Competing Influences Of The Tumor Microenvironment On CD26 And The Cancer Phenotype Of Colorectal Carcinoma Cells

by

Kristin Tweel

Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
December 2011

DALHOUSIE UNIVERSITY

DEPARTMENT OF PHARMACOLOGY

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Competing Influences Of The Tumor Microenvironment On CD26 And The Cancer Phenotype Of Colorectal Carcinoma Cells" by Kristin Tweel in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: December 12, 2011

External Examiner: _____

Research Supervisor: _____

Examining Committee: _____

_____

Departmental Representative: _____

DALHOUSIE UNIVERSITY

DATE: December 12, 2011

AUTHOR: Kristin Tweel

TITLE: Competing Influences Of The Tumor Microenvironment On CD26 And The Cancer Phenotype Of Colorectal Carcinoma Cells

DEPARTMENT OR SCHOOL: Department of Pharmacology

DEGREE: PhD CONVOCATION: May YEAR: 2012

_____

Signature of Author

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

In Canada, colorectal cancer is the second leading cause of cancer death for both men and women. There are many different factors that contribute to the progression and spread of the disease. However, increasing evidence now suggests that the tumor microenvironment plays a paramount role in these processes.

CD26 is a multifunctional, cell-surface glycoprotein that has intrinsic enzyme activity, binds adenosine deaminase and interacts with the extracellular matrix. Through its various functions it serves to constrain cancer progression. For example, it is known to cleave CXCL12, the ligand for CXCR4. The CXCL12:CXCR4 axis is normally involved in cancer metastasis by promoting cancer cell migration, invasion and proliferation. Down-regulation of CD26 is observed in certain cancers - this has been shown *in vitro* to occur in response to certain soluble mediators.

The first part of this study looked at the effects of glucose and its metabolic product lactate on CD26 expression in colorectal carcinoma cells. Our study showed that CD26 expression is lower in cancer cells that are grown in low-glucose, high-lactate conditions, which replicates the situation within a tumor.

The second part of this study examined the effect of adenosine, a purine nucleoside, on colorectal carcinoma cells and supportive stromal cells - cancer-associated HS675.T fibroblasts (CAFs) and Met-5a mesothelial cells. Adenosine increased the proliferation of CAFs and increased CXCL12 mRNA in both stromal cell lines. It also increased MMP-13 mRNA in stromal cells as well as colorectal cancer cells, suggesting that adenosine may promote progression and metastasis through various mechanisms.

The last section focused on the ability of cellular products and 3-dimensional tissue topology to coordinate and affect the behaviour of the different cell populations. Here we show that secretory products from colorectal cancer cells promote CAF proliferation but inhibit mesothelial cell proliferation, and are also able to modulate MMP-13 expression. Finally, certain responses are enhanced in multicellular spheroids.

In conclusion, the tumor microenvironment represents a major consideration in the treatment of solid tumors. Our data suggest that various soluble mediators, such as adenosine, may have therapeutic implications in cancer treatment and might represent novel targets for future research.

LIST OF ABBREVIATIONS AND SYMBOLS USED

5-FU	5-Fluorouracil
AC	Adenylate Cyclase
ACF	Aberrant Crypt Foci
ADA	Adenosine Deaminase
ADAbp	Adenosine Deaminase Binding Protein
ADAcp	Adenosine Deaminase Complexing Protein
AK	Adenosine Kinase
ANOVA	Analysis of Variance
AP-1	Activated Protein 1
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
BRE	Butyrate Response Element
CAF	Carcinoma Associated Fibroblast
CD	Cluster of Differentiation
CEA	Carcinoembryonic Antigen
CPM	Counts Per Minute
CRC	Colorectal Cancer
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DPPIV	Dipeptidyl Peptidase IV
dT	deoxythymidine
DTT	Dithiothreitol

dUTP	deoxyUridine TriPhosphate
EC50	Concentration producing half the maximum effect
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EHNA	Erythro-9-(2-Hydroxy-3-nonyl)-Adenine Hydrochloride
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-to-Mesenchymal Transition
EPC	Endothelial Progenitor Cell
EPR	Enhanced Permeability and Retention
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FSP-1	Fibroblast Specific Protein 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GIP	Gastric Inhibitory Peptide
GIST	Gastrointestinal Stromal Tumor
GLP-1	Glucagon-Like Peptide 1
GPCR	G-Protein-Coupled Receptor
GPI	Glycophosphatidylinositol
HIF	Hypoxia-Inducible Factor
HGF	Hepatocyte Growth Factor
HUVEC	Human Umbilical Vascular Endothelial Cell
IFP	Interstitial Fluid Pressure
IgG	Immunoglobulin G

IL-1β	Interleukin 1 Beta
IL-6	Interleukin 6
IT	Irinotecan
LDH	Lactate Dehydrogenase
LLOQ	Lower Limit of Quantification
mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MEM	Modified Eagle Medium
MHC	Major Histocompatibility Complex
M-MLV	Murine-Maloney Leukemia Virus
MMR	Mismatch Repair
mRNA	Messenger Ribonucleic Acid
NCS	Newborn Calf Serum
NECA	5′-(N-EthylCarboxamido) Adenosine
NF-kB	Nuclear Factor-kappa B
OX	Oxaliplatin
PBS	Phosphate-Buffered Saline
PDGFR	Platelet-Derived Growth Factor Receptor
Pgp	P-glycoprotein
PLC	Phospholipase C
pVHL	Von Hippel Lindau tumor suppressor protein
R-PIA	(−)-N^6-(2-phenylisopropyl)adenosine
RNA	Ribonucleic Acid

RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SAH	S-adenosylhomocysteine
SCID	Severe Combined Immunodeficiency Disease
SDF	Stromal cell-Derived Factor
SDS	Sodium Dodecyl Sulfate
α-SMA	Alpha Smooth Muscle Actin
TAE	Tris-acetate-EDTA
TBS-T	Tris-Buffered Saline Tween 20
TCA	Trichloroacetic Acid
TCF	T-cell Factor
TCR	T-cell Receptor
TGF-β	Transforming Growth Factor Beta
TIMP	Tissue Inhibitor of Metalloprotease
TNM	Tumor Node Metastasis
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
QPCR	Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction

ACKNOWLEDGMENTS

Thanks to everyone in the Department of Pharmacology for making my time here so memorable. To the ladies in the office: Janet, Luisa and Sandi, thank-you for all the financial/administrative help! You're always there for the students, making lives easier. To my advisory committee members Drs. Howlett, Goralski, and Denovan-Wright, thank you for the guidance and support.

Thank you to the Cancer Research Training Program and Beatrice Hunter Cancer Research Institute for the financial support. Through my interactions with people I met and events I attended, it's made a lasting impact on my life. Cindy and Shannon, your smiling faces always made my day that much better!

To all the Blay labbies past and present as well as my peers: Matt, Sebastian, BB, Jennie, Erica, Michelle, Emilie, Julia, Kelsie and Murray - thank you! Going through the same things made life easier. I don't know what I would have done without our girls' nights! Jibby (JB), I want to thank you for all of your support and encouragement over the past five years. Rob, thanks for finding time to help each of us when we needed you.

Manny, you were always there to encourage me and offer up a hug when needed. What would I do without you? Lastly, thank you to my parents for all their love and support. Without all your help, I would not have made it this far.

CHAPTER 1 Introduction

1.1 An Overview of Colorectal Cancer

1.1.1 Colorectal cancer incidence and mortality in Canada

Colorectal cancer (CRC) begins as a small growth, or polyp, in the epithelial lining of the colon or rectum, and over time some of these growths develop the malignant characteristics that turn them into a cancer (Shaw and Cantley 2006). CRC generally occurs as an adenocarcinoma (i.e. cancer of the secretory epithelial lining of the colon and rectum), although it may also be present less commonly as a carcinoid tumor, gastrointestinal stromal tumor (GIST), or a lymphoma (American Society of Clinical Oncology, 2007).

In 2010, it was estimated that 22500 Canadians were afflicted by colorectal cancer. CRC is the second leading cause of cancer death in men and women combined, surpassed only by lung cancer (Canadian Cancer Society, 2011). The rates of CRC incidence and mortality in Canada are highest in the eastern provinces (Canadian Cancer Society, 2011). Recently, a screening program has been implemented in some parts of eastern Canada to screen for individuals that are at a high risk for developing CRC (Desch, Benson et al. 1999). Early screening and detection are important, as CRC is largely treatable if detected sufficiently early on (Burt 2000).

Colorectal cancer is observed to fall into three different patterns of occurrence: sporadic, inherited or familial. The **sporadic** form of CRC occurs in people with no familial history or genetic disposition to CRC. It accounts for 70% of CRCs and is most likely to affect persons over the age of 50 years (Calvert and Frucht 2002). **Inherited**

CRCs are a result of familial adenomatous polyposis or hamartomatous polyposis syndromes. They account for less than 10% of CRCs. The **familial** CRCs are the least understood of the three types (Calvert and Frucht 2002). In some families CRC develops too quickly or frequently to be sporadic, yet does not occur in a pattern consistent with the inherited form of CRC (Calvert and Frucht 2002).

1.1.2 Risk factors for colorectal cancer

A number of factors contribute to the risk of developing CRC. Risk factors known to play a role in disease development include physical inactivity, obesity, smoking, and a diet deficient in fruit and vegetables (Giovannucci 2002). There is a 5 to 10-fold variation in the occurrence of CRC in different geographical regions worldwide (Parkin, Bray et al. 2005). The recent increasing incidence of CRC that is seen in the Czech Republic and other countries is thought to be a result of 'Westernization', as it accompanies rising rates of obesity and an increased consumption of fatty calorie-rich foods (Popkin 2004; Knai, Suhrcke et al. 2007; Center, Jemal et al. 2009).

As for many diseases, genetics can play a role in the development of CRC. As indicated above, less than 30% of all cases are believed to be hereditary, involving various genetic abnormalities (Calvert and Frucht 2002). Inflammatory diseases of the bowel,including ulcerative colitis and irritable bowel disease (IBD) are known to predispose patients to the pathogenesis of CRC (Harpaz and Polydorides 2010). For example, people with IBD are 3 to 5 times more likely to develop a colonic neoplasia in their lifetime (Eaden and Mayberry 2002).

1.1.3 Colorectal cancer development

The earliest known histopathological lesions associated with CRC are referred to as aberrant crypt foci (ACF) (Kim, Jen et al. 1994). Aberrant crypt foci are considered the precursors of colonic polyps and consequent carcinogenetic change. Patients with CRC often have an increased number of ACF compared with patients that did not have colon cancer or predisposing conditions (Pretlow, Barrow et al. 1991; Pretlow, Brasitus et al. 1993; Takayama, Katsuki et al. 1998). Additionally, studies have shown that up to 70% of ACFs from patients with CRC showed increased proliferative ability and evidence of K-ras mutations (Pretlow, Brasitus et al. 1993; Vivona, Shpitz et al. 1993; Yamashita, Minamoto et al. 1995; Takayama, Katsuki et al. 1998).

ACF, as putative precursors for malignant disease, have been suggested as a surrogate marker for CRC chemoprevention studies. In rat models of carcinogen-induced CRC with the use of 1,2-dimethylhydrazine, treatment with chemopreventative agents such as aspirin and sulindac suppressed ACF formation as compared to animals that did not receive those agents (Mereto, Frencia et al. 1994; Sutherland and Bird 1994; Takayama, Katsuki et al. 1998).

The dysplasia –cancer sequence for molecular events leading to CRC is well defined, perhaps better than for most other cancers. The accepted scheme (**Figure 1.1**) involves both genetic and epigenetic changes that together facilitate the transition of a normal cell to a malignant cell. The overall process involves both a loss-of-function (tumor suppressor) and gain-of-function (oncogenes) mutations within the cancer cell themselves (Calvert and Frucht 2002). Of the genetic mutations described to date, the critical determinants of the malignant phenotype in CRC are inactivation of the tumor

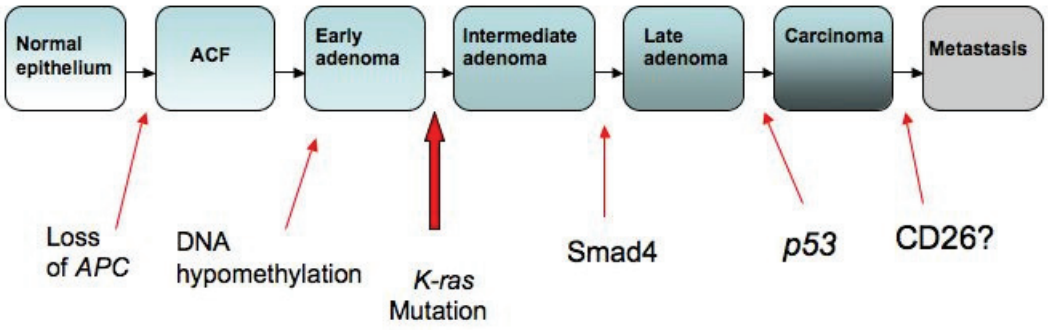


Figure 1.1 The model for multistep colorectal carcinogenesis.
Based on a series of genetic and epigenetic changes proposed by Fearon and Vogelstein. Figure adapted from (Fearon and Vogelstein 1990).

suppressors APC and p53, along with the activation of the oncogene K-ras (Knudson 1985; Fearon and Vogelstein 1990; Markowitz, Wang et al. 1995; Calvert and Frucht 2002)

The K-Ras protein acts as a molecular switch to transduce growth factor signals into the cell. Mutations are thought to be an early event in colorectal carcinogenesis (Fearon and Vogelstein 1990; Tortola, Marcuello et al. 1999). Mutation results in the overactivity of K-ras, promoting an increase in cell signaling through the ras pathway and has a variety of mitogenic effects on the cell (Villa, Dugani et al. 1996; Tortola, Marcuello et al. 1999).

Mutations in the APC gene result introduce a stop codon in the mRNA for APC and a deletion of the carboxy-terminal of the APC protein. Truncation alters the functions of the APC protein resulting in the loss of β-catenin and axin binding (Hamilton 1993; Su, Vogelstein et al. 1993). This is significant as β-catenin plays an important role in cell-cell adhesion, particular for epithelial cell layers. When the association of β-catenin and the APC protein is lost, β-catenin translocates to the nucleus and becomes part of transcriptional complex with T-cell transcription factor 4 (TCF-4) (Morin, Sparks et al. 1997). Early loss of APC functionality is proposed to be a key event in facilitating the transition of epithelial cells into the adenoma-carcinoma pathway (Hamilton 1993; Su, Vogelstein et al. 1993).

Additionally, mutations in DNA mismatch repair (MMR) enzymes are common in CRC (Fearon and Vogelstein 1990; Bronner, Baker et al. 1994). It is estimated that the risk may be as high as 85% (Calvert and Frucht 2002). Mutations in p53 generally occur

late in the adenoma formation process, but are considered to be important in the ultimate transformation to a malignant adenoma (Baker, Bronner et al. 1995).

1.1.4 Staging colorectal cancer

Tumors are graded on a scale of I-IV depending on the degree of cellular differentiation of the tumor (Compton and Greene 2004). Well-differentiated tumors (grade I) tend to grow more slowly and are less likely to spread. Poorly differentiated tumors are high-grade tumors that grow rapidly and are likely to spread. Moderately-differentiated tumors lie in between well-differentiated and poorly-differentiated tumors (**Table 1.1**) (Compton and Greene 2004).

Tumor staging is considered the most important prognostic factor (Chapuis, Dent et al. 1985; Newland, Dent et al. 1994; Compton and Greene 2004). Tumor staging follows a system known as the Tumor-Node-Metastasis (TNM) system (**Table 1.2**). The TNM staging system is the most commonly used tumor staging system. The process of staging a tumor from stage 0 – IV allows physicians to describe the degree to which a cancer has progressed through the layers of the colon (**Figure 1.2**) and whether or not it has metastasized. Staging a cancer allows health care providers to select the best postoperative adjuvant therapy (Compton and Greene 2004)

1.1.5 Colorectal cancer treatment strategies

The first step in CRC treatment is generally surgery. 'Adjuvant therapy' is defined as any treatment given to a patient after they have received primary treatment. Patients with resectable disease are treated with postoperative adjuvant therapy to eradicate any microscopic residual disease, thereby increasing the chance of a cure (Saltz and Minsky 2002). Advanced disease (stage II and above) is treated with radiation therapy and chemotherapy. Newer biological therapies, such as monoclonal antibodies and the tyrosine kinase inhibitors, are usually combined with conventional (cytotoxic) chemotherapeutics. Combining several therapies with different mechanisms of action helps reduce the risk of the cancer developing some form of resistance to any one therapy (Calvo, Cortes et al. 2002; Miles, von Minckwitz et al. 2002).

There are different potential chemotherapy regimens for CRC. The best regimen is chosen based on the individual properties of the cancer, including disease stage. The chemotherapeutics administered during a regimen generally have different mechanisms of action and therefore different cellular targets. This underlying principle of chemotherapy suggests that combining agents at maximal feasible doses (the drugs tend to differ in their dose-limiting effects) results in superior outcomes (DeVita and Schein 1973; Mayer 2009).

The most common and most effective first-line chemotherapeutic used is 5-Fluorouracil (5-FU) (Buyse, Thirion et al. 2000; de Gramont, Figer et al. 2000). 5-FU has been available for approximately half a century, and is referred to as an anti-metabolite drug (Gradishar and Vokes 1990).

Table 1.1 Colorectal Cancer Stage Groupings

	TNM		
Stage 0	**Tis**	**N0**	**M0**
Stage I	**T1**	**N0**	**M0**
	T2	**N0**	**M0**
Stage IIA	**T3**	**N0**	**M0**
Stage IIB	**T4**	**N0**	**M0**
Stage IIIA	**T1, T2**	**N1**	**M0**
Stage IIIB	**T3, T4**	**N1**	**M0**
Stage IIIC	**Any T**	**N2**	**M0**
Stage IV	**Any T**	**Any N**	**M1**

Adapted from (Compton and Greene 2004)

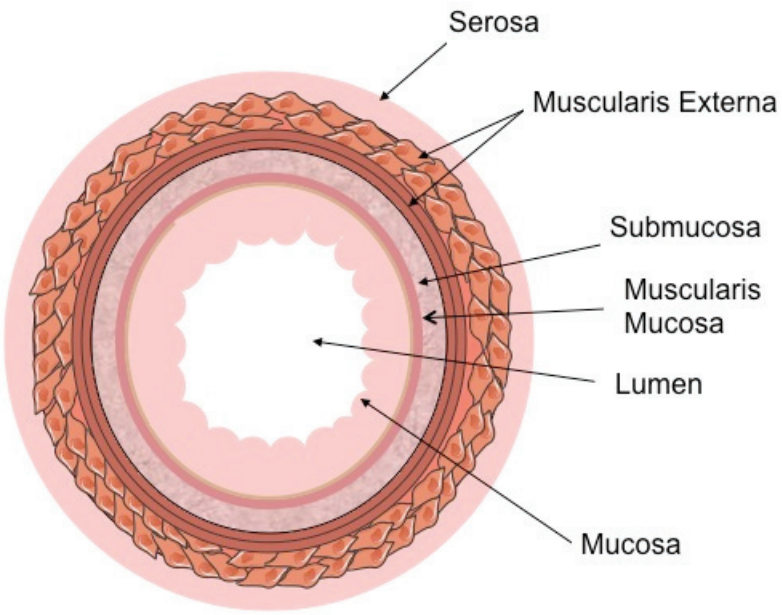


Figure 1.2 The anatomy of a normal colon. Colorectal cancer most commonly begins in the mucosa and invades through the various layers towards the serosa. Figure was produced using Servier Medical Art.

Table 1.2 TNM staging in colorectal cancer

	T/N/M	Description of Staging
Primary Tumor (T)	TX	Primary tumor cannot be assessed
	T0	No evidence of primary tumor
	Tis	Carcinoma in situ
	T1	Tumor invades submucosa
	T2	Tumor invades the muscularis propria
	T3	Tumor invades through the muscularis propria into the subserosa or into the nonperitonealized pericolic or perirectal tissues
	T4	Tumor directly invades other organs or structures or perforates the visceral peritoneum
Regional lymph nodes (N)	NX	Regional lymph nodes cannot be assessed
	N0	No regional lymph nodes metastasis
	N1	Metastasis in one to three lymph nodes
	N2	Metastasis in four or more lymph nodes
Distant metastasis (M)	MX	Presence of distant metastasis cannot be assessed
	M0	No distant metastasis
	M1	Distant metastasis present

Adapted from (Compton and Greene 2004).

The main mechanism of action of 5-FU is thought to be through its inhibition of thymidylate synthase, thereby reducing levels of thymidine (Peters, van Triest et al. 2000; De Angelis, Svendsrud et al. 2006). It is often given with leucovorin, a tetrahydrofolate precursor, to increase the response rate (Buyse, Thirion et al. 2000; Arkenau, Bermann et al. 2003). Leucovorin also inhibits the enzyme thymidylate synthase, synergistically enhancing the action of 5-FU and so making it more effective (Arkenau, Bermann et al. 2003). As an antimetabolite, the structure of 5-FU is similar to the pyrimidine base uracil of RNA. It is considered an S-phase drug, meaning that it has no activity when cells are in the G0 or G1 phase of the cell cycle (Shah and Schwartz 2001; De Angelis, Svendsrud et al. 2006). 5-FU also causes double- and single-strand breaks in DNA during the S-phase through the incorporation of FdUTP (a 5-FU metabolite) into DNA (Curtin, Harris et al. 1991; Peters, van Triest et al. 2000; De Angelis, Svendsrud et al. 2006).

Proliferating cells are susceptible to 5-FU, providing they do not have innate resistance to the drug. Some patients may have tumors that are already resistant to 5-FU; this is referred to as primary resistance. However, others receiving chemotherapy for cancer treatment may develop resistance afterwards. This is referred to as secondary resistance (Guo, Li et al. 2000; Peters, van Triest et al. 2000).

Until a randomized study combining leucovorin and 5-FU with oxaliplatin was shown to prolong survival and disease free survival over leucovorin and 5-FU alone, 5-FU was the only effective first-line treatment for non-resectable CRC (de Gramont, Figer et al. 2000). Now, 5-FU is most often given with oxaliplatin and/or irinotecan as a first-line strategy for metastatic CRC (Douillard, Cunningham et al. 2000). Oxaliplatin is a platinum-based drug that forms adducts with double-stranded DNA, inhibiting DNA

replication and transcription (Raymond, Faivre et al. 2002). Reactive platinum complexes are believed to inhibit DNA synthesis by forming intrastrand and interstrand cross-links in DNA molecules (Raymond, Faivre et al. 2002; Saltz and Minsky 2002). The active metabolite of irinotecan, SN-38, inhibits the topoisomerase I enzyme. Topoisomerase I plays a role in unwinding DNA during DNA replication, facilitating DNA relaxation (Champoux 2001; Koster, Palle et al. 2007). Loss of topoisomerase I function as a result of irinotecan being present causes G2 cycle arrest, thereby inhibiting cell division (Xu and Villalona-Calero 2002). Like 5-FU, irinotecan is an S-phase specific drug. *In vitro* studies have shown an additive interaction between SN-38, oxaliplatin and 5-FU (Raymond, Buquet-Fagot et al. 1997; Mans, Grivicich et al. 1999; Zeghari-Squalli, Raymond et al. 1999; Masi, Allegrini et al. 2004), supporting the combined use of these drugs against CRC.

1.2 The Solid Tumor Microenvironment

The tumor microenvironment is acknowledged to be an important factor in cancer progression. It is a complex milieu that is physiologically very different from that in normal tissue. It is an environment that is stressful to cells but it supports many different cell types, including cancer cells, fibroblasts, and endothelial cells.

Solid tumors are known to have a poorly formed and leaky vasculature. This leads to increased interstitial fluid pressure (IFP), regions of hypoxia and low extracellular pH (Cairns, Papandreou et al. 2006).

1.2.1 Tumor vasculature

Like normal tissues, tumors depend on their network of vasculature to supply them with nutrients and to remove waste. Tumor vasculature lacks the proper hierarchical structure and is functionally abnormal (Fukumura and Jain 2007). In contrast to normal blood vessels, tumor vessels are often dilated, saccular and have an irregular distribution, along with having increased vascular permeability (Jain 1998; Fukumura and Jain 2007).

Tumor growth is highly dependent on new vessel formation, or angiogenesis (Brown and Giaccia 1998). If a tumor is to expand beyond several millimeters in size, it must acquire a blood supply. While angiogenesis is normally a tightly-regulated process, it is thought that an imbalance between pro-angiogenic and anti-angiogenic factors contributes to the formation of dysfunctional vasculature (Jain 1998). When there is a switch in the balance of angiogenic factors toward a proangiogenic state in a tumor, it is referred to as the angiogenic switch (**Figure 1.3**) (Bergers and Benjamin 2003).
Solid tumors have increased vascular permeability, which is thought to support the high nutritional demands of rapidly growing tumors (Maeda, Fang et al. 2003). The increased vascular permeability results from poorly formed, leaky vessels. This is sometimes exploited in the treatment of solid tumors because of the tendency to take up and retain particulates, a phenomenon known as the enhanced permeability and retention effect (EPR) (Matsumura and Maeda 1986; Maeda, Fang et al. 2003). Matsumura first described the EPR effect in 1986 (Matsumoto, Yamamoto et al. 1986). As a result of the EPR effect, tumor tissues show increased extravasation and retention of macromolecular drugs such as liposomal and other polymer-drug conjugates (Maeda, Fang et al. 2003; Fang, Nakamura et al. 2011).

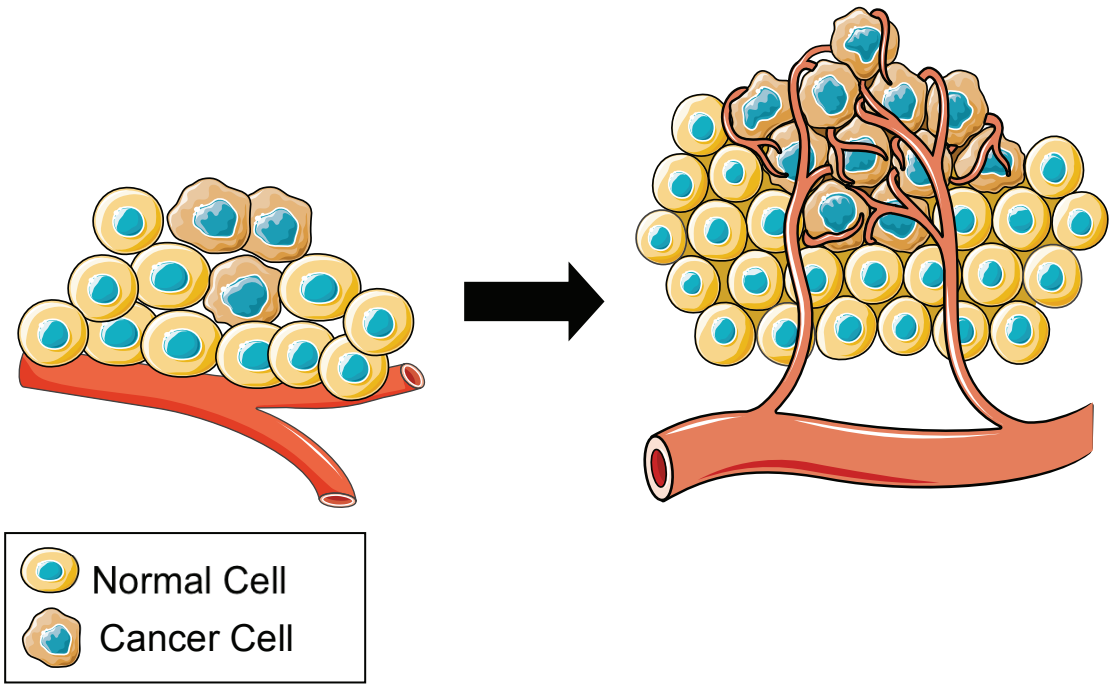


Figure 1.3 A representation of a tumor's dependence on the angiogenic switch. Tumors begin as small avascular neoplasms and can only continue to grow beyond a certain size by acquiring a source of blood. Adapted from (Bergers and Benjamin 2003). Figure was produced using Servier Medical Art.

1.2.2 Tumor acidity

Otto Warburg measured the levels of lactate in human tumor and normal tissue slices and found that the former produced much higher levels of lactate (Warburg 1956). He hypothesized that cancer was a result of defects in oxidative phosphorylation or aerobic respiration in the mitochondria (Warburg 1956; Favier, Briere et al. 2009). This would explain why tumor cells predominantly utilize glycolysis, even in the presence of oxygen (Favier, Briere et al. 2009) More recent evidence has shown that mitochondrial function is not impaired in the majority of cancer cells (Weinhouse 1976; Fantin, St-Pierre et al. 2006; Moreno-Sanchez, Rodriguez-Enriquez et al. 2007). A particular splice isoform of the enzyme pyruvate kinase M2 (PKM2) may play a role in initiation of the Warburg Effect (Christofk, Vander Heiden et al. 2008). Christofk et al showed that altered PKM2 and its altered association with phospho-tyrosine motifs enabled cancer cells to utilize aerobic glycolysis as opposed to oxidative phosphorylation (Christofk, Vander Heiden et al. 2008).

One of the major differences between normal tissue and tumor tissue is indeed the metabolic environment (Rotin, Steele-Norwood et al. 1989; Tannock and Rotin 1989). Several factors cooperate to produce the abnormal tissue physiology. The dysfunctional vasculature leads to glucose and oxygen gradients within regions of a tumor. Aerobic glycolysis and the production of lactic acid contribute to the low pH of the extracellular (pHe) fluid surrounding a tumor. Studies have shown that the external pH (pHe) of normal subcutaneous and muscle tissues ranged from 7.00 to 8.06. However, the pHe of a range of human and rodent tumors ranged from 5.5 to 7.6 (Wike-Hooley, Haveman et al.

1984; Kallinowski, Vaupel et al. 1988; Sevick and Jain 1989; Vaupel, Kallinowski et al. 1989).

Acidosis, or low pH, is a physiological response to ischemia (insufficient blood flow). Insufficient blood flow to an organ leads to an inadequate supply of glucose and oxygen in various conditions leading to a buildup of metabolic by-products (Vornov, Thomas et al. 1996). Mild acidosis has been shown to play a protective role in numerous systems. Low pHe during the recovery phase of a cerebral infarction has been shown to be protective (Tang, Dichter et al. 1990; Traynelis and Cull-Candy 1990; Vornov, Thomas et al. 1996), while low pHi has been shown to play a protective role in cardiac myocytes in ischemia reperfusion (Bing, Brooks et al. 1973). While lactic acidosis is a well-acknowledged feature of the tumor microenvironment, little is known about how it may influence cancer cells although it is thought to select for certain cell populations in the tumor (Fang, Gillies et al. 2008; Moellering, Black et al. 2008; Tang, Lucas et al. 2012).

The value of the pHe is a clinically relevant issue, as a low pHe reduces the efficacy of some chemotherapeutics. This is related to the pK_a or pK_b of a drug. Weakly basic drugs, such as doxorubicin, will be more ionized and relatively trapped in the acidic microenvironment, affecting their influx into the cell (Teicher, Holden et al. 1990; Gerweck, Vijayappa et al. 2006). Conversely, low pHe reduces the ionization and enhances the influx of weakly acidic drugs that are then more membrane permeable (Teicher, Holden et al. 1990). Alterations of pH will also affect the interaction of the drug with extracellular (such as matrix proteins) and intracellular (such as DNA) targets

(Menozzi and Arcamone 1978; Dalmark and Johansen 1982; Gerweck, Vijayappa et al. 2006).

Unlike the extracellular pH of tumors, the intracellular pH (pH_i) of tumors is the same as that of normal tissue, or is slightly alkaline (Gerweck and Seetharaman 1996; Gerweck, Vijayappa et al. 2006; Becelli, Renzi et al. 2007). Tumor cells have the ability to upregulate proteins such as proton transporters that are involved in pH regulation (Garcia-Canero, Trilla et al. 1999). Studies done by Strazzabosco et al in human liver tumor derived cell lines indicated that the Na^+/H^+ ion exchanger plays a major role in the regulating pHi (Rotin, Steele-Norwood et al. 1989; Strazzabosco and Boyer 1996; Garcia-Canero, Trilla et al. 1999). The vacuolar proton pump (V-ATPase) is expressed in the membrane of organelles and the plasma membrane of cells (Izumi, Torigoe et al. 2003). It pumps protons from the cytoplasm into the lumen of vacuoles or the extracellular space, using ATP energy (Izumi, Torigoe et al. 2003). The proton pump plays a variety of roles in the cell, such as endocytosis and pH homeostasis. Other families of regulators are known to be involved in this process, including the proton pump family, the monocarboxylate transporter family and the bicarbonate transporter family (Tannock and Rotin 1989; Boyer and Tannock 1992). These unique adaptations allow tumor cells to survive the acidic conditions resulting from an excess of anaerobic glycolysis.

1.2.3 Tumor hypoxia

Cells within a solid tumor have a variable access to a blood supply, depending on their location. As a result, localized regions of hypoxia, areas of low oxygen, occur within the poorly vascularized tissue of a tumor (Jain 2005).

Tumor hypoxia has been inversely correlated with patient survival (Bush, Jenkin et al. 1978; Becker, Stadler et al. 2000). It negatively impacts overall outcome by selecting for cells that can adapt to the harsh microenvironment—allowing for their survival and continued disease progression (Vaupel and Harrison 2004).

Hypoxia-inducible factor (HIF) signaling is activated during hypoxia. Under normoxic conditions, the hypoxia inducible factor alpha subunit (HIF-1α) is targeted by prolyl hydroxylases, marking it for degradation by E3 ubiquitin ligases (Huang and Bunn 2003; Huang, Bindra et al. 2007). Several mechanisms in the hypoxic state prevent degradation and stabilize HIF-1α, allowing it to translocate to the nucleus and heterodimerize with the HIF-1β subunit (Brahimi-Horn, Chiche et al. 2007). This dimer then acts as a transcription factor for many genes (**Figure 1.4**) (George and Kaelin 2003; Semenza 2003; Pouyssegur, Dayan et al. 2006; Brahimi-Horn, Chiche et al. 2007). Of particular importance for the situation in a solid tumor, HIF-1 regulates genes involved in energy metabolism, neovascularization, survival, migration and intracellular pH regulation (Semenza 2003). Changes resulting from hypoxia can influence both tumor cells and surrounding stromal cells to adapt to, and survive, conditions of nutrient deprivation (Hockel and Vaupel 2001). A transcriptional program activated during hypoxia, as a result of HIF-1α stabilization, leads to an increase in various glucose transporters (GLUT1 and GLUT3), glycolytic enzymes, pro-survival and pro-angiogenic factors (such as VEGF), as well as proteolytic enzymes (MMPs) (Hance, Robin et al. 1980; Brown and Giaccia 1998; Giaccia, Brown et al. 1998).

Conversely, hypoxia can also induce apoptosis in normal and neoplastic cells. The severity of hypoxia dictates whether a cell dies or whether it is able to adapt to and

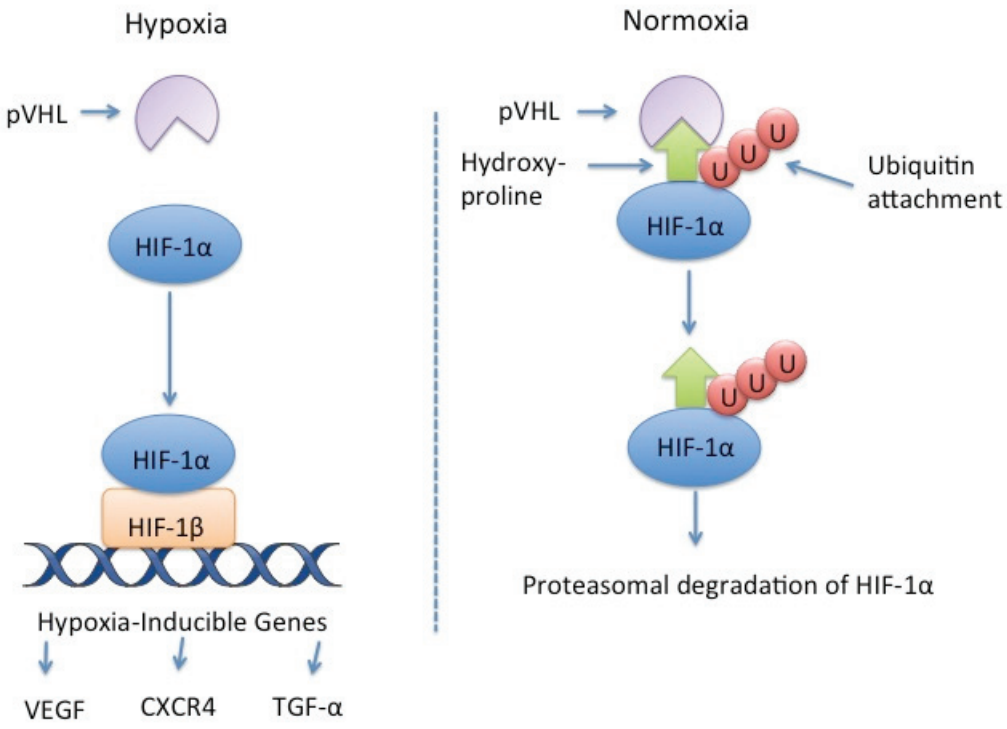


Figure 1.4 In the presence and absence of oxygen, HIF-1α plays very different roles in the cell. Left: the role of HIF-1α in the absence of oxygen. Right: HIF-1α is degraded in the presence of oxygen. Adapted from (George and Kaelin 2003)

survive in hypoxic conditions (Greijer and van der Wall 2004). Cancer cells are often exposed to hypoxic stress and adapt with repeated exposure to select for a hypoxia-resistant phenotype (Dong, Wang et al. 2003). During periods of hypoxia, pro-apoptotic proteins (e.g. BNIP3) are downregulated, while anti-apoptotic proteins (e.g. IAPs, XIAPs) are upregulated (Dong, Nishiyama et al. 2002; Greijer and van der Wall 2004). This selection process often results in a more resistant and aggressive phenotype (Harris 2002).

1.2.4 Therapeutic efficacy of chemotherapeutics in hypoxia

Tumor cells that are resistant to hypoxia and apoptosis are also likely to be more resistant to chemotherapy as well (Hickman, Potten et al. 1994; Liu, Ning et al. 2008). Solid tumors with a low growth fraction are generally resistant to chemotherapy (Sartorelli 1988). A diverse number of factors play a role in this resistance, including altered proliferation kinetics but also many of the influences that result from the physiological differences in tumor tissue. Hypoxia plays a profound role in cellular resistance in solid tumors. It has been long known that hypoxia protects cells from ionizing radiation (Moulder and Rockwell 1987). The radioresistance conferred in hypoxia has been shown to be either HIF-1 independent or HIF-1 dependent, depending on the model (Arvold, Guha et al. 2005). Many proteins that are implicated in cancer resistance to chemotherapeutics are under the control of HIF-1 and therefore, in hypoxia, are upregulated (Liu, Ning et al. 2008). Such proteins include MDR1, p-glycoprotein, and lung resistance protein (Wartenberg, Gronczynska et al. 2005; Liu, Ning et al. 2008).

1.3 Role of the Stroma in Cancer Progression

The role of the stroma in cancer progression is widely acknowledged although not fully understood (Bhowmick, Ghiassi et al. 2001; De Wever and Mareel 2003; Bhowmick, Neilson et al. 2004). In cancer, the changes in the stromal compartment are thought to help drive invasion and metastasis (De Wever and Mareel 2003). The interaction between cancer cells and the cells in their surrounding environment is attributed to molecular crosstalk, that is, the transmission of contextual cues through molecular mediators. Using various mouse models of tumor progression it has been shown that stromal cells, including vascular cells, fibroblasts and inflammatory cells, each contribute to tumorigenesis (Elenbaas, Spirio et al. 2001; Bhowmick, Neilson et al. 2004; Orimo, Gupta et al. 2005). These effects are mediated through secreted molecules that include growth factors, chemokines, cytokines, ECM components and proteases (Hazan, Kang et al. 1997; De Wever and Mareel 2003).

1.3.1 Carcinoma associated fibroblasts (CAFs)

One particular cell type that is thought to be key in facilitating cancer progression is the carcinoma-associated fibroblast (CAF) (Shimoda, Mellody et al.). In this context, the term fibroblast refers to stromal cells with a mesenchymal phenotype (Franco, Shaw et al. 2010). CAFs reside next to tumor cells and, along with other cell types, make up the heterogeneous community of stromal cells. CAFs are similar to myofibroblasts, or the fibroblasts seen in wound healing. CAFs are thought to be constitutively active, meaning that they secrete various growth factors and cytokines. Additionally, they have prominent cytoplasmic actin stress fiber formation and indented nuclei, like myofibroblasts (Dvorak

1986; Schurch, Seemayer et al. 1998; Xouri and Christian 2010). As a result of their similarities with myofibroblasts, they have a very different impact on neighboring cells than normal fibroblasts (Haviv, Polyak et al. 2009).

CAFs have been found to be highly heterogeneous in tumors (Schor and Schor 1987; Sugimoto, Mundel et al. 2006; Franco, Shaw et al. 2010). Subpopulations of stromal fibroblasts have been identified using various markers such as α-smooth muscle actin (α-SMA), platelet derived growth factor receptor (PDGFR) and fibroblast-specific protein (FSP-1).

CAFs have been shown in several cancer models to contribute to the various 'Hallmarks of Cancer' (Hanahan and Weinberg 2000; Pietras and Ostman 2010). Weinberg and Hanahan published the original Hallmarks of Cancer in 2000 and described a set of six acquired capabilities of cancer cells (Hanahan and Weinberg 2000). These include self-sufficiency in growth signals, insensitivity to anti-growth signals, the ability to evade apoptosis, limitless replicative potential, the ability to sustain angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2000). A decade later, Hanahan and Weinberg published an updated view of the Hallmarks of Cancer. The new Hallmarks encompass the role of the stroma in tumorigenesis, as the stroma is now acknowledged to significantly impact this process (Hanahan and Weinberg 2011). Realizing the complexity of the disease, two additional characteristics have emerged. The new Hallmarks, genome instability and tumor-promoting inflammation, are described as 'enabling' characteristics that facilitate the acquisition of the original Hallmarks of Cancer (Hanahan and Weinberg 2011).

Cancer cells may send signals to stromal cells, influencing the stromal cells to in-turn supply the cancer cells with growth factors (Bhowmick, Neilson et al. 2004; Hanahan and Weinberg 2011). CAFs are able to stimulate cancer cell growth directly through the release of various growth factors, including hepatocyte growth factor (HGF) and epidermal growth factor (EGF) (Franco, Shaw et al. 2010). Secretion of other factors, such as VEGF-A and PDGFβ, facilitates endothelial cell and pericyte recruitment, contributing to tumor angiogenesis (Anderberg, Li et al. 2009). Additionally, they secrete factors such as TGF-β2 that promote cancer cell survival and resistance to apoptosis through the activation of NF-κB (Lu, Burdelya et al. 2004; Danielpour 2005). TGF-β has also been shown to play a critical role in inducing epithelial to mesenchymal transition (EMT) in tumor cells, facilitating local invasion and metastasis (Pietras and Ostman 2010).

1.3.2 The origin of CAFs

The origin of CAFs remains controversial. Initially they were thought to originate solely from local fibroblasts in the vicinity of tumor cells (Haviv, Polyak et al. 2009; Xouri and Christian 2010). This has since been shown to be cancer-type dependent as additional sources may contribute to local CAF populations (Franco, Shaw et al. 2010; Shimoda, Mellody et al. 2010). It is likely that some populations evolve as a result of molecular crosstalk from soluble factors in the tumor microenvironment derived from cancer cells. Second, there are some studies supporting the formation of CAFs by undergoing a transdifferentiation process, or an EMT (or an Endothelial MT (EnMT)). A wide variety of cell types, including adipocytes, pericytes and smooth muscle cells have

been shown to have the ability to transdifferentiate into activated fibroblasts(Xouri and Christian 2010). A variety of growth factors, in particular TGF-β, are able to induce EMT through the transcriptional activation of factors like Snail, Slug and Twist (Kunz-Schughart and Knuechel 2002; Zeisberg, Potenta et al. 2007). The cells of origin may be an epithelial-type cell (a normal cell or a tumor cell), an endothelial cell or some other progenitor cell (Orimo, Gupta et al. 2005; Xouri and Christian 2010). While the exact origin of CAFs is unclear, they are thus known to be highly heterogeneous and likely arise from multiple sources.

1.3.3 CAFs and the tumor-promoting roles of CXCL12

Among the many factors over-produced by CAFs, SDF-1/CXCL12 plays several roles in promoting tumor cell growth and invasiveness (Orimo, Gupta et al. 2005; Shimoda, Mellody et al. 2010).CXCL12 acts in a paracrine and endocrine manner by binding to the CXCR4 receptor to promote tumor cell proliferation, migration and neoangiogenesis (Ao, Franco et al. 2007; Franco, Shaw et al. 2010; Shimoda, Mellody et al. 2010). High levels of another factor produced by CAFs, TGF-β, have been shown to increase the expression of CXCR4. This has been demonstrated to occur in several different cancer cell types, suggesting that this may be a general mechanism to enhance the effects of CXCL12 (Ao, Franco et al. 2007; Mishra, Banerjee et al. 2010; Shimoda, Mellody et al. 2010).

