Changes in CXCL12/CXCR4-chemokine expression during onset of colorectal malignancies. *Tumor Biology, 32*(1), 189-196.

Fukasawa, K. M., Fukasawa, K., Sahara, N., Harada, M., Kondo, Y., & Nagatsu, I. (1981). Immunohistochemical localization of dipeptidyl aminopeptidase IV in rat kidney, liver, and salivary glands. *J Histochem Cytochem, 29*(3), 337-343.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science, 294*(5548), 1871-1875.

Ghosh, N., Chaki, R., Mandal, V., & Mandal, S. C. (2010). COX-2 as a target for cancer chemotherapy. *Pharmacological Reports, 62*(2), 233-244.

Goidin, D., Mamessier, A., Staquet, M. J., Schmitt, D., & Berthier-Vergnes, O. (2001). Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and β-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Analytical biochemistry*, *295*(1), 17-21.

Goldyne, M. E. (1975). Prostaglandins and cutaneous inflammation. *J Invest Dermatol, 64*(6), 377-385.

Gorrell, M. D., Gysbers, V., & McCaughan, G. W. (2001). CD26: A multifunctional integral membrane and secreted protein of activated lymphocytes. *Scandinavian Journal of Immunology, 54*(3), 249-264.

Guleng, B., Tateishi, K., Ohta, M., Kanai, F., Jazag, A., Ijichi, H., . . . Omata, M. (2005). Blockade of the stromal cell-derived factor-1/CXCR4 axis attenuates in vivo tumor growth by inhibiting angiogenesis in a vascular endothelial growth factor-independent manner. *Cancer Res, 65*(13), 5864-5871.

Gupta, R. A., Sarraf, P., Brockman, J. A., Shappell, S. B., Raftery, L. A., Willson, T. M., & DuBois, R. N. (2003). Peroxisome proliferator-activated receptor gamma and transforming growth factor-beta pathways inhibit intestinal epithelial cell growth by regulating levels of TSC-22. *J Biol Chem, 278*(9), 7431-7438.

Gutman, M., & Fidler, I. J. (1995). Biology of Human Colon-Cancer Metastasis. *World Journal of Surgery, 19*(2), 226-234.

Guyon, A. (2014). CXCL12 chemokine and its receptors as major players in the interactions between immune and nervous systems. *Frontiers in Cellular Neuroscience, 8*.

Half, E., Bercovich, D., & Rozen, P. (2009). Familial adenomatous polyposis. *Orphanet Journal of Rare Diseases, 4*.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanski, C., Huhle, T., Gossrau, R., & Reutter, W. (1988). Direct evidence for the binding of rat liver DPP IV to collagen in vitro. *Exp Cell Res, 178*(1), 64-72.

Hartel, S., Gossrau, R., Hanski, C., & Reutter, W. (1988). Dipeptidyl Peptidase (Dpp)-Iv in Rat Organs - Comparison of Immunohistochemistry and Activity Histochemistry. *Histochemistry, 89*(2), 151-161.

Hashimoto, K., Farrow, B. J., & Evers, B. M. (2004). Activation and role of MAP kinases in 15d-PGJ(2)-induced apoptosis in the human pancreatic cancer cell line MIA PaCa-2. *Pancreas, 28*(2), 153-159.

Hasko, G., & Cronstein, B. N. (2004). Adenosine: an endogenous regulator of innate immunity. *Trends in Immunology, 25*(1), 33-39.

Havre, P. A., Abe, M., Urasaki, Y., Ohnuma, K., Morimoto, C., & Dang, N. H. (2008). The role of CD26/dipeptidyl peptidase IV in cancer. *Front Biosci, 13*, 1634-1645.

Heidemann, J., Ogawa, H., Rafiee, P., Lugering, N., Maaser, C., Domschke, W., . . . Dwinell, M. B. (2004). Mucosal angiogenesis regulation by CXCR4 and its ligand CXCL12 expressed by human intestinal microvascular endothelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 286*(6), 1059-1068.

Helbig, G., Christopherson, K. W., Bhat-Nakshatri, P., Kumar, S., Kishimoto, H., Miller, K. D., . . . Nakshatri, H. (2003). NF-kappa B promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *Journal of Biological Chemistry, 278*(24), 21631-21638.

Herrera, C., Morimoto, C., Blanco, J., Mallol, J., Arenzana, F., Lluis, C., & Franco, R. (2001). Comodulation of CXCR4 and CD26 in human lymphocytes. *J Biol Chem, 276*(22), 19532-19539.

Herschman, H. R. (1996). Prostaglandin synthase 2. *Biochim Biophys Acta, 1299*(1), 125-140.

Hillen, F., & Griffioen, A. W. (2007). Tumour vascularization: sprouting angiogenesis and beyond. *Cancer and Metastasis Reviews, 26*(3-4), 489-502.

Houghton, A. N., Albino, A. P., Cordon-Cardo, C., Davis, L. J., & Eisinger, M. (1988). Cell surface antigens of human melanocytes and melanoma. Expression of adenosine deaminase binding protein is extinguished with melanocyte transformation. *J Exp Med, 167*(1), 197-212.

Hovi, T., Smyth, J. F., Allison, A. C., & Williams, S. C. (1976). Role of adenosine deaminase in lymphocyte proliferation. *Clin Exp Immunol, 23*(3), 395-403.

Howell, S., Kenny, A. J., & Turner, A. J. (1992). A survey of membrane peptidases in two human colonic cell lines, Caco-2 and HT-29. *Biochem J, 284 (Pt 2)*, 595-601.

Howes, J. M., Bihan, D., Slatter, D. A., Hamaia, S. W., Packman, L. C., Knauper, V., . . . Farndale, R. W. (2014). The recognition of collagen and triple-helical toolkit peptides by MMP-13: sequence specificity for binding and cleavage. *J Biol Chem, 289*(35), 24091-24101.

Hsu, H. T., & Chi, C. W. (2014). Emerging role of the peroxisome proliferator-activated receptor-gamma in hepatocellular carcinoma. *J Hepatocell Carcinoma, 1*, 127-135.

Huhn, J., Ehrlich, S., Fleischer, B., & von Bonin, A. (2000). Molecular analysis of CD26-mediated signal transduction in T cells. *Immunology Letters, 72*(2), 127-132.

Inamoto, T., Yamada, T., Ohnuma, K., Kina, S., Takahashi, N., Yamochi, T., . . . Morimoto, C. (2007). Humanized anti-CD26 monoclonal antibody as a treatment for malignant mesothelioma tumors. *Clin Cancer Res, 13*(14), 4191-4200.

Inamoto, T., Yamochi, T., Ohnuma, K., Iwata, S., Kina, S., Inamoto, S., . . . Morimoto, C. (2006). Anti-CD26 monoclonal antibody-mediated G(1)-S arrest of human renal clear cell carcinoma Caki-2 is associated with retinoblastoma substrate dephosphorylation, cyclin-dependent kinase 2 reduction, p27(kip1) enhancement, and disruption of binding to the extracellular matrix. *Clinical Cancer Research, 12*(11), 3470-3477.

Itzkowitz, S. H., & Yio, X. Y. (2004). Inflammation and cancer - IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 287*(1), 7-17.

Iwakawa, H. O., & Tomari, Y. (2015). The Functions of MicroRNAs: mRNA Decay and Translational Repression. *Trends Cell Biol, 25*(11), 651-665.

Jain, S., Pulikuri, S., Zhu, Y., Qi, C., Kanwar, Y. S., Yeldandi, A. V., . . . Reddy, J. K. (1998). Differential expression of the peroxisome proliferator-activated receptor gamma (PPARgamma) and its coactivators steroid receptor coactivator-1 and PPAR-binding protein PBP in the brown fat, urinary bladder, colon, and breast of the mouse. *Am J Pathol, 153*(2), 349-354.

Jasperson, K. W., Tuohy, T. M., Neklason, D. W., & Burt, R. W. (2010). Hereditary and Familial Colon Cancer. *Gastroenterology, 138*(6), 2044-2058.

Jiang, Y. P., Wu, X. H., Shi, B., Wu, W. X., & Yin, G. R. (2006). Expression of chemokine CXCL12 and its receptor CXCR4 in human epithelial ovarian cancer: an independent prognostic factor for tumor progression. *Gynecol Oncol, 103*(1), 226-233.

Johnson, C. M., Wei, C. M., Ensor, J. E., Smolenski, D. J., Amos, C. I., Levin, B., & Berry, D. A. (2013). Meta-analyses of colorectal cancer risk factors. *Cancer Causes & Control, 24*(6), 1207-1222.

Kaifi, J. T., Yekebas, E. F., Schurr, P., Obonyo, D., Wachowiak, R., Busch, P., . . . Izbicki, J. R. (2005). Tumor-cell homing to lymph nodes and bone marrow and CXCR4 expression in esophageal cancer. *Journal of the National Cancer Institute, 97*(24), 1840-1847.

Kajiyama, H., Kikkawa, F., Suzuki, T., Shibata, K., Ino, K., & Mizutani, S. (2002). Prolonged survival and decreased invasive activity attributable to dipeptidyl peptidase IV overexpression in ovarian carcinoma. *Cancer Res, 62*(10), 2753-2757.

Kajiyama, H., Shibata, K., Ino, K., Nawa, A., Mizutani, S., & Kikkawa, F. (2007). Possible involvement of SDF-1alpha/CXCR4-DPPIV axis in TGF-beta1-induced enhancement of migratory potential in human peritoneal mesothelial cells. *Cell Tissue Res, 330*(2), 221-229.

Kameoka, J., Tanaka, T., Nojima, Y., Schlossman, S. F., & Morimoto, C. (1993). Direct Association of Adenosine-Deaminase with a T-Cell Activation Antigen, Cd26. *Science, 261*(5120), 466-469.

Keller, H. U., Wilkinson, P. C., Abercrombie, M., Becker, E. L., Hirsch, J. G., Miller, M. E., . . . Zigmond, S. H. (1977). A proposal for the definition of terms related to locomotion of leucocytes and other cells. *Clin Exp Immunol, 27*(3), 377-380.