CXCL12 has been shown to facilitate neoangiogenesis in tumors. Through its actions on CXCR4, CXCL12 serves as a chemoattractant for endothelial progenitor cells

(EPCs) thereby enabling the initiation of angiogenesis in solid tumors (Orimo, Gupta et al. 2005; Kryczek, Wei et al. 2007).

1.3.4 Mesothelial cells as supportive stroma in cancer

Mesothelial cells grow in tight monolayers and line the body's serous cavities and cover many organs. The layers formed by these cells are collectively referred to as the mesothelium and provide a protective surface that on examination is found to be slippery and non-adhesive (Mutsaers 2004). While the mesothelium was for a long time mainly thought of as simply a protective barrier, it has been found to have other functions including antigen presentation, solute transport and suppression of coagulation and promotion of fibrinolysis in serosal cavities (Holmdahl, Eriksson et al. 1998; Mutsaers 2002).

However, the mesothelium is also known to play a role in tumor cell adhesion and metastasis in certain cancers where the cellular behaviour changes, particularly ovarian carcinomas (Cunliffe and Sugarbaker 1989). Epithelial ovarian carcinomas typically have a poor prognosis as a result of their ability to seed and form new tumors in the peritoneum (Cannistra, Kansas et al. 1993). This has been attributed in some cases to the presence of the cell-surface protein CD44 on tumor cells binding hyaluronan on the mesothelial cells surface, and the upregulation of other adhesion molecules as a result of inflammatory mediators (Cannistra, Kansas et al. 1993; Jones, Gardner et al. 1995; Mutsaers 2004).

In the case of CRC, peritoneal carcinomatosis occurs when colorectal carcinoma progresses and forms metastases on the peritoneal mesothelium (Jayne, Fook et al. 2002). It ultimately occurs in 10 - 30% of CRC patients and as it is considered a pre-terminal condition with a poor prognosis (Dawson, Russell et al. 1983; Chu, Lang et al. 1989; Jayne, Fook et al. 2002; Macri, Saladino et al.). In patients that eventually succumb to the disease, peritoneal carcinomatosis is evident in 40 - 80% of cases (Koppe, Boerman et al. 2006). Peritoneal carcinomatosis occurs in a predictable sequence of steps: the spread of cancer cells to the peritoneum, adhesion of cancer cells to the mesothelial layer, and finally, invasion of the subperitoneal layer (Confuorto, Giuliano et al. 2007).

Interestingly, mesothelial cells also play a role tissue repair. They secrete mediators that help to stimulate normal cell proliferation and migration, including EGF, bFGF, and HGF (Mutsaers 2002; Mutsaers 2004). Their role in tissue repair means that they also secrete proteolytic enzymes and ECM components to facilitate ECM re-modeling. While not many studies have been done looking at the interaction between mesothelial cells and colorectal carcinoma cells, a few studies have been conducted in rat models looking at the paracrine interactions between these two cell lines.

In a study by Van de Wal et al, mesothelial cells significantly inhibited the growth of a rat colon cancer cell line, whereas the colon cancer still stimulated the growth of the mesothelial cells (van der Wal, Hofland et al. 1997). The inhibition of rat colon cancer cell growth was attributed to the IGF-1 secreted by the mesothelial cells (van der Wal, Hofland et al. 1997). However, more recent *in vitro* studies suggest another perspective. Many researchers have suggested that in order for a cancer to colonize the peritoneum, tumor cells force their way through the mesothelium (see **Figure 1.5**). The CD44

receptor and ICAM-1 have been shown to be partially involved in the initial adherence of cancer cells to the mesothelial layer (Cannistra, Kansas et al. 1993; Schlaeppi, Ruegg et al. 1997). Ultimately, this leads to mesothelial cell retraction and disaggregation (Yonemura, Endou et al. 1997; Heath, Jayne et al. 2004). An *in vitro* study by Heath et al, suggests that colorectal carcinoma cells induce apoptosis in mesothelial cells through a Fas-Ligand/Fas interaction (Heath, Jayne et al. 2004). The mesothelial cells were shown to express a functional Fas receptor, that when stimulated with an anti-Fas antibody mediated mesothelial cell apoptosis (Heath, Jayne et al. 2004).

1.3.5 Multicellular tumor spheroids as a solid tumor model

Monolayer cell culture is still the most common technique when it comes to the majority of *in vitro* cancer cell culture systems. Studies using monolayer cultures do not recapitulate many of the aspects of solid tumors, which exist in 3 dimensions. Monolayer cell culture does have its advantages, as it is easier to grow most cell types in monolayer conditions, it is faster and less laborious. However, the correct topology can be generated by growing the cells as multicellular spheroids. Holtfreter et al pioneered the technique for growing spheroids over sixty years ago studying embryonic cells (Holtfreter 1947; Holtfreter 1951). Three-dimensional systems such as spheroids share similarities with solid tumors not seen in monolayer cultures (Sutherland 1988).

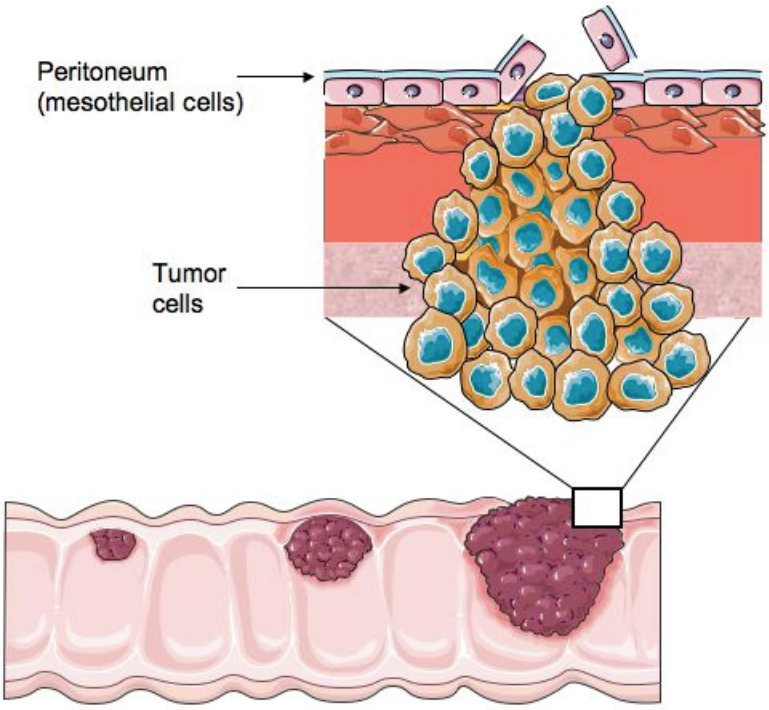


Figure 1.5 As colorectal cancer progresses, it invades through the layers of the colon. Eventually, it penetrates mesothelial lining leading to serosal ulceration. Figure produced using Servier Medical Art.

Cellular topology influences many factors of cell growth and behavior (Santini and Rainaldi 1999). It directly affects gene transcription, and cells grown in three dimensions will express a different subset of genes than those grown in monolayer (Dardousis, Voolstra et al. 2007). These genes include transcription factors that are known to play a role in tumorigenesis, and indeed inhibition of some of these genes ablates spheroid formation (Dardousis, Voolstra et al. 2007). Other relevant genes include those corresponding to the family of receptor tyrosine kinases, crucial for cancer growth (Dardousis, Voolstra et al. 2007). Diffusivity of nutrients and metabolites varies partly on cell type in spheroids. Predicting the diffusion of nutrients and metabolic waste, such as lactate, in spheroids can be done very generally using diffusion theory (Casciari, Sotirchos et al. 1988Groebe, 1991 #549; Groebe and Mueller-Klieser 1991). For example, glucose diffusion coefficients have been found to vary from 5.5×10^{-7} cm^2/s to 2.3×10^{-7} cm^2/s (Casciari, Sotirchos et al. 1988). As a result of differences in diffusion of various substances, multicellular spheroids also exhibit the nutrient and oxygen gradients seen in solid tumor tissue and may experience many of the same microenvironmental stresses (Sutherland and Durand 1973; Sutherland 1988).

In appropriate conditions of culture, cancer cells that have limited anchorage dependence begin to form loose cell aggregates and over time take a more disc-like shape and become spheroids. In time they develop into tightly aggregated structures where it is difficult to distinguish between individual cells (Holtfreter 1951). In contrast to monolayer cultures that grow exponentially, solid tumors have an early period of exponential growth, followed by a later period of growth maintenance. The second phase is dependent on tumor/spheroid size and nutritional restrictions of the cells in the interior

(Sutherland and Durand 1984). Similar to solid tumors, they may exhibit regions of necrosis in the center, as a result of limitations in oxygen and nutrient diffusion. The growth dynamics of spheroids have been shown to resemble those of solid tumors *in vivo* with respect to a number of different kinetic parameters (Laird 1964; McCredie JA 1965; Sutherland and Durand 1984; Santini and Rainaldi 1999; Santini, Rainaldi et al. 2004).

1.4 Adenosine

Adenosine (9-β -D-ribofuranosyladenine) is a purine nucleoside composed of a nitrogenous base and ribose sugar. Adenosine is known to play a role in many physiological processes, including energy metabolism and signal transduction (Merighi, Mirandola et al. 2002; Ledoux, Runembert et al. 2003; Klaasse, Ijzerman et al. 2008). It exists in many forms including as the core structure of adenine nucleotides - a mono-, di- or tri- phosphate ester of the nucleoside that we know as AMP, ADP, or ATP. In this context, adenosine plays a crucial role as one of the most important energy sources in the majority of cellular processes. Adenosine itself is ubiquitous throughout the body and has various further effects in different organs; it is known to regulate neurotransmission, inflammation, cardioprotection, and pain (Palmer and Stiles 1995; Yaar, Jones et al. 2005; Klaasse, Ijzerman et al. 2008).

1.4.1 Adenosine production and metabolism

Adenosine is produced both intracellularly and extracellularly, largely as a result of adenine nucleotide breakdown. Adenosine levels in cells and interstitial fluids are usually

in the nanomolar range under normal physiological conditions (Fredholm 2007). Adenosine is produced through two different pathways: (i) 5′-AMP is dephosphorylated by 5'-nucleotidase and (ii) S-adenosylhomocysteine (SAH) is hydrolyzed by SAH hydrolase to produce adenosine (**Figure 1.6**). Studies done on the heart have reported that during normoxia, 90% of the adenosine is produced and derived from SAH (Deussen, Lloyd et al. 1989; Kroll, Deussen et al. 1992), but this is likely an exception and in other tissues the 5'-nucleotidase pathway is probably the principal route for adenosine generation.

1.4.2 Adenosine levels in hypoxia

Adenosine production is increased in stressful conditions such as inflammation. Its production is also increased in hypoxia as a result of increased nucleotide breakdown, and efflux from the cell (Fredholm 2007). The 5'-nucleotidase pathway is thought to be the major contributor of adenosine during stress as a result of the increased activity of this pathway in hypoxia (Ledoux, Runembert et al. 2003; Gorlach 2005). Casanello et al showed that extracellular adenosine increased in HUVECs exposed to hypoxia (Casanello, Torres et al. 2005). However, the effect was attributed to a reduced uptake of adenosine in cells due to a diminished number of ENT-1 transporters on the cell surface in a hypoxic state (Casanello, Torres et al. 2005; Gorlach 2005). Hypoxic downregulation of ENT-1 has also been shown in mouse cardiomyocytes, potentially leading to decreased uptake of extracellular adenosine and therefore greater extracellular concentrations (Casanello, Torres et al. 2005; Gorlach 2005).

Blay et al has reported that higher levels of adenosine are found in the extracellular fluid in models of experimental human and murine solid tumors (Blay, White et al. 1997). High levels of extracellular adenosine have been shown to be both mitogenic and immunosuppressive in the tumor microenvironment (Blay, White et al. 1997; Mujoomdar, Hoskin et al. 2003).

1.4.3 Adenosine deaminase

Adenosine deaminase (ADA) is an enzyme in purine metabolism. It catalyzes the irreversible hydrolytic deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine (Vistoli, Pedretti et al. 2009). It exists as a 43-kDa protein and is found in both cytosolic and extracellular locations. ADA is a high-capacity and high-Km enzyme (Jackson, Morris et al. 1978). The extracellular form of the enzyme is referred to as ecto-ADA. It was found to bind the T-cell activation antigen, CD26, directly on the surface of Jurkat cells (Kameoka, Tanaka et al. 1993), and subsequently to have the same principal cell-surface binding site in most tissues. It has been suggested that additional ADA binding to adenosine receptors (A2A, A2B) increases agonist affinity for the receptor (Ciruela, Saura et al. 1996). Gracia et al showed that in neurons, ADA might act as an allosteric modulator to enhance the ligand affinity and function of A2A receptors (Gracia, Perez-Capote et al.). ADA is expressed in most of the tissues of the body, but with the highest expression in lymphocytes and lymphoid tissues (Andy and Kornfeld 1982; Kameoka, Tanaka et al. 1993).

A deficiency in ADA results in severe combined immunodeficiency, or SCID, which is the result of an autosomal recessive disorder. Despite the fact that ADA is

absent in every cell in the body, only the immune system is significantly affected by this deficiency (Giblett, Anderson et al. 1972; Resta, Hooker et al. 1997; Resta and Thompson 1997). This indicates the overwhelming role ADA plays in the regulation of the immune system. In the absence of ADA there is a build-up of ADA substrates, which include adenosine and deoxyadenosine. These nucleosides are toxic to developing lymphocytes, leading to a virtual absence of T cells and significant decrease in B cells (Giblett, Anderson et al. 1972; Gessi, Varani et al. 2000).

1.4.4 Adenosine kinase

Adenosine kinase (AK) is another enzyme that plays a major role in adenosine and purine metabolism. AK catalyzes a phosphorylation event using ATP or GTP as the phosphate donor and can be inhibited by high concentrations of adenosine (Lavoinne, Claeyssens et al. 1990; von Schwartzenberg, Kruse et al. 1998). It is an important regulator of adenosine both intra- and extra-cellularly, catalyzing the exchange reaction of adenosine with ATP to form ADP and AMP. AK has a much lower capacity and K_m for adenosine than ADA, and is the predominant enzyme at lower adenosine concentrations. In situations where ADA is not functional or absent, the capacity of AK is overcome and levels of adenosine rise (Lloyd and Fredholm 1995; Decking, Schlieper et al. 1997).

1.4.5 Adenosine receptors

Adenosine elicits its effects through four different G-protein coupled receptors (GPCRs). The receptors were initially discovered as a result of caffeine's ability to antagonize the adenosine effect on the heart (Degubareff and Sleator 1965). As with other GPCRs, these are composed of seven α-helices arranged perpendicularly to the plasma membrane. The N-terminus is located extracellularly while the C-terminus is located intracellularly and contains palmitoylation and phosphorylation sites, which are involved in receptor desensitization and internalization (Klaasse, Ijzerman et al. 2008). Phosphorylation of the third intracellular loop is involved in desensitization and internalization of adenosine receptors (Palmer and Stiles 1995; Yaar, Jones et al. 2005; Fredholm 2007).

Each receptor has a different pharmacological profile and effector coupling (**Table 1.5**). These receptors are named A1, A2A, A2B, and A3. The receptors were initially classified based on the effect of cAMP production in different tissues, ie, a stimulatory versus an inhibitory effect on adenylyl cyclase (AC) (Londos, Cooper et al. 1980; Fredholm, AP et al. 2001). The A1 receptor inhibited AC and the A2 receptors stimulated AC (Fredholm, Abbracchio et al. 1994). It was not until 1993 that Salvatore et al identified a further adenosine receptor gene, for the A3 receptor, from a human striatal cDNA library (Salvatore, Jacobson et al. 1993). Stimulation of the A3 receptor

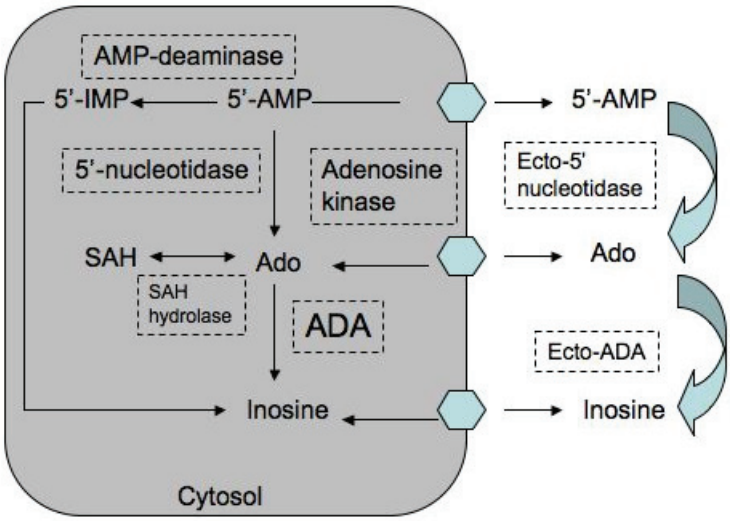


Figure 1.6 Pathways and enzymes of adenosine metabolism. Ado: Adenosine, ADA: adenosine deaminase, SAH hydrolase: S-adenosylhomocysteine hydrolase. Adapted from (Frobert, Haink et al. 2006)

Table 1.3 Adenosine Receptors and their G-Proteins

Receptor Subtype	G-Protein	Effectors of G-Protein Coupling
A1	$G_{i/o}$	↓cAMP ↑IP_3/DAG Ca^{2+} Channels K^+ Channels
A2A	G_s, G_{olf}	↑cAMP ↑IP_3/DAG
A2B	G_s, $G_{q/11}$	↑cAMP ↑IP_3/DAG
A3	$G_{i/q}$	↓cAMP ↑IP_3/DAG K^+ ATP Channels Cl^- Channels

Adapted from (Fredholm, AP et al. 2001; Linden 2001).

is similar to the A1 receptor in that the effects of its G-protein result in a decrease in cAMP (Palmer and Stiles 2000; Linden 2001).

The receptor subtypes are expressed variably in different tissues. The A1 receptor is highly expressed in certain regions of the brain, including the cortex and hippocampus, and the adrenal gland and atria. The A1 receptor couples to $G_{i/o}$, resulting in an inhibitory effect on AC, activation of phospholipase C (PLC) signaling, opening of K^+ channels, and closing of various types of Ca^{2+} channels (Dolphin, Forda et al. 1986; Munshi, DeBernardi et al. 1993; Akbar, Dasari et al. 1996). The A1 receptor is also known to have the highest affinity for adenosine of all four receptor subtypes (Palmer and Stiles 1995).

High levels of the A2A receptor are seen in the spleen, thymus and leukocytes, reflecting its strong involvement with the immune system (Sitkovsky, Lukashev et al. 2004). Signaling through the A2A receptor occurs through G_s or G_{olf} and results in an increase in cAMP (Dionisotti, Ongini et al. 1997; Olah 1997).

Interestingly, the A2B receptor is most highly expressed in the cecum, colon and bladder (Stehle, Rivkees et al. 1992; Feoktistov and Biaggioni 1997; Schulte and Fredholm 2000). This suggests that this particular receptor may be important for any adenosine-mediated effects in CRC progression. It is considered a low-affinity adenosine receptor (Linden 2005). Similar to A2A, A2B couples to G_s, but also to G_q. This results in an increase in cAMP and PLC signaling, Ca^{2+} mobilization and mitogen-activated protein kinase (MAPK) activation (Pierce, Furlong et al. 1992; Yakel, Warren et al. 1993; Linden 2005).

The expression of the A3 receptor is not well characterized in humans, but there is

low-level expression in the colon, regions of the brain, and the testes (Palmer and Stiles 1995). The A3 receptor couples to G_i and G_q G-proteins, leading to a decrease in cAMP.

1.4.6 Adenosine as an imunosuppressant

Adenosine is considered an important stress hormone and is released in large amounts during periods of cellular or systemic stress (Hasko, Deitch et al. 2002). Immune cells are generally found in the vicinity of such stress, exposing them to high levels of adenosine. Adenosine at concentrations of 3μM or above has been shown to be immunosuppressive, inhibiting various components of the immune system (Hasko, Deitch et al. 2002; Mandapathil, Hilldorfer et al. 2010) . In inflammatory conditions adenosine concentrations have been reported to be as high as 100μM (Cronstein 1994; Hasko, Deitch et al. 2002). In solid tumors, the extracellular concentration of adenosine is often elevated in hypoxic microregions at sufficient concentrations to cause immunosuppression (Blay, White et al. 1997). This suggests that adenosine may play a role in immune evasion by tumor cells.

Macrophages and lymphocytes have been shown to express all four adenosine receptors (Sajjadi, Takabayashi et al. 1996; Mirabet, Herrera et al. 1999; Gessi, Varani et al. 2000; Khoa, Montesinos et al. 2001). In macrophages, adenosine has been shown to attenuate antibacterial activity by reducing their ability to produce superoxide and nitric oxide (Edwards, Watts et al. 1994; Sajjadi, Takabayashi et al. 1996; Xaus, Mirabet et al. 1999). This action of adenosine has been attributed to the activation of A2 receptors (Sajjadi, Takabayashi et al. 1996; Hasko, Deitch et al. 2002). Adenosine has been shown to decrease inflammatory cytokine production by macrophages through the A2A and A3

receptors. In lymphocytes, adenosine has been shown to impair T-cell activation by inhibiting the production of IL-2 (DosReis, Nobrega et al. 1986; Hasko, Deitch et al. 2002). Activation of A2 receptors is thought to inhibit granule exocytosis in natural killer cells, contributing to tumor cell evasion of the immune system (Williams, Manzer et al. 1997; Hoskin, Butler et al. 2002).

1.4.7 Adenosine receptors and cancer

The role of adenosine receptors in cancer is not entirely clear. It has been reported that all four receptors are upregulated in various cancers, with some receptors playing a role in promoting tumor progression and others playing a role in inhibiting tumor progression (Fishman, Bar-Yehuda et al. 2004; Borea, Gessi et al. 2009).

The A1 receptor's role in cancer is poorly understood even against that background. Stimulation of the A1 receptor in different types of colorectal carcinoma cell lines has been shown to either inhibit or stimulate cell proliferation (Kohno, Sei et al. 1996). It has also been reported to play a role in the estrogen responsiveness in breast cancer proliferation (Lin, Yin et al. 2009). Lin et al suggest that the A1 receptor is a novel target for estrogen action through the ERα receptor, increasing transcription of the A1 receptor in the presence of estradiol (Lin, Yin et al. 2009). Little further data exist on this receptor subtype in the context of cancer.

The A2A receptor plays a role in suppressing the immune response to solid hypoxic tumors, as previously discussed. Additionally, A2A receptor agonists have been shown to increase tumor cell and endothelial cell growth and facilitate angiogenesis in different models (Montesinos, Gadangi et al. 1997; Sexl, Mancusi et al. 1997; Merighi, Mirandola et al. 2002). Conversely, activation of the A2A receptor has also been shown

to induce apoptosis in different cancer cells through the activation of caspase-9 and caspase-3 (Yasuda, Saito et al. 2009).

The A2B receptor is considered a low affinity adenosine receptor that is only activated in the presence of pathophysiological concentrations of adenosine. Evidence suggests its expression is high in adenocarcinomas of the colon and colorectal carcinoma cell lines (Ma, Kondo et al. 2010). A2B is notably important as it is transcriptionally upregulated by hypoxia, and is likely to be up regulated in hypoxic sections of a solid tumor (Kong, Westerman et al. 2006). Reports suggest that the A2B receptor is largely responsible for adenosine-mediated cell proliferation and/or differentiation (Dubey, Gillespie et al. 2002; Panjehpour and Karami-Tehrani 2007; Ma, Kondo et al. 2010). Use of the specific A2B antagonist MR1754 was shown to inhibit CRC cell growth in a dose-dependent manner (Ma, Kondo et al. 2010). A2B agonists can induce endothelial cell proliferation and induce neovascularization in tumor tissue (Merighi, Mirandola et al. 2002; Ma, Kondo et al. 2010). Interpretation of A2B data remains challenging as a result of the fact that it is a low-affinity receptor and specific A2B agonists are not widely available.

There is also evidence for the anti-cancer of effects of A3R modulation using specific A3R agonists. IB-MECA and Cl-IB-MECA are two adenosine analogs that act as agonists and have a high affinity for the A3R. Investigations using such agonists in various tumor cell lines (melanoma, lymphoma, etc) suggest that A3R antagonists inhibit cell proliferation in these tumor cell lines through the de-regulation of the Wnt signaling pathway, and cause the cells to arrest in G_o/G1 (Fishman, Bar-Yehuda et al. 2000; Fishman, Bar-Yehuda et al. 2002; Fishman, Bar-Yehuda et al. 2004).

1.5 CD26

1.5.1 Introduction and structure

CD26 is a cell-surface glycoprotein of approximately 110kDa that exists in the plasma membrane principally as homodimers. The protein has a variety of functions and for this reason is often referred to as a 'moonlighting' protein. CD26 has roles in glucose homeostasis, immune function, and cancer progression (Marguet, Baggio et al. 2000; Dang and Morimoto 2002; Kobayashi, Hosono et al. 2002). CD26 is widely expressed on different cells throughout the body, with strong expression in epithelial cell types; it is also expressed on fibroblasts, endothelial cells and some leukocytes (Dinjens, ten Kate et al. 1989; De Meester, Korom et al. 1999; Lambeir, Durinx et al. 2003).

The CD26 protein is comprised of 766 amino acids arranged into three segments: a small cytoplasmic segment, a transmembrane segment, and a complex extracellular segment, which is by far the largest of the three segments **(Figure 1.7)**. While the cytoplasmic domain is not well characterized, it is known to associate with CD45, a protein tyrosine phosphatase, in T-cells (Torimoto, Dang et al. 1991; Ishii, Ohnuma et al. 2001). The transmembrane segment is a small hydrophobic segment following this short cytoplasmic sequence and linking the extracellular segment to the cytoplasmic domain. The transmembrane segment encompasses residues 7-28. The extracellular segment of CD26 has intrinsic peptidase activity referred to as dipeptidyl peptidase 4 (DPP4), and as a result, CD26 is often synonymously referred to as DPPIV or DPP4 (Tanaka, Camerini et al. 1992; Fan, Meng et al. 1997). Additionally, the extracellular segment binds the

enzyme ADA as well as interacting with collagen and fibronectin proteins in the extracellular matrix. The extracellular segment comprises residues 29-766.

The extracellular domain can be divided into three protein structural domains: a membrane proximal glycosylation domain, an intermediate cysteine-rich domain and a catalytic domain, and a serine protease (**Figure 1.7**) (Engel, Hoffmann et al. 2006). The membrane proximal region is post-translationally modified (N-linked glycosylation) while the protein is en route to the plasma membrane. None of the nine sites of glycosylation have been shown to be necessary for homodimerization, ADA binding or enzyme activity (Aertgeerts, Ye et al. 2004). The cysteine-rich domain is from residues 290-492 and includes each of the three functionalities for protein binding. Specifically, residues 340-343 are thought to be necessary for ADA binding, residues 238-495 for collagen binding, and residues 469-479 for fibronectin binding (Gonzalez-Gronow, Gawdi et al. 1994; Dong, Tachibana et al. 1997; Morimoto and Schlossman 1998).

CD26 belongs to a clan of serine peptidases containing α/β hydrolase folds, the SC clan (Lambeir, Durinx et al. 2003). Within the SC clan, it is a member of the S9 family, subfamily B. This subfamily all shares a C-terminal catalytic domain with a high degree of sequence similarity (Lambeir, Durinx et al. 2003).

The gene for CD26 is located on chromosome 2 (2q24.3), spanning approximately 70kb and comprising 26 exons (Abbott, Baker et al. 1994; Boonacker, Wierenga et al. 2002). The 5' end features a 300bp sequence rich in cytosine and guanosine and has several transcription factor binding sites including NF-κB, AP2, Sp1 (GC boxes) and a butyrate-responsive element (BRE). (Bohm, Gum et al. 1995; Boonacker, Wierenga et al. 2002)

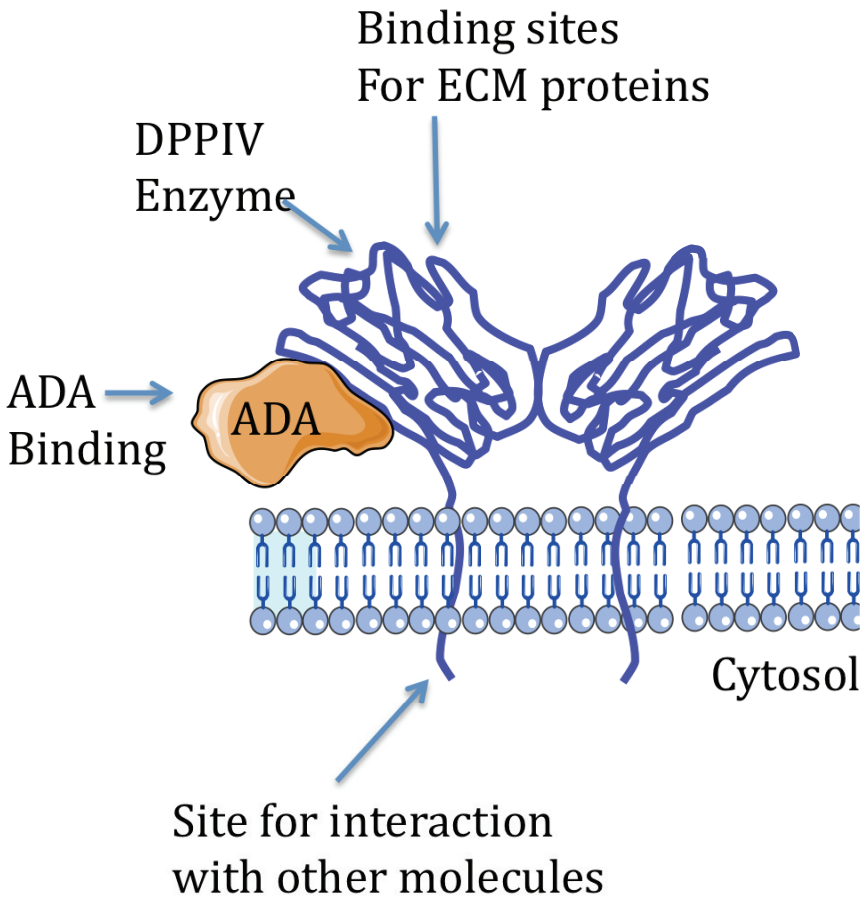


Figure 1.7 The domains and functions of CD26. Along with having intrinsic enzyme activity, CD26 forms homodimers and interacts with other proteins intra- and extra-cellularly. Produced with Servier Medical Art.

1.5.2 CD26 in T-cell activation

CD26 was initially studied as a marker of T-cell activation in the early 90's and as ADA complexing protein (ADACP) in other tissues (Dinjens, ten Kate et al. 1989; Schrader, West et al. 1994; von Bonin, Huhn et al. 1998). Direct evidence for its stimulatory properties in T-cells has been found using anti-CD26 antibodies against particular CD26 epitopes (von Bonin, Huhn et al. 1998). These antibodies, depending on the particular target, have been shown to either stimulate or suppress mitogen-activated T-cell proliferation *in vitro*. CD26 has also been shown to interact with CD45, a tyrosine phosphatase also involved in T-cell activation (Morimoto and Schlossman 1998). These various studies suggest that in lymphocytes, CD26 may play a role in regulating T-cell signaling through its interactions with CD45 (Torimoto, Dang et al. 1991; Tanaka, Kameoka et al. 1993; von Bonin, Huhn et al. 1998).

Levels of surface CD26 are generally very low on resting T-cells but increase 5-10 fold in response to antigenic, mitogen or interleukin stimulation (Hafler, Fox et al. 1986; von Bonin, Huhn et al. 1998; Gorrell, Gysbers et al. 2001). Activation of T-cells requires two independent signals, the first through the T cell receptor (TCR) complex and MHC complex on antigen presenting cells. The second signal is provided though a receptor-ligand interaction on T cells and the antigen-presenting cell (Ishii, Ohnuma et al. 2001). CD26 has been shown to be capable of generating the co-stimulatory signal on T cells (Dang, Torimoto et al. 1990; Tanaka, Kameoka et al. 1993). Cross-linking CD26 and CD3, a protein complex that forms a portion of the TCR complex, on T cells has been shown to induce co-stimulation of T cells and lead to IL-2 production, a potent T cell growth factor (Tanaka, Camerini et al. 1992; Morimoto and Schlossman 1998).

CD26 also provides a co-stimulatory signal to T-cells along with ligation of CD3 and CD2 (Dang, Torimoto et al. 1990). Ultimately, CD26 contributes to T-cell expansion by increasing cytokine stimulation and cell proliferation, inducing differentiation and upregulating other activation effectors (Morimoto and Schlossman 1998; Gorrell, Gysbers et al. 2001). More specifically, higher expression of CD26 is associated with a Th1-type immune response (Gorrell, Gysbers et al. 2001).

1.5.3 CD26 intrinsic enzyme activity

CD26 has an intrinsic peptidase enzyme activity, a type IV dipeptidyl peptidase. Homodimerization of CD26 has been shown to be necessary for this enzyme activity (Engel, Hoffmann et al. 2006; Kuhn, Hennig et al. 2007). This enzyme activity is sometimes also used to refer to the whole protein, 'DPP4' or 'DPPIV'. DPP4 is a serine aminopeptidase that preferentially cleaves oligopeptides with an alanine or proline at the penultimate position at the N-terminus, releasing the N-terminal dipeptide (Engel, Hoffmann et al. 2006; Kuhn, Hennig et al. 2007). The DPP4 catalytic triad is composed of Ser-630, Asp-708, and His-740 with the active site nucleophile located on hydroxyl radical of the serine-630 residue (Kuhn, Hennig et al. 2007).

Many chemokines, growth factors and neuropeptides possess the necessary motif for cleavage and are therefore substrates for DPP4. The cleavage of different substrates by DPP4 may alter their bioactivity, rendering the substrate either more active or inactive thereby affecting their selectivity in subsequently acting upon receptors (Bauvois 2004).

1.5.4 Functions of CD26

1.5.4.1 CD26 binds ADA

CD26 acts as the major binding protein for ecto-ADA. This particular function of CD26 was identified over thirty years ago leading it to be called ADA-binding (or complexing) protein (ADABP/ADACP) up until that point (Daddona and Kelley 1978). The complex of ADA and CD26 allows both proteins to maintain their individual catalytic activity. Through a series of point mutation experiments, Dong and colleagues were able to show that residues 340-343 in the cysteine-rich region of CD26 were necessary for ADA binding (Dong, Tachibana et al. 1997; De Meester, Korom et al. 1999). There are several possible implications for the existence of the complex between CD26 and ADA. Proliferating cells (as in a tumor) accumulate high extracellular adenosine concentrations. These concentrations of adenosine may be toxic or affect cellular proliferation depending on the subset of adenosine receptors expressed (Cordero, Salgado et al. 2009). CD26 binding of ADA is known to regulate extracellular levels of adenosine, overcoming this potential toxicity. The binding of ADA also contributes to local immune regulation in the tumor microenvironment (Blay, White et al. 1997; Tan, Mujoomdar et al. 2004). As mentioned, CD26 is expressed on T-cells and is a co-stimulator of T-cell antigen receptor-mediated activation (Kameoka, Tanaka et al. 1993; Morimoto and Schlossman 1998). It is thought that ADA binding to CD26 may be necessary for the costimulatory properties of CD26 (Kameoka, Tanaka et al. 1993; Morimoto and Schlossman 1998).

1.5.4.2 CD26 binds the proteins of the extracellular matrix

As mentioned, CD26 binds the ECM components collagen and fibronectin, suggesting a role for CD26 as a cell adhesion molecule. CD26 was initially implicated in facilitating activated T-helper cells binding to collagen type I (Hanski, Huhle et al. 1988; Loster, Zeilinger et al. 1995; Dang and Morimoto 2002). CD26 was also shown to be involved in rat hepatocyte spreading on collagen (Loster, Zeilinger et al. 1995; Antczak, De Meester et al. 2001). Loster et al were able to show that collagen type I and type III were the two major binding collagen partners of CD26, and that they bind to the cysteine-rich portion of CD26 (Loster, Zeilinger et al. 1995). CD26 does bind to collagens II, IV and V, but to a lesser degree. Loster et al observed that the binding specificity of CD26 resembles the ligand specificity of the main collagen receptors, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin (Loster, Zeilinger et al. 1995).

The interaction of CD26 with fibronectin has been reported to have several consequences, including facilitating its own interactions with collagen and acting as an adhesive molecule in cancer progression. This would serve to facilitate colonization of micrometastases in various types of cancer. Bauvois et al demonstrated that DPPIV inhibitors opposed rat hepatocyte spreading on fibronectin matrices while Piazza et al showed that rat heptocyte CD26 directly binds to fibronectin, possibly with a higher affinity than CD26 binds collagen (Piazza, Callanan et al. 1989; Bauvois 2004)

1.5.4.3 CD26 in malignant transformation

Loss of CD26 expression has been reported in a number of different cancers, including colorectal, lung, hepatocellular carcinoma and melanoma (Trotta and Balis

1978; Piazza, Callanan et al. 1989; Sedo, Krepela et al. 1991; Wesley, Tiwari et al. 2004). The best evidence we have for CD26's involvement in cancer progression came from a study by Houghton and Wesley et al in 1999. Houghton et al showed that while normal melanocytes express CD26, malignant melanoma cells do not. They demonstrated, by transducing the cells with the v-Ha-ras oncogene, that the loss of expression occurs during the malignant transformation of melanocytes. Re-introduction of the CD26 gene in malignant melanocytes resulted in the reversal of the malignant phenotype, including a change in cell morphology, inhibition of anchorage-independent cell growth, and a return of serum-dependence (Morrison, Vijayasaradhi et al. 1993; Wesley, Tiwari et al. 2004).

More recent reports suggest that the lack of CD26 in many melanomas is a result of promoter hypermethylation (McGuinness and Wesley 2008). Studies by Wesley et al indicated that CD26 expression was lost at the RNA level, but could be rescued with the use of a methyltransferase inhibitor, 5-AZA-Cdr. This study suggests a role for CD26 in melanoma initiation and progression (Wesley, Tiwari et al. 2004).

We now know that CD26 expression is variable in different cancers and this is not always a result of promoter methylation. Down-regulation of CD26 has been seen in renal cancers and colorectal cancers, but is seen to be upregulated in other cancers, such as glioblastoma (**Table 1.3**) (Ten Kate, Dinjens et al. 1986; Darmoul, Lacasa et al. 1990; Varona, Blanco et al. 2010).

A recent study by Pang et al suggested that colorectal cancer tumors might possess a subpopulation of cancer stem cells that are CD26 positive ($CD26^{+}$) (Pang, Law et al. 2010). While the presence of CD26 on liver metastasis is not surprising, results indicated that CD26 was only found to be expressed on two of ten primary tumors,

whereas it was expressed on all ten of the liver metastases (Pang, Law et al. 2010). The role of CD26 on a metastasis is likely different on a liver metastasis than on a primary tumor. Additionally, there is evidence to suggest that CD26 expression can be modulated by the microenvironment, and as the microenvironment is entirely different in the primary tumor versus the metastasis (Tan, Richard et al. 2006). The research by Pang et al suggests that CD26 may be a valid marker for cancer stem cells but does not solidify a link between CD26 and cancer metastasis (Pang, Law et al. 2010).

1.5.4.4 CD26 modulates cancer cell behavior

Previous work by Lowthers and Blay (unpublished data, 2006) indicated that various antitumor drugs, including 5-FU, IT, and OX, were able to up-regulate cell-surface CD26 expression on various colorectal carcinoma cells *in vitro*. Additionally, Tan et al indicated that the cell-surface expression of CD26 on colorectal carcinoma cells could be regulated by factors present in the solid tumor microenvironment (Tan, Mujoomdar et al. 2004). Adenosine was found to down-regulate CD26 and up-regulate CXCR4 in colorectal cancer cells (Tan, Mujoomdar et al. 2004; Richard, Tan et al. 2006). As a result, Richard et al showed that these changes in CXCR4 directly affected a cancer cell's ability to migrate towards CXCL12, a chemoattractant implicated in cancer metastasis (Richard, Tan et al. 2006). Low dose chemotherapy has been shown to up-regulate CD26 expression *in vitro* and *in vivo,* thereby regulating cancer cell behavior and local cancer cell invasion (Lowthers and Blay, unpublished).

Table 1.3 CD26 Expression in Cancer. CD26 has been shown to play a role in a variety of different cancers, as indicated below. The level of expression relative to normal tissue is also listed.

Malignancy	Expression in Malignant Tissue	References
Colon	Variable	(Ten Kate, Wijnen et al. 1985; Ten Kate, Dinjens et al. 1986)
Ovarian	Decreased	(Kajiyama, Shibata et al. 2006; Kajiyama, Shibata et al.)
Thyroid carcinoma	Increased	(Kotani, Aratake et al. 1991)
Non-small cell lung cancer	Decreased	(Asada, Aratake et al. 1993; Wesley, Tiwari et al. 2004)
Melanoma	Decreased	(Houghton, Albino et al. 1988)
Mesothelioma	Increased	(Inamoto, Yamada et al. 2007; Ghani, Yamazaki et al. 2010)
Hepatocellular carcinoma	Variable	(Stecca, Nardo et al. 1997)
B-chronic lymphocytic leukemia	Increased	(Bauvois, De Meester et al. 1999)
Non-Hodgkin's Lymphomas	Increased	(Carbone, Cozzi et al. 1994)

1.5.6 Inhibition of CD26 enzyme activity

1.5.6.1 Endogenous DPPIV inhibitors

To date, only one endogenous inhibitor of CD26 enzyme activity has been identified *in vitro* (Davoodi, Kelly et al. 2007). Glypican-3 is a member of a family of six different glypicans. Glypicans are heparan-sulfate proteoglycans that are glycosylphosphatidyl inositol (GPI)-anchored to the plasma membrane, and are found to be upregulated in many tumor tissues (Baumhoer, Tornillo et al. 2008; Cordero, Salgado et al. 2009). Glypicans have been found to play a role in many cell processes, including cell adhesion, migration, proliferation etc (Woods, Oh et al. 1998; Song and Filmus 2002; Davoodi, Kelly et al. 2007). In fact, patients with Simpson-Golabi-Behmel syndrome, a syndrome in which glypican-3 is absent or mutated, are more susceptible to tumor development (Xuan, Besner et al. 1994; Okamoto, Yagi et al. 1999; Davoodi, Kelly et al. 2007).

1.5.6.2 DPPIV inhibitors in clinical use

CD26 has a diverse set of substrates including various incretins that are released in response to food and play a role in glucose homeostasis (Drucker, Easley et al. 2007). Two particular hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) stimulate glucose-dependent secretion of insulin from islet beta cells (Drucker, Shi et al. 1997; Drucker, Easley et al. 2007). The DPPIV activity of CD26 cleaves and inactivates GLP-1 and GIP, leading to a very short half-life of both incretins *in vivo* (Ahren, Simonsson et al. 2002). Inhibition of DPPIV will

therefore prolong GLP action and maintain insulin levels.

A variety of DPPIV inhibitors are currently on the market to help treat type-2 diabetes and are effective in lowering the blood glucose levels in patients. The first of these inhibitors is Sitagliptin (Januvia®), produced by Merck. Sitagliptin is a highly selective DPPIV inhibitor that exhibits very little affinity for two similar DPP enzymes, DPP8 and DPP9 (Lankas, Leiting et al. 2005). Sitagliptin incorporates a beta amino acid moiety that allows it to fit into the active site of DPPIV. It is a potent, reversible competitive inhibitor of DPPIV enzyme activity. DPPIV inhibitors have been shown to inhibit up to 90% of plasma DPPIV activity (Vilsboll, Krarup et al. 2001).

As a result of the involvment of CD26 in cancer progression, there is some speculation that use of DPPIV inhibitors may lead to an increased risk of certain cancers. In many cancers, CD26 acts to oppose tumor progression. At study by Wesley et al showed that the down-regulation of DPPIV enzyme activity might contribute to the loss of growth control in non-small cell lung carcinoma (NSCLC) (Wesley, Tiwari et al. 2004). This was also shown in prostate cancer, whereby DPPIV blocked the bFGF-signaling pathway (Wesley, Tiwari et al. 2004).

More recently, Elashoff et al published a study citing the hazards of using sitagliptin alongside GLP-1 therapy (Elashoff, Matveyenko et al. 2011). They found that there was an increased incidence of thyroid cancer in patients taking sitagliptin versus other therapies. Patients taking sitagliptin had a 6-fold increase in the odds ratio for developing pancreatitis, possibly increasing the likelihood of developing pancreatic cancer (Elashoff, Matveyenko et al. 2011).

1.5.7 CD26 regulates the CXCL12:CXCR4 axis

CD26, through its DPP4 activity, removes two amino acids from the N-terminus of its substrates. This cleavage often results in changing in the substrate's functional activity or affinity/binding to the receptor (Baggiolini 1998; Christopherson, Hangoc et al. 2002).