Kettmann, U., Humbel, B., & Holzhausen, H. J. (1992). Ultrastructural localization of dipeptidylpeptidase IV in the glomerulum of the rat kidney. *Acta Histochem, 92*(2), 225-227.

Khin, E. E., Kikkawa, F., Ino, K., Kajiyama, H., Suzuki, T., Shibata, K., . . . Mizutani, S. (2003). Dipeptidyl peptidase IV expression in endometrial endometrioid adenocarcinoma and its inverse correlation with tumor grade. *Am J Obstet Gynecol, 188*(3), 670-676.

Kikkawa, F., Kajiyama, H., Shibata, K., Ino, K., Nomura, S., & Mizutani, S. (2005). Dipeptidyl peptidase IV in tumor progression. *Biochim Biophys Acta, 1751*(1), 45-51.

Kim, I. K., Lee, J. H., Sohn, H. W., Kim, H. S., & Kim, S. H. (1993). Prostaglandin A2 and delta 12-prostaglandin J2 induce apoptosis in L1210 cells. *FEBS Lett, 321*(2-3), 209-214.

Kim, J., Takeuchi, H., Lam, S. T., Turner, R. R., Wang, H. J., Kuo, C., . . . Hoon, D. S. B. (2005). Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. *Journal of Clinical Oncology, 23*(12), 2744-2753.

Klebe, R. J. (1974). Isolation of a collagen-dependent cell attachment factor. *Nature, 250*(463), 248-251.

Klemann, C., Wagner, L., Stephan, M., & von Horsten, S. (2016). Cut to the chase: a review of CD26/dipeptidyl peptidase-4's (DPP4) entanglement in the immune system. *Clin Exp Immunol, 185*(1), 1-21.

Kodama, J., Hasengaowa, Kusumoto, T., Seki, N., Matsuo, T., Ojima, Y., . . . Hiramatsu, Y. (2007). Association of CXCR4 and CCR7 chemokine receptor expression and lymph node metastasis in human cervical cancer. *Ann Oncol, 18*(1), 70-76.

Kucia, M., Jankowski, K., Reca, R., Wysoczynski, M., Bandura, L., Allendorf, D. J., . . . Ratajczak, M. Z. (2004). CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. *Journal of Molecular Histology, 35*(3), 233-245.

Lakatos, P. L., & Lakatos, L. (2008). Risk for colorectal cancer in ulcerative colitis: Changes, causes and management strategies. *World Journal of Gastroenterology, 14*(25), 3937-3947.

Lam, C. S., Cheung, A. H., Wong, S. K., Wan, T. M., Ng, L., Chow, A. K., . . . Law, W. L. (2014). Prognostic significance of CD26 in patients with colorectal cancer. *PLoS One, 9*(5), e98582.

Lambeir, A. M., Proost, P., Durinx, C., Bal, G., Senten, K., Augustyns, K., . . . De Meester, I. (2001). Kinetic investigation of chemokine truncation by CD26/dipeptidyl peptidase IV reveals a striking selectivity within the chemokine family. *J Biol Chem, 276*(32), 29839-29845.

Landskron, G., De la Fuente, M., Thuwajit, P., Thuwajit, C., & Hermoso, M. A. (2014). Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res, 2014*, 149185.

Lea, M. A., Sura, M., & Desbordes, C. (2004). Inhibition of cell proliferation by potential peroxisome proliferator-activated receptor (PPAR) gamma agonists and antagonists. *Anticancer Res, 24*(5A), 2765-2771.

Leahy, K. M., Koki, A. T., & Masferrer, J. L. (2000). Role of cyclooxygenases in angiogenesis. *Curr Med Chem, 7*(11), 1163-1170.

Lee, S. J., Kim, M. S., Park, J. Y., Woo, J. S., & Kim, Y. K. (2008). 15-Deoxy-delta 12,14-prostaglandin J2 induces apoptosis via JNK-mediated mitochondrial pathway in osteoblastic cells. *Toxicology, 248*(2-3), 121-129.

Lee, Y., & Rio, D. C. (2015). Mechanisms and Regulation of Alternative Pre-mRNA Splicing. *Annu Rev Biochem, 84*, 291-323.

Leone, V., di Palma, A., Ricchi, P., Acquaviva, F., Giannouli, M., Di Prisco, A. M., . . . Acquaviva, A. M. (2007). PGE(2) inhibits apoptosis in human adenocarcinoma Caco-2 cell line through Ras-PI3K association and cAMP-dependent kinase A activation. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 293*(4), 673-681.

Leporrier, J., Maurel, J., Chiche, L., Bara, S., Segol, P., & Launoy, G. (2006). A population-based study of the incidence, management and prognosis of hepatic metastases from colorectal cancer. *British Journal of Surgery, 93*(4), 465-474.

Levine, H., & Rappel, W. J. (2013). The physics of eukaryotic chemotaxis. *Phys Today, 66*(2).

Li, M. Z., & Ransohoff, R. M. (2009). The roles of chemokine CXCL12 in embryonic and brain tumor angiogenesis. *Seminars in Cancer Biology, 19*(2), 111-115.

Liang, Z. X., Wu, T., Lou, H., Yu, X. W., Taichman, R. S., Lau, S. K., . . . Shim, H. (2004). Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. *Cancer Research, 64*(12), 4302-4308.

Liang, Z. X., Yoon, Y. H., Votaw, J., Goodman, M. M., Williams, L., & Shim, H. (2005). Silencing of CXCR4 blocks breast cancer metastasis. *Cancer Research, 65*(3), 967-971.

Licatalosi, D. D., & Darnell, R. B. (2010). RNA processing and its regulation: global insights into biological networks. *Nat Rev Genet, 11*(1), 75-87.

Liekens, S., Schols, D., & Hatse, S. (2010). CXCL12-CXCR4 axis in angiogenesis, metastasis and stem cell mobilization. *Curr Pharm Des, 16*(35), 3903-3920.

Loeb, K. R., & Loeb, L. A. (2000). Significance of multiple mutations in cancer. *Carcinogenesis, 21*(3), 379-385.

Lojda, Z. (1979). Studies on dipeptidyl(amino)peptidase IV (glycyl-proline naphthylamidase). II. Blood vessels. *Histochemistry, 59*(3), 153-166.

Loster, K., Zeilinger, K., Schuppan, D., & Reutter, W. (1995). The Cysteine-Rich Region of Dipeptidyl Peptidase-Iv (Cd 26) Is the Collagen-Binding Site. *Biochemical and Biophysical Research Communications, 217*(1), 341-348.

Luster, A. D. (1998). Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med, 338*(7), 436-445.

Maier, T., Guell, M., & Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. *FEBS Lett, 583*(24), 3966-3973.

Marnett, L. J., Rowlinson, S. W., Goodwin, D. C., Kalgutkar, A. S., & Lanzo, C. A. (1999). Arachidonic acid oxygenation by COX-1 and COX-2 - Mechanisms of catalysis and inhibition. *Journal of Biological Chemistry, 274*(33), 22903-22906.

Masunaga, R., Kohno, H., Dhar, D. K., Ohno, S., Shibakita, M., Kinugasa, S., . . . Nagasue, N. (2000). Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. *Clin Cancer Res, 6*(10), 4064-4068.

Matuchansky, C., & Bernier, J. J. (1973). Effect of prostaglandin E 1 on glucose, water, and electrolyte absorption in the human jejunum. *Gastroenterology, 64*(6), 1111-1118.

Mccaughan, G. W., Wickson, J. E., Creswick, P. F., & Gorrell, M. D. (1990). Identification of the Bile Canalicular Cell-Surface Molecule Gp110 as the Ectopeptidase Dipeptidyl Peptidase-Iv - an Analysis by Tissue Distribution, Purification and N-Terminal Amino-Acid-Sequence. *Hepatology, 11*(4), 534-544.

McClay, E. F., Winski, P. J., Jones, J. A., Jennerette, J., 3rd, & Gattoni-Celli, S. (1996). delta 12-Prostaglandin-J2 is cytotoxic in human malignancies and synergizes with both cisplatin and radiation. *Cancer Res, 56*(17), 3866-3869.

Meade, E. A., Smith, W. L., & DeWitt, D. L. (1993). Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J Biol Chem, 268*(9), 6610-6614.

Mentlein, R., Gallwitz, B., & Schmidt, W. E. (1993). Dipeptidylpeptidase-Iv Hydrolyzes Gastric-Inhibitory Polypeptide, Glucagon-Like Peptide-1(7-36)Amide, Peptide Histidine Methionine and Is Responsible for Their Degradation in Human Serum. *European Journal of Biochemistry, 214*(3), 829-835.

Meyer, B. J., Mann, N. J., Lewis, J. L., Milligan, G. C., Sinclair, A. J., & Howe, P. R. (2003). Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. *Lipids, 38*(4), 391-398.

Milton-Thompson, G. J., Cummings, J. H., Newman, A., Billings, J. A., & Misiewicz, J. J. (1975). Colonic and small intestinal response to intravenous prostaglandin F2 alpha and E2 in man. *Gut, 16*(1), 42-46.

Miyanishi, N., Suzuki, Y., Simizu, S., Kuwabara, Y., Banno, K., & Umezawa, K. (2010). Involvement of autocrine CXCL12/CXCR4 system in the regulation of ovarian carcinoma cell invasion. *Biochem Biophys Res Commun, 403*(1), 154-159.

Mizokami, Y., Kajiyama, H., Shibata, K., Ino, K., Kikkawa, F., & Mizutani, S. (2004). Stromal cell-derived factor-1alpha-induced cell proliferation and its possible regulation by CD26/dipeptidyl peptidase IV in endometrial adenocarcinoma. *Int J Cancer, 110*(5), 652-659.

Monneret, G., Li, H., Vasilescu, J., Rokach, J., & Powell, W. S. (2002). 15-Deoxy-delta 12,14-prostaglandins D2 and J2 are potent activators of human eosinophils. *J Immunol, 168*(7), 3563-3569.

Morrison, M. E., Vijayasaradhi, S., Engelstein, D., Albino, A. P., & Houghton, A. N. (1993). A Marker for Neoplastic Progression of Human Melanocytes Is a Cell-Surface Ectopeptidase. *Journal of Experimental Medicine, 177*(4), 1135-1143.