CD26 cleaves the chemokine CXCL12 (SDF-1α) by removing an N-terminal dipeptide (Christopherson, Hangoc et al. 2002). CXCL12 and its GPCR receptor, CXCR4, are involved in hematopoietic cell trafficking and lymphoid tissue structuring (Nagasawa, Tachibana et al. 1998). Different knockout models of either CXCR4 or CXCL12 display embryonic lethality or impaired development (Ma, Jones et al. 1998; Ratajczak, Zuba-Surma et al. 2006). In adults, the CXCR4:CXCL12 axis plays a major role in hematopoietic stem cell homing to the bone marrow and lymphocyte migration (Christopherson, Hangoc et al. 2002).

Together with its receptor CXCR4, CXCL12 has been shown to play a major role in tumorigenesis, progression and angiogenesis in many cancers (Wang, Deng et al. 1998; Schrader, Lechner et al. 2002). CXCL12 is constitutively expressed in bone marrow stromal cells, and a variety of organs such as the brain, lungs, liver and kidney that are frequent sites of cancer metastasis (Nagasawa, Kikutani et al. 1994; Jo, Rafii et al. 2000; Schrader, Lechner et al. 2002). Disruption of the CXCL12:CXCR4 axis by CD26 enzyme activity has been shown to regulate prostate, breast and cancer metastasis (Sun, Pedersen et al. 2008)..

1.6 Matrix Metalloproteases

1.6.1 Introduction and structure

Physical changes in the tumor microenvironment that result from changes between cells and the ECM are known to regulate tumor cell behavior (Rapier, Huq et al.). These changes in the surrounding environment have been shown to regulate cell adhesion, migration, survival and cell growth (Lampin, Warocquier et al. 1997; Ranucci and Moghe 2001; Chung, Liu et al. 2003) through altering the interaction of integrins with the extracellular scaffolding surrounding cells. This scaffolding is referred to the extracellular matrix (ECM) and is composed of many proteins, including fibers such as fibronectin, collagen, and various types of proteoglycans. The process of ECM proteolysis and remodeling is tightly regulated in normal tissues (Radisky and Radisky). Dysregulation of this activity can contribute to disease pathogenesis.

Matrix metalloproteases, or MMPs, are a family of enzymes that are capable of cleaving ECM proteins, thereby regulating cell-matrix interactions. They are a family of zinc-dependent endopeptidases that is comprised of 23 members. There is a high degree of structural and functional similarity among members (Chakraborti, Mandal et al. 2003). MMPs contain several domains. The propeptide domain is approximately 80 amino acids in length and contains a conserved cysteine involved in enzyme activation (Bode, Gomis-Ruth et al. 1993). The catalytic domain is approximately 170 amino acids and contains a cysteine residue and zinc-binding motif. The catalytic domain links to a hemopexin domain through a hinge region. The hemopexin domain is approximately 200 amino acids long and MMPs-7, 23, 26 lack this domain (Knauper, Cowell et al. 1997; Murphy

and Knauper 1997; Nagase and Woessner 1999). This particular domain acts primarily as a recognition sequence for the substrates (Knauper, Cowell et al. 1997; Murphy and Knauper 1997). Two gelatinases, MMP-2 and MMP-9, contain fibronectin-like repeats allowing them to interact with denatured type I collagen and various other native collagens (Murphy and Knauper 1997; Klein and Bischoff 2011).

Based on their substrate specificity, MMPs can be categorized in six different groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and others (**Table 1.4**) (Kadoglou and Liapis 2004).

1.6.2 Regulation of MMP activity

As previously mentioned, dysregulation of MMP activity can contribute to the pathogenesis of many diseases and is therefore is tightly regulated. MMP activity is regulated at three different levels: the transcriptional level, at the level of pro-enzyme activation, and at inhibition of enzyme activity by complexing with their endogenous inhibitors such as the tissue inhibitors of metalloproteases (TIMPs) (Kadoglou and Liapis 2004).

Transcriptional regulation of MMPs can occur by a diverse number of factors. These factors include growth factors [TGF-β, EGF], cytokines [IL-6, IL-1β] and hormones [estrogen, corticosteroids] and are able to regulate MMP gene expression in many systems (Dollery, McEwan et al. 1995; Thompson and Parks 1996; Kadoglou and Liapis 2004).

Table 1.4 Matrix metalloproteases and their substrates.

MMPs	Enzyme	Substrates
Collagenases		
MMP-1	Collagenase-1	Collagens (I-III, VII,VIII,X), gelatin, MMP-2,-9
MMP-8	Collagenase-2	Collagens (I-III,V,VII,VIII, X), gelatin
MMP-13	Collagenase-3	Collagens (I-IV, IX,X,XIV), gelatin, fibronectin, MMP-9
MMP-18	Collagenase-4	Collagens (I-III)
Gelatinases		
MMP-2	Gelatinase A	Gelatin, collagens, elastin, fibronectin, MMP-1,-9,-13
MMP-9	Gelatinase B	Gelatin, collagens, elastin, fibronectin, plasminogen
Stromelysins		
MMP-3	Stromelysin-1	Collagens (III-V, IX,X), gelatin, fibronectin, MMP-1,-7,-8,-9,-13
MMP-10	Stromelysin-2	Collagens (III-V), gelatin, casein, MMP-1,-8
MMP-11	Stromelysin-3	Gelatin, collagen IV, fibronectin, casein
Matrilysins		
MMP-7	Matrilysin-1	Collagens (IV,X), gelatin, fibronectin, MMP-1,-2,-9, MMP-9/TIMP-1 complex
MMP-26	Matrilysin-2	Collagen IV, fibronectin, gelatin, proMMP-9, fibrinogen
Membrane Type (MT)		
MMP-14	MT1-MMP	Collagens (I-III), gelatin, elastin, casein, fibronectin, laminin, MMP-2,-13
MMP-15	MT2-MMP	Gelatin, fibronectin, laminin, MMP-2
MMP-16	MT3-MMP	Collagen III, gelatin, casein, fibronectin, MMP-2
MMP-17	MT4-MMP	Gelatin, proMMP-2
MMP-24	MT5-MMP	Proteoglycans, proMMP-2, collagen I, gelatin, fibronectin
MMP-25	MT6-MMP	Collagen IV, proMMP-8, proMMP-9

Adapted from Kadoglou and Liapis 2004.

The inhibition of MMP enzyme activity is achieved through the binding of MMPs to various endogenous inhibitors. The TIMPs are a family of homologous proteins and are the most important inhibitors of MMPs. The TIMPs bind either latent or active MMPs to inhibit their activity in a 1:1 stoichiometric ratio (Dollery, McEwan et al. 1995; Visse and Nagase 2003). A change in the balance of MMPs:TIMPs determines net proteolytic activity and may result in diseases associated with unregulated matrix turnover, including fibrosis, nephritis and cancer (Knox, Sukhova et al. 1997; Brew, Dinakarpandian et al. 2000; Kadoglou and Liapis 2004).

1.6.3 Tissue inhibitors of metalloproteases (TIMPs)

Currently, four known TIMPs (TIMPs 1-4) have been identified in vertebrates. They are a family of small proteins, ranging from 21-28kDa (Kleiner and Stetler-Stevenson 1999). TIMPs are proteins composed of two domains, with each domain is stabilized by three conserved disulfide bonds (Brew, Dinakarpandian et al. 2000). The N-terminal domain is the larger of the two at approximately 125 residues, while the C-terminal domains are generally much smaller, at 65 residues. They form tight, non-covalent bonds with MMPs to inhibit their proteolytic activity (Gomis-Ruth, Maskos et al. 1997).

While there is a high degree of sequence homology among the four TIMP isoforms, there are differences in their inhibitory properties (Liu, Wang et al. 1997; Amour, Slocombe et al. 1998). The affinity of the four TIMPs to the many MMPs also varies. For example, TIMP-1 binds to and inhibits most secreted MMPs, but poorly inhibits any

of membrane-type MMPs (MT-MMP) (Goldberg, Wilhelm et al. 1986; Cossins, Dudgeon et al. 1996).

Interestingly, the TIMPs have been shown to play MMP-independent roles in promoting and inhibiting cell proliferation as well as inhibiting and promoting apoptosis in different systems (Stetler-Stevenson 2008). TIMP-2 has been shown to play an MMP-independent role in angiogenesis. TIMP-2 was shown to inhibit endothelial cell proliferation *in vitro* and angiogenesis *in vivo* in the presence of VEGF or FGF-2. This was found to be a result of the activation of an integrin-signaling pathway; TIMP-2 interacting with a3b1 (Seo, Kim et al. 2008). TIMP-3 also has anti-angiogenic activity, although the mechanism is different from that of TIMP-2. TIMP-3 is thought to bind directly to the VEGFR, antagonizing the receptor (Bourboulia and Stetler-Stevenson).

TIMP-1 has been shown to be over-expressed in a variety of malignancies, including breast cancer and CRC. It promotes tumorigenesis through unknown mechanisms (Bigelow, Williams et al. 2009). Overexpression of TIMP-1 has been shown to enhance MAPK and subsequently, AKT phosphorylation in breast cancer cells *in vitro*. *In vivo*, over-expression of TIMP-1 was shown to increase tumor size and tumor vessel density (Bigelow, Williams et al. 2009).

1.6.4 Roles of MMPs in modulating growth factors and bioactive peptides

Many growth factors, such as TGF-β, VEGF and bFGF can be tethered to the ECM. In particular, these three growth factors have been shown to be released in their biologically active form by MMPs. TGF-β can be tethered to extracellular plasma

membrane in a complex formed of TGF-β-latency associated protein and latent TGF-β-binding protein (LTBP), which can be released by either MMP-2 or MMP-9 (Yu and Stamenkovic 2000; Page-McCaw, Ewald et al. 2007). MMP-9 was shown to enhance endothelial cell migration towards ovarian carcinoma conditioned medium as a result of increased release of the biologically active form of VEGF from the ECM (Coussens, Fingleton et al. 2002; Belotti, Paganoni et al. 2003). MMP-2 has been shown to release active bFGF from the ECM (Tholozan, Gribbon et al. 2007). Additionally, it has been found that MMPs may reveal cryptic sites in ECM and cellular proteins. Cleavage of proteins, such as E-Cadherin and CD44, result in the release of biologically active fragments that have been shown to promote tumor cell invasiveness (Noe, Fingleton et al. 2001; Page-McCaw, Ewald et al. 2007). The MMP-7 and MMP-3 cleave E-cadherin on the cell surface and release a soluble form, sE-CAD, into the extracellular fluid. This soluble fragment is thought to inhibit the functions of E-cadherin in a paracrine way. This was shown by inducing cell invasion into collagen type I and inhibit E-cadherin-dependent cell aggregation (Noe, Fingleton et al. 2001). Cleavage of several types of collagen by MMPs has been shown to reveal cryptic sites in the fragments of collagen produced. Collagen 18 and collagen type IV produce fragments following MMP proteolysis that have anti-angiogenic effects (Ortega and Werb 2002; Hamano, Zeisberg et al. 2003; Sund, Hamano et al. 2005). Additionally, collagen type II produces a fragment that antagonizes the bone morphogenic protein (BMP) signaling pathway (Larrain, Bachiller et al. 2000; Ortega and Werb 2002).

1.6.5 MMPs and TIMPs as biomarkers in colorectal cancer

Until relatively recently, one of the only validated plasma biomarker for colorectal carcinoma was carcinoembryonic antigen (CEA) (Duffy, van Dalen et al. 2007). Now, studies are looking at plasma levels and high tumoral levels of TIMPs and MMPs in correlation with poor outcome in CRC patients (Birgisson, Nielsen et al. 2010). High TIMP-1 levels are directly correlated with poor prognosis in breast cancer (Bigelow, Williams et al. 2009). Pre-operative plasma TIMP-1 levels have been shown to be a better prognostic indicator than CEA levels in CRC (Birgisson, Nielsen et al. 2010).

A recent study by Yamada et al indicated that TIMP-1 and MMP-7 gene expression levels are elevated in colorectal tissue, compared to normal adjacent tissue. MMP-13 activity has also been associated with poor prognosis in colorectal cancer (Leeman, McKay et al. 2002; Yamada, Oshima et al.). Additionally, elevated MMP-13 gene expression was shown to be predictive of liver metastases in patients with CRC (Yamada, Oshima et al. 2010).

1.6.6 MMP inhibitors in cancer therapy

As a result of the role they play in cancer progression and metastasis, MMPs would seem to be a good target for cancer therapy. Many pharmaceutical companies developed targeted MMP inhibitors and were tested in phase III clinical trials, only to have them fail. None of the trials exhibited evidence of significant efficacy (Fingleton 2008). Despite that, many researchers are concerned that the failure of the overall clinical trials may have over-shadowed some important findings (Dove 2002).

Studies have largely focused on the use of broad-spectrum MMP inhibitors to block angiogenesis, as the MMP inhibitors on the market were notoriously non-specific. While MMPs are involved in this process, there are many other enzymes that play a role in the angiogenesis, and it has been argued that only targeting one subset of enzymes is not the best approach.

In the past few years, there have been some major advances in the design of MMP inhibitors, with new hydroxamate-based inhibitors. Still, MMP inhibitors remain notoriously non-specific and there are few currently in use or in clinical trials.

Perspective

The influence of the tumor environment is multifaceted. The behavior of the cancer cells is influenced firstly by soluble factors released into that environment. Thus, several mediators such as chemokines, growth factors, and cytokines are released from different cell types in the tumor cell population and act to activate or inhibit cancer cell function. Fibroblasts and endothelial cells are thought to release substances that promote tumor growth and progression. In addition, other small-molecular-weight substances are released because of changes in cellular metabolism or in response to host defense mechanisms. These include such molecules as adenosine, lactic acid, and more complex families of products such as eicosanoids. In addition to these soluble mediators there are secreted molecules that are larger proteins with specific functions that act more locally to influence cell behavior. One key example of such a group is the MMPs and their endogenous inhibitors, the TIMPs. Finally, the actual topology of the cell population, and

the relationships between the different cell types are important. It cannot be presumed that cancer cells behave in a tumor in the same way that they do in a cultured cell monolayer.

These different influences are not independent, but can interact. For example, the presence of different cell types in the microenvironment contributes different types of soluble mediators. It is therefore important to study the tumor microenvironment in its whole context, with these different influences in one coherent perspective. In this research, I have therefore aspired to examine the different kinds of microenvironmental influences, hoping to gain some insight into the relative importance of different factors and how they might join together in promoting or hindering cancer progression.

Objectives

1. Determine how secreted products, including the substrates and by-products of glycolysis such as glucose and lactate, affect cell-surface expression of the multifunctional CD26 protein.

2. Determine if the production of adenosine, as a result of hypoxia, acts on different populations of cells in the tumor microenvironment to potentially facilitate or hinder cancer progression.

3. Determine if soluble mediators, such as those released by cell types in the tumor microenvironment, support stromal cell growth and the production of matrix metalloproteases, thereby priming the microenvironment for cancer progression.

4. Determine if the aforementioned soluble factors, together with a 3-dimensional cell topology, function together in affecting the cellular responses to chemotherapeutic drugs.

Hypothesis

Within the harsh local environment of a solid tumor, the extracellular scaffold and the fluid milieu produce a cooperative response to the adverse conditions amongst the different cell populations, promoting a metastatic phenotype in colorectal cancer cells.

CHAPTER 2 Materials and Methods

2.1 Materials

The HS675.T primary tumor associated fibroblasts, the Met-5a mesothelial cell line, and the HRT-18 and HT-29 colorectal carcinoma cells were purchased from American Tissue Type Collection (Manassas, VA). Cell culture vessels (NUNC) and other materials including media, sera, TRIzol® reagent, dNTP mix, 5X First strand buffer, M-MLV, DTT, oligo$(dT)_{12\text{-}18}$ primer and custom primers were purchased from Invitrogen (Burlington, ON, Canada). Brilliant SYBR Green QPCR kits were purchased from Stratagene (Cedar Creek, TX). Transwell® polycarbonate (8-µm pore) inserts were from Corning (Corning, NY). [^{3}H-*methyl*]-thymidine was from Amersham Biosciences (QC, Canada). Mouse anti-human CD26 antibody (clone M-A261) was from Cedarlane Laboratories Ltd (Burlington, ON, Canada). ^{125}I-labelled goat anti-mouse IgG was purchased from Perkin Elmer Life Sciences (Boston, MA). CXCL12 was from Chemicon International (Temecula, CA). Alexa fluor 488 goat anti-mouse was IgG was purchased from Molecular Probes (Eugene, OR). MMP-13 goat anti-mouse IgG was purchased from R&D Systems (Minneapolis, MN). Oxaliplatin, collagen Type IV and bovine serum albumen (BSA) were from Sigma-Aldrich (St. Louis, MO), and Irinotecan and 5-FU were from Mayne Pharma (Montreal, QC, Canada).

2.2 Cell lines

Four different cell lines were used: HS675.T primary tumor associated fibroblasts, Met-5a mesothelial cells, and HRT-18 and HT-29 colorectal carcinoma cells. According

to ATCC materials, HS675.T cells were isolated from a human colorectal tumor and the Met-5A cells were isolated from the ascites fluid of a healthy donor. HRT-18 cells were initially isolated from an ileocecal tumor while the HT-29 cells were derived from a colon tumor. Both colorectal carcinoma cell lines are poorly metastatic (Wagner, Toth et al. 1992; Haier, Nasralla et al. 1999).

2.3 Cell culture

Cells were cultured in 75-cm^2 flasks containing a total volume of 20 ml of Dulbecco's Modified Eagle Medium (DMEM). Colorectal carcinoma cells were grown with either 5% (v/v) or 10% (v/v) newborn calf serum (NCS), while both the Met-5A and HS675.T cells were grown in 10% (v/v) fetal calf serum (FCS). The cells were maintained at 37°C in a humidified atmosphere with an oxygenated gas mixture containing 10% CO_2. Cells were regularly sub-cultured following exposure to 5 ml of Tryple Express (Invitrogen).

Cells were seeded at varying densities for experiments. For binding assays in a 48-well plate, and real time quantitative RT-PCR in 6-well plates, cells were seeded at a density of 100,000 cells per ml in 0.5 ml (48-well plate) and in 2.0 ml (6-well plate) DMEM containing 10% (v/v) NCS. For spheroid binding assays with the colorectal carcinoma cells, cells were seeded at a density of 500,000 cells per ml in 0.2 ml DMEM in a 96-well plate.

2.4 Monolayer radioantibody binding assay

Cell-surface protein expression of CD26 in both monolayer cell culture and multicellular tumor spheroids was quantified using a radioantibody-binding assay. The assay was carried out at 4 ºC.

For monolayer cell cultures, the assays were carried out in 48-well plates, 48 h post-treatment. The cells were washed with cold phosphate-buffered saline (PBS) containing 0.2% (w/v) bovine serum albumin (BSA). Cells were then incubated for one hour in 125 µl of PBS containing 1% (w/v) BSA and 1 µg/ml of CD26 antibody (clone M-A261) or isotype control. Cells were washed twice with 250 µl PBS containing 0.2% (w/v) BSA. Following the washes, cells were incubated in the secondary I^{125}-conjugated antibody, 1µCi/ml, in 125 µl PBS containing 1% (w/v) BSA. Cells were washed twice with 250 µl PBS containing 0.2% (w/v) BSA, and then solubilized in 500 µl 0.5M NaOH at room temperature. Following solubilization, the NaOH was transferred to a tube and the radioactivity read in a gamma-counter. A parallel set of plates was used to perform cell counts to account for the toxicity of any of the reagents used. Cells were briefly exposed to 0.05% (w/v) trypsin containing 0.53 mM ethylenediamine tetraacetic acid (EDTA) and counted using a Coulter Model ZM30383 particle counter (Beckman Coulter, Mississauga, Canada). Subtracting the corresponding isotype controls and correcting for cell number determined the CD26-specific radioactivity.

2.5 Spheroid cell culture

U-bottom 96-well plates were coated with 50µl of 2.5% agarose in 0.5g/l glucose, 1% NCS medium. The plates were left uncovered in a biosafety cabinet for approximately 30 minutes to allow the agarose to solidify. The plates were then covered and placed in a cell culture incubator at 37°C for 1-2 h. HRT-18 or HT-29 cells were seeded at a density of 5 x 10^4 cells in 100µl of 0.5g/l glucose 1% (v/v) NCS DMEM. Plates were placed in the incubator until spheroid formation occurred 7-10 d later.

We did attempt to analyze the histology of our multicellular tumor spheroids using paraffin embedding but we were unable to get good sections because of the compactness of the tissue. However, we were able to section the spheroids for electron microscopy following resin embedding, and this showed that the spheroids had a multicellular exterior and necrotic center (see **Figure 2.1**).

2.6 Spheroid radioantibody binding assay

The spheroid assays were performed in a 96-well conical bottom plate, 72-h post-treatment. Six spheroids were pooled per replicate. One hundred microliters of TrypLE™ Express enzyme was added to each replicate well to allow the spheroids to dissociate. Once dissociated, cell suspensions were centrifuged at 4 ºC at 500×g for 10 min in a plate spinner. The enzyme was aspirated and the cell pellets were washed and re-suspended in 150µl PBS containing 0.2% (w/v) BSA, and centrifuged again. Cells were re-suspended in 125 µl of PBS containing 1% (w/v) BSA and 1 µg/ml of CD26 antibody or isotype control at 4ºC for one hour. Two washes of 150µl PBS containing 0.2% (w/v) BSA were performed as previously described. The cells were incubated in the secondary I^{125}-

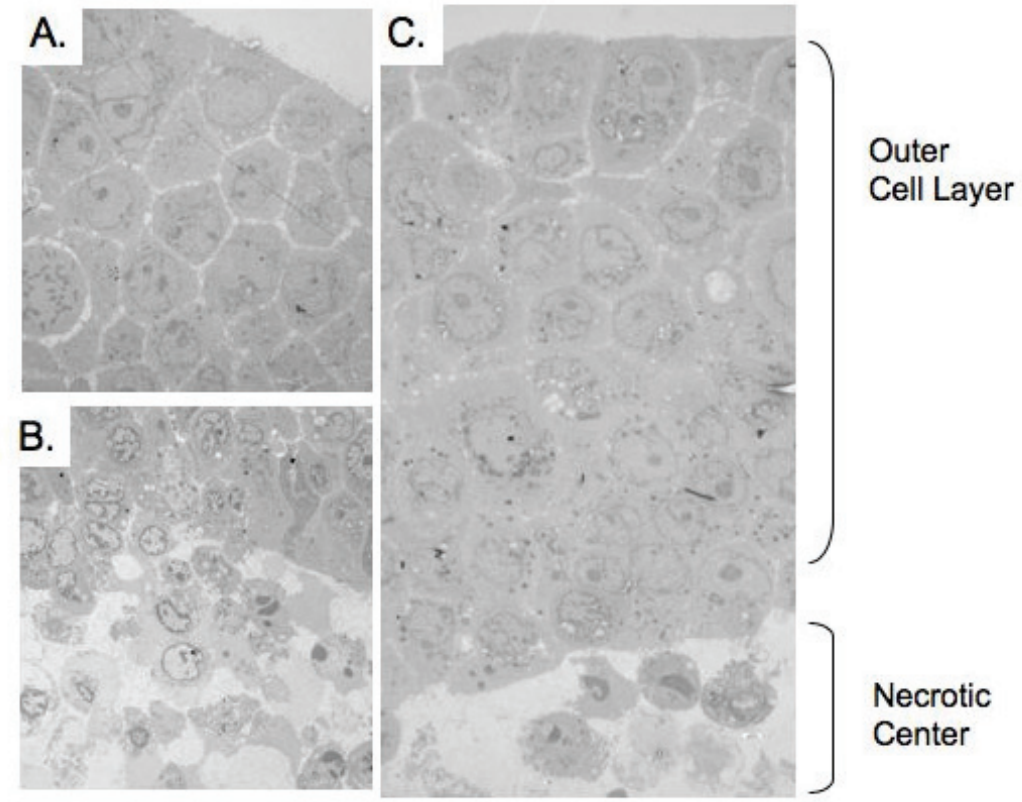


Figure 2.1 Transmission Electron Microscopy of different sections of an HRT-18 spheroid. Panel A) is a close-up of the layers of cells composing the outer rim of the spheroid, B) shows the span of the outer cell layer to the necrotic lumen and C) illustrates the necrotic lumen of a spheroid.

conjugated antibody, 1μCi/ml in 125μl PBS containing 1% (w/v) BSA for one hour and then washed three times. Cells were solubilized in 0.5M NaOH and the radioactivity read in a gamma counter. A parallel set of plates was used to perform cell counts to account for the toxicity of any of the reagents used. Cells were briefly exposed to 0.05% (w/v) trypsin containing 0.53 mM EDTA and counted using a Coulter Model ZM30383 particle counter (Beckman Coulter, Mississauga, Canada). Subtracting the corresponding isotype controls and correcting for cell number determined the CD26-specific radioactivity.

2.7 Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was harvested by following the TRIzol® RNA extraction protocol described by Chomczynski et al (Chomczynski and Sacchi 1987). Two micrograms of RNA was reverse transcribed to complementary DNA (cDNA) using oligo-dT_{12-18} primer and M-MLV reverse transcriptase. cDNA was frozen at -20 °C until further use.
For the polymerase chain reaction (PCR), the cDNA was combined with Omega 2X GoTaq, and forward and reverse primers for the gene of interest. A list of primer sequences follows. Samples were amplified for a pre-determined amount of cycles depending on the gene of interest. Samples were then run at 90V in a 1.5% (w/v) agarose gel in TAE buffer containing ethidium bromide. The gel was placed in a UV transilluminator and photographed. A change in the signal corresponding to band intensity under UV light was then quantified with ImageJ.

2.8 Real-time quantitative RT-PCR

RT-PCR for MMP-13 and CXCL12 mRNA was performed after a 72-hour treatment. Following time course experiments, it appeared that the effect of adenosine on MMP-13 mRNA peaked at 72 hours following treatment. One microgram of RNA was reverse transcribed to cDNA using M-MLV enzyme (Invitrogen, Oakville, Canada) as previously described. cDNA and Brilliant SYBR Green were combined and amplified in a Stratagene Mx3000P thermocycler (Cedar Creek, Texas). The following primer sets were used for CD26, MMP-13, MMP-7, CycA, GAPDH, CXCL12, A1, A2A, A2B, and A3.

GAPDH	F: 5'-CATGAGAAGTATGACAACAGCCT-3'
	R: 5'-AGTCCTTCCACGATACC-3'
CXCL12	F: 5'-GAGCTACAGATGCCCATGC-3'
	R: 5'-CTTTAGCTTCGGGTCAATGC-3'
CXCR4	F: 5'-GCCTGAGTGCTCCAGTAGCC-3'
	R: 5'-TGGAGTCATAGTCCCCT-3'
MMP-13	F: 5'- TTGAGCTGGACTCATTGTCG-3'
	R: 5'- GGAGCCTCTCAGTCATGGAG-3'
CD26	F: 5'-CTGACAGTCGCAAAACTTACACT-3'
	R: 5'-TGAGCTGTTTCCATATTCAGCAT-3'
CycA	F: 5'- TTCATCTGCACTGCCAAGAC-3'
	R: 5'- TCGAGTTGTCCACAGTCAGC-3'
A1	F: 5'-CATTGGGCCACAGACCTACT-3'
	R: 5'-AGTAGGTCTGTGGCCCAATG-3'

A2A	F: 5’-GGGGTACCAGTGGAGGGAGTGC-3’
	R: 5’-CCTTAGGAAGGGGCAAACTC-3’
A2B	F: 5’-AATGAAAGCTGCTGCCTTGT-3’
	R: 5’-GCTGGCYGGAAAAGAGTGAC-3’
A3	F: 5’-ACGGTGAGGTACCACAGCTTGTG-3’
	R: 5’-ATGTAAAAATCCCTTGGCCC-3’

Relative gene expression was analyzed using Ct values from the manufacturer’s software and standardized to GAPDH or CycA, depending on the experiment. These values were normalized to the appropriate control using the ΔΔCt method and graphed using Prism (Livak and Schmittgen 2001).

2.9 Cell proliferation assay

Cell growth was measured using a [*methyl*-^{3}H]- thymidine incorporation assay. Two days after seeding, cells were downshifted to a 1% (v/v) serum DMEM for two days. On day four, cells were treated with a reagent or the appropriate control. Radiolabeled thymidine and non-labeled thymidine were added to the wells at concentrations of 1μCi/ml and 1μM, respectively. Cells were assayed 36-48 h following treatment. Treatment time varied depending on the doubling time of the cells. Cells with a shorter doubling time, such as mesothelial cells, were incubated with thymidine for a shorter period of time than cells with a long doubling time, such as the HS675.T cells. It is the cumulative increase or decrease in radioactive thymidine over the time period that is measured.

The plates were removed from the incubator, placed on ice and allowed to cool. Medium was aspirated from the wells and the cells were washed twice with ice-cold PBS. The PBS was aspirated and 10% (w/v) trichloroacetic acid (TCA) was added to each well and left for one hour on ice. The TCA was aspirated and the wells were washed in 100% EtOH to dissolve any remaining TCA. After allowing the wells to dry, 500µl of 0.5µM 1% SDS (w/v) was added to each well placed on a shaker at RT for approximately an hour. The contents of the wells were transferred to a vial containing 2.5ml of acidified scintillation fluid. Vials were shaken and the radioactivity was read in a Beckman LS5000TA scintillation counter (Beckman Coulter Canada).

2.10 Conditioned medium

Conditioned medium was derived from a variety of cell types, including colon carcinoma cells HT29 and HRT-18, primary CAFs HS675.T, primary endothelial HUVECs, and mesothelial Met-5a cells. Cells were seeded in the appropriate growth medium and allowed to grow to 60% confluence. The medium was aspirated and replaced with fresh 0.2% (v/v) NCS or 0.2% (v/v) FCS DMEM. After 24 h, the medium was collected in and frozen at -80°C until use. Conditioned media from HUVECs were generated by Bassma Bseso and frozen at -80°C until use.

2.11 Immunofluorescence assays for CD26

Immunofluorescence was used to visually assess CD26 expression. HT-29 or Met-5a cells were seeded 35,000 cells per well in an 8–well chamber slide. When the cells reached 60% confluence they were assayed.

Cells were placed on ice and rinsed with cold PBS. PBS was aspirated and 120µl of the primary antibody, mouse anti-human α-CD26 (clone M-A261), or control, mouse IgG1 (Serotec MCA928), in PBS containing 1% (w/v) BSA. Cells were incubated for one hour at room temperature and washed three times with PBS. Each well received 120 µl per well of Alexa 488-antimouse and incubated for one hour at RT. Wells were washed with PBS and mounted.

2.12 Statistical analysis

Each figure shows a representative result of several experiments that were repeated on at least three independent occasions. Data are represented as the mean values +/- SEM. For unpaired data, results were analyzed using a two-tailed Student's *t*-test. Where appropriate, an analysis of variance (ANOVA) was performed followed by a Dunnet's or Bonferroni post-test to confirm statistical significance in relation to control samples. All analyses were done using Prism Graphpad software.

CHAPTER 3 Results

3.1 The Role of the Microenvironment

The physiology of tumor tissue differs from that of normal tissue, with irregular vasculature, localized regions of hypoxia and low nutrient levels; this often leads to regions of low glucose. With tumor cells preferentially utilizing glycolysis instead of oxidative phosphorylation, this leads to an accumulation of lactate (Warburg 1956). This shifts the local pH resulting in slightly acidic extracellular fluid and an additional stress on the cells (Mahoney, Raghunand et al. 2003; Walenta and Mueller-Klieser 2004).

3.1.1 High glucose conditions increase basal levels of CD26 cell-surface expression in CRC cells

Culture of cancer cells in DMEM provides an ambient glucose concentration of 4.5 g/l. This is high compared to physiological conditions where blood glucose is usually in the 1g/l range (D'Souza, Shertzer et al. 2003), and much higher than in established solid tumors (Toyokuni, Okamoto et al. 1995; Brown and Bicknell 2001). I wished to examine whether different glucose levels affected CRC cell behavior, particularly in the context of CD26, which is the central regulator of interest in our studies. HRT-18 or HT-29 cells were therefore grown in media containing either 0.5g/l (low), 1.5.g/l (intermediate) or 4.5g/ (high) glucose and CD26 cell-surface expression was assessed.

The upper panel of **Figure 3.1.1** shows that CD26 cell-surface expression on HT-29 cells increases significantly with the higher concentration of glucose. Similarly for HRT-18 cells (**Figure 3.1.1** lower panel) there is a significant increase in CD26 cell-surface expression in high glucose conditions. Growing HT-29 and HRT-18 cells in low-

glucose medium typically reduced CD26 protein expression by 45% and 30%, respectively, compared with our normal culture conditions.

3.1.2 High lactate has no effect on basal levels of CD26 cell-surface expression in CRC cells grown in monolayer

Excess lactate is produced in an hypoxic environment as oxygen-deficient cells utilize anaerobic glycolysis (Hsu and Sabatini 2008). This results in an excess of lactate and low pH locally in the extracellular fluid (Izumi, Torigoe et al. 2003).

I therefore investigated the CD26 expression on HRT-18 or HT-29 cells that were grown in medium supplemented with different concentrations of lactate. **Figure 3.1.2** shows the results for HT-29 cells grown in monolayers. The CD26 protein level at the cell surface was not affected by lactate concentrations up to 20 mM (**Figure 3.1.2**, HT-29 cells) or 100mM for HRT-18 cells (data not shown). Elevating the concentration of lactate to levels at or beyond the levels present in solid tumors (Brizel, Schroeder et al. 2001; Walenta and Mueller-Klieser 2004) therefore has no effect on CD26 cell-surface protein on CRCs grown in monolayer.

3.1.3 Lactate decreases basal levels of CD26 cell-surface expression in a three-dimensional model of colorectal carcinoma

Parallel studies within my project looked at the behavior of CRCs in a spheroid culture model. A three-dimensional model is more topologically relevant than a typical monolayer model. This approach revealed an interesting topology dependence of the influence of lactate on this aspect of cell behavior. HRT-18 or HT-29 cells were grown as multicellular spheroids and CD26 expression was studied in the context of different lactate concentrations. Lactate decreased CD26 cell-surface expression in spheroid

cultures of HRT-18 cells (**Figure 3.1.3, top**). Even at the lowest concentration of 5mM lactate, cell-surface expression of CD26 decreased by approximately 50% in HRT-18 cells (**Figure 3.1.3, top**) and by ~40% in HT-29 cells (**bottom, Figure 3.1.3**). At the higher level of 20 mM, which approximates to lactate concentrations in the tumor microenvironment (Walenta and Mueller-Klieser 2004; Koukourakis, Pitiakoudis et al. 2006), lactate decreased surface CD26 expression by approximately 60% in both HT-29 and HRT-18 cells.

3.1.4 Varying levels of pyruvate have no effect on CD26 cell surface expression in a three-dimensional model of CRC

Having looked at the effect of lactate on CD26 in CRC spheroids, we decided to see if another metabolic intermediate, pyruvate, had any effect on CD26 cell-surface expression in spheroids. However, results indicate that increasing concentrations of pyruvate had no effect on CD26 in HRT-18 (**Figure 3.1.4**) or HT-29 cells (data not shown).

3.2 Chemotherapeutics Increase CD26 Expression in Conditions that Mimic the Tumor Microenvironment

Having established that glucose concentrations (monolayer culture) and lactate levels (spheroid culture) could affect CD26 levels at the cancer cell surface, I was interested to know whether this might be relevant to the cellular response to chemotherapeutic agents. Our laboratory has shown that many antitumor drugs, including 5-FU, IT, and OX, are able to up-regulate cell-surface CD26 expression on colorectal carcinoma cells in monolayer and three-dimensional models (Lowthers and Blay, unpublished).

3.2.1 Chemotherapeutics increase CD26 in low glucose conditions in monolayer CRC cell culture

The HT-29 cells used in these experiments proved less sensitive with respect to the response in altered CD26 expression to chemotherapeutic agents than the cells used previously in comparable studies in the laboratory. For that reason, I was unable to confirm the expected enhancement of CD26 levels by treatment of HT-29 cells with 20 μg/ml of either irinotecan or 5-fluorouracil (**Figure 3.2.1.1**) at 4.5g/l glucose. However, this proved advantageous from my perspective in that treatment of the same cells (in the same experiment) with identical additions of these two drugs at a lower concentration of glucose (0.5 g/l) provided a clear and statistically significant enhancement of CD26 levels (**Figure 3.2.1.1**). Thus, not only have I confirmed that the anticipated chemotherapeutic drug-induced upregulation of CD26 can occur in conditions of low glucose, but these conditions – which more closely resemble those within the actual tumor – actually work together to enhance this effect.

Interestingly, HRT-18 CRCs showed a completely different behavior (**Figure 3.2.1.2**). Firstly, in neither low- or high-glucose medium was there an accentuation of CD26 levels after 5-fluorouracil treatment. Irinotecan, on the other hand, produced a robust response in both conditions. In this case, and in contrast to the situation with HT-29 cells, the effect of irinotecan was equally apparent between the low- and high-glucose situations (**Figure 3.2.1.2**).

3.2.2 Low-glucose conditions do not significantly alter the cytotoxicity of chemotherapeutics in CRC cells grown in monolayer

As the levels of glucose affected the chemotherapeutic up-regulation of CD26 in cells grown in monolayer, we investigated whether it had an effect on the cytotoxicity of the drugs. The HT-29 cells showed no significant change in cell death when cultured in varying glucose conditions with the addition of 20μg/ml chemotherapeutic agent (**top, Figure 3.2.2**). The HRT-18 cells had a similar response, but cytotoxicity was significantly reduced when the cells were cultured in high glucose medium (**bottom, Figure 3.2.2**). However, the increase in cytotoxicity is modest and may not be biologically significant.

3.2.3 Chemotherapeutics increase CD26 in low glucose/high lactate conditions in spheroid CRC cell culture

High lactate concentrations, representative of tumor physiology, are capable of suppressing basal levels of CD26 on CRCs when grown in spheroid culture (**Figure 3.1.3**). It therefore remained possible that the further addition of lactate might suppress or eliminate the chemotherapeutic drug-induced elevation in CD26, particularly in the 3-dimensional tissue. I extended the approach of **Figure 3.1.3** by both reducing glucose levels *and* supplementing with lactate, in HRT-18 cell spheroids.

The results are shown in **Figure 3.2.3**. In order to elicit a consistent response it was necessary to use a high dose of irinotecan (200 μg/ml). This concentration produced an exceptionally robust effect on CD26, and did so either in the presence of low glucose alone, or with both low glucose and supplementation with a high concentration (100 mM) of lactate. This shows that the authentic circumstances of a tumor (low glucose, high

lactate, 3-D topology) not only allow the drug response, but in fact work to support the most dramatic CD26 response seen in the context of irinotecan treatment.

3.2.4 Chemotherapeutics increase CD26 on CRC spheroids grown in conditioned medium

In order to mimic the nutrient-depleted milieu of a tumor, we treated spheroids with conditioned medium from their respective cell lines in monolayer culture. Two days prior to treatment with irinotecan the medium was aspirated from the spheroids and replaced with conditioned medium.

Our results indicated that the irinotecan-induced upregulation of CD26 still occurs in both HT-29 and HRT-18 (**Figure 3.2.4**) spheroids, and in some cases is enhanced (**top panel, Figure 3.2.4**).

3.3 Adenosine and Related Responses

3.3.1 The effect of adenosine on colorectal carcinoma cells

Our laboratory has found that the purine nucleoside adenosine, which is present at around 10^{-4}M concentrations in the tumor extracellular fluid (Blay et al 1997), has effects on both the proliferation (Mujoomdar et al 2003) and CD26 expression (Tan et al 2004) of colorectal carcinoma cells. I therefore examined adenosine receptor expression and the effects on cell proliferation and potential cell-ECM interaction through MMPs, firstly in colorectal cancer cell lines. I focused on the HT-29 and HRT-18 cell lines grown in monolayer.

3.3.1.1 Relative expression of mRNAs for adenosine receptors

The HT-29 cell line is derived from colorectal adenocarcinoma (American Tissue Type Collection, ATCC.org). The HRT-18 cell line is identical to the line termed HCT-8, and derived from the colon in a case of ileocecal colorectal adenocarcinoma (American Tissue Type Collection, ATCC.org). In both cell lines, the A2B receptor mRNA was the most abundant (about 90-700-fold the expression level for A3 receptor), while the A3 receptor mRNA was negligibly expressed in either HT-29 or HRT-18 cells (**Figure 3.3.1.1**). In both cell lines, the A1 and A2A mRNAs were expressed comparably; in HRT-18 cells the A1 and A2A receptor mRNA expression was 2-3 fold higher than the A3 receptor and in HT-29 cells, 5-6 fold higher than the A3 receptor.

3.3.1.2 Adenosine increases MMP-13 mRNA in HRT-18 cells

Our laboratory has shown that adenosine can act upon colorectal carcinoma cells to modify behaviours that are consistent with the metastatic phenotype. For example, adenosine has been shown to down-regulate CD26 while up-regulating CXCR4 on the surface of CRC cell lines (Richard, Tan et al. 2006; Tan, Richard et al. 2006). I was curious to see if adenosine also had effects on MMPs that are known to play a role in cancer metastasis. After various preliminary experiments, my experiments focused on MMP-13. MMP-13 activity has been associated with poor prognosis in colorectal cancer and has also been found to be an indicator of liver metastasis (Leeman, McKay et al. 2002; Yamada, Oshima et al.).

MMP-13 mRNA expression was measured using quantitative real-time reverse transcriptase PCR (QPCR). Adenosine was shown to increase MMP-13 mRNA in both colorectal carcinoma cell lines; data are shown here for HRT-18 cells. I found that with a

treatment time of 72h, adenosine (at a concentration that can exist within the solid tumor microenvironment) caused up to a 5-fold elevation in the level of MMP-13 mRNA (**Figure 3.3.1.2**). A series of three independent experiments indicated that MMP-13 mRNA consistently peaked at 10 μM adenosine and declined at higher concentrations (e.g. **Figure 3.3.1.2**). The upregulation of MMP-13 mRNA averaged 53% across all experiments with an EC_{50} of approximately 8 μM adenosine.

3.3.1.3 The A2-selective adenosine receptor agonist NECA regulates MMP-13 mRNA

Cellular biological responses to adenosine are usually linked to the expression profiles of its cell-surface receptors and the receptor density. In order to establish that adenosine was acting through receptors and not simply perturbing adenine nucleotide pathways, I used two different adenosine analogs, NECA and R-PIA, which have different affinities for different adenosine receptors. R-PIA has been shown to have a much higher affinity for A1 receptors than the A2 receptors (Fredholm, Abbracchio et al. 1994). In contrast, NECA has a much higher affinity for both A2A and A2B receptors as opposed to the A1 receptor (Fredholm, Abbracchio et al. 1994). Dose-response analyses were performed on HRT-18 cells in monolayer culture with both NECA and R-PIA and MMP-13 mRNA expression was analyzed.

Treating HRT-18 cells with the A1R-selective agent R-PIA resulted in no change in MMP-13 mRNA expression (**Figure 3.3.1.3**). However, the relatively A2-selective agent NECA was able to stimulate a substantial (15-fold) increase in MMP-13 mRNA with a maximal effect at 1μM (**Figure 3.3.1.3**), and following a bell-shaped curve with a decline in effect at NECA concentrations of 3μM and above. The results with NECA and

R-PIA suggested that adenosine may be regulating MMP-13 mRNA through the A2 receptors.

3.3.1.4 The A2A agonist CGS21680, but not the A1 agonist CHA, stimulates MMP-13 mRNA

In order to determine which adenosine receptor subtype might be acting to stimulate an increase in MMP-13 mRNA, I looked at more specific receptor agonists. As expected from the result with R-PIA, the A1 receptor agonist CHA had no statistically significant effect on MMP-13 mRNA expression (**Figure 3.3.1.4**). In contrast, the specific A2A agonist CGS21680 had a progressive and significant effect on MMP-13 mRNA (**Figure 3.3.1.4**). The maximum effect for the response is seen at 1μM CGS21680.

3.3.2 The effect of adenosine on carcinoma-associated fibroblasts

3.3.2.1 Relative expression of mRNAs for adenosine receptors

HS675.T primary colorectal tumor-associated fibroblasts (American Tissue Type Collection, ATCC.org) were grown in standard cell culture conditions until the cells were approximately 60-70% confluent, and analyzed for adenosine receptor expression using QPCR. As with the HT-29 and HRT-18 cancer cells, the A2B receptor had the most highly expressed mRNA, while the A3 receptor mRNA was negligibly expressed (**Figure 3.3.2.1**). In contrast to the colorectal cancer cells, however, the A1 receptor was relatively (about 10-fold) more highly expressed than the A2A receptor (**Figure 3.3.2.1**).

3.3.2.2 Adenosine increases cell proliferation in HS675.T cells

Our laboratory has previously shown that adenosine is mitogenic in both colorectal and breast cancer cell lines over a range of 1μM-300μM (Mujoomdar, Hoskin et al. 2003; Mujoomdar, Bennett et al. 2004). However, the contribution of adenosine to expansion of the stromal compartment is not clear. I wanted to see if adenosine had a similar effect on CAFs that would also contribute to overall tumor expansion. I used the thymidine incorporation assay to measure DNA synthesis as a surrogate indicator for cell growth.

Adenosine significantly increased HS675.T DNA synthesis in a dose-dependent jmanner, with a maximum being reached at about 100μM adenosine with an EC_{50} in the region of 10μM (**Figure 3.3.2.2**). The degree of stimulation was typically no more than 30% and reached statistical significance throughout the range 3-300μM (**Figure 3.3.2.2**).

3.3.2.3 Adenosine increases MMP-13 mRNA in HS675.T cells

We were interested to see if adenosine could regulate MMP-13 production in stromal cells, as I had found it able to do with the carcinoma cells. I therefore used QPCR to establish if adenosine up-regulated MMP-13 mRNA in HS675.T cells.

Although the dose-response relationship did not follow the smooth curve expected, adenosine increased MMP-13 mRNA in HS675.T cells (**Figure 3.3.2.3**), and even did so to a greater degree (about 10-fold compared with 5-fold) than it does in carcinoma cells. On average, there was a 10-fold increase in MMP-13 mRNA in HS675.T cells following adenosine treatment.

Zymography and immunoblotting to demonstrate MMP activity and protein secretion was performed extensively to confirm the expression data but although the results were promising (**Appendix A**), variability in responses prevents these data from providing conclusive findings. This may have been related to the method used to isolate secreted protein (TCA precipitation) or the high rate of MMP-13 proteolysis. Using a better method to concentrate the protein samples may have yielded better, cleaner, results.

3.3.2.4 Adenosine increases CXCL12 mRNA in HS675.T cells

Carcinoma-associated fibroblasts are known to up-regulate CXCL12, which promotes tumor expansion and migration in the tumor microenvironment. As adenosine has been shown to up-regulate various factors conducive to tumor progression including the receptor CXCR4, we looked to see if it had the same effect on CXCL12 mRNA in HS675.T cells.

Adenosine increased CXCL12 mRNA production in a dose-dependent manner (**Figure 3.3.2.4**). The greatest effect was seen at the highest concentration of adenosine used (300μM), at which point CXCL12 gene expression was increased 2.5 fold. Several experiments were carried out to attempt to quantify CXCL12 protein secretion. Unfortunately, these were not conclusive as the levels present were below the LLOQ for LC-MS/MS. However, some preliminary data suggest that HS675.T cells do secrete a soluble factor(s) that enhance chemotaxis in HT-29 cells (see **Appendix B**). Further attempts to quantify CXCL12 are needed using a different method, such as an ELISA.

3.3.2.5 Adenosine increases cell-surface CD26 on HS675.T cells

Our laboratory has previously shown that adenosine down-regulates CD26 cell-surface expression on colorectal carcinoma cells (Tan, Mujoomdar et al. 2004). The down-regulation of CD26 on cancer cells is thought to benefit cancer progression. We sought to determine the effect of adenosine on CD26 expression in CAFs.

Adenosine increased CD26 cell-surface expression in HS675.T cells (**Figure 3.3.2.5**). The dose-response of adenosine resulted in a bell-shaped curve, with the cell-surface expression peaking at 30μM, with approximately a 35% increase in CD26 expression, and decreasing thereafter.

3.3.2.6 HS675.T cells do not express CXCR4

I looked to see if HS675.T cells express the chemokine CXCL12 or its receptor, CXCR4. This would help determine whether the HS675.T cells were facilitating cancer cell migration through the secretion of CXCL12, or whether they were sensitive to the effects of SDF-1 through its actions on its receptor, CXCR4.

We performed an RT-PCR for CXCR4 and there was no detectable signal for CXCR4 mRNA, suggesting that the receptor is not expressed (data not shown). To verify further that CXCR4 is not expressed on HS675.T cells, we treated the cells with a CXCL12 dose response and performed a thymidine incorporation assay. Results indicated no change in thymidine incorporation (**Figure 3.3.2.6**).

3.3.3 The effect of adenosine on mesothelial cells

3.3.3.1 Relative expression of mRNAs for adenosine receptors

A third fixed cell population that is relevant to colorectal cancer is that of mesothelial cells, which form a barrier to escape into the peritoneal cavity and are different in phenotype to the vascular (blood or lymphatic) endothelial cells that have been widely studied in the context of vascular extrusion. I used Met-5a cells as a model of the mesothelial cell phenotype; these are an immortalized but non-tumorigenic cell line derived from the human pleural mesothelium (American Tissue Type Collection). As before, cells were grown in typical cell culture conditions until the cells were approximately 60-70% confluent and gene expression was analyzed using QPCR.

As for both the CRC and CAF cells studied, I found that Met-5a cells expressed the highest amounts of mRNA for the A2B adenosine receptor, while the A3 receptor mRNA was negligibly expressed in all cases (**Figure 3.3.3.1**). In contrast to the other cell lines tested however, Met-5a cells had relatively high expression of the A2A receptor mRNA, about 40-fold that of the A1 receptor and 1000-fold that of the A3 receptor.

3.3.3.2 Adenosine has no effect on cell proliferation in Met-5A cells

Having established in this and other work that adenosine was mitogenic for CRC and CAF cells, we were interested to see if adenosine treatment had any effect on the growth of mesothelial cells. The cell culture approach, adenosine-dosing regimen and assay procedures were the same as with the other cell types.

Surprisingly, extensive work failed to show any effect of adenosine on cell proliferation or DNA synthesis in Met-5a mesothelial cells. The top panel of **Figure**

3.3.3.2 shows the result of one such experiment using the DNA synthesis assay. I also repeated the experiment and counted the number of cells. These results were the same as the thymidine experiments and showed that there was no change in cell number with increasing concentrations of adenosine over 48 h (data not shown). Other nucleosides also failed to elicit any sign of a proliferative response (**Figure 3.3.3.2**, lower panel).

3.3.3.3 Adenosine increases MMP-13 mRNA in Met-5a cells

As mentioned in the context of results with the HS675.T cells, the adenosine induced up-regulation of MMP-13 mRNA is much greater in the CAFs than in carcinoma cells. Since adenosine had the ability to up-regulate MMP-13 mRNA in both CRCs and CAFs, I also looked to see if it up-regulated MMP-13 mRNA in Met-5A cells. Adenosine did indeed up-regulate MMP-13 mRNA in Met-5a cells, achieving a maximal effect at around 30 μM of adenosine with an EC_{50} of 7.3 μM. (**Figure 3.3.3.3**). This profile appears intermediate between that of CRCs, which have a maximum around 10 μM but the same 'bell-shaped' dose-response curve (**Figure 3.3.3.3**) and the HS675.T cells, which respond to adenosine at 100μM adenosine and above (**Figure 3.3.3.3**).

As indicated earlier, zymography and immunoblotting to demonstrate MMP activity and protein secretion was performed extensively to confirm the expression data but although the results were promising (**Appendix A**), variability in responses prevent these data being used in conclusive findings.

3.3.3.4 Adenosine increases CXCL12 mRNA in Met-5a cells

The mesothelium is a frequent site of metastasis in ovarian cancer, for which there is a strong anatomical reason for cancer cell seeding on the mesothelium. It is not implausible to suggest that mesothelial cells may play a role in secreting chemoattractants to promote local tumor cell invasion in colorectal carcinoma, when these cells cross the wall of the intestine to reach the peritoneal cavity. As adenosine is able to modulate the production of MMP-13 in Met-5a cells we inferred that they might also respond to adenosine in other aspects of an invasive phenotype. Given our interests in the CXCR4 pathway, I therefore looked for an effect on CXCL12 expression.

Adenosine increased CXCL12 mRNA substantially (up to ~3-fold) in Met-5a cells, with a maximal effect on expression at approximately 30μM, with an EC_{50} of 1.45μM (**Figure 3.3.3.4**).

Several experiments were carried out to quantify CXCL12 protein secretion. Unfortunately, these were not conclusive as the levels present were below the LLOQ for LC-MS/MS (data not shown). As mentioned, more experiments are needed using new methods, such as an ELISA, to quantify CXCL12 secretion.

3.3.4 Met-5a mesothelial cells do not express CD26 protein

CD26 is known to play a role in malignant mesothelioma (Inamoto, Yamada et al. 2007). Studies have shown that CD26 is only expressed on malignant mesothelial cells and not their 'normal' counterparts. In order to investigate whether our 'normal' Met-5a cells followed this pattern, I performed a radioantibody-binding assay to assess cell-surface CD26 protein levels. The radioantibody-binding assay showed that the cells

incubated with anti-CD26 antibody had the same final radioactivity levels as the isotype control, indicating that the CD26, if present, was below the level of detection of the assay (data not shown). I also used immunofluorescence to look for both the intra- and extra-cellular protein expression. HT-29 cells were used as a positive control as they are known to express CD26 on the cell-surface. However, the immunofluorescence studies showed that the degree of fluorescence detected in the anti-CD26 antibody-stained cells was no different to the isotype controls (**Fig. 3.3.4**). Met-5a cells therefore do not express detectable CD26 protein, consistent with their putative nature as 'normal' mesothelial cells.

3.3.5 Colorectal cancer cells do not express CXCL12 mRNA

One of the effects of adenosine is to regulate CXCR4, the receptor for CXCL12 (Richard, Tan et al. 2006). Cancer cells that express CXCR4 migrate towards a CXCL12 gradient. CXCL12-producing tissues in the body are the frequent sites of colorectal cancer metastasis. It was of interest to determine whether our HT-29 and HRT-18 cells expressed CXCL12, since the published data on these two cell lines are contradictory (Wendt, Johanesen et al. 2006). We looked at the CXCL12 mRNA expression in two colorectal carcinoma cell lines (HRT-18, HT-29). cDNA samples were amplified with three different primer sets to assess CXCL12 mRNA expression by PCR and visualization on agarose gel. There was no detectable signal for CXCL12 mRNA with the primers used (**Figure 3.3.5**).

3.4 Potential for Cellular Crosstalk Through More Complex Interactions

Adenosine acts on CRCs, CAFs and mesothelial cells and, at different stages of the cancer process, may modulate processes that are common to the different cell types such as MMP and CXCL12 secretion and cell proliferation. My experiments tested the effect of adenosine in the separate cell models. I wished to examine directly whether these particular representatives of the different cell populations showed behavior in coculture that could be fitted to such cooperative interactions.

Using conditioned media (media incubated with viable cells to become enriched with their secretory products) provides an opportunity for the indirect measurement of cell-cell communication. Previous studies in the Blay laboratory had been performed looking at the effect of various conditioned media (from stromal cell types) on colorectal carcinoma cells. Conditioned media derived from Met-5a, HS675.T and HUVECs had been shown to decrease DNA synthesis in HT-29 cells (Bseso et al, unpublished data). I looked to see how the conditioned media affected proliferation in the different stromal/supportive cell types.

3.4.1 Conditioned MEM increases cell proliferation in HS675.T cells

HS675.T cells were seeded and two days later, the growth medium was replaced with fresh medium containing 20% medium conditioned by the other cell types: control, Met-5a, HS675.T, HT-29, HRT-18, HUVEC. Changes in DNA synthesis of the HS675.T cells were assessed with a thymidine incorporation assay. Conditioned medium from HT-29 CRC cells substantially stimulated (about 2.5-fold) the proliferation of HS675.T cells

(**Figure 3.4.1**). In addition, media conditioned by Met-5a cells also increase thymidine incorporation in HS675.T cells by nearly 2-fold (**Figure 3.4.1**).

3.4.2 Addition of ADA or EHNA to conditioned MEM does not alter the increase in cell proliferation in HS675.T cells

Having seen an increase in thymidine incorporation in HS675.T cells following treatment with Met-5a or HT-29 conditioned media, we were interested to see if the response may be adenosine-mediated. We treated the cells with either ADA, to deplete adenosine in the medium, or 20μM EHNA, an inhibitor of ADA, to augment the levels of adenosine.

Neither the addition of EHNA or ADA altered the increase in cell proliferation induced by CRC cell or mesothelial cell medium in HS675.T cells (**Figure 3.4.2**).

3.4.3 Conditioned media decreases cell proliferation in Met-5a cells

Using the same panel of conditioned media, results indicated that treatment of Met-5a cells with conditioned media from HRT-18 cells or HT-29 cells significantly inhibited DNA synthesis (**Figure 3.4.3**). HT-29 cell-conditioned medium inhibited Met-5a thymidine incorporation by an average of 24% while HRT-18 cell-conditioned medium inhibited it by an average of 35% (**Figure 3.4.3**).

3.4.4 Conditioned medium regulates MMP-13 mRNA in HS675.T cells

The release of various factors functions as a means of cellular crosstalk. Not only do they support cell growth, they help in priming the local environment for invasion. The stroma supports this process by being the major producer of proteolytic enzymes. A series of conditioned media were tested to see how they influenced MMP-13 mRNA in HS675.T cells.

Results were analyzed using QPCR. The data shows that HRT-18 CRC cell and HS675.T cell media increases MMP-13 mRNA significantly over control (**Figure 3.4.4**).

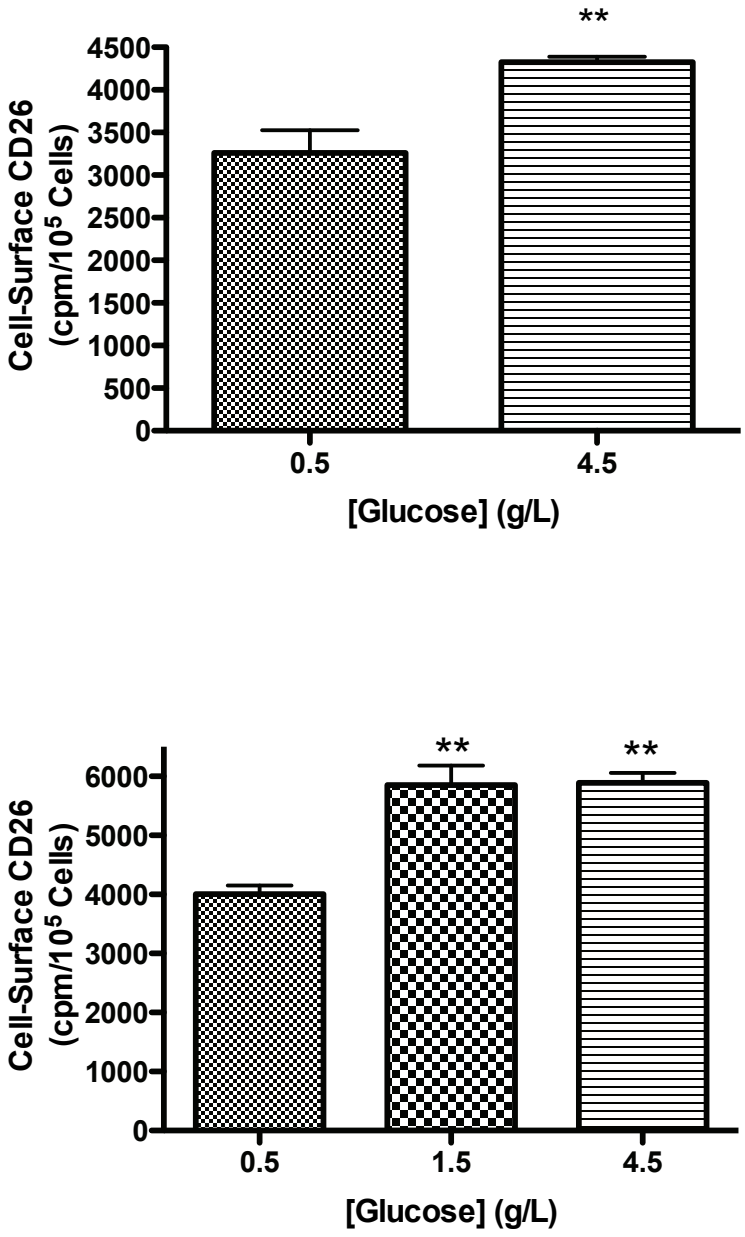


Figure 3.1.1 CD26 cell-surface expression is increased when HT-29 (top) or HRT-18 (bottom) cells are cultured in monolayer with higher glucose medium. Cells received either 0.5 g/l glucose or 4.5 g/l and were assayed 48 hours later for CD26 expression. Data are represented as the mean values +/- SEM (n=4). Change compared to control, for 0.5 g/l, $p<0.01$. **

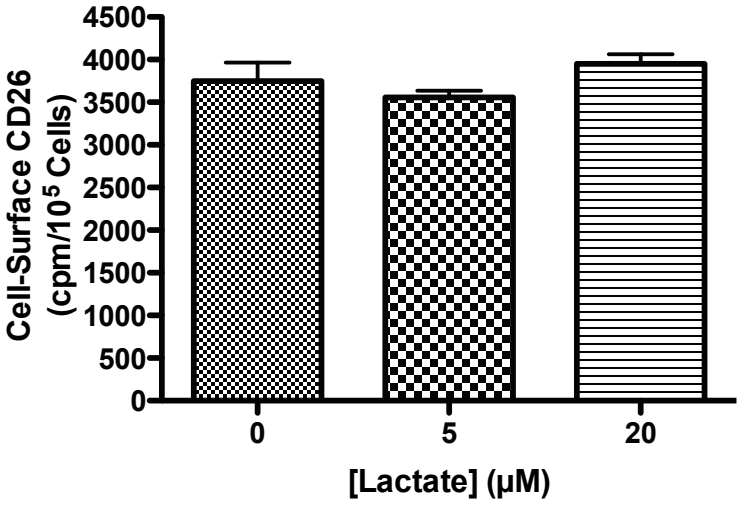


Figure 3.1.2 Lactate has no effect on CD26 cell-surface expression in HT-29 cells grown in monolayer. Cells were treated with 0, 5, or 20 mM lactate and assayed for CD26 expression at 48h. Data are represented as the mean values +/- SEM (n=4). One-way analysis of variance $p > 0.05$.

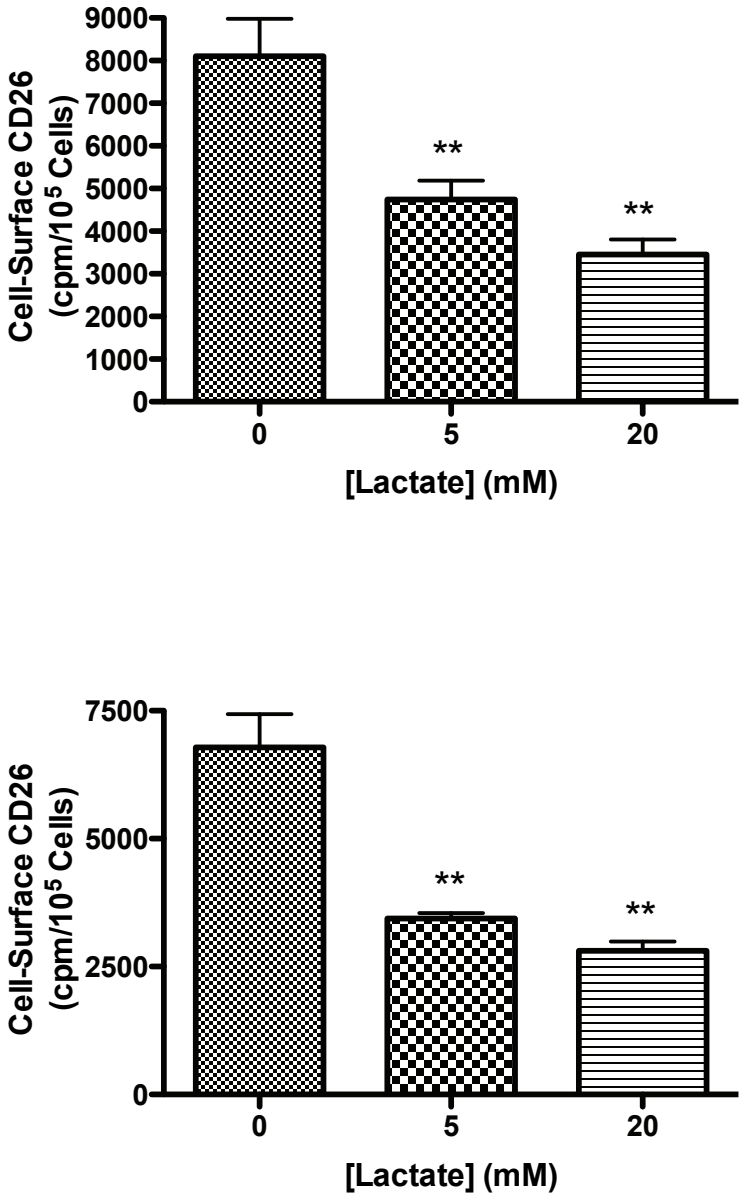


Figure 3.1.3 Lactate decreases basal levels of CD26 in an HRT-18 (top) and HT-29 (bottom) spheroid model of cell culture. Spheroids were treated with 0, 5, or 20mM lactate. Cells were assayed 72hrs later. Data are represented as the mean values +/- SEM (n=6). One-way analysis of variance $p < 0.01$; **significant change compared to control, Dunnet's multiple comparison post-test $p < 0.01$.

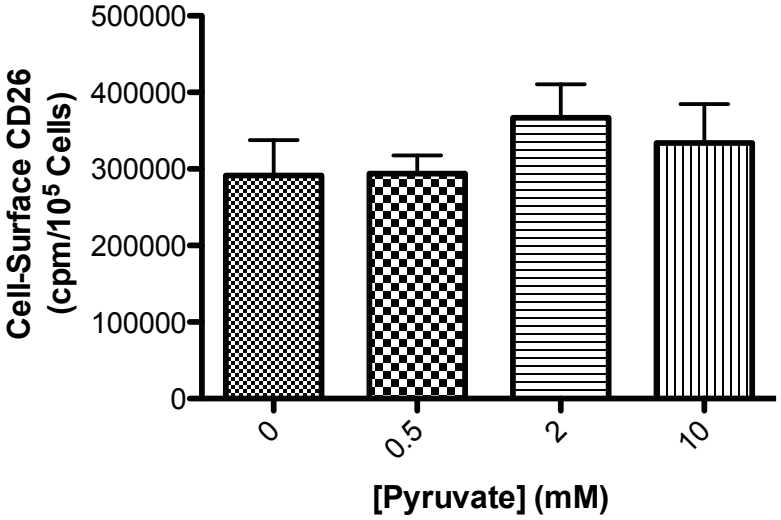


Figure 3.1.4 Pyruvate has no effect on CD26 cell-surface expression in HRT-18 spheroids. Spheroids were treated with 0, 0.5, 2, or 10 mM sodium pyruvate. Cells were assayed 72 hrs later for CD26 expression. Data are represented as the mean values +/- SEM (n=5). One-way analysis of variance $p > 0.05$.

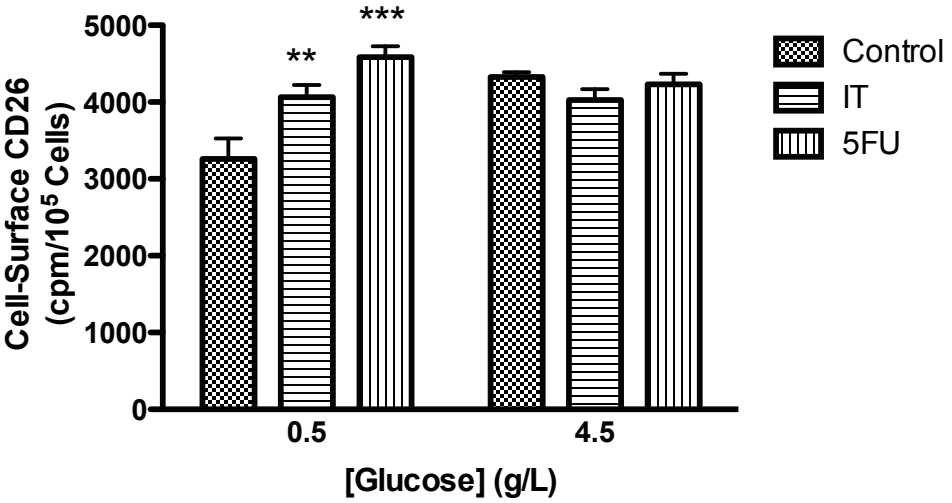


Figure 3.2.1.1 Chemotherapeutics increase CD26 in low glucose conditions in HT-29 monolayer cell culture. HT-29 cells received either 0.5 g/L glucose or 4.5 g/L and were then treated with 20 μg/mL of the indicated chemotherapeutic. Data are represented as the mean values +/- SEM (n=4). Two-way analysis of variance $p < 0.01$; **significant change compared to control, Bonferroni post-test $p < 0.01$.Change compared to control, for 0.5 g/L, $p<0.001$, ***.

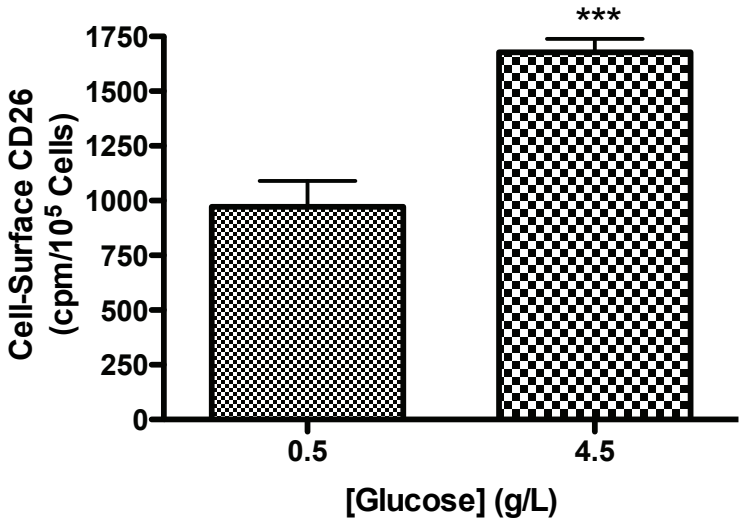


Figure 3.2.1.2 Chemotherapeutics increase CD26 in low glucose conditions in HRT-18 monolayer cell culture. HRT-18 cells received either 0.5 g/L glucose or 4.5 g/L and were then treated with 20 μg/mL of the indicated chemotherapeutic. Data are represented as the mean values +/- SEM (n=4). Two-way analysis of variance $p < 0.01$; **significant change compared to control, Bonferroni post-test $p < 0.01$.Change compared to control, for 0.5 g/L, $p<0.001$,*** .

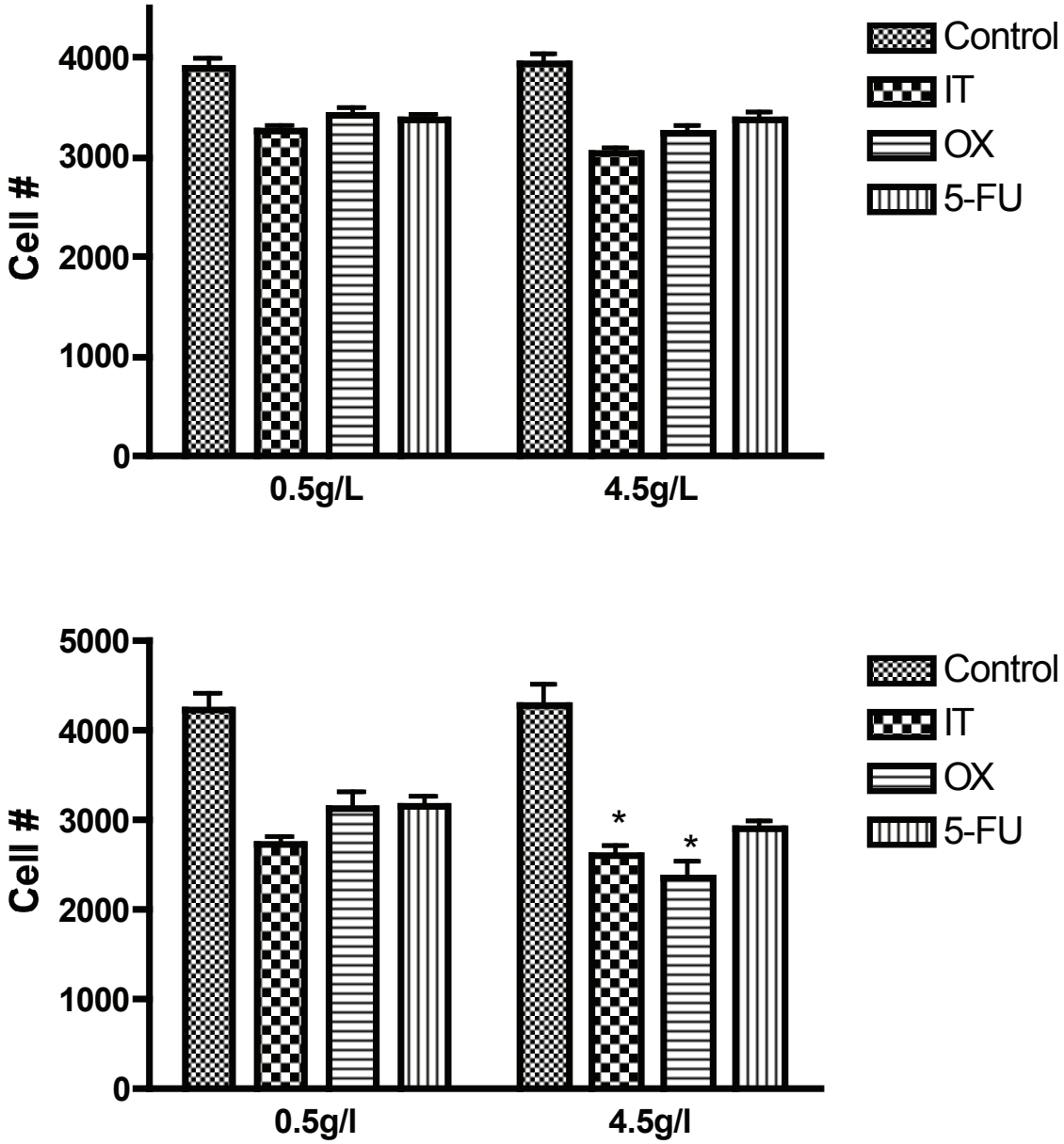


Figure 3.2.2 The effect of different concentrations of glucose on chemotherapeutic toxicity in HT-29 (top) and HRT-18 (bottom) cells grown in monolayer. Chemotherapeutics were added to a final concentration of 20 µg/ml. Data are represented as the mean values +/- SEM (n=4). Two-way analysis of variance $p < 0.05$; *significant change compared to control, Bonferroni post-test $p < 0.05$.

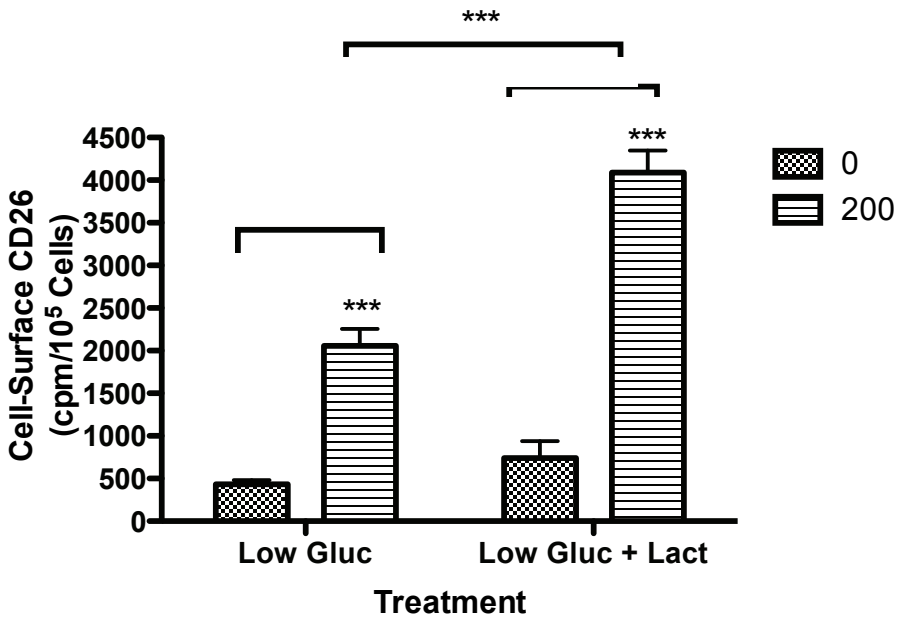


Figure 3.2.3 High lactate enhances the irinotecan-induced up-regulation of CD26 on the cell-surface of HRT-18 spheroids. HRT-18 Spheroids were grown in the presence of absence of 100mM lactate then treated to a final concentration of 200µg/mL IT. Data are represented as the mean values +/- SEM (n=6).. Two-way analysis of variance $p < 0.01$; **significant change compared to control, Bonferroni post-test $p < 0.01$.Change compared to control, for 0.5 g/L, $p<0.001$,

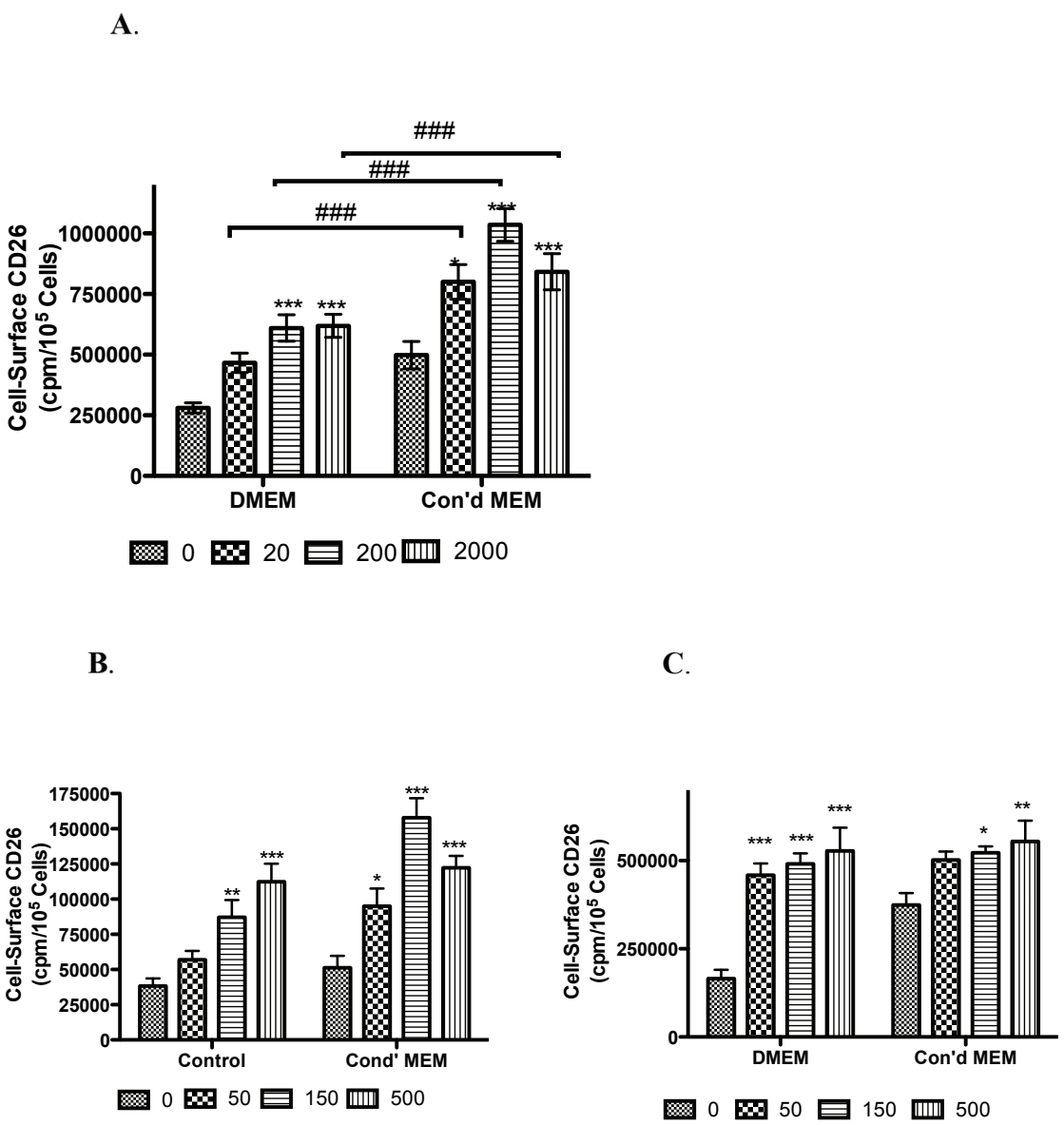
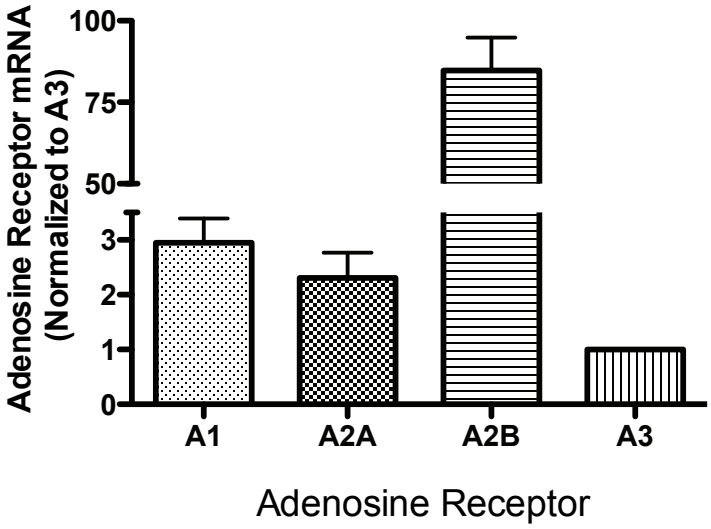


Figure 3.2.4 The IT-induced up-regulation in CD26 occurs and is enhanced in conditioned medium on HT-29 spheroids (A). 5-FU increases CD26 expression in HT-29 (B) and HRT-18 (C) spheroids when grown in normal and conditioned medium. Data are represented as the mean values +/- SEM (n=6). Two-way analysis of variance $p < 0.001$; ***significant change compared to control, Bonferroni post-test $p < 0.01$.

A. HT-29



B. HRT-18

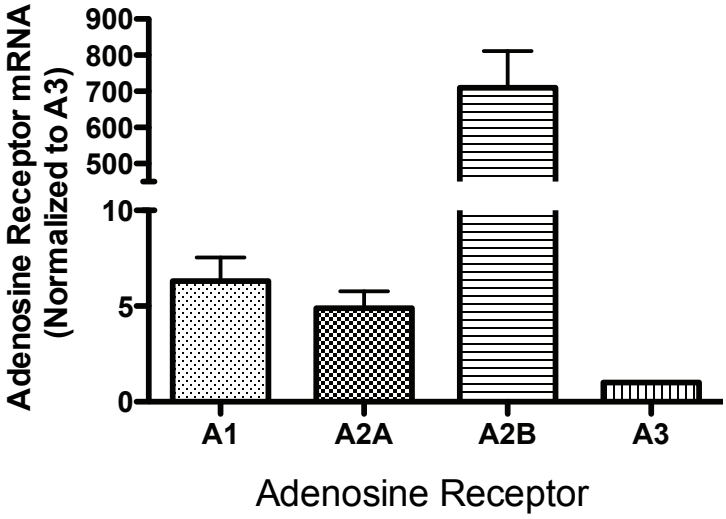


Figure 3.3.1.1 Adenosine receptor mRNA expression profile (relative to the A3 receptor) in HT-29 (top) and HRT-18 cells (bottom) grown in monolayer. RNA was isolated from HT-29 and HRT-18 cells in basal monolayer cell culture conditions to look at relative adenosine receptor mRNA levels. Values are represented as the mean +/- SEM (n=4).

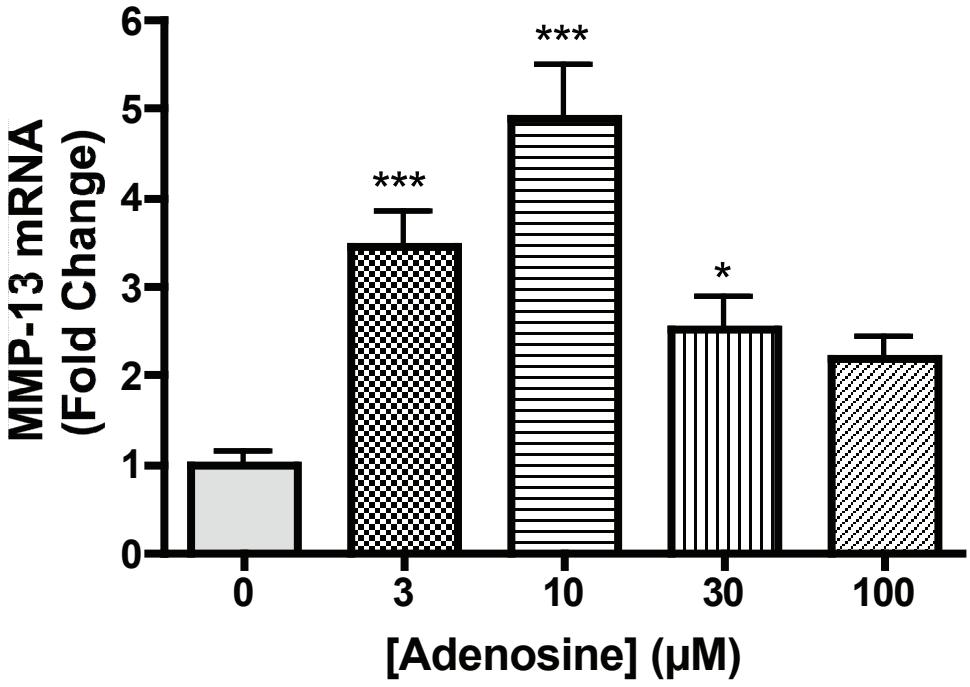


Figure 3.3.1.2 Adenosine increases MMP-13 mRNA in colorectal carcinoma cells grown in monolayer. HRT-18 cells were treated with the vehicle or the indicated dose of adenosine. Data are represented as mean values +/- SEM (n=3). One-way analysis of variance $p < 0.01$; ***significant change compared to control, Dunnet's multiple comparison post-test $p < 0.01$,* $p < 0.05$.