Mujoomdar, M., Bennett, A., Hoskin, D., & Blay, J. (2004). Adenosine stimulation of proliferation of breast carcinoma cell lines: evaluation of the [3H]thymidine assay system and modulatory effects of the cellular microenvironment in vitro. *J Cell Physiol, 201*(3), 429-438.

Mujoomdar, M., Hoskin, D., & Blay, J. (2003). Adenosine stimulation of the proliferation of colorectal carcinoma cell lines - Roles of cell density and adenosine metabolism. *Biochemical Pharmacology, 66*(9), 1737-1747.

Muller, A., Homey, B., Soto, H., Ge, N. F., Catron, D., Buchanan, M. E., . . . Zlotnik, A. (2001). Involvement of chemokine receptors in breast cancer metastasis. *Nature, 410*(6824), 50-56.

Muller, P. A. J., & Vousden, K. H. (2013). p53 mutations in cancer. *Nature Cell Biology, 15*(1), 2-8.

Munkholm, P. (2003). Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease. *Alimentary Pharmacology & Therapeutics, 18*, 1-5.

Namkoong, S., Lee, S. J., Kim, C. K., Kim, Y. M., Chung, H. T., Lee, H., . . . Kim, Y. M. (2005). Prostaglandin E2 stimulates angiogenesis by activating the nitric oxide/cGMP pathway in human umbilical vein endothelial cells. *Exp Mol Med, 37*(6), 588-600.

Narumiya, S., & Fukushima, M. (1985). delta 12-Prostaglandin J2, an ultimate metabolite of prostaglandin D2 exerting cell growth inhibition. *Biochem Biophys Res Commun, 127*(3), 739-745.

Naumann, U., Cameroni, E., Pruenster, M., Mahabaleshwar, H., Raz, E., Zerwes, H. G., . . . Thelen, M. (2010). CXCR7 Functions as a Scavenger for CXCL12 and CXCL11. *PLoS One, 5*(2).

Olah, M. E., & Caldwell, C. C. (2003). Adenosine receptors and mammalian toll-like receptors: synergism in macrophages. *Mol Interv, 3*(7), 370-374.

Orimo, A., Gupta, P. B., Sgroi, D. C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., . . . Weinberg, R. A. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell, 121*(3), 335-348.

Ottaiano, A., di Palma, A., Napolitano, M., Pisano, C., Pignata, S., Tatangelo, F., . . . Scala, S. (2005). Inhibitory effects of anti-CXCR4 antibodies on human colon cancer cells. *Cancer Immunology Immunotherapy, 54*(8), 781-791.

Pagliacci, M. C., Spinozzi, F., Migliorati, G., Fumi, G., Smacchia, M., Grignani, F., . . . Nicoletti, I. (1993). Genistein inhibits tumour cell growth in vitro but enhances mitochondrial reduction of tetrazolium salts: a further pitfall in the use of the MTT assay for evaluating cell growth and survival. *Eur J Cancer, 29A*(11), 1573-1577.

Pai, R., Szabo, I. L., Soreghan, B. A., Atay, S., Kawanaka, H., & Tarnawski, A. S. (2001). PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. *Biochem Biophys Res Commun, 286*(5), 923-928.

Pethiyagoda, C. L., Welch, D. R., & Fleming, T. P. (2001). Dipeptidyl peptidase IV (DPPIV) inhibits cellular invasion of melanoma cells. *Clinical & Experimental Metastasis, 18*(5), 391-400.

Piazza, G. A., Callanan, H. M., Mowery, J., & Hixson, D. C. (1989). Evidence for a Role of Dipeptidyl Peptidase-Iv in Fibronectin-Mediated Interactions of Hepatocytes with Extracellular-Matrix. *Biochemical Journal, 262*(1), 327-334.

Poulogiannis, G., Frayling, I. M., & Arends, M. J. (2010). DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. *Histopathology, 56*(2), 167-179.

Pugh, S., & Thomas, G. A. O. (1994). Patients with Adenomatous Polyps and Carcinomas Have Increased Colonic Mucosal Prostaglandin E(2). *Gut, 35*(5), 675-678.

Rajakariar, R., Hilliard, M., Lawrence, T., Trivedi, S., Colville-Nash, P., Bellingan, G., . . . Gilroy, D. W. (2007). Hematopoietic prostaglandin D2 synthase controls the onset and resolution of acute inflammation through PGD2 and 15-deoxyDelta12 14 PGJ2. *Proc Natl Acad Sci U S A, 104*(52), 20979-20984.

Reid, G., Wielinga, P., Zelcer, N., van der Heijden, I., Kuil, A., de Haas, M., . . . Borst, P. (2003). The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci U S A, 100*(16), 9244-9249.

Reinherz, E. L., & Schlossman, S. F. (1980). The differentiation and function of human T lymphocytes. *Cell, 19*(4), 821-827.

Ricciotti, E., & FitzGerald, G. A. (2011). Prostaglandins and Inflammation. *Arteriosclerosis Thrombosis and Vascular Biology, 31*(5), 986-1000.