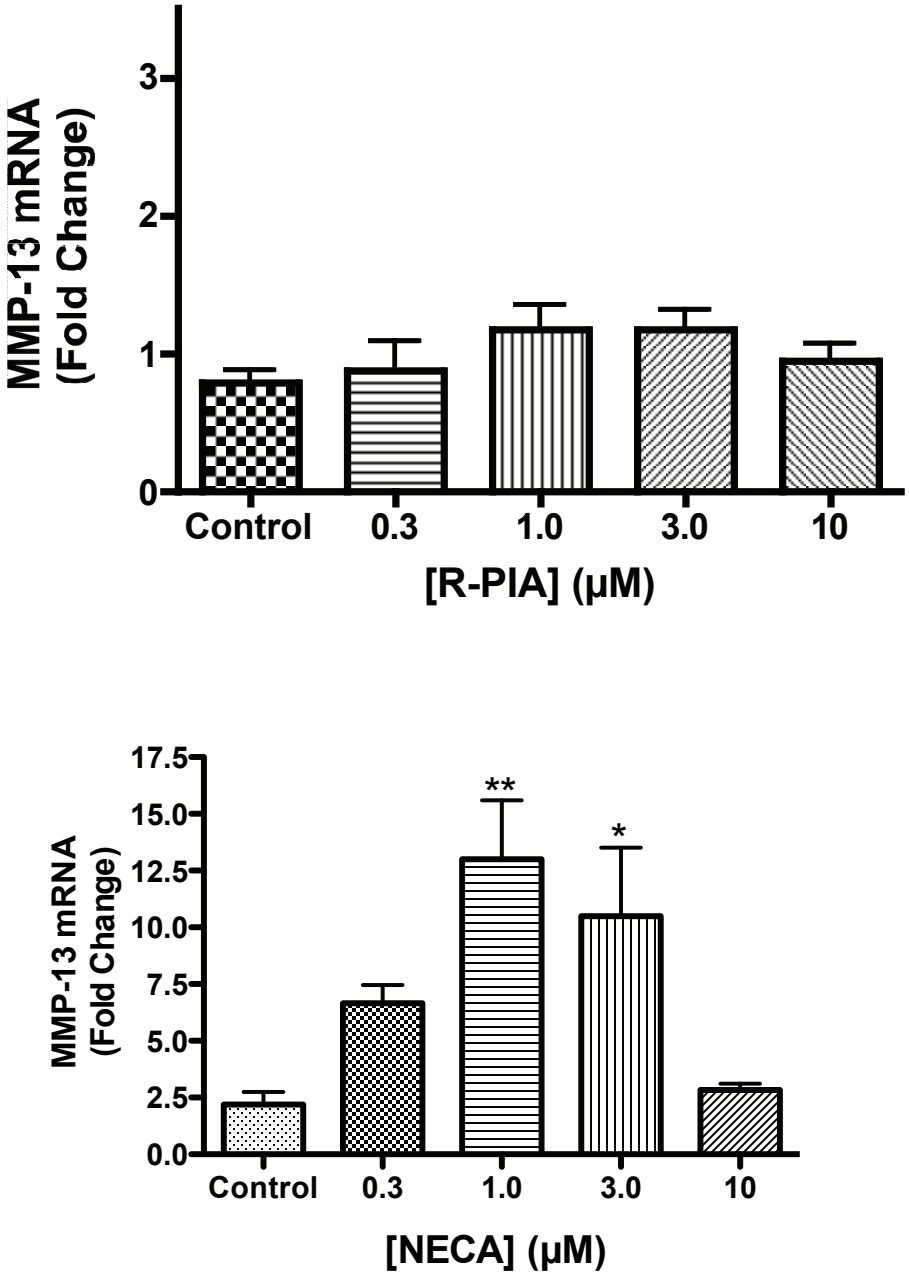


Figure 3.3.1.3 NECA, but not R-PIA increases MMP-13 mRNA in HRT-18 cells grown in monolayer. HRT-18 cells were treated with the vehicle or the indicated concentration of NECA or R-PIA. Data are represented as mean values +/- SEM (n=3). One-way analysis of variance $p < 0.01$; **significant change compared to control, Dunnet's multiple comparison post-test $p < 0.01$, * $p < 0.05$

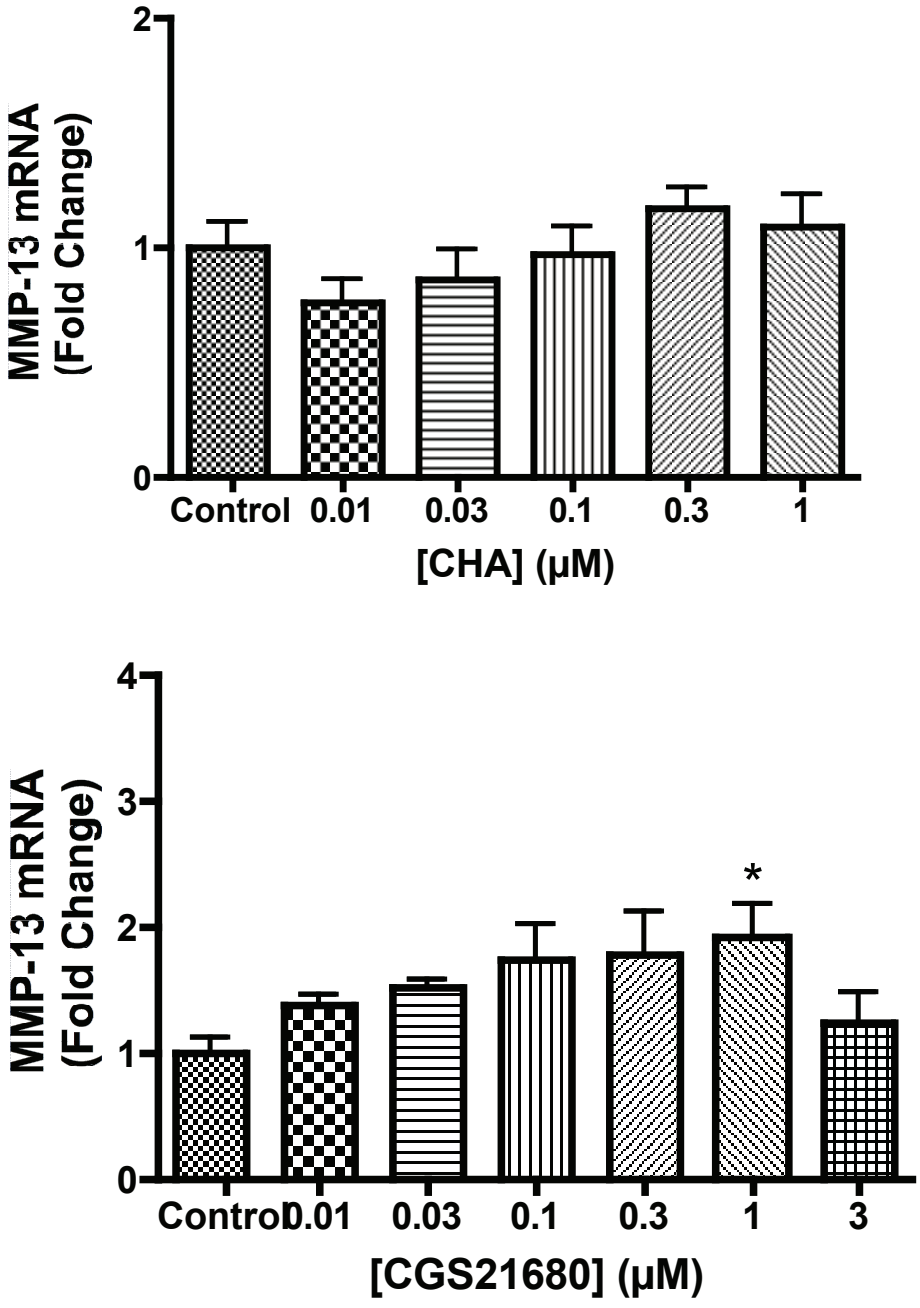


Figure 3.3.1.4 CGS21680, but not CHA, increases MMP-13 mRNA expression in HRT-18 cells grown in monolayer. HRT-18 cells were treated with the vehicle control or the indicated concentration of adenosine receptor agonist. Data are represented as the mean values +/- SEM (n=6). One-way analysis of variance $p < 0.01$; * Dunnet's multiple comparison post-test $p < 0.05$.

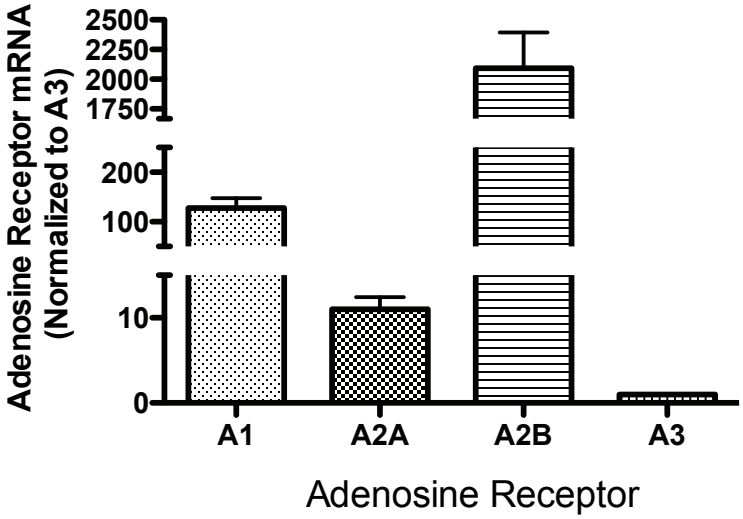


Figure 3.3.2.1 Relative expression of mRNAs for adenosine receptors in HS675.T cells. RNA was isolated from HS675.T cells in basal cell culture conditions to look at relative adenosine receptor mRNA levels. Data are represented as mean values +/- SEM (n=3).

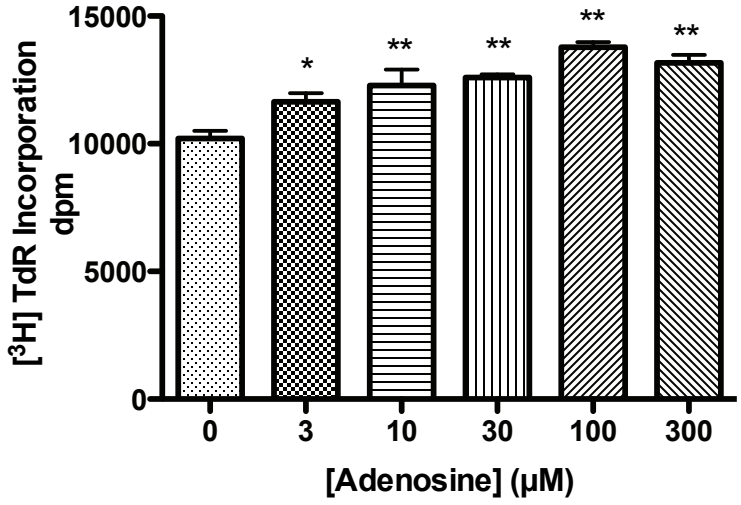


Figure 3.3.2.2 Adenosine increases thymidine incorporation in HS675.T cells. HS675.T cells were treated with the concentration of adenosine indicated. Cell proliferation was assessed with the thymidine incorporation assay. Data are represented as the mean values +/- SEM (n=6). One-way analysis of variance, $p < 0.001$; *, change compared to control, Dunnet's multiple comparison post-test, $p < 0.05$; **, $p < 0.01$.

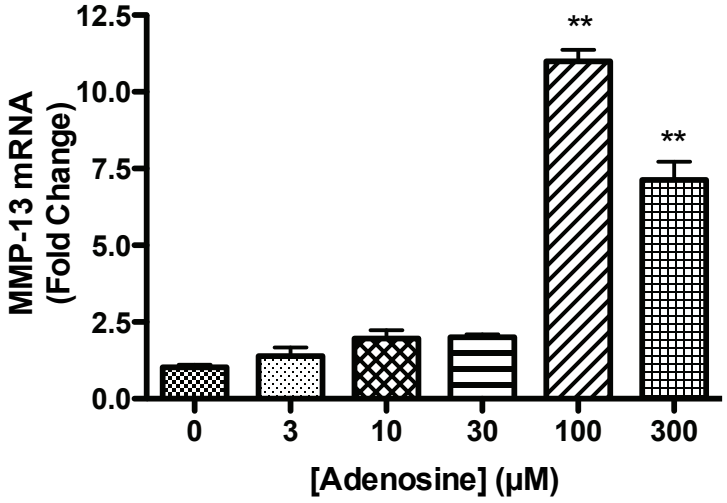


Figure 3.3.2.3 Adenosine increases MMP-13 mRNA expression in HS675.T cells. Adenosine was added to HS675.T cells at the indicated concentration. Relative fold change compared to control was measured (n=4). One-way analysis of variance $p < 0.01$; **significant change compared to control, Dunnet's multiple comparison post-test $p < 0.01$, * $p < 0.05$

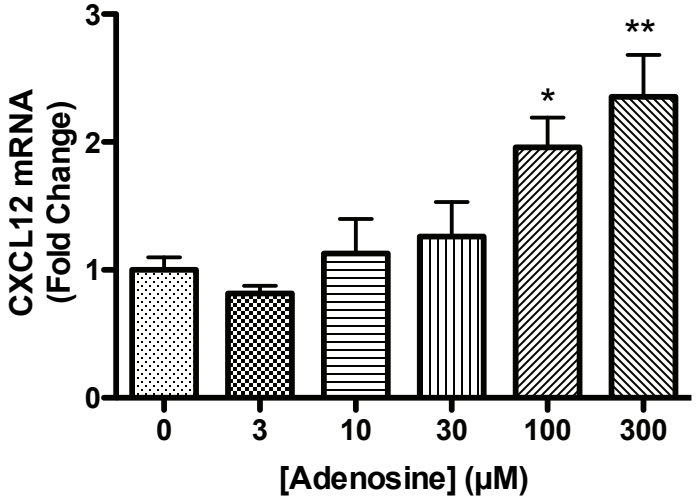


Figure 3.3.2.4 Adenosine increases CXCL12 mRNA expression in HS675.T cells. HS675.T cells were treated with the concentration of adenosine indicated. Data are represented as the mean values +/- SEM (n=3). One-way analysis of variance $p<0.01$; **significant change compared to control, Dunnet's multiple comparison post-test $p< 0.01$.

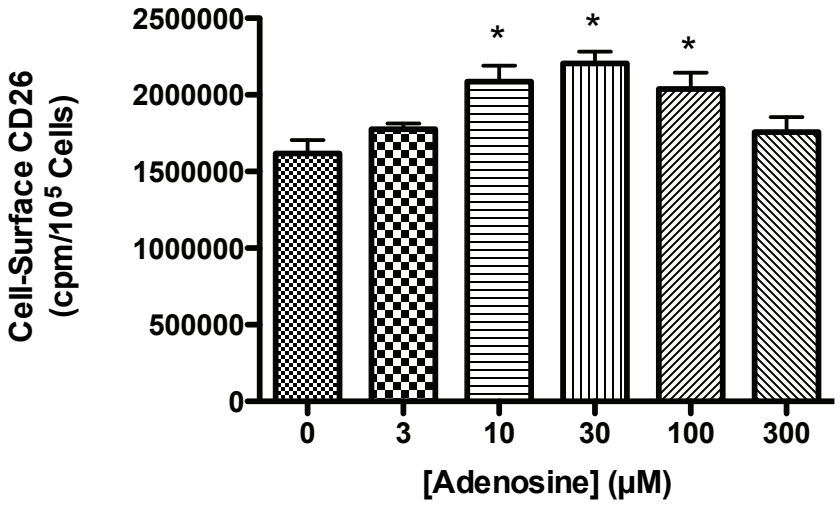


Figure 3.3.2.5 Adenosine increases CD26 cell-surface expression in HS675.T cells. HS675.T cells were treated with the concentration of adenosine indicated. Data are represented as the mean values +/- SEM (n=3). One-way analysis of variance $p<0.05$; * Dunnet's multiple comparison post-test $p < 0.05$.

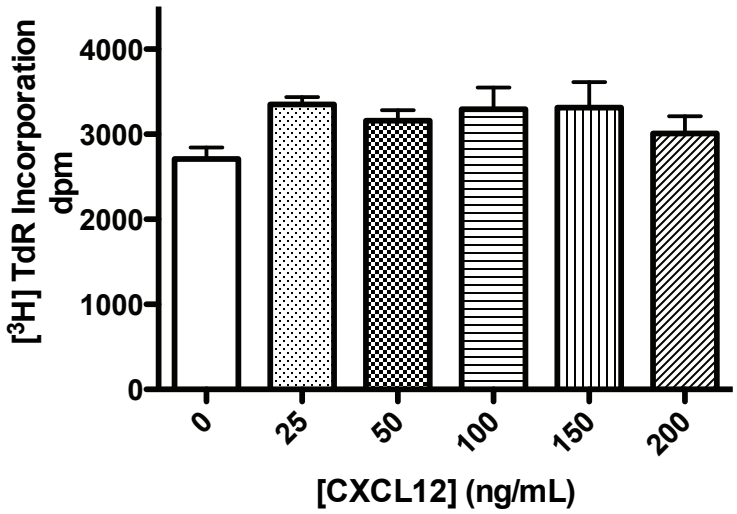


Figure 3.3.2.6 CXCL12 has no effect on thymidine incorporation in HS675.T cells. Data are represented as the mean values +/- SEM (n=3). One-way analysis of variance $p>0.05$.

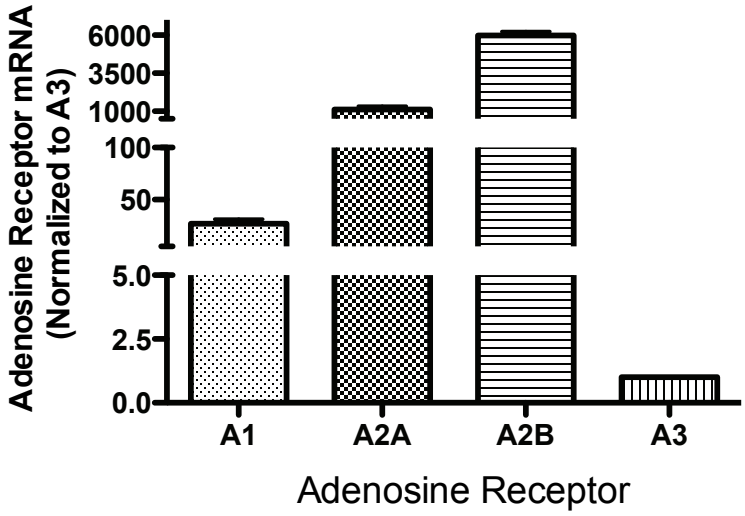


Figure 3.3.3.1 Relative expression of mRNAs for adenosine receptors. RNA was isolated from Met-5a cells in basal cell culture conditions to look at relative adenosine receptor mRNA levels. Data are represented as mean values +/- SEM (n=3).

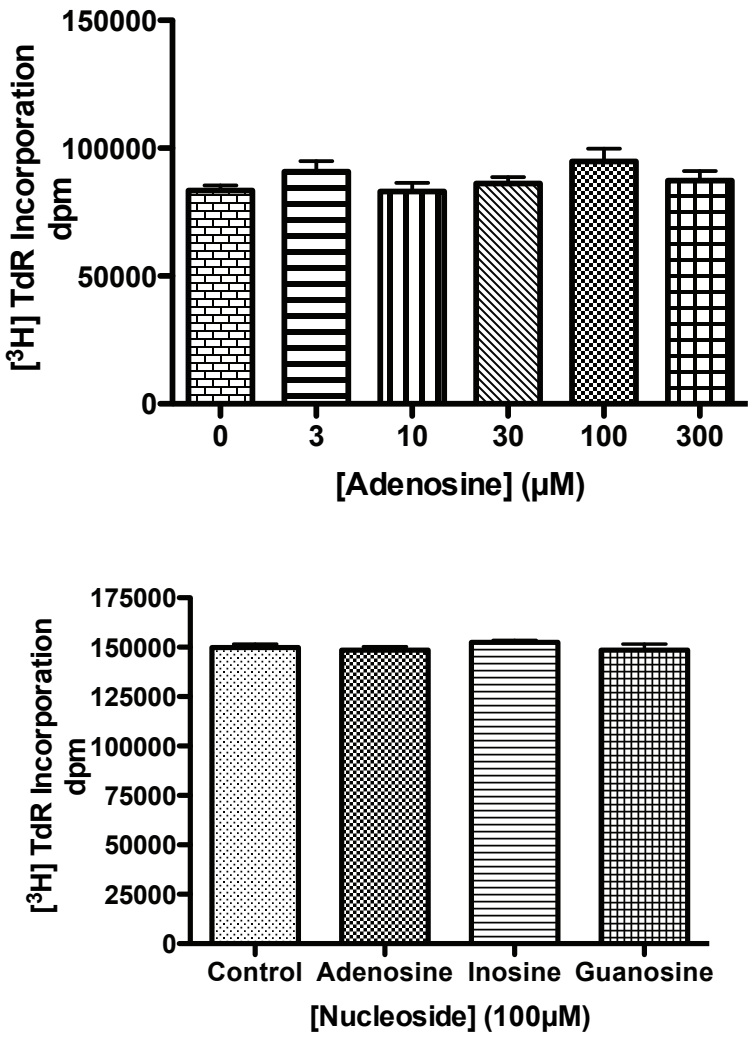


Figure 3.3.3.2 Adenosine (top) and other nucleosides (bottom) fail to stimulate a change in thymidine incorporation in Met-5a cells. Met-5a cells were treated with 100µM of the nucleoside indicated. Data are represented as mean values +/- SEM (n=6). One-way analysis of variance $p > 0.05$.

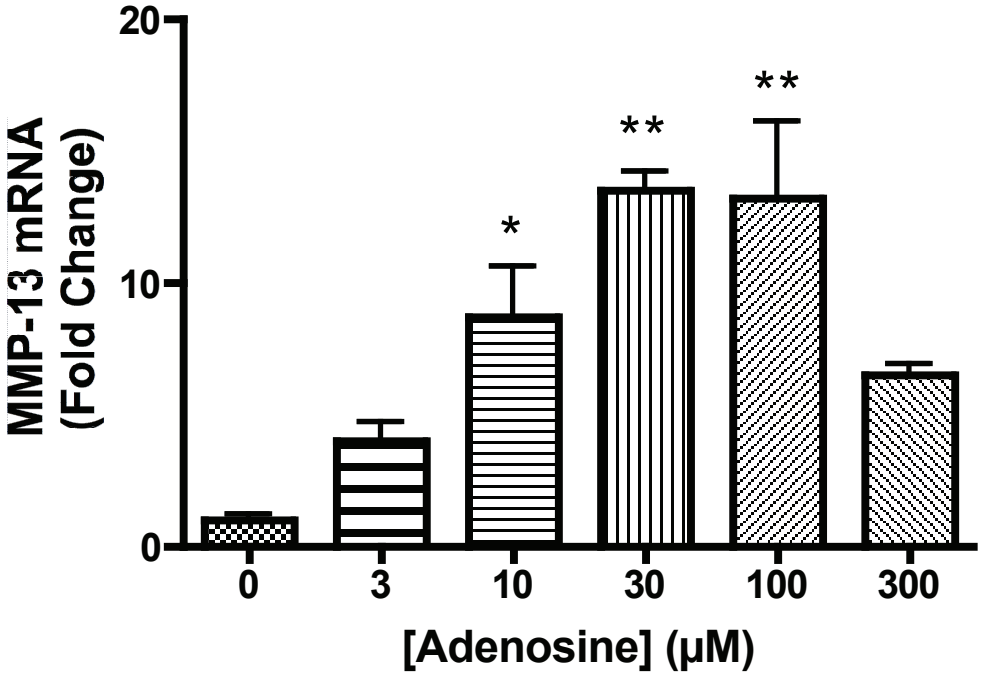


Figure 3.3.3.3 Adenosine increases MMP-13 mRNA expression in Met-5A cells. Met-5A cells were treated with the concentration of adenosine indicated for 72 hours. Data are represented as the mean values +/- SEM (n=3). One-way analysis of variance $p<0.01$; * significant change compared to control, Dunnet's multiple comparison post-test $p<0.05$; ** $p< 0.01$.

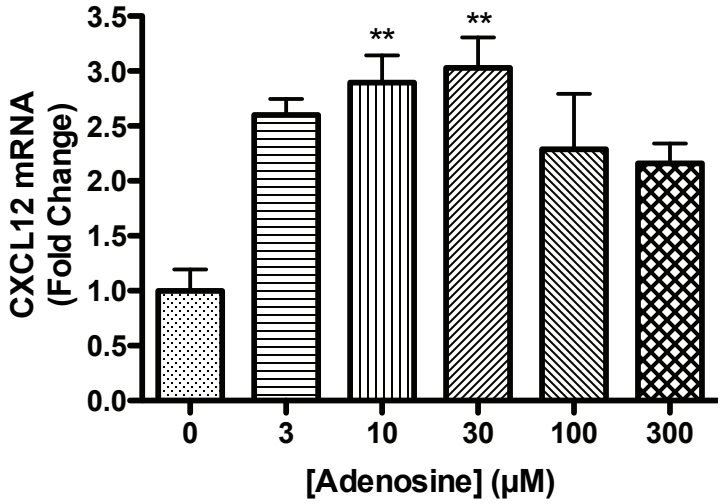


Figure 3.3.3.4 Adenosine increases CXCL12 mRNA expression in Met-5a cells. Met-5a cells were treated with the concentration of adenosine indicated for 72 hours. Data are represented as the mean values +/- SEM (n=3). One-way analysis of variance $p<0.01$; ** significant change compared to control, Dunnet's multiple comparison post-test $p< 0.01$.

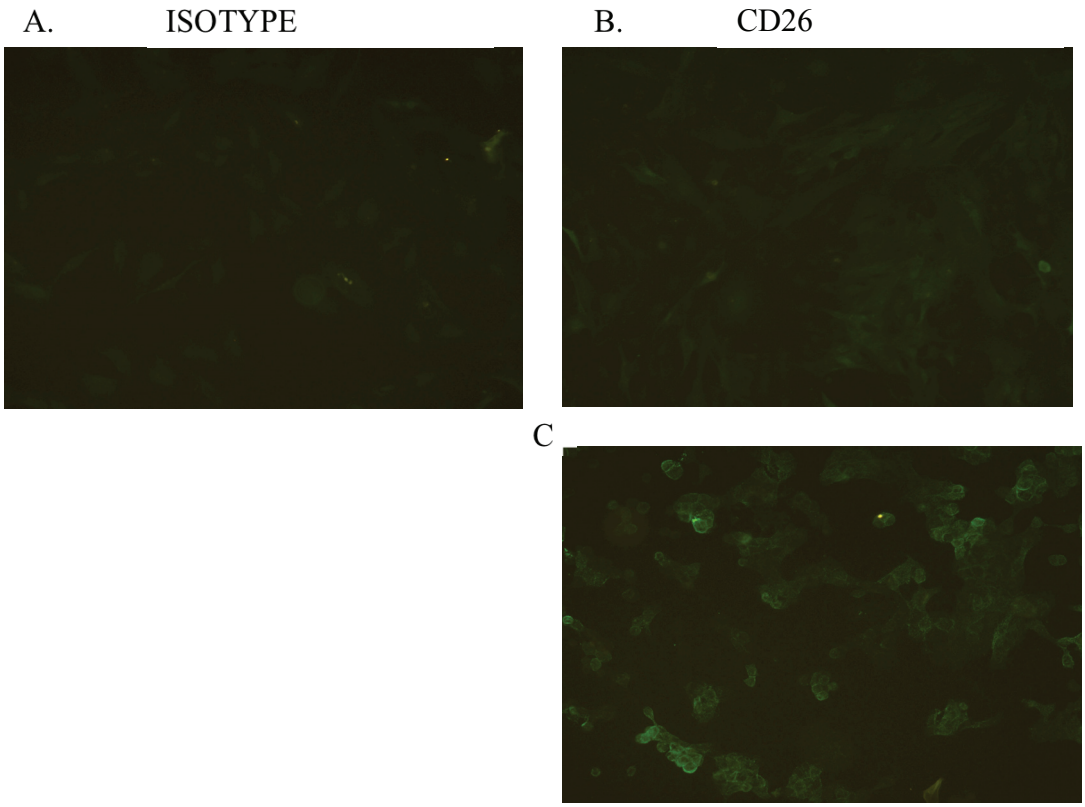


Figure 3.3.4 CD26 expression in Met-5A (A,B) cells and HT-29 (C) cells. Met-5A and HT-29 cells were permeabilized with formalin to look at total cellular CD26 expression and then stained with an isotype (left) or anti-CD26 antibody. HT-29 cells were used a positive control, suggesting Met-5A cells do

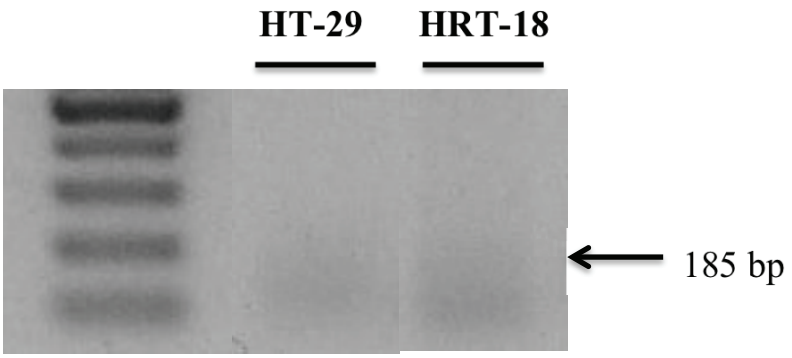


Figure 3.3.5 Colorectal carcinoma cells (HT-29 and HRT-18) do not appear to express CXCL12 mRNA based on the primers used to amplify CXCL12 mRNA. Anticipated product size, 185 base pairs (bp).

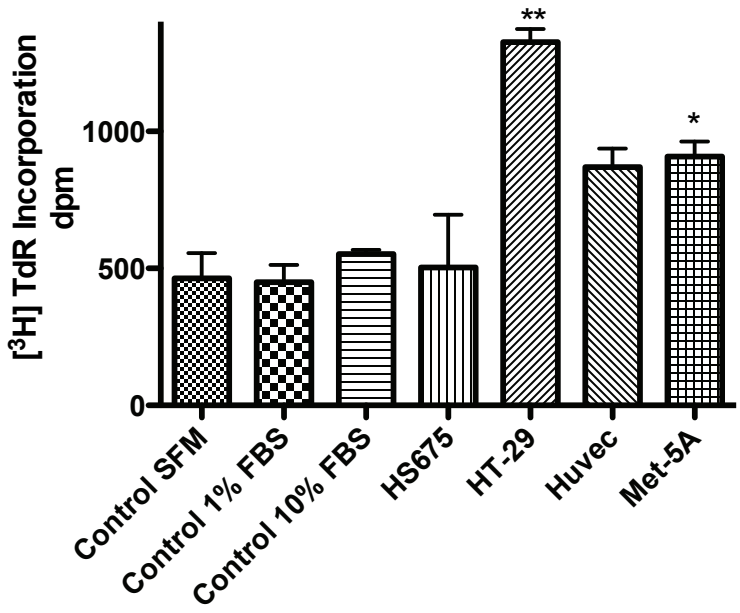
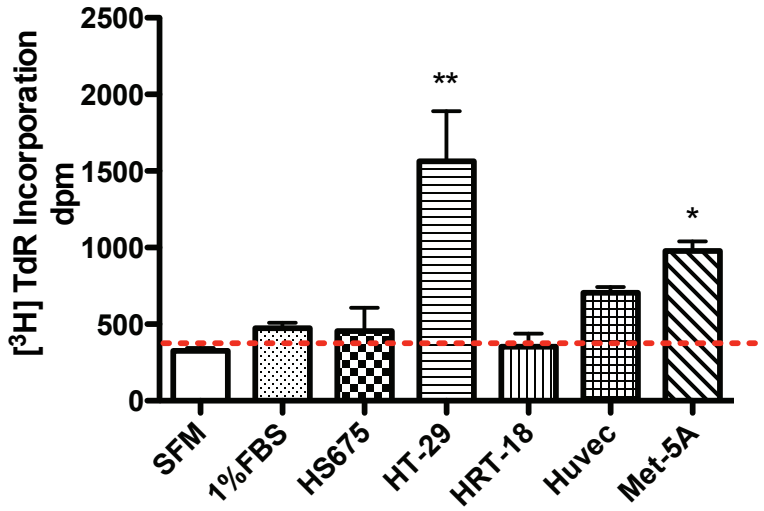


Figure 3.4.1 Conditioned mesothelial cell and colorectal carcinoma cell media stimulates an increase in thymidine incorporation in HS675.T cells. HS675.T cells were treated with the indicated medium and [^{3}H]-thymidine. [^{3}H]thymidine incorporation was measured after 36h. Data are represented as mean values +/- SEM (n=6). One-way analysis of variance $p < 0.01$; **significant change compared to control, Dunnet's multiple comparison post-test $p < 0.01$, * $p < 0.05$.

A. ADA



B. EHNA

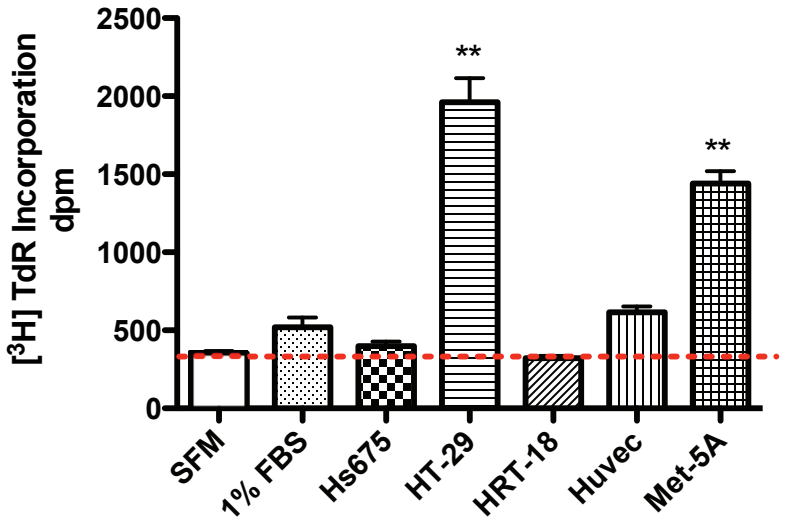


Figure 3.4.2 Neither the addition of ADA A) or EHNA B) affects the HT-29 or Met-5A induced increase in HS675.T thymidine incorporation. Data are represented as the mean values +/- SEM (n=6). One-way analysis of variance $p<0.01$; * * significant change compared to control, Dunnet's multiple comparison post-test $p<0.01$, * $p<0.05$.

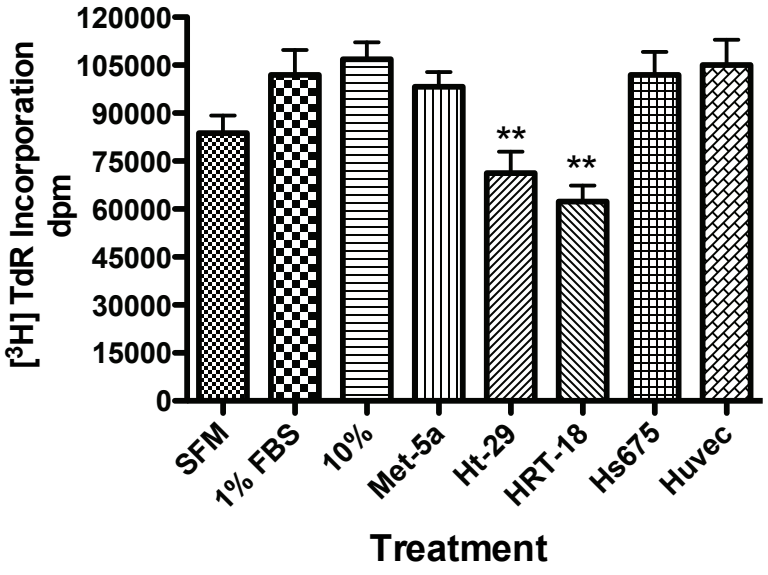


Figure 3.4.3 Conditioned media from colorectal carcinoma cells decreases thymidine incorporation in Met-5a cells. Met-5a cells were treated with the indicated media and $[H]^3$ thymidine. $[H]^3$ thymidine incorporation was measured after 36 h. Data are represented as mean values +/- SEM (n=6). One-way analysis of variance $p<0.01$; ** significant change compared to control, Dunnet's multiple comparison post-test $p< 0.01$.

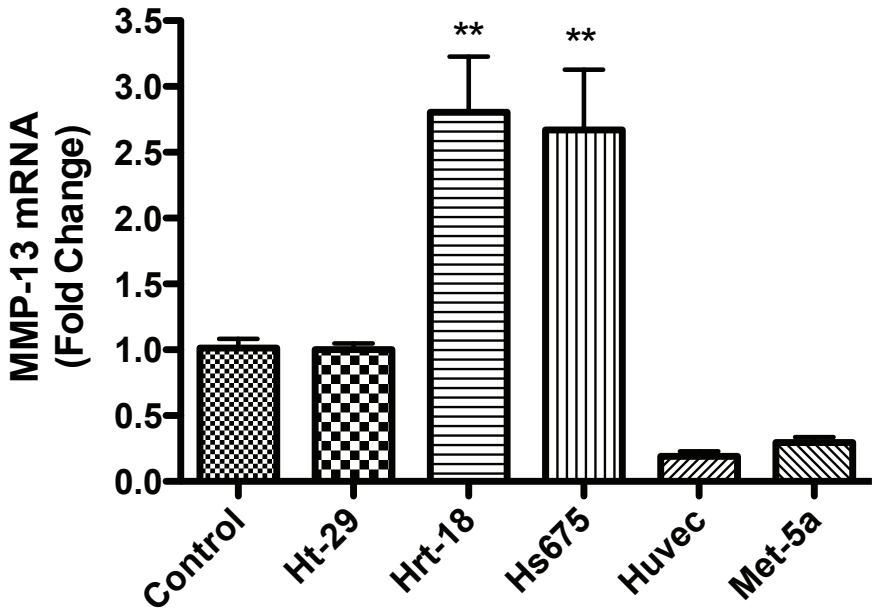


Figure 3.4.4 Conditioned medium derived from HRT-18 and HS675 cells increase MMP-13 mRNA expression in HS675 cells. HS675.T cells were treated with 20% conditioned medium from the indicated cell lines, or a serum-free control. Data is expressed as the mean values +/-SEM (n=3). One-way analysis of variance $p < 0.01$; ** significant change compared to control, Dunnet's multiple comparison post-test $p< 0.01$.

CHAPTER 4 Discussion

4.1 The effect of metabolites on CD26 expression in CRC cells

As a tumor grows it is constantly expanding beyond the diffusion limits of oxygen and its compromised vasculature; this leads to local regions of hypoxia and low levels of nutrients and leads to the accumulation of waste products within the tumor. The inefficient process of acquiring ATP through glycolysis results in cells with a higher-than-average demand for glucose and an excess production of lactate (Denko 2008). As a result, tumors often have localized regions of low glucose, low oxygen levels and high levels of lactate. CD26 cell-surface expression has been shown to be highly variable in tumors (Ten Kate, Dinjens et al. 1986; Stecca, Nardo et al. 1997; Wesley, Tiwari et al. 2004; Kajiyama, Shibata et al. 2010) and to depend on cell culture conditions (Abe, Havre et al. 2011). This may be due to areas of hypoxia in dense monolayer culture, related to oxygen diffusion (Metzen, Wolff et al. 1995). We were interested to see if conditions that mimic various aspects of the tumor microenvironment affected cell-surface levels of CD26. In particular, we looked at whether low glucose or high lactate conditions would affect CD26 expression.

The results indicate that CD26 cell-surface expression is decreased on CRC cells in low (0.5 g/l) glucose conditions. CD26 cell-surface expression was decreased by approximately one-third, as indicated by radioactive counts, when the cells were cultured in low glucose (0.5g/l) conditions. When the cells were maintained in 1.5g/l or 4.5g/l (normal culture conditions) of glucose, CD26 expression was significantly higher.

While we have not determined a mechanism for this effect, these findings are consistent with a study by Gu et al that showed that CD26 could be regulated by glucose

levels *in vitro* (Gu, Tsuda et al. 2008). Gu et al demonstrated that the glucose regulation of CD26 gene expression could be mediated by the presence of hepatocyte nuclear factor one alpha (HNF-1α) in the colorectal carcinoma cell line, Caco-2 (Gu, Tsuda et al. 2008). They showed that CD26 expression was not regulated by glucose in the absence of a functional HNF-1α by using an *in vitro* model expressing a dominant negative form of HNF-1α (Gu, Tsuda et al. 2008). Further work would be required to determine if the same mechanism occurs in our cell lines.

As we saw a change in CD26 expression resulting from changes in glucose concentrations, we investigated whether lactate had any effect of CD26 basal levels in colorectal carcinoma cells. Unlike glucose, levels of lactate tend to be high in the tumor microenvironment (20mM) and can also affect the extracellular pH of the fluid tumor microenvironment (Brizel, Schroeder et al. 2001; Walenta and Mueller-Klieser 2004). Lactate at concentrations up to 100mM had no effect on cell-surface CD26 expression in CRC cells if grown in monolayer culture. Research has shown that many proteins can be regulated by lactate, but almost all of these are cytoplasmic or nuclear instead of cell-surface proteins (Hashimoto, Hussien et al. 2007; Vegran, Boidot et al. 2011). Additionally, this is in agreement with the findings of Abe et al that acidic pH had no effect on CD26 expression in colorectal carcinoma cells (Abe, Havre et al. 2011).

However, our results were dramatically different for a three dimensional model. CRC cells grown as spheroids showed a 30%-50% decrease in cell-surface CD26 with lactate treatment, depending on the experiment and cell line. While there was a decrease in cell number as a result of increasing levels of lactate, cell death was taken into account and caused a maximum of 60% cell kill in monolayer cultures and in spheroid cultures.

Previous work in the Blay lab suggests that low-glucose culture conditions facilitate CRC cell spheroid formation (unpublished observation) and is therefore incorporated as part of the protocol for spheroid culture. The results of my spheroid assays represent the effect of the cells being cultured in low-glucose conditions. However, the effect of low-glucose culture conditions was not evaluated compared to normal culture conditions.

Multicellular tumor spheroids are considered to be more representative of tumors *in vivo* (Sutherland and Durand 1976; Sutherland, MacDonald et al. 1977; Sutherland 1988; Santini and Rainaldi 1999). My results, taken together, indicate that in conditions such as the tumor microenvironment where there are microregions of low glucose and higher levels of lactate, cell-surface CD26 levels may well be decreased on cancer cells.

Tumor lactate levels have been shown to be an indicator of poor prognosis in various solid tumors, including colorectal cancer tumors (Brizel, Schroeder et al. 2001; Koukourakis, Giatromanolaki et al. 2006; Koukourakis, Pitiakoudis et al. 2006). The cell-surface level of CD26 has been linked to changes in cancer cell morphology, cell adhesion and migration (Kajiyama, Shibata et al. 2006). Furthermore, the acidic microenvironment has been shown to affect the protonation of weakly basic drugs, decreasing their influx into the cell, and by association, the regulation of CD26 (Raghunand, He et al. 1999; Raghunand, Mahoney et al. 2003; Gerweck, Vijayappa et al. 2006). This effectively alters the bioavailability of cytotoxic agents leading to the idea that tumor acidity may need to be combated with the use of proton-pump inhibitors and other regulators of extracellular pH (Raghunand, He et al. 1999; Luciani, Spada et al. 2004). Such work needs to take into account the influence of the high levels of lactate

within a tumor. As a result, we sought to determine whether these conditions altered the chemotherapeutic-induced upregulation of CD26.

Our results indicate that the irinotecan-induced upregulation of CD26 occurs in low-glucose conditions when cells are cultured in monolayer. While this effect was not seen with 5-FU, this may be a result of the drugs having different mechanisms of action. Cell cytotoxicity following the addition of a chemotherapeutic in our CRC cells was not greatly altered as a result of low or high glucose conditions.

In contrast, when CRC spheroids were grown in low-glucose and high lactate conditions, the irinotecan-induced upregulation of CD26 occurred but was enhanced in the presence of lactate. When CRC spheroids were grown in nutrient-depleted conditioned medium, the chemotherapeutic-induced upregulation of CD26 was maintained and in some case enhanced.

Why would the response to lactate vary in spheroid versus monolayer culture? The answer may be as simple as cellular topology. In monolayer culture the cells have equal and unrestricted access to nutrients, such as glucose, in the medium. In spheroids, glucose and oxygen gradients exist within the different layers of cells (Casciari, Sotirchos et al. 1988). This creates nutrient-deficient hypoxic microregions. Hypoxia has been reported to upregulate the lactate transporter MCT-1 (Perez de Heredia, Wood et al. 2009). Thus it is possible that there is an upregulation of MCT-1, increasing lactate transport into the cells for use as energy. Additionally, increased lactate levels in the spheroid may lead to the activation or repression of lactate sensitive transcription factors and may be the reason spheroids respond so differently to lactate, with respect to CD26 expression (Formby and Stern 2003; Hashimoto, Hussien et al. 2007).

Together, these results suggest that the presence of lactate and low-glucose in a solid tumor serve to enhance colorectal tumor progression through their actions on CD26 (see **Figure 4.1**) (Dong, Tachibana et al. 1997; De Meester, Korom et al. 1999; Dang and Morimoto 2002). Additionally, preliminary data suggests they may play a role in regulating the expression of some MMPs involved in cancer progression (see **Appendix C**).

4.2 Cellular products and their effects on cell proliferation

The tumor microenvironment plays a critical role in tumor initiation and cancer progression. There are many cell types that secrete a variety of soluble factors that enhance processes such as tumor growth, angiogenesis and the epithelial-to-mesenchymal transition (EMT) (Sasaki, Nakamura et al. 2008).

The conditioned medium taken from each cell type contributes a variety of soluble mediators that influence the various cell types in the tumor microenvironment. The different mediators range in size from small metabolites and lipid-based mediators such as eicosanoids and other hormones, to larger polypeptides such as growth factors and cytokines. I wanted to see if any of the conditioned media affected the proliferation of our two stromal cell lines, HS675.T and Met-5a. The fibroblast and mesothelial cells responded very differently to the conditioned media.

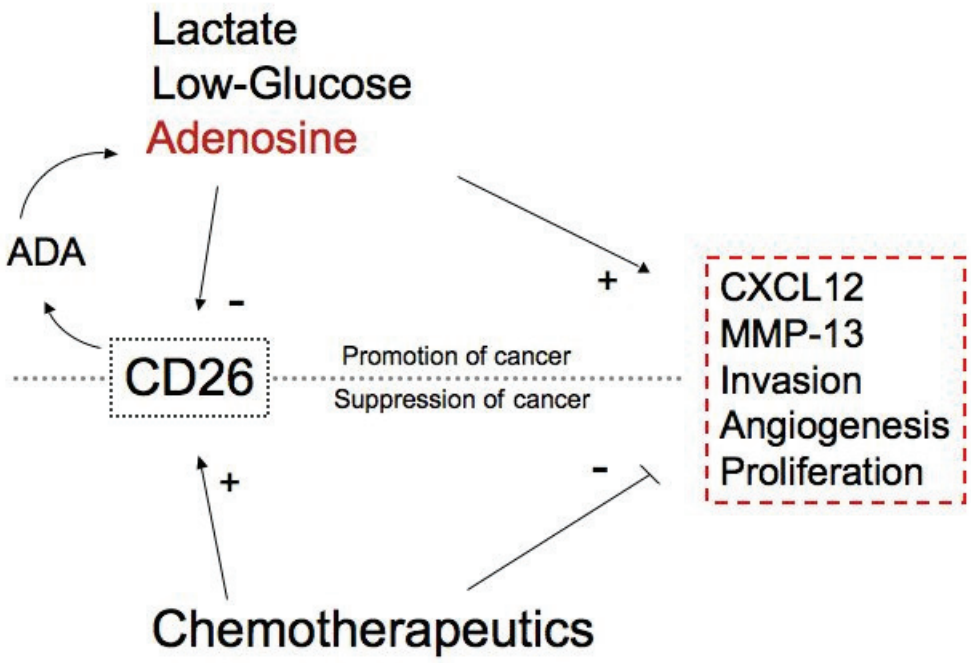


Figure 4.1. Soluble mediators (adenosine, lactate, glucose) play a role in mediating tumor promotion through their actions on CD26 and other molecules involved in cancer progression. Chemotherapeutics oppose this progression, partially by up-regulating CD26, and partly through their effects on other pro-tumorigenic processes.

Mesothelial (Met-5a) cell DNA synthesis was significantly decreased by conditioned medium from the CRC cell lines. HT-29 and HRT-18 cell conditioned media decreased thymidine incorporation by 18%-45% depending on the experiment. The implications of cancer cells suppressing cell growth in mesothelial cells may be directly related to their ability to penetrate the serosal layer. Penetration through the different layers of the colon (submucosa, muscularis externa etc) and finally the serosa leads to lymph node involvement and metastasis in the later stages of the disease. Diagnosis of serosal ulceration is thought to be an exceedingly important prognostic marker, perhaps even more important than TNM staging (Shepherd, Baxter et al. 1997; Compton 2003).

Unlike the literature on CAF involvement in cancer, less is known about the role of mesothelial cells in colorectal carcinoma progression. Yonemura et al theorized that tumor soluble factors cause mesothelial cells to retract and disaggregate to penetrate the mesothelial layer (Yonemura, Endo et al. 1996; Yonemura, Endou et al. 1997). Serosal perforation, ulceration, or an inflammatory reaction is often seen on the peritoneal lining of the large bowel in the later stages of colon cancer (Shepherd, Baxter et al. 1997). It is acknowledged that serosal involvement is consistent with a poor prognosis in patients with CRC, but it is not understood how they penetrate the serosal layer.

Our results indicate that tumor soluble factors are able to decrease the proliferation of mesothelial cells. This might limit the ability of the mesothelium to repair itself and therefore facilitate tumor penetration of the serosal layer. To further expand on these studies, I would have liked to assess markers of apoptosis following CRC medium treatment in our Met-5A cells. Heath et al. determined in an *in vitro* model that tumor

cells were able to adhere and induce apoptosis in a FasL/Fas dependent manner (Schlaeppi, Ruegg et al. 1997; Strobel and Cannistra 1999; Heath, Jayne et al. 2004).

In contrast, HT-29 medium increased thymidine incorporation in HS675.T cells by 2.9 fold whereas the Met-5a medium increased thymidine incorporation by 1.5-2 fold in HS675.T cells. CAFs and their various roles in promoting tumor growth and invasion have been well documented in the literature (De Wever and Mareel 2003; Franco, Shaw et al. 2010; Shimoda, Mellody et al. 2010; Xouri and Christian 2010). The majority of studies focus on the effect of CAFs to promote cancer cell growth and tumorigenic progression (Orimo, Gupta et al. 2005; De Wever, Demetter et al. 2008). Conversely, my results suggest that cancer cells themselves secrete soluble factors promoting the growth of CAFs. Whether the cancer cells recruit and transform fibroblasts, or whether mutated fibroblasts induce neoplastic growth first, is not clear, but there is literature suggesting both may occur (Bhowmick, Ghiassi et al. 2001; Bhowmick, Neilson et al. 2004; Martinez-Outschoorn, Pavlides et al. 2010). The mechanism surrounding CAF transformation is not well understood. For example, tumor cell-derived soluble factors have been shown to induce the CAF phenotype in normal mammary-derived fibroblasts (Martinez-Outschoorn, Pavlides et al.). The finding that CRC cells may also regulate CAF growth is consistent with the fact that cancer cells secrete mediators to prime the microenvironment for optimal growth and progression. Cancer cells may secrete various mediators, such as TGF-ß, IGF-1 and PDGF, which would support fibroblast activation and proliferation (Barcellos-Hoff and Ewan 2000; Bauer, Su et al. 2010). However, many of these studies identifying these mediators are conducted in regular cell culture

conditions that do not mimic the tumor microenvironment (Roberts, Tian et al. 2006; Chen, Yang et al. 2009). Further work would be required to pinpoint a mediator.

In addition, I found that mesothelial cell conditioned medium also affected CAF proliferation. The interaction between these cell types is not often considered in the context of cancer research. Mesothelial cells have the ability to release various mediators, such as FGF-2, when they become activated (Topley, Brown et al. 1993; Li, Davenport et al. 1998; Basok, Shnaider et al. 2001; Sako, Kitayama et al. 2003). It is plausible that the Met-5A cells induce proliferation in an FGF-2 dependent manner. RT-PCR analysis has confirmed that Met-5A cells express FGF-2 and that HS675.T cells express the relevant receptors (See **Appendix D**). Further work is required to confirm this hypothesis.

We wondered if adenosine may be present in the conditioned media of Met-5a and HT-29 cells, and if it may be in part responsible for this increase in thymidine incorporation in HS675.T cells (Mujoomdar, Hoskin et al. 2003; Mujoomdar, Bennett et al. 2004). It is also possible that adenosine may be mediating the decrease in proliferation seen in Met-5A cells (Huffaker, Corcoran et al. 1984; Dubey, Gillespie et al. 1996; Merighi, Benini et al. 2005). Sufficient levels of ADA were added to degrade feasible levels of adenosine. This did not result in any decreases in thymidine incorporation as would be expected had the effect been adenosine-mediated. Use of the ADA inhibitor EHNA also failed to significantly alter the effect of the conditioned medium on thymidine incorporation in HS675.T cells. The HPLC work failed to demonstrate the presence of adenosine in the conditioned media but indicated the presence of a structurally related molecule that we were unable to properly identify (data not shown).

We hypothesized that it could be inosine, a metabolite of adenosine. Thymidine incorporation assays with inosine indicated that inosine significantly increased proliferation in HS675.T cells, but had no effect on the proliferation of Met-5A cells.

The complexity of conditioned medium makes it mechanistically difficult to pinpoint the mediators of a biological effect. Identifying mediators of these effects was not the goal of this project and represents a very prodigious undertaking. Treating one cell type with conditioned medium from another is not always the best way to assess cellular communication between two cell types. However, should I continue with the project the next logical step would be to assess if these changes in proliferation occur in co-culture models using Transwell® inserts. This would be a more accurate representation of the cellular crosstalk that occurs *in vivo*.

4.3 The role of adenosine as a key cellular product

Adenosine has been found to be mitogenic in certain carcinoma cell lines (Mujoomdar, Hoskin et al. 2003; Mujoomdar, Bennett et al. 2004). There is also a body of literature supporting the fact that adenosine may be cytotoxic to certain cell types, including neutrophils, murine neuroblastoma cells, lymphoblasts, and various other cell types (Snyder, Hershfield et al. 1978; Mirkin, O'Dea et al. 1987; Seetulsingh-Goorah 2006). Our studies indicated that adenosine might play a pro-tumorigenic role. For a summary of our findings on the adenosine effect on different cell lines, see **Figure 4.2**.

Adenosine treatment resulted in an average of a 30% increase in thymidine incorporation in HS675.T cells. The shape of the DNA synthesis curve was consistent and exhibited dose dependent increases in thymidine incorporation up to 300μM of adenosine. However, this contrasts with the normal fibroblast cell line, Detroit-551 (see

Appendix E). Preliminary data suggests that the growth of these normal fibroblasts is not stimulated by adenosine. Further work to determine how adenosine elicits its effects on cell proliferation in various cell lines to see if perhaps adenosine primarily elicits its effects on the transformed cell types in the tumor microenvironment.

The stimulatory effect of adenosine on DNA synthesis was not seen in Met-5a cells. Similar to experiments on HS675.T cells, the Met-5a cells were grown in 10% FBS. The cells were also downshifted to low serum conditions to see if the serum starvation would reveal an adenosine effect or make the cells more responsive to adenosine. There was no change in thymidine incorporation in either condition. The basal level of cell growth in Met-5a cells as indicated by the level thymidine incorportation were significantly higher than the basal level of cell growth in primary fibroblasts.

It is possible that cell turnover rate plays a role in the adenosine response; the mesothelial cells used are phenotypically very different from the primary fibroblasts used in our studies. Mesothelial cell lines in culture have a much shorter doubling time and as a result they may not be as sensitive to growth altering agents (Tweel and Blay, unpublished observation). Alternately, the sensitivity of mesothelial cells to growth altering agents may not be reduced, but any additional mitogenic effects of a treatment may not be noticeable since the cells proliferate so rapidly. Conversely, *in vivo*, the mesothelial lining is a slowly renewing tissue, with less than one percent of the cells undergoing division at any one time (Mutsaers, Whitaker et al. 2002). After insult or injury, the number of cells actively preparing for division may be as high as eighty percent (Mutsaers, Whitaker et al. 2002), suggesting the regulation of mesothelial cell growth is complex and tightly regulated *in vivo*.

While the cell types used for my studies expressed all four adenosine receptors, mesothelial cells had different levels of the receptor mRNA expression, and this may have played a role in regulating the response to adenosine. This does not necessarily reflect the level of adenosine receptor expression on the cell surface, so further work to assess cell-surface levels of the adenosine receptors would be necessary to confirm that this was a factor.

Mesothelial cells may be commonly exposed to adenosine in periods of acute peritonitis. Adenosine levels are upregulated in peritonitis and the peritoneal mesothelial cells are thought to play an important role in regulating inflammation (Yao, Platell et al. 2003; Rogachev, Ziv et al. 2006). During acute peritonitis, adenosine levels are known to increase significantly, up to 2.5μM in mouse models (Rogachev, Ziv et al. 2006). This suggests that mesothelial cells have the ability to tolerate these pathological concentrations of adenosine. Mujoomdar et al found that the adenosine-mediated increase in cell proliferation in CRC cell lines was a result of A2 receptor activation (Mujoomdar, Hoskin et al. 2003). In addition, it is the A2B and A3 receptors that most frequently implicated in regulating cell proliferation (Grant, Davis et al. 2001; Merighi, Benini et al. 2005; Ma, Kondo et al. 2010). During acute peritonitis, mesothelial cells respond by quickly increasing A1 receptor levels (Rogachev, Ziv et al. 2006). While A2A and A2B receptor protein levels steadily increase for 72 h from the onset of peritonitis, the level of expression has been shown to be significantly lower than the A1 receptor (Rogachev, Ziv et al. 2006). The induction of the A1 receptor facilitates the inflammatory stage of peritonitis, promoting the release of inflammatory mediators and recruiting leukocytes (Nakav, Chaimovitz et al. 2008). A1 receptor levels increase and are

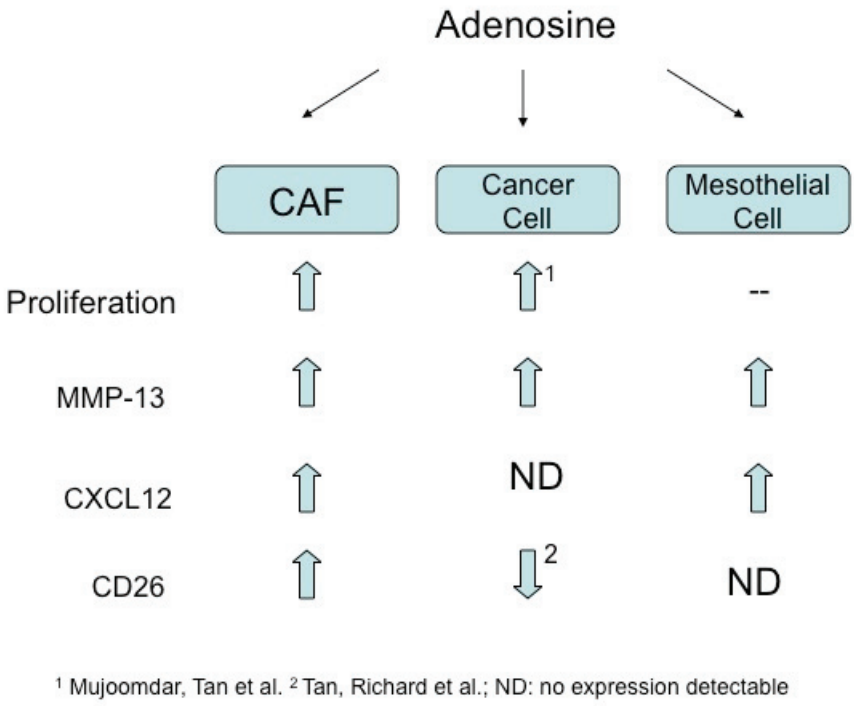
activated, thereby preventing the proliferation of Met-5A cells. Further work would be required to validate this in our cell line. An important follow-up would be to assess the effects of chronic adenosine levels on adenosine receptor expression in Met-5A cells. It is possible that chronic adenosine treatment would lead to an eventual downregulation of the A1 receptor over time, and induction of the A2A receptor, as in peritonitis (Nakav, Naamani et al. 2010), thereby modifying the response to adenosine.

4.4 Adenosine may play a role in promoting chemotaxis and angiogenesis

Cells overexpressing the CXCL12 receptor, CXCR4, travel towards a CXCL12 gradient (Terada, Yamamoto et al. 2003; Sun, Pedersen et al. 2008). Small changes in a CXCL12 gradient are able to stimulate a chemotactic response (Scotton, Wilson et al. 2001; Scotton, Wilson et al. 2002). Richard et al showed that pre-treatment with adenosine increased HT-29 cell invasion towards a CXCL12 gradient through the up-regulation of the CXCR4 receptor (Richard, Tan et al. 2006).

Interestingly, we were able to show that our CRC cells do not express CXCL12. We used reverse transcriptase PCR (RT-PCR) to detect a CXCL12 mRNA signal in CRC cells. Neither the HT-29 cells nor the HRT-18 cells had any detectable transcript product. This may be a result of promoter hypermethylation, although that was not directly tested in our cells. Previous work by Wendt et al reported that the CXCL12 gene was epigenetically modified in colorectal cancer cells. Further research revealed that the gene was hypermethylated in the CRC cells and as a result it was not expressed (Wendt, Cooper et al. 2008).

Figure 4.2 A Summary of the effect of adenosine on various cell types used in our studies.



[1] Mujoomdar, Tan et al. [2] Tan, Richard et al.; ND: no expression detectable

In our stromal cell lines (HS675.T, Met-5A), we assessed changes in CXCL12 mRNA expression. At 300μM, adenosine increased CXCL12 mRNA in HS675.T cells by an average of two-fold over control. In Met-5a cells, CXCL12 mRNA expression was increased by three-fold over control at 30μM adenosine.

The induction of CXCL12 gene expression by an inflammatory mediator, adenosine, in two reactive cell types (fibroblasts and mesothelial cells) is not surprising considering CXCL12's various roles in cell trafficking and tissue repair (Santiago, Calonge et al. 2010). CXCL12 regulation in this context is typically attributed to typical inflammatory regulated signaling pathways, such as MAPK or NF-κB (Amin, Mansfield et al. 2007). Adenosine has been shown to suppress NF-κB and enhance AP-1 DNA binding (Li, Ha et al. 2000). CXCL12 expression is AP-1 dependent, so it is possible that adenosine increases CXCL12 mRNA through enhanced AP-1 binding (Florin, Hummerich et al. 2004; Hess, Angel et al. 2004). Further work would be required to determine how adenosine mediates its effects on CXCL12 mRNA. The mechanism may be receptor dependent or may rely on the transport of adenosine into the cell.

CXCL12 expression can be induced during hypoxia by HIF-1 (Ceradini, Kulkarni et al. 2004). There are local microregions within a solid tumor that are hypoxic and trabecular regions containing stromal cells, including fibroblasts. The increase of CXCL12 mRNA by adenosine supports a novel hypoxic mechanism for inducing CXCL12 expression in the tumor microenvironment. The concentration of adenosine required to evoke a maximal increase of CXCL12 mRNA in Met-5A cells versus HS675.T cells differs by an order of magnitude. This suggests that adenosine may be facilitating different processes through its actions on CXCL12 mRNA. CXCL12 mRNA

is significantly increased in HS675.T at higher concentrations of adenosine that are characteristic of necrosis (la Sala, Ferrari et al. 2003). Regions of necrosis are found in hypoxic and poorly vascularized regions of a tumor. It is plausible that the production of CXCL12 by HS675.T in severely hypoxic regions serves to recruit endothelial progenitor cells (EPCs) and enhance angiogenesis, leading to a more conducive environment for tumor growth (Orimo, Gupta et al. 2005).

In Met-5A cells, CXCL12 mRNA peaks between 10-30μM adenosine and decreases with increasing concentrations of adenosine. As reported by Blay et al (Blay, White et al. 1997), the extracellular fluid in tumors approaches upwards of 20μM of adenosine. As the tumor invades through the layers of the colon, adenosine may provide a stimulatory chemotactic signal by inducing CXCL12 production in the mesothelium, directing cancer cell migration to the serosa (**Figure 4.2**)(Yasumoto, Koizumi et al. 2006).

It is therefore possible that the initial stimulation of cancer cell chemotaxis in poorly vascularized regions of tumor may come directly from proximal stromal cells. For some preliminary migration assay data, see **Appendix B**. Our data suggests that the conditioned medium from both Met-5A and HS675.T cells (untreated) enhances HT-29 cell migration. Further studies to determine if this conditioned medium is chemotactic to endothelial cells would prove interesting and worthwhile.

We were curious as to whether the HS675.T were producing CXCL12 to act in an autocrine or paracrine manner, or both. We confirmed that HS675.T cells do not express the CXCR4 receptor, nor are they responsive to CXCL12. These results suggest that

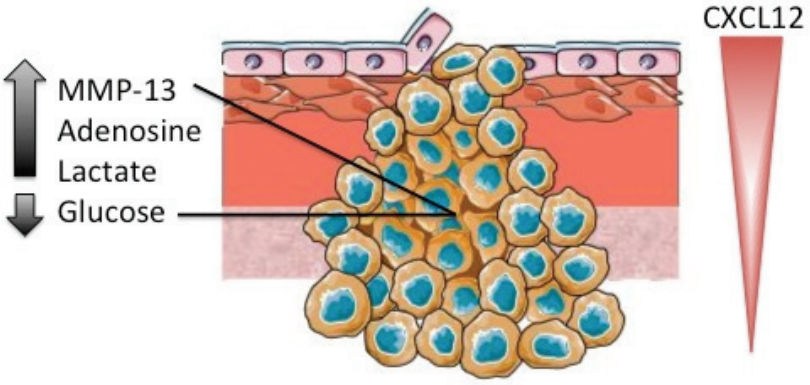


Figure 4.3 As the tumor grows and invades through the colon the different cell types and soluble mediators influence its progression. Figure produced using Servier Medical Art.

CXCL12 is not acting through its receptor, CXCR4, on HS675.T cells. CXCL12 produced by HS675.T cells is not likely acting in an autocrine fashion but rather as a juxtacrine or paracrine factor, being secreted from the fibroblasts and acting upon other adjacent cells such as the cancer cells themselves. Additionally, Tan et al showed that adenosine regulated the cell-surface expression of CXCR4 on colorectal cancer cells, suggesting that adenosine plays a dual role in regulating the CXCL12:CXCR4 axis (Tan, Mujoomdar et al. 2004; Richard, Tan et al. 2006).

In a clinical context, the regulatory effects of adenosine relate to three important processes: tumor growth, angiogenesis and cell migration/metastasis. Classical cytotoxic therapy is still used therapeutically to target rapidly growing tumors. However, it is not always successful, particularly in targeting the hypoxic fraction of cells. The use of different adenosine receptor modulators in cancer treatment has been suggested. As mentioned in the introduction, Mujoomdar et al discovered that the A2 receptors were involved in the adenosine-induced proliferation of colorectal carcinoma cells (Mujoomdar, Hoskin et al. 2003). Additionally, they are known to stimulate angiogenesis and may possibly regulate MMP-13 mRNA in colorectal carcinoma cells (Montesinos, Gadangi et al. 1997; Sexl, Mancusi et al. 1997; Ma, Kondo et al.). Adenosine has also been shown to suppress the immune response through the stimulation of the A2 receptors *in vivo* (Lukashev, Ohta et al. 2004; Sitkovsky, Lukashev et al. 2004). Adenosine A2 receptor antagonists may diminish some of the angiogenic, proliferative and other tumor-promoting effects of hypoxia. Treatment modalities may some day include novel anti-adenosinergic approaches to target processes like angiogenesis and tumor growth.

There are several *in vivo* models of colorectal cancer metastases that could be used to evaluate whether adenosine had any effect on the invasive, or metastatic, phenotype of our cells. Orthotopic xenograft models are often utilized, however, many established cell lines are often exhibit variable metastatic capacities (Flatmark, Maelandsmo et al. 2004; Haier, Nasralla et al. 1999). For this reason, *in vivo* models to induce metastases in specific organs or locations have evolved. For example, two *in vivo* models commonly used to mimic liver metastases involve microinjecting colorectal carcinoma cells into the tail vein or spleen of immunocompromised mice (Giavazzi, Jessup et al. 1986; Garofalo, Chirivi et al. 1993; Cespedes, Espina et al. 2007). Following the microinjection of cancer cells, the ability of these cells to form metastases can be evaluated by counting the number of micrometastases or colonies formed (Heijstek, Kranenburg et al. 2005).

4.5 The contribution of different cell types and soluble mediators in the production of MMPs

MMP-13 is one of many proteases that are over-expressed in colorectal carcinomas and is also a marker of poor prognosis in colorectal cancer (Leeman, Curran et al. 2002; Yamada, Oshima et al. 2010). MMP-13 up-regulation in cancer is known to be a result of transcriptional regulation as opposed to gene amplification, and therefore we needed to quantitatively analyze these changes (Benbow and Brinckerhoff 1997). Current literature suggests that MMP-13 gene expression in colorectal tumor tissue positively correlates with liver metastases (Yamada, Oshima et al. 2010).

We found that adenosine treatment increased MMP-13 mRNA in all of our cell lines. MMP-13 mRNA expression in Met-5a cells peaked at 30μM adenosine with gene expression increasing 15-fold over control. MMP-13 mRNA expression in HS675.T cells increased an average of 10-fold at 300μM. The most sensitive response to adenosine was seen in HRT-18 cells, with MMP-13 mRNA reaching a maximum at 10μM with a 5-fold increase over control.

Comparing the sensitivities of the MMP-13 response to adenosine in each cell type is interesting. For the colorectal cancer cells, the most effective concentration of adenosine is 10 μM and the response declines at higher concentrations (**Figure 3.3.1b**). In HS675.T cells, however, there is very little sign of an effect on MMP-13 mRNA expression at 10uM adenosine, but a dramatic response was detected at 100μM adenosine and above (**Figure 3.3.2c**). These contrasting and in fact complementary dose-response relationships raise the question of whether the regulation of MMP-13 is differentially handled by different cell types in the environment of a tumor. In a typical hypoxic area, where the adenosine concentration reaches approximately 20μM (Blay, White et al. 1997), MMP-13 would likely be produced mainly by the cancer cells. However, in areas close to necrosis where the adenosine concentration can range up to 100μM (Blay, White et al. 1997; la Sala, Ferrari et al. 2003), it may be the CAFs that are the main responders, with MMP expression possibly favoring angiogenesis and local invasion. Western blot analysis and zymography were performed extensively to confirm the expression data but the findings were not conclusive (See **Appendix A** for representative data). The data suggests that adenosine does increase MMP-7 secretion in CRC cells and may enhance the secretion of MMP-13 in CRC and HS675.T cells.

The adenosine response on MMP-13 mRNA in the Met-5a and HRT-18 cells increases in a dose-dependent manner until it reaches peak levels followed by a decrease in MMP-13 mRNA expression with increasing concentrations of adenosine. This gives the overall curve a bell shape. As previously described, the adenosine receptors are all GPCRs. Bell-shaped curves may arise from ligand-induced receptor interconversion between high-affinity and low-affinity states associated with G-protein coupling or interaction between receptor oligomers (Jarv 1995; Avlani, May et al. 2004; Christopoulos, May et al. 2004).

To identify which adenosine receptor(s) may be contributing to the increase in MMP-13 mRNA expression in HRT-18 cells, we used receptor agonists and antagonists. These results were difficult to interpret as the synthetic compounds were solubilized in DMSO, a solvent that was found to increase MMP-13 mRNA expression in CRC cell lines (**Appendix F**). However, use of two adenosine agonists, NECA (A2) and CGS21680 (A2A) suggested to us that it was MMP-13 mRNA was at least in part regulated by the A2 receptors. We were not able to stimulate the A2B receptor separately due to the unavailability of selective agonists. Studies have shown that use of synthetic adenosine agonists do not always reproduce the biological effects of adenosine but may be helpful in identifying contributing receptors (Merighi, Mirandola et al. 2002; Fishman, Bar-Yehuda et al. 2003).

We saw that the adenosine induced fold-change of MMP-13 mRNA expression in stromal cells is greater than in colorectal carcinoma cells. Additionally, we can infer from the control threshold values for MMP-13 mRNA in each cell line that both stromal cell lines have higher basal levels of expression of MMP-13 than the HRT-18s. This is

consistent with the literature suggesting that the supportive stroma contributes the majority of proteolytic enzymes during local cancer cell invasion (Hazan, Kang et al. 1997; De Wever and Mareel 2003). Promoting changes in cell-ECM interaction through the excess production of MMPs facilitates migration of cancer cells through the underlying stroma.

The conditioned media results indicated to us that there were some soluble mediators influencing cell proliferation in HS675.T and Met-5a cells. We took a quantitative approach and looked at whether or not some of these soluble mediators were able to change MMP-13 mRNA expression in HS675.T cells.

Results showed that conditioned medium from HRT-18 CRC cells and conditioned medium from HS675.T cells significantly increases MMP-13 mRNA 2-3 fold over control and 2-fold over control, respectively. This implies that the increase in MMP-13 mRNA can occur in an autocrine fashion in HS675.T cells, and that the mechanism by which HRT-18 and HS675.T increase MMP-13 mRNA may be completely different. The autocrine effect could be explained by the enormous variety of growth factors that CAFs are reported to produce including FGF-2 (Giulianelli, Cerliani et al. 2008; Fabris, Sahores et al. 2010). HS7675.T cells express both FGF-2 and its receptors (see **Appendix D**). FGF-2 has been shown to induce the expression of MMP-13 in several systems through the activation MAPK and NF-κB by stimulating FGFR1 (Wang and Keiser 1998; Aharinejad, Krenn et al. 2005).

The regulation of MMPs in supportive stroma by tumor cells is likely regulated by complex crosstalk involving not one, but a variety of soluble mediators. Stuelten et al showed that MMP-9 expression in stromal fibroblasts could be induced by several

factors, mainly TGF-β, TNF-α secreted by breast cancer cells (Stuelten, DaCosta Byfield et al. 2005). The induction of MMP by TNF-α has been shown in other tumor infiltrating cells, such as macrophages (Hagemann, Robinson et al. 2004). Many cell types express CD147, also known as the extracellular matrix metalloproteinase inducer (EMMPRIN). EMMPRIN is enriched on the surface of many tumor cells and has been shown to induce the production of collagenase in human gingival fibroblasts (Prescott, Troccoli et al. 1989; Nabeshima, Lane et al. 1991; Biswas, Zhang et al. 1995; Guo, Zucker et al. 1997; Lim, Martinez et al. 1998; Davidson, Goldberg et al. 2003). We have not examined the expression of these mediators in our cell lines, and further research is required to identify the specific factors contributing to the observed effects on MMP-13 expression.

CHAPTER 5 Summary and Conclusions

Adenosine is one of many possible soluble mediators found in the extracellular fluid of the tumor microenvironment. It acts as regulatory molecule in the tumor microenvironment. The body of literature supporting this follows two schools of thought; the first suggests that adenosine is anti-tumorigenic and induces apoptosis in tumor cells (Fishman, Bar-Yehuda et al. 2004; Madi, Ochaion et al. 2004; Borea, Gessi et al. 2009). The second school of thought supports a pro-tumorigenic role for adenosine through various mechanisms (Blay, White et al. 1997; MacKenzie, Hoskin et al. 2002; Mujoomdar, Hoskin et al. 2003). My work in this thesis supports the latter. Previous evidence for the pro-tumorigenic role of adenosine in the tumor microenvironment focuses on the tumor cells and the cells of the immune system. My work on adenosine focuses on both the tumor cells and supportive fibroblasts and mesothelial cells. The data suggests that local extracellular concentrations of adenosine may play a role in facilitating colorectal cancer progression and metastasis. This may occur through the production of proteolytic enzymes, MMPs, and chemoattractants, CXCL12, leading to successful tissue invasion. As described, adenosine has been previously shown to down-regulate CD26 and up-regulate CXCR4, further promoting the chemotactic response to CXCL12 production. As levels of adenosine surge during hypoxia, these data may represent additional mechanisms, which may be HIF-1 independent, by which the hypoxic state influences tumor growth.

The tumor microenvironment is rich in factors, produced by heterogeneous populations of cells, influencing tumor progression. These factors contribute to disease progression by affecting several mechanisms. The conditioned media studies support this.

This suggests that cancer cells release factors that support the growth of stromal cells while also stimulating them release MMPs, thereby facilitating local cancer cell invasion. Conversely, colorectal carcinoma cell conditioned medium decreased cell proliferation of Met-5a cells. This fits with literature that suggests cancer cells are able to induce apoptosis in mesothelial cells in order to invade through the peritoneal layers. In colorectal cancer, cancer cell invasion through peritoneal layers occurs in the later stages of disease, when the tumor breaks through the mesothelial lining of the colon and then when it eventually progresses to peritoneal carcinomatosis.

Lastly, we believe that the loss of CD26 expression on the CRC cell-surface contributes to colorectal cancer progression. Downregulation of CD26 expression can occur as result of changes in the local microenvironment (Ten Kate, Wijnen et al. 1985; Ten Kate, Dinjens et al. 1986). Loss of CD26 expression can lead to decreased cell-adhesion and immune surveillance and an increase in adenosine levels, angiogenesis, and chemotaxis (Hanski, Huhle et al. 1985; Hanski, Huhle et al. 1988; Dong, Tachibana et al. 1997; Richard, Tan et al. 2006). It is clear that the tumor microenvironment plays a significant role in the disease pathogenesis.

References

Abbott, C. A., E. Baker, et al. (1994). "Genomic organization, exact localization, and tissue expression of the human CD26 (dipeptidyl peptidase IV) gene." Immunogenetics **40**(5): 331-338.

Abe, M., P. A. Havre, et al. (2011). "Mechanisms of confluence-dependent expression of CD26 in colon cancer cell lines." BMC Cancer **11**: 51.

Aertgeerts, K., S. Ye, et al. (2004). "N-linked glycosylation of dipeptidyl peptidase IV (CD26): effects on enzyme activity, homodimer formation, and adenosine deaminase binding." Protein Sci **13**(1): 145-154.

Aharinejad, S., K. Krenn, et al. (2005). "Differential role of TGF-beta1/bFGF and ET-1 in graft fibrosis in heart failure patients." Am J Transplant **5**(9): 2185-2192.

Ahren, B., E. Simonsson, et al. (2002). "Inhibition of dipeptidyl peptidase IV improves metabolic control over a 4-week study period in type 2 diabetes." Diabetes Care **25**(5): 869-875.

Akbar, G. K., V. R. Dasari, et al. (1996). "Molecular cloning of a novel P2 purinoceptor from human erythroleukemia cells." J Biol Chem **271**(31): 18363-18367.

Amin, M. A., P. J. Mansfield, et al. (2007). "Interleukin-18 induces angiogenic factors in rheumatoid arthritis synovial tissue fibroblasts via distinct signaling pathways." Arthritis Rheum **56**(6): 1787-1797.

Amour, A., P. M. Slocombe, et al. (1998). "TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3." FEBS Lett **435**(1): 39-44.

Anderberg, C., H. Li, et al. (2009). "Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts." Cancer Res **69**(1): 369-378.

Andy, R. J. and R. Kornfeld (1982). "The adenosine deaminase binding protein of human skin fibroblasts is located on the cell surface." J Biol Chem **257**(14): 7922-7925.

Antczak, C., I. De Meester, et al. (2001). "Ectopeptidases in pathophysiology." Bioessays **23**(3): 251-260.

Ao, M., O. E. Franco, et al. (2007). "Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium." Cancer Res **67**(9): 4244-4253.

Arkenau, H. T., A. Bermann, et al. (2003). "5-Fluorouracil plus leucovorin is an effective adjuvant chemotherapy in curatively resected stage III colon cancer: long-term follow-up results of the adjCCA-01 trial." Ann Oncol **14**(3): 395-399.

Arvold, N. D., N. Guha, et al. (2005). "Hypoxia-induced radioresistance is independent of hypoxia-inducible factor-1A in vitro." Int J Radiat Oncol Biol Phys **62**(1): 207-212.

Asada, Y., Y. Aratake, et al. (1993). "Expression of dipeptidyl aminopeptidase IV activity in human lung carcinoma." Histopathology **23**(3): 265-270.

Avlani, V., L. T. May, et al. (2004). "Application of a kinetic model to the apparently complex behavior of negative and positive allosteric modulators of muscarinic acetylcholine receptors." J Pharmacol Exp Ther **308**(3): 1062-1072.

Baggiolini, M. (1998). "Chemokines and leukocyte traffic." Nature **392**(6676): 565-568.

Baker, S. M., C. E. Bronner, et al. (1995). "Male mice defective in the DNA mismatch repair gene PMS2 exhibit abnormal chromosome synapsis in meiosis." Cell **82**(2): 309-319.

Barcellos-Hoff, M. H. and K. B. Ewan (2000). "Transforming growth factor-beta and breast cancer: Mammary gland development." Breast Cancer Res **2**(2): 92-99.

Basok, A., A. Shnaider, et al. (2001). "CD40 is expressed on human peritoneal mesothelial cells and upregulates the production of interleukin-15 and RANTES." J Am Soc Nephrol **12**(4): 695-702.

Bauer, M., G. Su, et al. (2010). "Heterogeneity of gene expression in stromal fibroblasts of human breast carcinomas and normal breast." Oncogene **29**(12): 1732-1740.

Baumhoer, D., L. Tornillo, et al. (2008). "Glypican 3 expression in human nonneoplastic, preneoplastic, and neoplastic tissues: a tissue microarray analysis of 4,387 tissue samples." Am J Clin Pathol **129**(6): 899-906.

Bauvois, B. (2004). "Transmembrane proteases in cell growth and invasion: new contributors to angiogenesis?" Oncogene **23**(2): 317-329.

Bauvois, B., I. De Meester, et al. (1999). "Constitutive expression of CD26/dipeptidylpeptidase IV on peripheral blood B lymphocytes of patients with B chronic lymphocytic leukaemia." Br J Cancer **79**(7-8): 1042-1048.

Becelli, R., G. Renzi, et al. (2007). "Intracellular and extracellular tumor pH measurement in a series of patients with oral cancer." J Craniofac Surg **18**(5): 1051-1054.

Becker, A., P. Stadler, et al. (2000). "Severe anemia is associated with poor tumor oxygenation in head and neck squamous cell carcinomas." Int J Radiat Oncol Biol Phys **46**(2): 459-466.

Belotti, D., P. Paganoni, et al. (2003). "Matrix metalloproteinases (MMP9 and MMP2) induce the release of vascular endothelial growth factor (VEGF) by ovarian carcinoma cells: implications for ascites formation." Cancer Res **63**(17): 5224-5229.

Benbow, U. and C. E. Brinckerhoff (1997). "The AP-1 site and MMP gene regulation: what is all the fuss about?" Matrix Biol **15**(8-9): 519-526.

Bergers, G. and L. E. Benjamin (2003). "Tumorigenesis and the angiogenic switch." Nat Rev Cancer **3**(6): 401-410.

Bhowmick, N. A., M. Ghiassi, et al. (2001). "Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism." Mol Biol Cell **12**(1): 27-36.

Bhowmick, N. A., E. G. Neilson, et al. (2004). "Stromal fibroblasts in cancer initiation and progression." Nature **432**(7015): 332-337.

Bigelow, R. L., B. J. Williams, et al. (2009). "TIMP-1 overexpression promotes tumorigenesis of MDA-MB-231 breast cancer cells and alters expression of a subset of cancer promoting genes in vivo distinct from those observed in vitro." Breast Cancer Res Treat **117**(1): 31-44.

Bing, O. H., W. W. Brooks, et al. (1973). "Heart muscle viability following hypoxia: protective effect of acidosis." Science **180**(4092): 1297-1298.

Birgisson, H., H. J. Nielsen, et al. (2010). "Preoperative plasma TIMP-1 is an independent prognostic indicator in patients with primary colorectal cancer: a prospective validation study." Eur J Cancer **46**(18): 3323-3331.

Biswas, C., Y. Zhang, et al. (1995). "The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily." Cancer Res **55**(2): 434-439.

Blay, J., T. D. White, et al. (1997). "The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine." Cancer Res **57**(13): 2602-2605.

Bode, W., F. X. Gomis-Ruth, et al. (1993). "Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'." FEBS Lett **331**(1-2): 134-140.

Bohm, S. K., J. R. Gum, Jr., et al. (1995). "Human dipeptidyl peptidase IV gene promoter: tissue-specific regulation from a TATA-less GC-rich sequence characteristic of a housekeeping gene promoter." Biochem J **311 (Pt 3)**: 835-843.

Boonacker, E. P., E. A. Wierenga, et al. (2002). "CD26/DPPIV signal transduction function, but not proteolytic activity, is directly related to its expression level on human Th1 and Th2 cell lines as detected with living cell cytochemistry." J Histochem Cytochem **50**(9): 1169-1177.

Borea, P. A., S. Gessi, et al. (2009). "A3 adenosine receptor: pharmacology and role in disease." Handb Exp Pharmacol(193): 297-327.

Bourboulia, D. and W. G. Stetler-Stevenson (2010). "Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion." Semin Cancer Biol **20**(3): 161-168.

Boyer, M. J. and I. F. Tannock (1992). "Regulation of intracellular pH in tumor cell lines: influence of microenvironmental conditions." Cancer Res **52**(16): 4441-4447.

Brahimi-Horn, M. C., J. Chiche, et al. (2007). "Hypoxia and cancer." J Mol Med **85**(12): 1301-1307.

Brew, K., D. Dinakarpandian, et al. (2000). "Tissue inhibitors of metalloproteinases: evolution, structure and function." Biochim Biophys Acta **1477**(1-2): 267-283.

Brizel, D. M., T. Schroeder, et al. (2001). "Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer." Int J Radiat Oncol Biol Phys **51**(2): 349-353.

Bronner, C. E., S. M. Baker, et al. (1994). "Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer." Nature **368**(6468): 258-261.

Brown, J. M. and A. J. Giaccia (1998). "The unique physiology of solid tumors: opportunities (and problems) for cancer therapy." Cancer Res **58**(7): 1408-1416.

Brown, N. S. and R. Bicknell (2001). "Hypoxia and oxidative stress in breast cancer. Oxidative stress: its effects on the growth, metastatic potential and response to therapy of breast cancer." Breast Cancer Res **3**(5): 323-327.

Burt, R. W. (2000). "Colon cancer screening." Gastroenterology **119**(3): 837-853.

Bush, R. S., R. D. Jenkin, et al. (1978). "Definitive evidence for hypoxic cells influencing cure in cancer therapy." Br J Cancer Suppl **3**: 302-306.

Buyse, M., P. Thirion, et al. (2000). "Relation between tumour response to first-line chemotherapy and survival in advanced colorectal cancer: a meta-analysis. Meta-Analysis Group in Cancer." Lancet **356**(9227): 373-378.

Cairns, R., I. Papandreou, et al. (2006). "Overcoming physiologic barriers to cancer treatment by molecularly targeting the tumor microenvironment." Mol Cancer Res **4**(2): 61-70.

Calvert, P. M. and H. Frucht (2002). "The genetics of colorectal cancer." Ann Intern Med **137**(7): 603-612.

Calvo, E., J. Cortes, et al. (2002). "Combined irinotecan, oxaliplatin and 5-fluorouracil in patients with advanced colorectal cancer. a feasibility pilot study." Oncology **63**(3): 254-265.

Cannistra, S. A., G. S. Kansas, et al. (1993). "Binding of ovarian cancer cells to peritoneal mesothelium in vitro is partly mediated by CD44H." Cancer Res **53**(16): 3830-3838.

Carbone, A., M. Cozzi, et al. (1994). "CD26/dipeptidyl peptidase IV expression in human lymphomas is restricted to CD30-positive anaplastic large cell and a subset of T-cell non-Hodgkin's lymphomas." Hum Pathol **25**(12): 1360-1365.

Casanello, P., A. Torres, et al. (2005). "Equilibrative nucleoside transporter 1 expression is downregulated by hypoxia in human umbilical vein endothelium." Circ Res **97**(1): 16-24.

Casciari, J. J., S. V. Sotirchos, et al. (1988). "Glucose diffusivity in multicellular tumor spheroids." Cancer Res **48**(14): 3905-3909.

Center, M. M., A. Jemal, et al. (2009). "Worldwide variations in colorectal cancer." CA Cancer J Clin **59**(6): 366-378.

Ceradini, D. J., A. R. Kulkarni, et al. (2004). "Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1." Nat Med **10**(8): 858-864.

Cespedes, M. V., C. Espina, et al. (2007). "Orthotopic microinjection of human colon cancer cells in nude mice induces tumor foci in all clinically relevant metastatic sites." Am J Pathol **170**(3): 1077-1085.

Chakraborti, S., M. Mandal, et al. (2003). "Regulation of matrix metalloproteinases: an overview." Mol Cell Biochem **253**(1-2): 269-285.

Champoux, J. J. (2001). "DNA topoisomerase I-mediated nicking of circular duplex DNA." Methods Mol Biol **95**: 81-87.

Chapuis, P. H., O. F. Dent, et al. (1985). "A multivariate analysis of clinical and pathological variables in prognosis after resection of large bowel cancer." Br J Surg **72**(9): 698-702.

Chen, H., W. W. Yang, et al. (2009). "TGF-beta induces fibroblast activation protein expression; fibroblast activation protein expression increases the proliferation,

adhesion, and migration of HO-8910PM [corrected]." Exp Mol Pathol **87**(3): 189-194.

Chomczynski, P. and N. Sacchi (1987). "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction." Anal Biochem **162**(1): 156-159.

Christofk, H. R., M. G. Vander Heiden, et al. (2008). "The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth." Nature **452**(7184): 230-233.

Christopherson, K. W., 2nd, G. Hangoc, et al. (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.

Christopoulos, A., L. T. May, et al. (2004). "G-protein-coupled receptor allosterism: the promise and the problem(s)." Biochem Soc Trans **32**(Pt 5): 873-877.

Chu, D. Z., N. P. Lang, et al. (1989). "Peritoneal carcinomatosis in nongynecologic malignancy. A prospective study of prognostic factors." Cancer **63**(2): 364-367.

Chung, T. W., D. Z. Liu, et al. (2003). "Enhancement of the growth of human endothelial cells by surface roughness at nanometer scale." Biomaterials **24**(25): 4655-4661.

Ciruela, F., C. Saura, et al. (1996). "Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors." FEBS Lett **380**(3): 219-223.

Compton, C. C. (2003). "Colorectal carcinoma: diagnostic, prognostic, and molecular features." Mod Pathol **16**(4): 376-388.

Compton, C. C. and F. L. Greene (2004). "The staging of colorectal cancer: 2004 and beyond." CA Cancer J Clin **54**(6): 295-308.

Confuorto, G., M. E. Giuliano, et al. (2007). "Peritoneal carcinomatosis from colorectal cancer: HIPEC?" Surg Oncol **16 Suppl 1**: S149-152.

Cordero, O. J., F. J. Salgado, et al. (2009). "On the origin of serum CD26 and its altered concentration in cancer patients." Cancer Immunol Immunother **58**(11): 1723-1747.

Cossins, J., T. J. Dudgeon, et al. (1996). "Identification of MMP-18, a putative novel human matrix metalloproteinase." Biochem Biophys Res Commun **228**(2): 494-498.

Coussens, L. M., B. Fingleton, et al. (2002). "Matrix metalloproteinase inhibitors and cancer: trials and tribulations." Science **295**(5564): 2387-2392.

Cronstein, B. N. (1994). "Adenosine, an endogenous anti-inflammatory agent." J Appl Physiol **76**(1): 5-13.

Cunliffe, W. J. and P. H. Sugarbaker (1989). "Gastrointestinal malignancy: rationale for adjuvant therapy using early postoperative intraperitoneal chemotherapy." Br J Surg **76**(10): 1082-1090.

Curtin, N. J., A. L. Harris, et al. (1991). "Mechanism of cell death following thymidylate synthase inhibition: 2'-deoxyuridine-5'-triphosphate accumulation, DNA damage, and growth inhibition following exposure to CB3717 and dipyridamole." Cancer Res **51**(9): 2346-2352.

D'Souza, V. M., H. G. Shertzer, et al. (2003). "High glucose concentration in isotonic media alters caco-2 cell permeability." AAPS PharmSci **5**(3): E24.

Daddona, P. E. and W. N. Kelley (1978). "Human adenosine deaminase binding protein. Assay, purification, and properties." J Biol Chem **253**(13): 4617-4623.

Dalmark, M. and P. Johansen (1982). "Molecular association between doxorubicin (adriamycin) and DNA-derived bases, nucleosides, nucleotides, other aromatic compounds, and proteins in aqueous solution." Mol Pharmacol **22**(1): 158-165.