Richard, C. L., & Blay, J. (2008). CXCR4 in Cancer and Its Regulation by PPAR gamma. *Ppar Research*. *2008*, 769413.

Richard, C. L., Lowthers, E. L., & Blay, J. (2007). 15-Deoxy-Delta(12,14)-prostaglandin J(2) down-regulates CXCR4 on carcinoma cells through PPAR gamma- and NF kappa B-mediated pathways. *Experimental Cell Research, 313*(16), 3446-3458.

Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L. Cell viability assays. In: Sittampalam GS G-EN, Arkin M, et al., editors. Assay Guidance Manual. Bethesda (MD), USA: Eli Lilly & Co and National Centre for Advancing Translational Sciences; 2012

Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., & Santoro, M. G. (2000). Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of I kappa B kinase. *Nature, 403*(6765), 103-108.

Rostom, A.; Dube, C.; Lewin, G. Use of aspirin and NSAIDs to prevent colorectal cancer. An Evidence Synthesis. AHRQ Publication:Rockville, 2007.

Rutter, M. D., Saunders, B. P., Wilkinson, K. H., Rumbles, S., Schofield, G., Kamm, M. A., . . . Forbes, A. (2004). Cancer surveillance in longstanding ulcerative colitis: endoscopic appearances help predict cancer risk. *Gut, 53*(12), 1813-1816.

Ryan, G. B., & Majno, G. (1977). Acute-Inflammation - Review. *American Journal of Pathology, 86*(1), 183-276.

Sahara, N., & Suzuki, K. (1984). Ultrastructural-Localization of Dipeptidyl Peptidase-Iv in Rat Salivary-Glands by Immunocytochemistry. *Cell and Tissue Research, 235*(2), 427-432.

Sakai, T., Aoike, A., Marui, N., Kawai, K., Nishino, H., & Fukushima, M. (1989). Protection by cycloheximide against cytotoxicity induced by vincristine, colchicine, or delta 12-prostaglandin J2 on human osteosarcoma cells. *Cancer Res, 49*(5), 1193-1196.

Sakai, Y., Fujita, K., Sakai, H., & Mizuno, K. (2001). Prostaglandin E2 regulates the expression of basic fibroblast growth factor messenger RNA in normal human fibroblasts. *Kobe J Med Sci, 47*(1), 35-45.

Sakamoto, J., Watanabe, T., Teramukai, S., Akiyama, S., Morimoto, T., Takagi, H., . . . Takahashi, T. (1993). Distribution of Adenosine-Deaminase Binding-Protein in Normal and Malignant-Tissues of the Gastrointestinal-Tract Studied by Monoclonal-Antibodies. *Journal of Surgical Oncology, 52*(2), 124-134.

Sandler, R. S., Halabi, S., Baron, J. A., Budinger, S., Paskett, E., Keresztes, R., . . . Schilsky, R. (2003). A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. *New England Journal of Medicine, 348*(10), 883-890.

Sarraf, P., Mueller, E., Jones, D., King, F. J., DeAngelo, D. J., Partridge, J. B., . . . Spiegelman, B. M. (1998). Differentiation and reversal of malignant changes in colon cancer through PPARgamma. *Nat Med, 4*(9), 1046-1052.

Scala, S., Ottaiano, A., Ascierto, P. A., Cavalli, M., Simeone, E., Giuliano, P., . . . Castello, G. (2005). Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma. *Clinical Cancer Research, 11*(5), 1835-1841.

Scher, J. U., & Pillinger, M. H. (2005). 15d-PGJ(2): The anti-inflammatory prostaglandin? *Clinical Immunology, 114*(2), 100-109.

Schimanski, C. C., Bahre, R., Gockel, I., Muller, A., Frerichs, K., Horner, V., . . . Moehler, M. (2006). Dissemination of hepatocellular carcinoma is mediated via chemokine receptor CXCR4. *British Journal of Cancer, 95*(2), 210-217.

Schimanski, C. C., Schwald, S., Simiantonaki, N., Jayasinghe, C., Gonner, U., Wilsberg, V., . . . Moehler, M. (2005). Effect of chemokine receptors CXCR4 and CCR7 on the metastatic behavior of human colorectal cancer. *Clin Cancer Res, 11*(5), 1743-1750.

Schrader, A. J., Lechner, O., Templin, M., Dittmar, K. E., Machtens, S., Mengel, M., . . . Lauber, J. (2002). CXCR4/CXCL12 expression and signalling in kidney cancer. *Br J Cancer, 86*(8), 1250-1256.

Shacter, E., & Weitzman, S. A. (2002). Chronic inflammation and cancer. *Oncology-New York, 16*(2), 217-229.

Shen, B., Chu, E. S., Zhao, G., Man, K., Wu, C. W., Cheng, J. T., . . . Yu, J. (2012). PPARgamma inhibits hepatocellular carcinoma metastases in vitro and in mice. *Br J Cancer, 106*(9), 1486-1494.

Shibata, T., Kondo, M., Osawa, T., Shibata, N., Kobayashi, M., & Uchida, K. (2002). 15-deoxy-delta 12,14-prostaglandin J2. A prostaglandin D2 metabolite generated during inflammatory processes. *J Biol Chem, 277*(12), 10459-10466.