Dang, N. H. and C. Morimoto (2002). "CD26: an expanding role in immune regulation and cancer." Histol Histopathol **17**(4): 1213-1226.

Dang, N. H., Y. Torimoto, et al. (1990). "Human CD4 helper T cell activation: functional involvement of two distinct collagen receptors, 1F7 and VLA integrin family." J Exp Med **172**(2): 649-652.

Dang, N. H., Y. Torimoto, et al. (1990). "Cell surface modulation of CD26 by anti-1F7 monoclonal antibody. Analysis of surface expression and human T cell activation." J Immunol **145**(12): 3963-3971.

Danielpour, D. (2005). "Functions and regulation of transforming growth factor-beta (TGF-beta) in the prostate." Eur J Cancer **41**(6): 846-857.

Dardousis, K., C. Voolstra, et al. (2007). "Identification of differentially expressed genes involved in the formation of multicellular tumor spheroids by HT-29 colon carcinoma cells." Mol Ther **15**(1): 94-102.

Darmoul, D., M. Lacasa, et al. (1990). "Isolation of a cDNA probe for the human intestinal dipeptidylpeptidase IV and assignment of the gene locus DPP4 to chromosome 2." Ann Hum Genet **54**(Pt 3): 191-197.

Davidson, B., I. Goldberg, et al. (2003). "EMMPRIN (extracellular matrix metalloproteinase inducer) is a novel marker of poor outcome in serous ovarian carcinoma." Clin Exp Metastasis **20**(2): 161-169.

Davoodi, J., J. Kelly, et al. (2007). "The Simpson-Golabi-Behmel syndrome causative glypican-3, binds to and inhibits the dipeptidyl peptidase activity of CD26." Proteomics **7**(13): 2300-2310.

Dawson, L. E., A. H. Russell, et al. (1983). "Adenocarcinoma of the sigmoid colon: sites of initial dissemination and clinical patterns of recurrence following surgery alone." J Surg Oncol **22**(2): 95-99.

De Angelis, P. M., D. H. Svendsrud, et al. (2006). "Cellular response to 5-fluorouracil (5-FU) in 5-FU-resistant colon cancer cell lines during treatment and recovery." Mol Cancer **5**: 20.

de Gramont, A., A. Figer, et al. (2000). "Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer." J Clin Oncol **18**(16): 2938-2947.

De Meester, I., S. Korom, et al. (1999). "CD26, let it cut or cut it down." Immunol Today **20**(8): 367-375.

De Wever, O., P. Demetter, et al. (2008). "Stromal myofibroblasts are drivers of invasive cancer growth." Int J Cancer **123**(10): 2229-2238.

De Wever, O. and M. Mareel (2003). "Role of tissue stroma in cancer cell invasion." J Pathol **200**(4): 429-447.

Decking, U. K., G. Schlieper, et al. (1997). "Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release." Circ Res **81**(2): 154-164.

Degubareff, T. and W. Sleator, Jr. (1965). "Effects of Caffeine on Mammalian Atrial Muscle, and Its Interaction with Adenosine and Calcium." J Pharmacol Exp Ther **148**: 202-214.

Denko, N. C. (2008). "Hypoxia, HIF1 and glucose metabolism in the solid tumour." Nat Rev Cancer.

Desch, C. E., A. B. Benson, 3rd, et al. (1999). "Recommended colorectal cancer surveillance guidelines by the American Society of Clinical Oncology." J Clin Oncol **17**(4): 1312.

Deussen, A., H. G. Lloyd, et al. (1989). "Contribution of S-adenosylhomocysteine to cardiac adenosine formation." J Mol Cell Cardiol **21**(8): 773-782.

DeVita, V. T. and P. S. Schein (1973). "The use of drugs in combination for the treatment of cancer: rationale and results." N Engl J Med **288**(19): 998-1006.

Dinjens, W. N., J. ten Kate, et al. (1989). "Distribution of adenosine deaminase complexing protein (ADCP) in human tissues." J Histochem Cytochem **37**(12): 1869-1875.

Dionisotti, S., E. Ongini, et al. (1997). "Characterization of human A2A adenosine receptors with the antagonist radioligand [3H]-SCH 58261." Br J Pharmacol **121**(3): 353-360.

Dollery, C. M., J. R. McEwan, et al. (1995). "Matrix metalloproteinases and cardiovascular disease." Circ Res **77**(5): 863-868.

Dolphin, A. C., S. R. Forda, et al. (1986). "Calcium-dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue." J Physiol **373**: 47-61.

Dong, R. P., K. Tachibana, et al. (1997). "Determination of adenosine deaminase binding domain on CD26 and its immunoregulatory effect on T cell activation." J Immunol **159**(12): 6070-6076.

Dong, Z., J. Nishiyama, et al. (2002). "Gene promoter of apoptosis inhibitory protein IAP2: identification of enhancer elements and activation by severe hypoxia." Biochem J **364**(Pt 2): 413-421.

Dong, Z., J. Z. Wang, et al. (2003). "Apoptosis-resistance of hypoxic cells: multiple factors involved and a role for IAP-2." Am J Pathol **163**(2): 663-671.

DosReis, G. A., A. F. Nobrega, et al. (1986). "Purinergic modulation of T-lymphocyte activation: differential susceptibility of distinct activation steps and correlation with intracellular 3',5'-cyclic adenosine monophosphate accumulation." Cell Immunol **101**(1): 213-231.

Douillard, J. Y., D. Cunningham, et al. (2000). "Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial." Lancet **355**(9209): 1041-1047.

Dove, A. (2002). "MMP inhibitors: glimmers of hope amidst clinical failures." Nat Med **8**(2): 95.

Drucker, D., C. Easley, et al. (2007). "Sitagliptin." Nat Rev Drug Discov **6**(2): 109-110.

Drucker, D. J., Q. Shi, et al. (1997). "Regulation of the biological activity of glucagon-like peptide 2 in vivo by dipeptidyl peptidase IV." Nat Biotechnol **15**(7): 673-677.

Dubey, R. K., D. G. Gillespie, et al. (2002). "A(2B) adenosine receptors stimulate growth of porcine and rat arterial endothelial cells." Hypertension **39**(2 Pt 2): 530-535.

Dubey, R. K., D. G. Gillespie, et al. (1996). "Adenosine inhibits growth of rat aortic smooth muscle cells. Possible role of A2b receptor." Hypertension **27**(3 Pt 2): 786-793.

Duffy, M. J., A. van Dalen, et al. (2007). "Tumour markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines for clinical use." Eur J Cancer **43**(9): 1348-1360.

Dvorak, H. F. (1986). "Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing." N Engl J Med **315**(26): 1650-1659.

Eaden, J. A. and J. F. Mayberry (2002). "Guidelines for screening and surveillance of asymptomatic colorectal cancer in patients with inflammatory bowel disease." Gut **51 Suppl 5**: V10-12.

Edwards, C. K., 3rd, L. M. Watts, et al. (1994). "Effect of the carbocyclic nucleoside analogue MDL 201,112 on inhibition of interferon-gamma-induced priming of Lewis (LEW/N) rat macrophages for enhanced respiratory burst and MHC class II Ia+ antigen expression." J Leukoc Biol **56**(2): 133-144.

Elashoff, M., A. V. Matveyenko, et al. (2011). "Pancreatitis, pancreatic, and thyroid cancer with glucagon-like Peptide-1-based therapies." Gastroenterology **141**(1): 150-156.

Elenbaas, B., L. Spirio, et al. (2001). "Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells." Genes Dev **15**(1): 50-65.

Engel, M., T. Hoffmann, et al. (2006). "Rigidity and flexibility of dipeptidyl peptidase IV: crystal structures of and docking experiments with DPIV." J Mol Biol **355**(4): 768-783.

Fabris, V. T., A. Sahores, et al. (2010). "Inoculated mammary carcinoma-associated fibroblasts: contribution to hormone independent tumor growth." BMC Cancer **10**: 293.

Fan, H., W. Meng, et al. (1997). "Domain-specific N-glycosylation of the membrane glycoprotein dipeptidylpeptidase IV (CD26) influences its subcellular trafficking, biological stability, enzyme activity and protein folding." Eur J Biochem **246**(1): 243-251.

Fang, J., H. Nakamura, et al. (2011). "The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect." Adv Drug Deliv Rev.

Fang, J. S., R. D. Gillies, et al. (2008). "Adaptation to hypoxia and acidosis in carcinogenesis and tumor progression." Semin Cancer Biol **18**(5): 330-337.

Fantin, V. R., J. St-Pierre, et al. (2006). "Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance." Cancer Cell **9**(6): 425-434.

Favier, J., J. J. Briere, et al. (2009). "The Warburg effect is genetically determined in inherited pheochromocytomas." PLoS One **4**(9): e7094.

Fearon, E. R. and B. Vogelstein (1990). "A genetic model for colorectal tumorigenesis." Cell **61**(5): 759-767.

Feoktistov, I. and I. Biaggioni (1997). "Adenosine A2B receptors." Pharmacol Rev **49**(4): 381-402.

Fingleton, B. (2008). "MMPs as therapeutic targets--still a viable option?" Semin Cell Dev Biol **19**(1): 61-68.

Fishman, P., S. Bar-Yehuda, et al. (2003). "Targeting the A3 adenosine receptor for cancer therapy: inhibition of prostate carcinoma cell growth by A3AR agonist." Anticancer Res **23**(3A): 2077-2083.

Fishman, P., S. Bar-Yehuda, et al. (2002). "A3 adenosine receptor as a target for cancer therapy." Anticancer Drugs **13**(5): 437-443.

Fishman, P., S. Bar-Yehuda, et al. (2004). "An agonist to the A3 adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3 beta and NF-kappa B." Oncogene **23**(14): 2465-2471.

Fishman, P., S. Bar-Yehuda, et al. (2000). "Adenosine acts as an inhibitor of lymphoma cell growth: a major role for the A3 adenosine receptor." Eur J Cancer **36**(11): 1452-1458.

Florin, L., L. Hummerich, et al. (2004). "Identification of novel AP-1 target genes in fibroblasts regulated during cutaneous wound healing." Oncogene **23**(42): 7005-7017.

Formby, B. and R. Stern (2003). "Lactate-sensitive response elements in genes involved in hyaluronan catabolism." Biochem Biophys Res Commun **305**(1): 203-208.

Franco, O. E., A. K. Shaw, et al. (2010). "Cancer associated fibroblasts in cancer pathogenesis." Semin Cell Dev Biol **21**(1): 33-39.

Fredholm, B. B. (2007). "Adenosine, an endogenous distress signal, modulates tissue damage and repair." Cell Death Differ **14**(7): 1315-1323.

Fredholm, B. B., M. P. Abbracchio, et al. (1994). "Nomenclature and classification of purinoceptors." Pharmacol Rev **46**(2): 143-156.

Fredholm, B. B., I. J. AP, et al. (2001). "International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors." Pharmacol Rev **53**(4): 527-552.

Frobert, O., G. Haink, et al. (2006). "Adenosine concentration in the porcine coronary artery wall and A2A receptor involvement in hypoxia-induced vasodilatation." J Physiol **570**(Pt 2): 375-384.

Fukumura, D. and R. K. Jain (2007). "Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization." Microvasc Res **74**(2-3): 72-84.

Garcia-Canero, R., C. Trilla, et al. (1999). "Na+ :H+ exchange inhibition induces intracellular acidosis and differentially impairs cell growth and viability of human and rat hepatocarcinoma cells." Toxicol Lett **106**(2-3): 215-228.

Garofalo, A., R. G. Chirivi, et al. (1993). "Comparative study on the metastatic behavior of human tumors in nude, beige/nude/xid and severe combined immunodeficient mice." Invasion & metastasis **13**(2): 82-91.

George, D. J. and W. G. Kaelin, Jr. (2003). "The von Hippel-Lindau protein, vascular endothelial growth factor, and kidney cancer." N Engl J Med **349**(5): 419-421.

Gerweck, L. E. and K. Seetharaman (1996). "Cellular pH gradient in tumor versus normal tissue: potential exploitation for the treatment of cancer." Cancer Res **56**(6): 1194-1198.

Gerweck, L. E., S. Vijayappa, et al. (2006). "Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics." Mol Cancer Ther **5**(5): 1275-1279.

Gessi, S., K. Varani, et al. (2000). "A(2A) adenosine receptors in human peripheral blood cells." Br J Pharmacol **129**(1): 2-11.

Ghani, F. I., H. Yamazaki, et al. (2010). "Identification of cancer stem cell markers in human malignant mesothelioma cells." Biochem Biophys Res Commun **404**(2): 735-742.

Giaccia, A. J., J. M. Brown, et al. (1998). "Cancer therapy and tumor physiology." Science **279**(5347): 12-13.

Giavazzi, R., J. M. Jessup, et al. (1986). "Experimental nude mouse model of human colorectal cancer liver metastases." J Natl Cancer Inst **77**(6): 1303-1308.

Giblett, E. R., J. E. Anderson, et al. (1972). "Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity." Lancet **2**(7786): 1067-1069.

Giovannucci, E. (2002). "Obesity, gender, and colon cancer." Gut **51**(2): 147.

Giulianelli, S., J. P. Cerliani, et al. (2008). "Carcinoma-associated fibroblasts activate progesterone receptors and induce hormone independent mammary tumor growth: A role for the FGF-2/FGFR-2 axis." Int J Cancer **123**(11): 2518-2531.

Goldberg, G. I., S. M. Wilhelm, et al. (1986). "Human fibroblast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein." J Biol Chem **261**(14): 6600-6605.

Gomis-Ruth, F. X., K. Maskos, et al. (1997). "Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1." Nature **389**(6646): 77-81.

Gonzalez-Gronow, M., G. Gawdi, et al. (1994). "Characterization of the plasminogen receptors of normal and rheumatoid arthritis human synovial fibroblasts." J Biol Chem **269**(6): 4360-4366.

Gorlach, A. (2005). "Control of adenosine transport by hypoxia." Circ Res **97**(1): 1-3.

Gorrell, M. D., V. Gysbers, et al. (2001). "CD26: a multifunctional integral membrane and secreted protein of activated lymphocytes." Scand J Immunol **54**(3): 249-264.

Gracia, E., K. Perez-Capote, et al. (2011). "A2A adenosine receptor ligand binding and signaling is allosterically modulated by adenosine deaminase." Biochem J.

Gradishar, W. J. and E. E. Vokes (1990). "5-Fluorouracil cardiotoxicity: a critical review." Ann Oncol **1**(6): 409-414.

Grant, M. B., M. I. Davis, et al. (2001). "Proliferation, migration, and ERK activation in human retinal endothelial cells through A(2B) adenosine receptor stimulation." Invest Ophthalmol Vis Sci **42**(9): 2068-2073.

Greijer, A. E. and E. van der Wall (2004). "The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis." J Clin Pathol **57**(10): 1009-1014.

Groebe, K. and W. Mueller-Klieser (1991). "Distributions of oxygen, nutrient, and metabolic waste concentrations in multicellular spheroids and their dependence on spheroid parameters." European biophysics journal : EBJ **19**(4): 169-181.

Gu, N., M. Tsuda, et al. (2008). "Glucose regulation of dipeptidyl peptidase IV gene expression is mediated by hepatocyte nuclear factor-1alpha in epithelial intestinal cells." Clin Exp Pharmacol Physiol **35**(12): 1433-1439.

Guo, H., R. Li, et al. (2000). "EMMPRIN (CD147), an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase to the tumor cell surface." Cancer Res **60**(4): 888-891.

Guo, H., S. Zucker, et al. (1997). "Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells." J Biol Chem **272**(1): 24-27.

Hafler, D. A., D. A. Fox, et al. (1986). "Antigen reactive memory T cells are defined by Ta1." J Immunol **137**(2): 414-418.

Hagemann, T., S. C. Robinson, et al. (2004). "Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteases." Carcinogenesis **25**(8): 1543-1549.

Haier, J., M. Nasralla, et al. (1999). "Different adhesion properties of highly and poorly metastatic HT-29 colon carcinoma cells with extracellular matrix components: role of integrin expression and cytoskeletal components." Br J Cancer **80**(12): 1867-1874.

Hamano, Y., M. Zeisberg, et al. (2003). "Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin." Cancer Cell **3**(6): 589-601.

Hamilton, S. R. (1993). "The molecular genetics of colorectal neoplasia." Gastroenterology **105**(1): 3-7.

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

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hance, A. J., E. D. Robin, et al. (1980). "Regulation of glycolytic enzyme activity during chronic hypoxia by changes in rate-limiting enzyme content. Use of monoclonal antibodies to quantitate changes in pyruvate kinase content." J Clin Invest **66**(6): 1258-1264.

Hanski, C., T. Huhle, et al. (1988). "Direct evidence for the binding of rat liver DPP IV to collagen in vitro." Exp Cell Res **178**(1): 64-72.

Hanski, C., T. Huhle, et al. (1985). "Involvement of plasma membrane dipeptidyl peptidase IV in fibronectin-mediated adhesion of cells on collagen." Biol Chem Hoppe Seyler **366**(12): 1169-1176.

Harpaz, N. and A. D. Polydorides (2010). "Colorectal dysplasia in chronic inflammatory bowel disease: pathology, clinical implications, and pathogenesis." Arch Pathol Lab Med **134**(6): 876-895.

Harris, A. L. (2002). "Hypoxia--a key regulatory factor in tumour growth." Nat Rev Cancer **2**(1): 38-47.

Hashimoto, T., R. Hussien, et al. (2007). "Lactate sensitive transcription factor network in L6 cells: activation of MCT1 and mitochondrial biogenesis." Faseb J **21**(10): 2602-2612.

Hasko, G., E. A. Deitch, et al. (2002). "Adenosine: a potential mediator of immunosuppression in multiple organ failure." Curr Opin Pharmacol **2**(4): 440-444.

Haviv, I., K. Polyak, et al. (2009). "Origin of carcinoma associated fibroblasts." Cell Cycle **8**(4): 589-595.

Hazan, R. B., L. Kang, et al. (1997). "N-cadherin promotes adhesion between invasive breast cancer cells and the stroma." Cell Adhes Commun **4**(6): 399-411.

Heath, R. M., D. G. Jayne, et al. (2004). "Tumour-induced apoptosis in human mesothelial cells: a mechanism of peritoneal invasion by Fas Ligand/Fas interaction." Br J Cancer **90**(7): 1437-1442.

Heijstek, M. W., O. Kranenburg, et al. (2005). "Mouse models of colorectal cancer and liver metastases." Digestive surgery **22**(1-2): 16-25.

Hess, J., P. Angel, et al. (2004). "AP-1 subunits: quarrel and harmony among siblings." J Cell Sci **117**(Pt 25): 5965-5973.

Hickman, J. A., C. S. Potten, et al. (1994). "Apoptosis and cancer chemotherapy." Philos Trans R Soc Lond B Biol Sci **345**(1313): 319-325.

Hockel, M. and P. Vaupel (2001). "Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects." J Natl Cancer Inst **93**(4): 266-276.

Holmdahl, L., E. Eriksson, et al. (1998). "Depression of peritoneal fibrinolysis during operation is a local response to trauma." Surgery **123**(5): 539-544.

Holtfreter, J. (1947). "Neural induction in explants which have passed through a sublethal cytolysis." J Exp Zool **106**(2): 197-222.

Holtfreter, J. (1951). "Some aspects of embryonic induction." Growth(Suppl 10): 117-152.

Hoskin, D. W., J. J. Butler, et al. (2002). "Adenosine acts through an A3 receptor to prevent the induction of murine anti-CD3-activated killer T cells." Int J Cancer **99**(3): 386-395.

Houghton, A. N., A. P. Albino, et al. (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.

Hsu, P. P. and D. M. Sabatini (2008). "Cancer cell metabolism: Warburg and beyond." Cell **134**(5): 703-707.

Huang, L. E., R. S. Bindra, et al. (2007). "Hypoxia-induced genetic instability--a calculated mechanism underlying tumor progression." J Mol Med **85**(2): 139-148.

Huang, L. E. and H. F. Bunn (2003). "Hypoxia-inducible factor and its biomedical relevance." J Biol Chem **278**(22): 19575-19578.

Huffaker, T., T. Corcoran, et al. (1984). "Adenosine inhibits cell division and promotes neurite extension in PC12 cells." J Cell Physiol **120**(2): 188-196.

Inamoto, T., T. Yamada, et al. (2007). "Humanized anti-CD26 monoclonal antibody as a treatment for malignant mesothelioma tumors." Clin Cancer Res **13**(14): 4191-4200.

Ishii, T., K. Ohnuma, et al. (2001). "CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO." Proc Natl Acad Sci U S A **98**(21): 12138-12143.

Izumi, H., T. Torigoe, et al. (2003). "Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy." Cancer Treat Rev **29**(6): 541-549.

Jackson, R. C., H. P. Morris, et al. (1978). "Adenosine deaminase and adenosine kinase in rat hepatomas and kidney tumours." Br J Cancer **37**(5): 701-713.

Jain, R. K. (1998). "Delivery of molecular and cellular medicine to solid tumors." J Control Release **53**(1-3): 49-67.

Jain, R. K. (2005). "Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy." Science **307**(5706): 58-62.

Jarv, J. (1995). "A model of non-exclusive binding of agonist and antagonist on G-protein coupled receptors." J Theor Biol **175**(4): 577-582.

Jayne, D. G., S. Fook, et al. (2002). "Peritoneal carcinomatosis from colorectal cancer." Br J Surg **89**(12): 1545-1550.

Jo, D. Y., S. Rafii, et al. (2000). "Chemotaxis of primitive hematopoietic cells in response to stromal cell-derived factor-1." J Clin Invest **105**(1): 101-111.

Jones, L. M., M. J. Gardner, et al. (1995). "Hyaluronic acid secreted by mesothelial cells: a natural barrier to ovarian cancer cell adhesion." Clin Exp Metastasis **13**(5): 373-380.

Kadoglou, N. P. and C. D. Liapis (2004). "Matrix metalloproteinases: contribution to pathogenesis, diagnosis, surveillance and treatment of abdominal aortic aneurysms." Curr Med Res Opin **20**(4): 419-432.

Kajiyama, H., K. Shibata, et al. (2010). "The expression of dipeptidyl peptidase IV (DPPIV/CD26) is associated with enhanced chemosensitivity to paclitaxel in epithelial ovarian carcinoma cells." Cancer Sci **101**(2): 347-354.

Kajiyama, H., K. Shibata, et al. (2006). "Involvement of DPPIV/CD26 in epithelial morphology and suppressed invasive ability in ovarian carcinoma cells." Ann N Y Acad Sci **1086**: 233-240.

Kallinowski, F., P. Vaupel, et al. (1988). "Glucose uptake, lactate release, ketone body turnover, metabolic micromilieu, and pH distributions in human breast cancer xenografts in nude rats." Cancer Res **48**(24 Pt 1): 7264-7272.

Kameoka, J., T. Tanaka, et al. (1993). "Direct association of adenosine deaminase with a T cell activation antigen, CD26." Science **261**(5120): 466-469.

Khoa, N. D., M. C. Montesinos, et al. (2001). "Inflammatory cytokines regulate function and expression of adenosine A(2A) receptors in human monocytic THP-1 cells." J Immunol **167**(7): 4026-4032.

Kim, H., J. Jen, et al. (1994). "Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences." Am J Pathol **145**(1): 148-156.

Klaasse, E. C., A. P. Ijzerman, et al. (2008). "Internalization and desensitization of adenosine receptors." Purinergic Signal **4**(1): 21-37.

Klein, T. and R. Bischoff (2011). "Physiology and pathophysiology of matrix metalloproteases." Amino Acids **41**(2): 271-290.

Kleiner, D. E. and W. G. Stetler-Stevenson (1999). "Matrix metalloproteinases and metastasis." Cancer Chemother Pharmacol **43 Suppl**: S42-51.

Knai, C., M. Suhrcke, et al. (2007). "Obesity in Eastern Europe: an overview of its health and economic implications." Econ Hum Biol **5**(3): 392-408.

Knauper, V., S. Cowell, et al. (1997). "The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity, and tissue inhibitor of metalloproteinase interaction." J Biol Chem **272**(12): 7608-7616.

Knox, J. B., G. K. Sukhova, et al. (1997). "Evidence for altered balance between matrix metalloproteinases and their inhibitors in human aortic diseases." Circulation **95**(1): 205-212.

Knudson, A. G., Jr. (1985). "Hereditary cancer, oncogenes, and antioncogenes." Cancer Res **45**(4): 1437-1443.

Kobayashi, H., O. Hosono, et al. (2002). "Reduction of serum soluble CD26/dipeptidyl peptidase IV enzyme activity and its correlation with disease activity in systemic lupus erythematosus." J Rheumatol **29**(9): 1858-1866.

Kohno, Y., Y. Sei, et al. (1996). "Induction of apoptosis in HL-60 human promyelocytic leukemia cells by adenosine A(3) receptor agonists." Biochem Biophys Res Commun **219**(3): 904-910.

Kong, T., K. A. Westerman, et al. (2006). "HIF-dependent induction of adenosine A2B receptor in hypoxia." Faseb J **20**(13): 2242-2250.

Koppe, M. J., O. C. Boerman, et al. (2006). "Peritoneal carcinomatosis of colorectal origin: incidence and current treatment strategies." Ann Surg **243**(2): 212-222.

Koster, D. A., K. Palle, et al. (2007). "Antitumour drugs impede DNA uncoiling by topoisomerase I." Nature **448**(7150): 213-217.

Kotani, T., Y. Aratake, et al. (1991). "Expression of dipeptidyl aminopeptidase IV activity in thyroid carcinoma." Cancer Lett **57**(3): 203-208.

Koukourakis, M. I., A. Giatromanolaki, et al. (2006). "Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma." Cancer Res **66**(2): 632-637.

Koukourakis, M. I., M. Pitiakoudis, et al. (2006). "Oxygen and glucose consumption in gastrointestinal adenocarcinomas: correlation with markers of hypoxia, acidity and anaerobic glycolysis." Cancer Sci **97**(10): 1056-1060.

Kroll, K., A. Deussen, et al. (1992). "Comprehensive model of transport and metabolism of adenosine and S-adenosylhomocysteine in the guinea pig heart." Circ Res **71**(3): 590-604.

Kryczek, I., S. Wei, et al. (2007). "Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis." Am J Physiol Cell Physiol **292**(3): C987-995.

Kuhn, B., M. Hennig, et al. (2007). "Molecular recognition of ligands in dipeptidyl peptidase IV." Curr Top Med Chem **7**(6): 609-619.

Kunz-Schughart, L. A. and R. Knuechel (2002). "Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression?" Histol Histopathol **17**(2): 599-621.

la Sala, A., D. Ferrari, et al. (2003). "Alerting and tuning the immune response by extracellular nucleotides." J Leukoc Biol **73**(3): 339-343.

Laird, A. K. (1964). "Dynamics of Tumor Growth." Br J Cancer **13**: 490-502.

Lambeir, A. M., C. Durinx, et al. (2003). "Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV." Crit Rev Clin Lab Sci **40**(3): 209-294.

Lampin, M., C. Warocquier, et al. (1997). "Correlation between substratum roughness and wettability, cell adhesion, and cell migration." J Biomed Mater Res **36**(1): 99-108.

Lankas, G. R., B. Leiting, et al. (2005). "Dipeptidyl peptidase IV inhibition for the treatment of type 2 diabetes: potential importance of selectivity over dipeptidyl peptidases 8 and 9." Diabetes **54**(10): 2988-2994.

Larrain, J., D. Bachiller, et al. (2000). "BMP-binding modules in chordin: a model for signalling regulation in the extracellular space." Development **127**(4): 821-830.

Lavoinne, A., S. Claeyssens, et al. (1990). "Metabolism of adenosine through adenosine kinase inhibits gluconeogenesis in isolated rat hepatocytes." Eur J Biochem **187**(2): 403-407.

Ledoux, S., I. Runembert, et al. (2003). "Hypoxia enhances Ecto-5'-Nucleotidase activity and cell surface expression in endothelial cells: role of membrane lipids." Circ Res **92**(8): 848-855.

Leeman, M. F., S. Curran, et al. (2002). "The structure, regulation, and function of human matrix metalloproteinase-13." Crit Rev Biochem Mol Biol **37**(3): 149-166.

Leeman, M. F., J. A. McKay, et al. (2002). "Matrix metalloproteinase 13 activity is associated with poor prognosis in colorectal cancer." J Clin Pathol **55**(10): 758-762.

Li, C., T. Ha, et al. (2000). "Adenosine prevents activation of transcription factor NF-kappa B and enhances activator protein-1 binding activity in ischemic rat heart." Surgery **127**(2): 161-169.

Li, F. K., A. Davenport, et al. (1998). "Leukocyte migration across human peritoneal mesothelial cells is dependent on directed chemokine secretion and ICAM-1 expression." Kidney Int **54**(6): 2170-2183.

Lim, M., T. Martinez, et al. (1998). "Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38." FEBS Lett **441**(1): 88-92.

Lin, Z., P. Yin, et al. (2009). "Adenosine A1 receptor, a target and regulator of estrogen receptoralpha action, mediates the proliferative effects of estradiol in breast cancer." Oncogene **29**(8): 1114-1122.

Linden, J. (2001). "Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection." Annu Rev Pharmacol Toxicol **41**: 775-787.

Linden, J. (2005). "Adenosine in tissue protection and tissue regeneration." Mol Pharmacol **67**(5): 1385-1387.

Liu, L., X. Ning, et al. (2008). "Hypoxia-inducible factor-1 alpha contributes to hypoxia-induced chemoresistance in gastric cancer." Cancer Sci **99**(1): 121-128.

Liu, Y. E., M. Wang, et al. (1997). "Preparation and characterization of recombinant tissue inhibitor of metalloproteinase 4 (TIMP-4)." J Biol Chem **272**(33): 20479-20483.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lloyd, H. G. and B. B. Fredholm (1995). "Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices." Neurochem Int **26**(4): 387-395.

Londos, C., D. M. Cooper, et al. (1980). "Subclasses of external adenosine receptors." Proc Natl Acad Sci U S A **77**(5): 2551-2554.

Loster, K., K. Zeilinger, et al. (1995). "The cysteine-rich region of dipeptidyl peptidase IV (CD 26) is the collagen-binding site." Biochem Biophys Res Commun **217**(1): 341-348.

Lu, T., L. G. Burdelya, et al. (2004). "Secreted transforming growth factor beta2 activates NF-kappaB, blocks apoptosis, and is essential for the survival of some tumor cells." Proc Natl Acad Sci U S A **101**(18): 7112-7117.

Luciani, F., M. Spada, et al. (2004). "Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs." J Natl Cancer Inst **96**(22): 1702-1713.

Lukashev, D., A. Ohta, et al. (2004). "Targeting hypoxia--A(2A) adenosine receptor-mediated mechanisms of tissue protection." Drug Discov Today **9**(9): 403-409.

Ma, D. F., T. Kondo, et al. (2010). "Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells." Hum Pathol **41**(11): 1550-1557.

Ma, Q., D. Jones, et al. (1998). "Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice." Proc Natl Acad Sci U S A **95**(16): 9448-9453.

MacKenzie, W. M., D. W. Hoskin, et al. (2002). "Adenosine suppresses alpha(4)beta(7) integrin-mediated adhesion of T lymphocytes to colon adenocarcinoma cells." Exp Cell Res **276**(1): 90-100.

Macri, A., E. Saladino, et al. (2010). "Peritoneal carcinomatosis of colorectal origin." World J Gastrointest Oncol **2**(2): 98-101.

Madi, L., A. Ochaion, et al. (2004). "The A3 adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition." Clin Cancer Res **10**(13): 4472-4479.

Maeda, H., J. Fang, et al. (2003). "Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications." Int Immunopharmacol **3**(3): 319-328.

Mahoney, B. P., N. Raghunand, et al. (2003). "Tumor acidity, ion trapping and chemotherapeutics. I. Acid pH affects the distribution of chemotherapeutic agents in vitro." Biochem Pharmacol **66**(7): 1207-1218.

Mandapathil, M., B. Hilldorfer, et al. (2010). "Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells." J Biol Chem **285**(10): 7176-7186.

Mans, D. R., I. Grivicich, et al. (1999). "Sequence-dependent growth inhibition and DNA damage formation by the irinotecan-5-fluorouracil combination in human colon carcinoma cell lines." Eur J Cancer **35**(13): 1851-1861.

Marguet, D., L. Baggio, et al. (2000). "Enhanced insulin secretion and improved glucose tolerance in mice lacking CD26." Proc Natl Acad Sci U S A **97**(12): 6874-6879.

Markowitz, S., J. Wang, et al. (1995). "Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability." Science **268**(5215): 1336-1338.

Martinez-Outschoorn, U. E., S. Pavlides, et al. (2010). "Tumor cells induce the cancer associated fibroblast phenotype via caveolin-1 degradation: Implications for breast cancer and DCIS therapy with autophagy inhibitors." Cell Cycle **9**(12).

Masi, G., G. Allegrini, et al. (2004). "First-line treatment of metastatic colorectal cancer with irinotecan, oxaliplatin and 5-fluorouracil/leucovorin (FOLFOXIRI): results of a phase II study with a simplified biweekly schedule." Ann Oncol **15**(12): 1766-1772.

Matsumoto, K., T. Yamamoto, et al. (1986). "Enhancement of vascular permeability upon serratial infection: activation of Hageman factor--kallikrein--kinin cascade." Advances in experimental medicine and biology **198 Pt B**: 71-78.

Matsumura, Y. and H. Maeda (1986). "A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs." Cancer Res **46**(12 Pt 1): 6387-6392.

Mayer, R. J. (2009). "Targeted therapy for advanced colorectal cancer--more is not always better." N Engl J Med **360**(6): 623-625.

McCredie JA, I. W., Kruuv J (1965). The rate of tumor growth in animals. Growth. **29:** 331-347.

McGuinness, C. and U. V. Wesley (2008). "Dipeptidyl peptidase IV (DPPIV), a candidate tumor suppressor gene in melanomas is silenced by promoter methylation." Front Biosci **13**: 2435-2443.

Menozzi, M. and F. Arcamone (1978). "Binding of adriamycin to sulphated mucopolysaccharides." Biochem Biophys Res Commun **80**(2): 313-318.

Mereto, E., L. Frencia, et al. (1994). "Effect of aspirin on incidence and growth of aberrant crypt foci induced in the rat colon by 1,2-dimethylhydrazine." Cancer Lett **76**(1): 5-9.

Merighi, S., A. Benini, et al. (2005). "A3 adenosine receptor activation inhibits cell proliferation via phosphatidylinositol 3-kinase/Akt-dependent inhibition of the extracellular signal-regulated kinase 1/2 phosphorylation in A375 human melanoma cells." J Biol Chem **280**(20): 19516-19526.

Merighi, S., P. Mirandola, et al. (2002). "Adenosine receptors as mediators of both cell proliferation and cell death of cultured human melanoma cells." J Invest Dermatol **119**(4): 923-933.

Metzen, E., M. Wolff, et al. (1995). "Pericellular PO2 and O2 consumption in monolayer cell cultures." Respir Physiol **100**(2): 101-106.

Miles, D., G. von Minckwitz, et al. (2002). "Combination versus sequential single-agent therapy in metastatic breast cancer." Oncologist **7 Suppl 6**: 13-19.

Mirabet, M., C. Herrera, et al. (1999). "Expression of A2B adenosine receptors in human lymphocytes: their role in T cell activation." J Cell Sci **112 (Pt 4)**: 491-502.

Mirkin, B. L., R. F. O'Dea, et al. (1987). "Cytotoxic action of adenosine nucleoside and dialdehyde analogues on murine neuroblastoma in tissue culture: structure-activity relationships." Cancer Res **47**(14): 3650-3655.

Mishra, P., D. Banerjee, et al. (2010). "Chemokines at the crossroads of tumor-fibroblast interactions that promote malignancy." J Leukoc Biol **89**(1): 31-39.

Moellering, R. E., K. C. Black, et al. (2008). "Acid treatment of melanoma cells selects for invasive phenotypes." Clin Exp Metastasis **25**(4): 411-425.

Montesinos, M. C., P. Gadangi, et al. (1997). "Wound healing is accelerated by agonists of adenosine A2 (G alpha s-linked) receptors." J Exp Med **186**(9): 1615-1620.

Moreno-Sanchez, R., S. Rodriguez-Enriquez, et al. (2007). "Energy metabolism in tumor cells." The FEBS journal **274**(6): 1393-1418.

Morimoto, C. and S. F. Schlossman (1998). "The structure and function of CD26 in the T-cell immune response." Immunol Rev **161**: 55-70.

Morin, P. J., A. B. Sparks, et al. (1997). "Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC." Science **275**(5307): 1787-1790.

Morrison, M. E., S. Vijayasaradhi, et al. (1993). "A marker for neoplastic progression of human melanocytes is a cell surface ectopeptidase." J Exp Med **177**(4): 1135-1143.

Moulder, J. E. and S. Rockwell (1987). "Tumor hypoxia: its impact on cancer therapy." Cancer Metastasis Rev **5**(4): 313-341.

Mujoomdar, M., A. Bennett, et al. (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., D. Hoskin, et al. (2003). "Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism." Biochem Pharmacol **66**(9): 1737-1747.

Munshi, R., M. A. DeBernardi, et al. (1993). "P2U-purinergic receptors on C6-2B rat glioma cells: modulation of cytosolic Ca2+ and cAMP levels by protein kinase C." Mol Pharmacol **44**(6): 1185-1191.

Murphy, G. and V. Knauper (1997). "Relating matrix metalloproteinase structure to function: why the "hemopexin" domain?" Matrix Biol **15**(8-9): 511-518.

Mutsaers, S. E. (2002). "Mesothelial cells: their structure, function and role in serosal repair." Respirology **7**(3): 171-191.

Mutsaers, S. E. (2004). "The mesothelial cell." Int J Biochem Cell Biol **36**(1): 9-16.

Mutsaers, S. E., D. Whitaker, et al. (2002). "Stimulation of mesothelial cell proliferation by exudate macrophages enhances serosal wound healing in a murine model." Am J Pathol **160**(2): 681-692.

Nabeshima, K., W. S. Lane, et al. (1991). "Partial sequencing and characterization of the tumor cell-derived collagenase stimulatory factor." Arch Biochem Biophys **285**(1): 90-96.

Nagasawa, T., H. Kikutani, et al. (1994). "Molecular cloning and structure of a pre-B-cell growth-stimulating factor." Proc Natl Acad Sci U S A **91**(6): 2305-2309.

Nagasawa, T., K. Tachibana, et al. (1998). "A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection." Semin Immunol **10**(3): 179-185.

Nagase, H. and J. F. Woessner, Jr. (1999). "Matrix metalloproteinases." J Biol Chem **274**(31): 21491-21494.

Nakav, S., C. Chaimovitz, et al. (2008). "Anti-inflammatory preconditioning by agonists of adenosine A1 receptor." PLoS One **3**(5): e2107.

Nakav, S., O. Naamani, et al. (2010). "Regulation of adenosine system at the onset of peritonitis." Nephrol Dial Transplant **25**(3): 931-939.

Newland, R. C., O. F. Dent, et al. (1994). "Pathologic determinants of survival associated with colorectal cancer with lymph node metastases. A multivariate analysis of 579 patients." Cancer **73**(8): 2076-2082.

Noe, V., B. Fingleton, et al. (2001). "Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1." J Cell Sci **114**(Pt 1): 111-118.

Okamoto, N., M. Yagi, et al. (1999). "A clinical and molecular study of a patient with Simpson-Golabi-Behmel syndrome." J Hum Genet **44**(5): 327-329.

Olah, M. E. (1997). "Identification of A2a adenosine receptor domains involved in selective coupling to Gs. Analysis of chimeric A1/A2a adenosine receptors." J Biol Chem **272**(1): 337-344.

Orimo, A., P. B. Gupta, et al. (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.

Ortega, N. and Z. Werb (2002). "New functional roles for non-collagenous domains of basement membrane collagens." J Cell Sci **115**(Pt 22): 4201-4214.

Page-McCaw, A., A. J. Ewald, et al. (2007). "Matrix metalloproteinases and the regulation of tissue remodelling." Nat Rev Mol Cell Biol **8**(3): 221-233.

Palmer, T. M. and G. L. Stiles (1995). "Adenosine receptors." Neuropharmacology **34**(7): 683-694.

Palmer, T. M. and G. L. Stiles (2000). "Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A(3) adenosine receptor." Mol Pharmacol **57**(3): 539-545.

Pang, R., W. L. Law, et al. (2010). "A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer." Cell stem cell **6**(6): 603-615.

Panjehpour, M. and F. Karami-Tehrani (2007). "Adenosine modulates cell growth in the human breast cancer cells via adenosine receptors." Oncol Res **16**(12): 575-585.

Parkin, D. M., F. Bray, et al. (2005). "Global cancer statistics, 2002." CA Cancer J Clin **55**(2): 74-108.

Perez de Heredia, F., I. S. Wood, et al. (2009). "Hypoxia stimulates lactate release and modulates monocarboxylate transporter (MCT1, MCT2, and MCT4) expression in human adipocytes." Pflugers Arch **459**(3): 509-518.

Peters, G. J., B. van Triest, et al. (2000). "Molecular downstream events and induction of thymidylate synthase in mutant and wild-type p53 colon cancer cell lines after treatment with 5-fluorouracil and the thymidylate synthase inhibitor raltitrexed." Eur J Cancer **36**(7): 916-924.

Piazza, G. A., H. M. Callanan, et al. (1989). "Evidence for a role of dipeptidyl peptidase IV in fibronectin-mediated interactions of hepatocytes with extracellular matrix." Biochem J **262**(1): 327-334.

Pierce, K. D., T. J. Furlong, et al. (1992). "Molecular cloning and expression of an adenosine A2b receptor from human brain." Biochem Biophys Res Commun **187**(1): 86-93.

Pietras, K. and A. Ostman (2010). "Hallmarks of cancer: interactions with the tumor stroma." Exp Cell Res **316**(8): 1324-1331.

Popkin, B. M. (2004). "The nutrition transition: an overview of world patterns of change." Nutr Rev **62**(7 Pt 2): S140-143.

Pouyssegur, J., F. Dayan, et al. (2006). "Hypoxia signalling in cancer and approaches to enforce tumour regression." Nature **441**(7092): 437-443.

Prescott, J., N. Troccoli, et al. (1989). "Coordinate increase in collagenase mRNA and enzyme levels in human fibroblasts treated with the tumor cell factor, TCSF." Biochem Int **19**(2): 257-266.

Pretlow, T. P., B. J. Barrow, et al. (1991). "Aberrant crypts: putative preneoplastic foci in human colonic mucosa." Cancer Res **51**(5): 1564-1567.

Pretlow, T. P., T. A. Brasitus, et al. (1993). "K-ras mutations in putative preneoplastic lesions in human colon." J Natl Cancer Inst **85**(24): 2004-2007.

Radisky, E. S. and D. C. Radisky (2010). "Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer." J Mammary Gland Biol Neoplasia **15**(2): 201-212.

Raghunand, N., X. He, et al. (1999). "Enhancement of chemotherapy by manipulation of tumour pH." Br J Cancer **80**(7): 1005-1011.

Raghunand, N., B. P. Mahoney, et al. (2003). "Tumor acidity, ion trapping and chemotherapeutics. II. pH-dependent partition coefficients predict importance of ion trapping on pharmacokinetics of weakly basic chemotherapeutic agents." Biochem Pharmacol **66**(7): 1219-1229.

Ranucci, C. S. and P. V. Moghe (2001). "Substrate microtopography can enhance cell adhesive and migratory responsiveness to matrix ligand density." J Biomed Mater Res **54**(2): 149-161.

Rapier, R., J. Huq, et al. (2010). "The extracellular matrix microtopography drives critical changes in cellular motility and Rho A activity in colon cancer cells." Cancer Cell Int **10**: 24.

Ratajczak, M. Z., E. Zuba-Surma, et al. (2006). "The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis." Leukemia **20**(11): 1915-1924.

Raymond, E., C. Buquet-Fagot, et al. (1997). "Antitumor activity of oxaliplatin in combination with 5-fluorouracil and the thymidylate synthase inhibitor AG337 in human colon, breast and ovarian cancers." Anticancer Drugs **8**(9): 876-885.

Raymond, E., S. Faivre, et al. (2002). "Cellular and molecular pharmacology of oxaliplatin." Mol Cancer Ther **1**(3): 227-235.

Resta, R., S. W. Hooker, et al. (1997). "Insights into thymic purine metabolism and adenosine deaminase deficiency revealed by transgenic mice overexpressing ecto-5'-nucleotidase (CD73)." J Clin Invest **99**(4): 676-683.

Resta, R. and L. F. Thompson (1997). "SCID: the role of adenosine deaminase deficiency." Immunol Today **18**(8): 371-374.

Richard, C. L., E. Y. Tan, et al. (2006). "Adenosine upregulates CXCR4 and enhances the proliferative and migratory responses of human carcinoma cells to CXCL12/SDF-1alpha." Int J Cancer **119**(9): 2044-2053.

Roberts, A. B., F. Tian, et al. (2006). "Smad3 is key to TGF-beta-mediated epithelial-to-mesenchymal transition, fibrosis, tumor suppression and metastasis." Cytokine Growth Factor Rev **17**(1-2): 19-27.

Rogachev, B., N. Y. Ziv, et al. (2006). "Adenosine is upregulated during peritonitis and is involved in downregulation of inflammation." Kidney Int **70**(4): 675-681.