Shimada, T., Kojima, K., Yoshiura, K., Hiraishi, H., & Terano, A. (2002). Characteristics of the peroxisome proliferator activated receptor gamma (PPARgamma) ligand induced apoptosis in colon cancer cells. *Gut, 50*(5), 658-664.

Shimizu, N., & Nakamura, T. (1985). Prostaglandins as hormones. *Dig Dis Sci, 30*(11 Suppl), 109-113.

Shimizu, T., Ohto, T., & Kita, Y. (2006). Cytosolic phospholipase A2: biochemical properties and physiological roles. *IUBMB Life, 58*(5-6), 328-333.

Shin, S. W., Seo, C. Y., Han, H., Han, J. Y., Jeong, J. S., Kwak, J. Y., & Park, J. I. (2009). 15d-PGJ(2) Induces Apoptosis by Reactive Oxygen Species-mediated Inactivation of Akt in Leukemia and Colorectal Cancer Cells and Shows In vivo Antitumor Activity. *Clinical Cancer Research, 15*(17), 5414-5425.

Shioda, T., Kato, H., Ohnishi, Y., Tashiro, K., Ikegawa, M., Nakayama, E. E., . . . Nagai, Y. (1998). Anti-HIV-1 and chemotactic activities of human stromal cell-derived factor 1alpha (SDF-1alpha) and SDF-1beta are abolished by CD26/dipeptidyl peptidase IV-mediated cleavage. *Proc Natl Acad Sci U S A, 95*(11), 6331-6336.

Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T., & Honjo, T. (1995). Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. *Genomics, 28*(3), 495-500.

Siegel, R., DeSantis, C., & Jemal, A. (2014). Colorectal cancer statistics, 2014. *Ca-a Cancer Journal for Clinicians, 64*(2), 104-117.

Silver, M. J., & Smith, J. B. (1975). Prostaglandins as intracellular messengers. *Life Sci, 16*(11), 1635-1648.

Singh, S., Singh, U. P., Grizzle, W. E., & Lillard, J. W. (2004). CXCL12-CXCR4 interactions modulate prostate cancer cell migration, metalloproteinase expression and invasion. *Laboratory Investigation, 84*(12), 1666-1676.

Smith, W. L. (1989). The eicosanoids and their biochemical mechanisms of action. *Biochem J, 259*(2), 315-324.

Soares, A. F., Nosjean, O., Cozzone, D., D'Orazio, D., Becchi, M., Guichardant, M., . . . Geloen, A. (2005). Covalent binding of 15-deoxy-delta12,14-prostaglandin J2 to PPARgamma. *Biochem Biophys Res Commun, 337*(2), 521-525.

Sonoshita, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., . . . Taketo, M. M. (2001). Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice. *Nature Medicine, 7*(9), 1048-1051.

Spencer, N., Hopkinson, D. A., & Harris, H. (1968). Adenosine Deaminase Polymorphism in Man. *Annals of Human Genetics, 32*(1), 9-14

Stange, T., Kettmann, U., & Holzhausen, H. J. (1996). Immunoelectron microscopic single and double labelling of aminopeptidase N (CD 13) and dipeptidyl peptidase IV (CD 26). *Acta Histochemica, 98*(3), 323-331.

Stecca, B. A., Nardo, B., Chieco, P., Mazziotti, A., Bolondi, L., & Cavallari, A. (1997). Aberrant dipeptidyl peptidase IV (DPP IV/CD26) expression in human hepatocellular carcinoma. *J Hepatol, 27*(2), 337-345.

Sun, Y. X., Pedersen, E. A., Shiozawa, Y., Havens, A. M., Jung, Y., Wang, J., . . . Taichman, R. S. (2008). CD26/dipeptidyl peptidase IV regulates prostate cancer metastasis by degrading SDF-1/CXCL12. *Clin Exp Metastasis, 25*(7), 765-776.

Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., . . . Nagasawa, T. (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature, 393*(6685), 591-594.

Taki, M., Higashikawa, K., Yoneda, S., Ono, S., Shigeishi, H., Nagayama, M., & Kamata, N. (2008). Up-regulation of stromal cell-derived factor-1 alpha and its receptor CXCR4 expression accompanied with epithelial-mesenchymal transition in human oral squamous cell carcinoma. *Oncology Reports, 19*(4), 993-998.

Teicher, B. A., & Fricker, S. P. (2010). CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res, 16*(11), 2927-2931.

Tenkate, J., Vandeningh, H. F. G. M., Khan, P. M., & Bosman, F. T. (1986). Adenosine-Deaminase Complexing Protein (Adcp) Immunoreactivity in Colorectal Adenocarcinoma. *International Journal of Cancer, 37*(4), 479-485.

Tenkate, J., Wijnen, J. T., Boldewijn, J., Khan, P. M., & Bosman, F. T. (1985). Immunohistochemical Localization of Adenosine-Deaminase Complexing Protein in Intestinal-Mucosa and in Colorectal Adenocarcinoma as a Marker for Tumor-Cell Heterogeneity. *Histochemical Journal, 17*(1), 23-31.

Tsujimoto, Y. (1998). Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells, 3*(11), 697-707.

Ulmer, A. J., Mattern, T., & Flad, H. D. (1992). Expression of Cd26 (Dipeptidyl Peptidase-Iv) on Memory and Naive Lymphocytes-T. *Scandinavian Journal of Immunology, 35*(5), 551-559.

Vandercappellen, J., Van Damme, J., & Struyf, S. (2008). The role of CXC chemokines and their receptors in cancer. *Cancer Letters, 267*(2), 226-244.

Vane, J. R., Bakhle, Y. S., & Botting, R. M. (1998). Cyclooxygenases 1 and 2. *Annual Review of Pharmacology and Toxicology, 38*, 97-120.

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet, 13*(4), 227-232.

Wang, D. Z., Wang, H. B., Shi, Q., Katkuri, S., Walhi, W., Desvergne, B., . . . DuBois, R. N. (2004). Prostaglandin E-2 promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. *Cancer Cell, 6*(3), 285-295.

Wesley, U. V., McGroarty, M., & Homoyouni, A. (2005). Dipeptidyl peptidase inhibits malignant phenotype of prostate cancer cells by blocking basic fibroblast growth factor signaling pathway. *Cancer Res, 65*(4), 1325-1334.

Wesley, U. V., Tiwari, S., & Houghton, A. N. (2004). Role for dipeptidyl peptidase IV in tumor suppression of human non small cell lung carcinoma cells. *International Journal of Cancer, 109*(6), 855-866.

Williams, K. I., & Higgs, G. A. (1988). Eicosanoids and inflammation. *J Pathol, 156*(2), 101-110.

Wilson, M. J., Ruhland, A. R., Quast, B. J., Reddy, P. K., Ewing, S. L., & Sinha, A. A. (2000). Dipeptidylpeptidase IV activities are elevated in prostate cancers and adjacent benign hyperplastic glands. *J Androl, 21*(2), 220-226.

Woodhouse, E. C., Amanatullah, D. F., Schetz, J. A., Liotta, L. A., Stracke, M. L., & Clair, T. (1998). Adenosine receptor mediates motility in human melanoma cells. *Biochemical and Biophysical Research Communications, 246*(3), 888-894.

Xin, X. H., Yang, S. Y., Kowalski, J., & Gerritsen, M. E. (1999). Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and in vivo. *Journal of Biological Chemistry, 274*(13), 9116-9121.

Yamada, K., Hayashi, M., Du, W., Ohnuma, K., Sakamoto, M., Morimoto, C., & Yamada, T. (2009). Localization of CD26/DPPIV in nucleus and its nuclear translocation enhanced by anti-CD26 monoclonal antibody with anti-tumor effect. *Cancer Cell Int, 9*, 17.

Yen, C. C., Hsiao, C. D., Chen, W. M., Wen, Y. S., Lin, Y. C., Chang, T. W., . . . Fletcher, J. A. (2014). Cytotoxic effects of 15d-PGJ2 against osteosarcoma through ROS-mediated AKT and cell cycle inhibition. *Oncotarget, 5*(3), 716-725.

Yoshida, T., Ohki, S., Kanazawa, M., Mizunuma, H., Kikuchi, Y., Satoh, H., . . . Abe, R. (1998). Inhibitory effects of prostaglandin D-2 against the proliferation of human colon cancer cell lines and hepatic metastasis from colorectal cancer. *Surgery Today-the Japanese Journal of Surgery, 28*(7), 740-745.

Zeng, Y., Yi, R., & Cullen, B. R. (2003). MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci U S A, 100*(17), 9779-9784.

Zeng, Z. S., Cohen, A. M., & Guillem, J. G. (1999). Loss of basement membrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumorigenesis. *Carcinogenesis, 20*(5), 749-755.

Zeng, Z. S., Cohen, A. M., Hajdu, S., Sternberg, S. S., Sigurdson, E. R., & Enker, W. (1992). Serosal Cytologic Study to Determine Free Mesothelial Penetration of Intraperitoneal Colon Cancer. *Cancer, 70*(4), 737-740.

Zhang, H., Chen, Y., Keane, F. M., & Gorrell, M. D. (2013). Advances in understanding the expression and function of dipeptidyl peptidase 8 and 9. *Mol Cancer Res, 11*(12), 1487-1496.

Zhang, M. Z., Qiao, Y. H., & Suo, Z. H. (2008). [Correlation of DPPIV expression with clinicopathological features and prognosis in epithelial ovarian carcinoma]. *Zhonghua Zhong Liu Za Zhi, 30*(11), 848-852.

Zhao, B. S., Roundtree, I. A., & He, C. (2017). Post-transcriptional gene regulation by mRNA modifications. *Nat Rev Mol Cell Biol, 18*(1), 31-42.

Zhu, C., Bao, G., & Wang, N. (2000). Cell mechanics: mechanical response, cell adhesion, and molecular deformation. *Annu Rev Biomed Eng, 2*, 189-226.

Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., & Littman, D. R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature, 393*(6685), 595-599.