Rotin, D., D. Steele-Norwood, et al. (1989). "Requirement of the Na+/H+ exchanger for tumor growth." Cancer Res **49**(1): 205-211.

Sajjadi, F. G., K. Takabayashi, et al. (1996). "Inhibition of TNF-alpha expression by adenosine: role of A3 adenosine receptors." J Immunol **156**(9): 3435-3442.

Sako, A., J. Kitayama, et al. (2003). "Vascular endothelial growth factor synthesis by human omental mesothelial cells is augmented by fibroblast growth factor-2: possible role of mesothelial cell on the development of peritoneal metastasis." J Surg Res **115**(1): 113-120.

Saltz, L. B. and B. Minsky (2002). "Adjuvant therapy of cancers of the colon and rectum." Surg Clin North Am **82**(5): 1035-1058.

Salvatore, C. A., M. A. Jacobson, et al. (1993). "Molecular cloning and characterization of the human A3 adenosine receptor." Proc Natl Acad Sci U S A **90**(21): 10365-10369.

Santiago, B., E. Calonge, et al. (2010). "CXCL12 gene expression is upregulated by hypoxia and growth arrest but not by inflammatory cytokines in rheumatoid synovial fibroblasts." Cytokine **53**(2): 184-190.

Santini, M. T. and G. Rainaldi (1999). "Three-dimensional spheroid model in tumor biology." Pathobiology **67**(3): 148-157.

Santini, M. T., G. Rainaldi, et al. (2004). "MG-63 human osteosarcoma cells grown in monolayer and as three-dimensional tumor spheroids present a different metabolic profile: a (1)H NMR study." FEBS Lett **557**(1-3): 148-154.

Sartorelli, A. C. (1988). "Therapeutic attack of hypoxic cells of solid tumors: presidential address." Cancer Res **48**(4): 775-778.

Sasaki, T., T. Nakamura, et al. (2008). "Modification of the primary tumor microenvironment by transforming growth factor alpha-epidermal growth factor receptor signaling promotes metastasis in an orthotopic colon cancer model." Am J Pathol **173**(1): 205-216.

Schlaeppi, M., C. Ruegg, et al. (1997). "Role of integrins and evidence for two distinct mechanisms mediating human colorectal carcinoma cell interaction with peritoneal mesothelial cells and extracellular matrix." Cell Adhes Commun **4**(6): 439-455.

Schor, S. L. and A. M. Schor (1987). "Clonal heterogeneity in fibroblast phenotype: implications for the control of epithelial-mesenchymal interactions." Bioessays **7**(5): 200-204.

Schrader, A. J., O. Lechner, et al. (2002). "CXCR4/CXCL12 expression and signalling in kidney cancer." Br J Cancer **86**(8): 1250-1256.

Schrader, W. P., C. A. West, et al. (1994). "Subcellular distribution of adenosine deaminase and adenosine deaminase-complexing protein in rabbit kidney: implications for adenosine metabolism." J Histochem Cytochem **42**(6): 775-782.

Schulte, G. and B. B. Fredholm (2000). "Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2." Mol Pharmacol **58**(3): 477-482.

Schurch, W., T. A. Seemayer, et al. (1998). "The myofibroblast: a quarter century after its discovery." Am J Surg Pathol **22**(2): 141-147.

Scotton, C. J., J. L. Wilson, et al. (2001). "Epithelial cancer cell migration: a role for chemokine receptors?" Cancer Res **61**(13): 4961-4965.

Scotton, C. J., J. L. Wilson, et al. (2002). "Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer." Cancer Res **62**(20): 5930-5938.

Sedo, A., E. Krepela, et al. (1991). "Dipeptidyl peptidase IV, prolyl endopeptidase and cathepsin B activities in primary human lung tumors and lung parenchyma." J Cancer Res Clin Oncol **117**(3): 249-253.

Seetulsingh-Goorah, S. P. (2006). "Mechanisms of adenosine-induced cytotoxicity and their clinical and physiological implications." Biofactors **27**(1-4): 213-230.

Semenza, G. L. (2003). "Targeting HIF-1 for cancer therapy." Nat Rev Cancer **3**(10): 721-732.

Seo, D. W., S. H. Kim, et al. (2008). "TIMP-2 disrupts FGF-2-induced downstream signaling pathways." Microvasc Res **76**(3): 145-151.

Sevick, E. M. and R. K. Jain (1989). "Geometric resistance to blood flow in solid tumors perfused ex vivo: effects of tumor size and perfusion pressure." Cancer Res **49**(13): 3506-3512.

Sexl, V., G. Mancusi, et al. (1997). "Stimulation of the mitogen-activated protein kinase via the A2A-adenosine receptor in primary human endothelial cells." J Biol Chem **272**(9): 5792-5799.

Shah, M. A. and G. K. Schwartz (2001). "Cell cycle-mediated drug resistance: an emerging concept in cancer therapy." Clin Cancer Res **7**(8): 2168-2181.

Shaw, R. J. and L. C. Cantley (2006). "Ras, PI(3)K and mTOR signalling controls tumour cell growth." Nature **441**(7092): 424-430.

Shepherd, N. A., K. J. Baxter, et al. (1997). "The prognostic importance of peritoneal involvement in colonic cancer: a prospective evaluation." Gastroenterology **112**(4): 1096-1102.

Shimoda, M., K. T. Mellody, et al. (2010). "Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression." Semin Cell Dev Biol **21**(1): 19-25.

Sitkovsky, M. V., D. Lukashev, et al. (2004). "Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors." Annu Rev Immunol **22**: 657-682.

Snyder, F. F., M. S. Hershfield, et al. (1978). "Cytotoxic and metabolic effects of adenosine and adenine on human lymphoblasts." Cancer Res **38**(8): 2357-2362.

Song, H. H. and J. Filmus (2002). "The role of glypicans in mammalian development." Biochim Biophys Acta **1573**(3): 241-246.

Stecca, B. A., B. Nardo, et al. (1997). "Aberrant dipeptidyl peptidase IV (DPP IV/CD26) expression in human hepatocellular carcinoma." J Hepatol **27**(2): 337-345.

Stehle, J. H., S. A. Rivkees, et al. (1992). "Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype." Mol Endocrinol **6**(3): 384-393.

Stetler-Stevenson, W. G. (2008). "The tumor microenvironment: regulation by MMP-independent effects of tissue inhibitor of metalloproteinases-2." Cancer Metastasis Rev **27**(1): 57-66.

Strazzabosco, M. and J. L. Boyer (1996). "Regulation of intracellular pH in the hepatocyte. Mechanisms and physiological implications." J Hepatol **24**(5): 631-644.

Strobel, T. and S. A. Cannistra (1999). "Beta1-integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro." Gynecol Oncol **73**(3): 362-367.

Stuelten, C. H., S. DaCosta Byfield, et al. (2005). "Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-alpha and TGF-beta." J Cell Sci **118**(Pt 10): 2143-2153.

Su, L. K., B. Vogelstein, et al. (1993). "Association of the APC tumor suppressor protein with catenins." Science **262**(5140): 1734-1737.

Sugimoto, H., T. M. Mundel, et al. (2006). "Identification of fibroblast heterogeneity in the tumor microenvironment." Cancer Biol Ther **5**(12): 1640-1646.

Sun, Y. X., E. A. Pedersen, et al. (2008). "CD26/dipeptidyl peptidase IV regulates prostate cancer metastasis by degrading SDF-1/CXCL12." Clin Exp Metastasis **25**(7): 765-776.

Sund, M., Y. Hamano, et al. (2005). "Function of endogenous inhibitors of angiogenesis as endothelium-specific tumor suppressors." Proc Natl Acad Sci U S A **102**(8): 2934-2939.

Sutherland, L. A. and R. P. Bird (1994). "The effect of chenodeoxycholic acid on the development of aberrant crypt foci in the rat colon." Cancer Lett **76**(2-3): 101-107.

Sutherland, R. M. (1988). "Cell and environment interactions in tumor microregions: the multicell spheroid model." Science **240**(4849): 177-184.

Sutherland, R. M. and R. E. Durand (1973). "Hypoxic cells in an in vitro tumour model." Int J Radiat Biol Relat Stud Phys Chem Med **23**(3): 235-246.

Sutherland, R. M. and R. E. Durand (1976). "Radiation response of multicell spheroids--an in vitro tumour model." Curr Top Radiat Res Q **11**(1): 87-139.

Sutherland, R. M. and R. E. Durand (1984). "Growth and cellular characteristics of multicell spheroids." Recent Results Cancer Res **95**: 24-49.

Sutherland, R. M., H. R. MacDonald, et al. (1977). "Multicellular spheroids: a new model target for in vitro studies of immunity to solid tumor allografts." J Natl Cancer Inst **58**(6): 1849-1853.

Takayama, T., S. Katsuki, et al. (1998). "Aberrant crypt foci of the colon as precursors of adenoma and cancer." N Engl J Med **339**(18): 1277-1284.

Tan, E. Y., M. Mujoomdar, et al. (2004). "Adenosine down-regulates the surface expression of dipeptidyl peptidase IV on HT-29 human colorectal carcinoma cells: implications for cancer cell behavior." Am J Pathol **165**(1): 319-330.

Tan, E. Y., C. L. Richard, et al. (2006). "Adenosine downregulates DPPIV on HT-29 colon cancer cells by stimulating protein tyrosine phosphatase(s) and reducing ERK1/2 activity via a novel pathway." Am J Physiol Cell Physiol **291**(3): C433-444.

Tanaka, T., D. Camerini, et al. (1992). "Cloning and functional expression of the T cell activation antigen CD26." J Immunol **149**(2): 481-486.

Tanaka, T., J. Kameoka, et al. (1993). "The costimulatory activity of the CD26 antigen requires dipeptidyl peptidase IV enzymatic activity." Proc Natl Acad Sci U S A **90**(10): 4586-4590.

Tang, C. M., M. Dichter, et al. (1990). "Modulation of the N-methyl-D-aspartate channel by extracellular H+." Proc Natl Acad Sci U S A **87**(16): 6445-6449.

Tang, X., J. E. Lucas, et al. (2012). "Functional Interaction between Responses to Lactic Acidosis and Hypoxia Regulates Genomic Transcriptional Outputs." Cancer Res **72**(2): 491-502.

Tannock, I. F. and D. Rotin (1989). "Acid pH in tumors and its potential for therapeutic exploitation." Cancer Res **49**(16): 4373-4384.

Teicher, B. A., S. A. Holden, et al. (1990). "Classification of antineoplastic treatments by their differential toxicity toward putative oxygenated and hypoxic tumor subpopulations in vivo in the FSaIIC murine fibrosarcoma." Cancer Res **50**(11): 3339-3344.

Ten Kate, J., W. N. Dinjens, et al. (1986). "Adenosine deaminase complexing protein in cancer studies." Anticancer Res **6**(5): 983-988.

Ten Kate, J., J. T. Wijnen, et al. (1985). "Immunohistochemical localization of adenosine deaminase complexing protein in intestinal mucosa and in colorectal adenocarcinoma as a marker for tumour cell heterogeneity." Histochem J **17**(1): 23-31.

Terada, R., K. Yamamoto, et al. (2003). "Stromal cell-derived factor-1 from biliary epithelial cells recruits CXCR4-positive cells: implications for inflammatory liver diseases." Lab Invest **83**(5): 665-672.

Tholozan, F. M., C. Gribbon, et al. (2007). "FGF-2 release from the lens capsule by MMP-2 maintains lens epithelial cell viability." Mol Biol Cell **18**(11): 4222-4231.

Thompson, R. W. and W. C. Parks (1996). "Role of matrix metalloproteinases in abdominal aortic aneurysms." Ann N Y Acad Sci **800**: 157-174.

Topley, N., Z. Brown, et al. (1993). "Human peritoneal mesothelial cells synthesize interleukin-8. Synergistic induction by interleukin-1 beta and tumor necrosis factor-alpha." Am J Pathol **142**(6): 1876-1886.

Torimoto, Y., N. H. Dang, et al. (1991). "Coassociation of CD26 (dipeptidyl peptidase IV) with CD45 on the surface of human T lymphocytes." J Immunol **147**(8): 2514-2517.

Tortola, S., E. Marcuello, et al. (1999). "p53 and K-ras gene mutations correlate with tumor aggressiveness but are not of routine prognostic value in colorectal cancer." J Clin Oncol **17**(5): 1375-1381.

Toyokuni, S., K. Okamoto, et al. (1995). "Persistent oxidative stress in cancer." FEBS Lett **358**(1): 1-3.

Traynelis, S. F. and S. G. Cull-Candy (1990). "Proton inhibition of N-methyl-D-aspartate receptors in cerebellar neurons." Nature **345**(6273): 347-350.

Trotta, P. P. and M. E. Balis (1978). "Characterization of adenosine deaminase from normal colon and colon tumors. Evidence for tumor-specific variants." Biochemistry **17**(2): 270-278.

van der Wal, B. C., L. J. Hofland, et al. (1997). "Paracrine interactions between mesothelial and colon-carcinoma cells in a rat model." Int J Cancer **73**(6): 885-890.

Varona, A., L. Blanco, et al. (2010). "Expression and activity profiles of DPP IV/CD26 and NEP/CD10 glycoproteins in the human renal cancer are tumor-type dependent." BMC Cancer **10**: 193.

Vaupel, P. and L. Harrison (2004). "Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response." Oncologist **9 Suppl 5**: 4-9.

Vaupel, P., F. Kallinowski, et al. (1989). "Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review." Cancer Res **49**(23): 6449-6465.

Vegran, F., R. Boidot, et al. (2011). "Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kappaB/IL-8 pathway that drives tumor angiogenesis." Cancer Res **71**(7): 2550-2560.

Villa, E., A. Dugani, et al. (1996). "Identification of subjects at risk for colorectal carcinoma through a test based on K-ras determination in the stool." Gastroenterology **110**(5): 1346-1353.

Vilsboll, T., T. Krarup, et al. (2001). "Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients." Diabetes **50**(3): 609-613.

Visse, R. and H. Nagase (2003). "Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry." Circ Res **92**(8): 827-839.

Vistoli, G., A. Pedretti, et al. (2009). "Enhanced activity or resistance of adenosine derivatives towards adenosine deaminase-catalyzed deamination: Influence of ribose modifications." Bioorg Med Chem Lett **19**(10): 2877-2879.

Vivona, A. A., B. Shpitz, et al. (1993). "K-ras mutations in aberrant crypt foci, adenomas and adenocarcinomas during azoxymethane-induced colon carcinogenesis." Carcinogenesis **14**(9): 1777-1781.

von Bonin, A., J. Huhn, et al. (1998). "Dipeptidyl-peptidase IV/CD26 on T cells: analysis of an alternative T-cell activation pathway." Immunol Rev **161**: 43-53.

von Schwartzenberg, K., S. Kruse, et al. (1998). "Cloning and characterization of an adenosine kinase from Physcomitrella involved in cytokinin metabolism." Plant J **13**(2): 249-257.

Vornov, J. J., A. G. Thomas, et al. (1996). "Protective effects of extracellular acidosis and blockade of sodium/hydrogen ion exchange during recovery from metabolic inhibition in neuronal tissue culture." J Neurochem **67**(6): 2379-2389.

Wagner, H. E., C. A. Toth, et al. (1992). "Metastatic potential of human colon cancer cell lines: relationship to cellular differentiation and carcinoembryonic antigen production." Clin Exp Metastasis **10**(1): 25-31.

Walenta, S. and W. F. Mueller-Klieser (2004). "Lactate: mirror and motor of tumor malignancy." Semin Radiat Oncol **14**(3): 267-274.

Wang, H. and J. A. Keiser (1998). "Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1." Circ Res **83**(8): 832-840.

Wang, J. M., X. Deng, et al. (1998). "Chemokines and their role in tumor growth and metastasis." J Immunol Methods **220**(1-2): 1-17.

Warburg, O. (1956). "On the origin of cancer cells." Science **123**(3191): 309-314.

Wartenberg, M., S. Gronczynska, et al. (2005). "Regulation of the multidrug resistance transporter P-glycoprotein in multicellular prostate tumor spheroids by hyperthermia and reactive oxygen species." Int J Cancer **113**(2): 229-240.

Weinhouse, S. (1976). "The Warburg hypothesis fifty years later." Zeitschrift fur Krebsforschung und klinische Onkologie. Cancer research and clinical oncology **87**(2): 115-126.

Wendt, M. K., A. N. Cooper, et al. (2008). "Epigenetic silencing of CXCL12 increases the metastatic potential of mammary carcinoma cells." Oncogene **27**(10): 1461-1471.

Wendt, M. K., P. A. Johanesen, et al. (2006). "Silencing of epithelial CXCL12 expression by DNA hypermethylation promotes colonic carcinoma metastasis." Oncogene **25**(36): 4986-4997.

Wesley, U. V., S. Tiwari, et al. (2004). "Role for dipeptidyl peptidase IV in tumor suppression of human non small cell lung carcinoma cells." Int J Cancer **109**(6): 855-866.

Wike-Hooley, J. L., J. Haveman, et al. (1984). "The relevance of tumour pH to the treatment of malignant disease." Radiother Oncol **2**(4): 343-366.

Williams, B. A., A. Manzer, et al. (1997). "Adenosine acts through a novel extracellular receptor to inhibit granule exocytosis by natural killer cells." Biochem Biophys Res Commun **231**(2): 264-269.

Woods, A., E. S. Oh, et al. (1998). "Syndecan proteoglycans and cell adhesion." Matrix Biol **17**(7): 477-483.

Xaus, J., M. Mirabet, et al. (1999). "IFN-gamma up-regulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation." J Immunol **162**(6): 3607-3614.

Xouri, G. and S. Christian (2010). "Origin and function of tumor stroma fibroblasts." Semin Cell Dev Biol **21**(1): 40-46.

Xu, Y. and M. A. Villalona-Calero (2002). "Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity." Ann Oncol **13**(12): 1841-1851.

Xuan, J. Y., A. Besner, et al. (1994). "Mapping of Simpson-Golabi-Behmel syndrome to Xq25-q27." Hum Mol Genet **3**(1): 133-137.

Yaar, R., M. R. Jones, et al. (2005). "Animal models for the study of adenosine receptor function." J Cell Physiol **202**(1): 9-20.

Yakel, J. L., R. A. Warren, et al. (1993). "Functional expression of adenosine A2b receptor in Xenopus oocytes." Mol Pharmacol **43**(2): 277-280.

Yamada, T., T. Oshima, et al. (2010). "Overexpression of MMP-13 gene in colorectal cancer with liver metastasis." Anticancer Res **30**(7): 2693-2699.

Yamashita, N., T. Minamoto, et al. (1995). "Frequent and characteristic K-ras activation in aberrant crypt foci of colon. Is there preference among K-ras mutants for malignant progression?" Cancer **75**(6 Suppl): 1527-1533.

Yao, V., C. Platell, et al. (2003). "Role of peritoneal mesothelial cells in peritonitis." Br J Surg **90**(10): 1187-1194.

Yasuda, Y., M. Saito, et al. (2009). "Extracellular adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-9/-3 via A(2a) adenosine receptors." J Gastroenterol **44**(1): 56-65.

Yasumoto, K., K. Koizumi, et al. (2006). "Role of the CXCL12/CXCR4 axis in peritoneal carcinomatosis of gastric cancer." Cancer Res **66**(4): 2181-2187.

Yonemura, Y., Y. Endo, et al. (1996). "Mechanisms of the formation of the peritoneal dissemination in gastric cancer." Int J Oncol **8**(4): 795-802.

Yonemura, Y., Y. Endou, et al. (1997). "A possible role of cytokines in the formation of peritoneal dissemination." Int J Oncol **11**(2): 349-358.

Yu, Q. and I. Stamenkovic (2000). "Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis." Genes Dev **14**(2): 163-176.

Zeghari-Squalli, N., E. Raymond, et al. (1999). "Cellular pharmacology of the combination of the DNA topoisomerase I inhibitor SN-38 and the diaminocyclohexane platinum derivative oxaliplatin." Clin Cancer Res **5**(5): 1189-1196.

Zeisberg, E. M., S. Potenta, et al. (2007). "Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts." Cancer Res **67**(21): 10123-10128.

Appendix A. Adenosine increases MMP-7 and MMP-13 secretion in HT-29 and HS675.T cells.

Note: The data included in the appendices is intended to support the discussion. These data were not included in the thesis results as it is preliminary and the results were not conclusive (after a series of experiments) or they were not repeated a minimum of three times.

Several different approaches were used to evaluate MMP protein, as listed below. The results were, for the most part, inconclusive in that results varied between successive experiments and unique conclusions could not be made.

Some examples of data follow.

Immunoblot

Protein	Antibody	
Anti- proMMP-7	M7429	Sigma
Anti-MMP-7	IM71T	Calbiochem
Anti-MMP-13	IM78T	Calbiochem
Anti-MMP-13	MAB511	RnD

Zymography

Substrate	Protein evaluated
Gelatin	MMP-2, MMP-9
Casein	MMP-7, MMP-13

Secreted protein was isolated from HT-29 or HS675.T conditioned medium following adenosine treatment. The protein was precipitated (10% w/v TCA) at 4°C and centrifuged. The protein pellet was washed several times with 100% EtOH to dissolve any remaining TCA. The pellet was re-suspended in 1X Laemmli buffer and gently

heated to dissolve. Samples were electrophoresed, transferred and finally immunoblotted with α-proMMP-7 (M7429) or α-MMP-13 (MAB511) antibody.

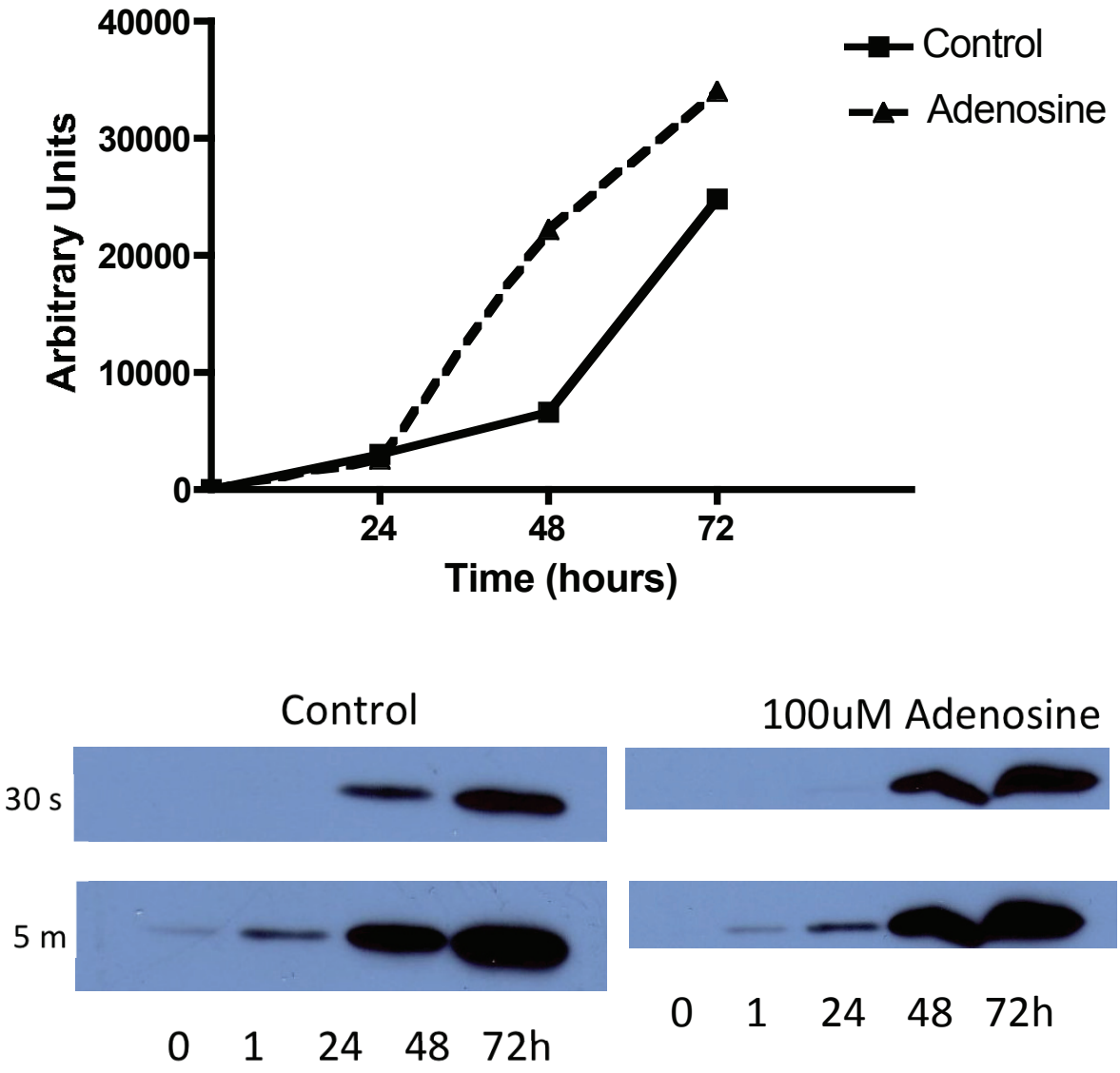


Figure A1. Adenosine (100μM) enhances proMMP-7 secretion over time in HT-29 cells.

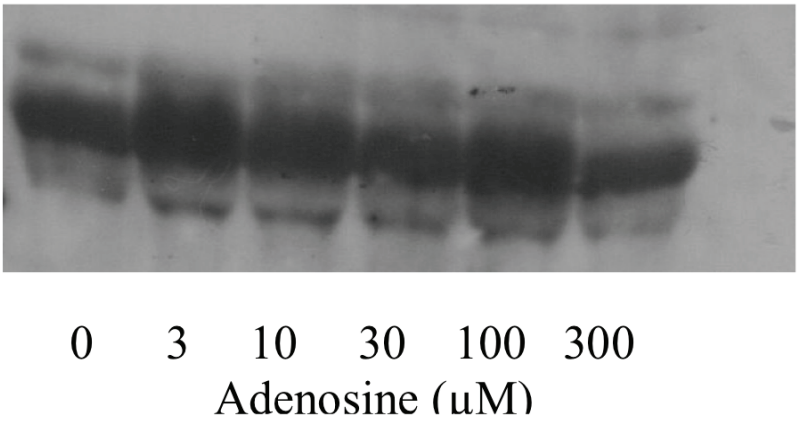


Figure A2. The effect of an adenosine dose-response approach on MMP-13 protein secretion in HS675.T cells (Immunoblot, MAB511).

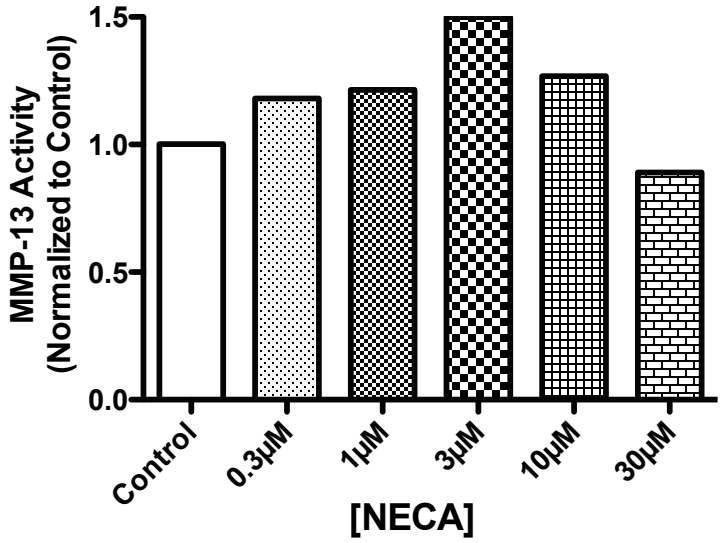


Figure A3. NECA increases MMP-13 proteolytic activity in HRT-18 cells (casein zymography).

These data suggest that adenosine not only regulates the expression of various MMPs in our CRC and stromal cell lines, but it may also increase the secretion of MMP protein.

Appendix B. Conditioned medium from HS675.T and Met-5A cells is chemotactic to HT-29 CRC cells, possibly as a result of CXCL12 production.

We sought to determine if our HT-29 cells would migrate in response to CXCL12 as well as the conditioned medium of our stromal cell lines. As CXCL12 mRNA is modulated by adenosine in our stromal cell lines, we wanted to see if the migratory response to Met-5A conditioned medium was enhanced by pre-treating Met-5A cells with adenosine 24h prior to collecting the medium.

Briefly, a chemoattractant (CXCL12, conditioned media) was placed in 24-well tissue culture plate. A Transwell® insert (6-8μm pores) coated in type IV collagen was placed in each well, and the HT-29 cells were seeded on top of the filter in the Transwell®. The next day the inserts were stained and the migrated cells were counted.

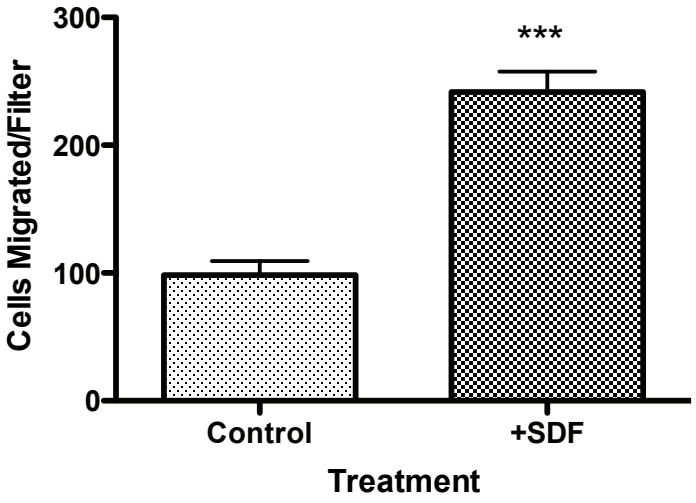


Figure B1. CXCL12 (100ng/ml) significantly enhances HT-29 cell migration.

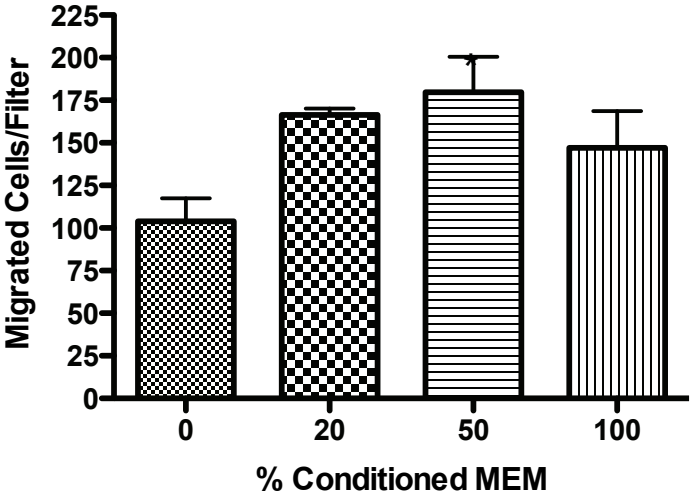


Figure B2. HS675.T cell conditioned medium elicits a chemotactic response in HT-29 cells in a dose-dependent manner.

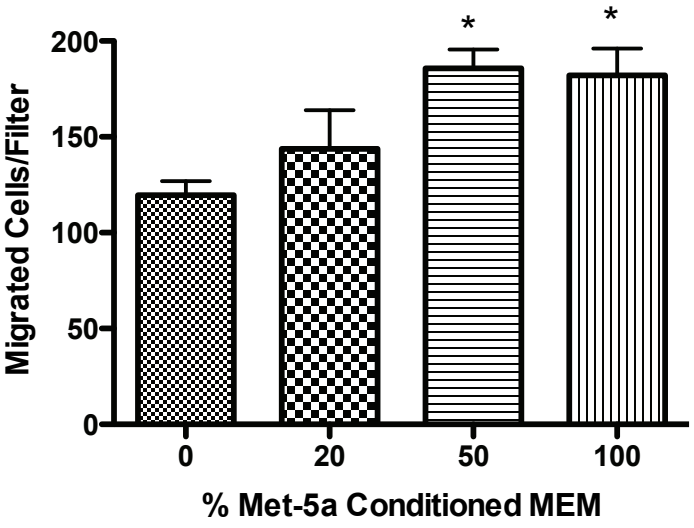


Figure B3. Met-5A conditioned medium elicits a chemotactic response in HT-29 cells in a dose-dependent manner.

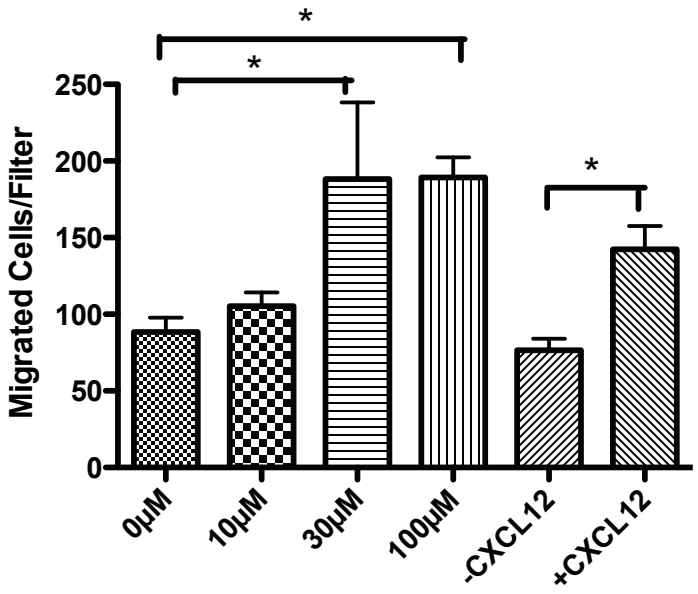


Figure B4. Adenosine enhances HT-29 migration to Met-5A conditioned medium (50% v/v). '+CXCL12' and '-CXCL12' are the positive migration control using pure CXCL12 (100ng/ml) only, and its vehicle control. The other bars represent the adenosine concentrations used to pretreat the Met-5A cells prior to collection of conditioned medium.

These preliminary results indicate that HT-29 cells migrate in response to CXCL12 as well as the conditioned media from Met-5A and HS675.T cells. Additionally, this shows that adenosine pre-treatment of Met-5A cells increased the chemotactic response of HT-29 cells to their conditioned medium, possibly through the release of some soluble mediator such as CXCL12.

Appendix C. Altered levels of lactate and glucose may increase MMP gene expression.

Our results have indicated that both glucose and lactate can downregulate CD26 cell-surface protein expression on CRC cells. We believe the downregulation of CD26 to be pro-tumorigenic and therefore tested to see if lactate and glucose had any effect on MMP mRNA expression in HT-29 cells.

RT-PCR was performed as previously described in 'Materials and Methods'. The following primer sets were used

MMP-7: F 5'-AAAGAGATCCCCCTGACATTT-3'
R 5'-GTGAGCATCTCCTCCGAGAC-3'

MMP-9: F 5'-CTCGAACTTTGACAGCGACA-3'
R 5'-GAAGTAGGTCGGAACCACCACGA-3'

TIMP-2: F 5'-ATTTGACCCAGAGTGGAACG
R 5'-AGACCAACGTGTGTGGATCA-3'

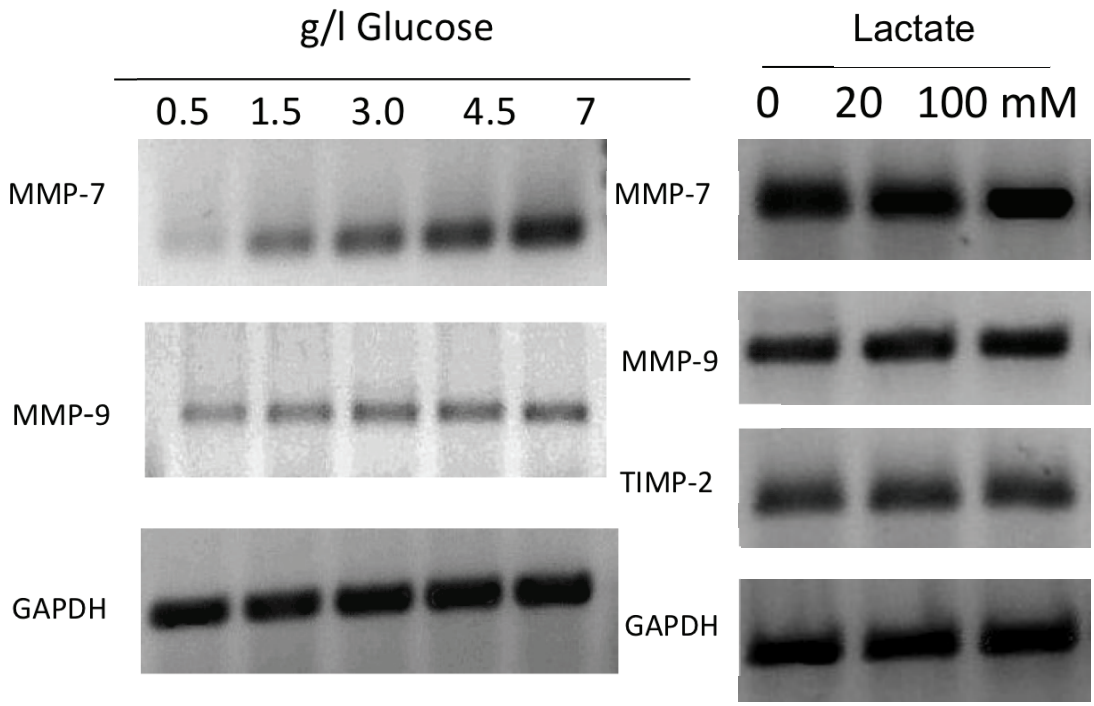


Figure C1. Glucose increases MMP-7 mRNA in HT-29 cells, while lactate has no effect on the various genes tested.

The results are preliminary (needing further replication) but suggest that glucose may play a role in regulation the mRNA expression of various MMPs. Further work would be required to assess the role of lactate in MMP regulation. Lactate and glucose may play a role in tumor progression through MMPs, in addition to the downregulation of CD26.

Appendix D. FGF-2 and FGF receptors are differently expressed in the various cell lines used in this study.

All of the cell lines used in our studies were assessed for FGF-2 and FGFR1-4 mRNA expression:

Cell line	FGF-2	FGFR1	FGFR1 (IIIc)	FGFR2	FGFR3	FGFR4
HRT-18	-	+++	+++	+	++	++
HT-29	-	+	-	+++	-	++
HS675.T	+++	+++	+	+	++	+
Met-5A	+++	+++	++	++	++	+

"-" Expression not detectable
"+" Expression detected
"++" Moderate expression
"+++" Strong expression

RT-PCR was performed as described in "Materials and Methods". The following primer sets were used:

FGF-2 F: 5′-AGAGCGACCCTCACATCAAG-3′
R: 5'-ACTGCCCAGTTCGTTTCAGT-3′

FGFR1 F: 5'-TAATGGACTCTGTGGTGCCCTC-3'
R: 5'-ATGTGTGGTTGATGCTGCCG-3'

FGFR1 (IIIc) F: 5'- AACCGCACCCGCATCACAGG-3'
R: 5'- TGTCCAATATGGAGCTACGGGGT-3'

FGFR2 F: 5'- CGCTGGTGAGGATAACAACACG-3'
R: 5'- TGGAAGTTCATACTCGGAGACCC-3'

FGFR3 F: 5'-GGCCCGAACAGCCGCTTCTT-3'
R: 5'-ACGCCGCTGAATGACACGCA-3'

FGFR4 F: 5'-GAGCCGGAACCCTTGTGGGC-3'
R: 5'-GGCCGGGGTGCTCTTTGGAC-3'

Some examples of expression data are shown in the two panels below. Lane 1: ladder, Lane 2: FGF-2 Lane 3: FGFR1, Lane 4: FGFR1 (IIIc), Lane 5: FGFR2, Lane 6: FGFR3, Lane 7: FGFR4

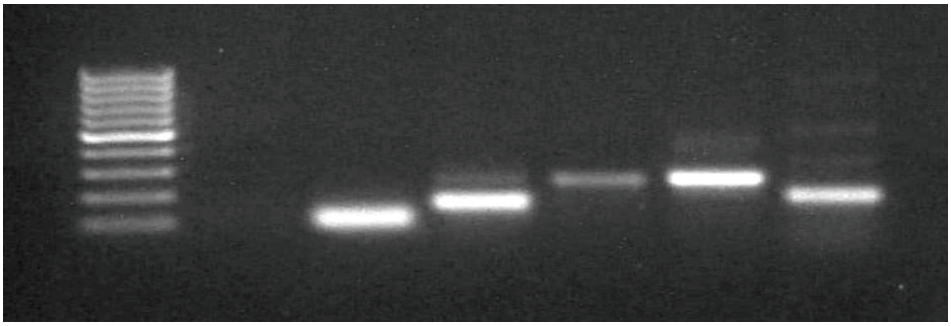


Figure D1. FGF-2 and FGFR1-4 mRNA expression in HRT-18 cells. Note the absence of signal in lane 2 for FGF-2.

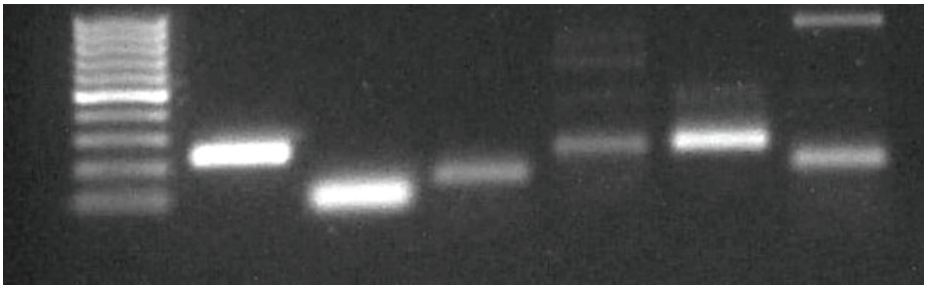


Figure D2. FGF-2 and FGFR1-4 mRNA expression in HS675.T cells.

Appendix E. Detroit-551 cells, a normal fibroblast cell line, respond differently to adenosine than HS675.T cells.

In our experiments HS675.T cells, which are CAFs, were found to respond to adenosine by means of an increase in cell proliferation and an increase in CD26 expression. We wanted to see if more normal fibroblasts (i.e. those from outside a tumor context) responded similarly.

Detroit-551 cells, a normal fibroblast cell line isolated from skin, were used for these studies. Thymidine incorporation assays and radioantibody binding assays were performed as is described in 'Materials and Methods'.

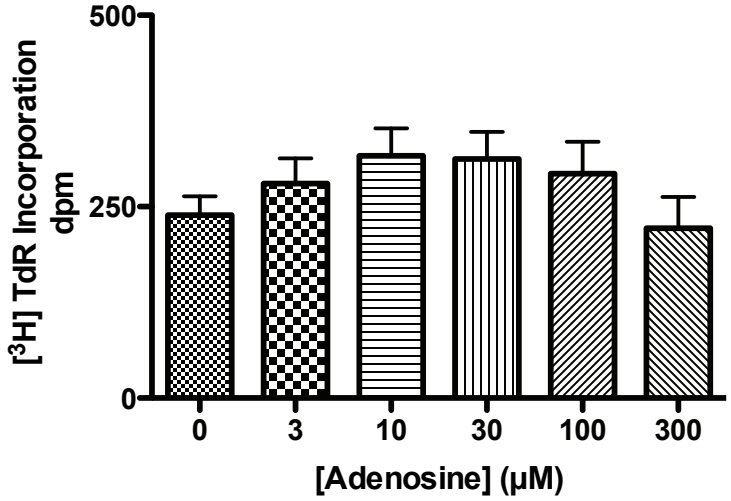


Figure E1. Adenosine has no effect on thymidine incorporation in Detroit-551 cells. Data are represented as the mean +/- SEM (n=6).

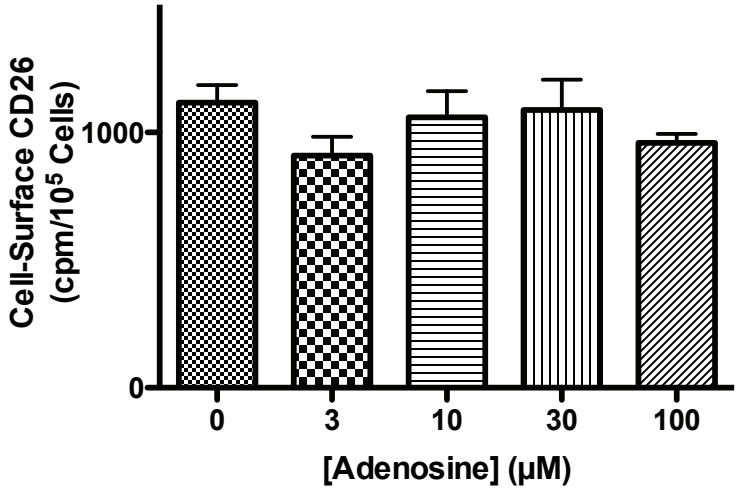


Figure E2. Adenosine has no effect on the cell-surface expression of CD26 in Detroit-551 cells. (In contrast, HS675.T cells had responded with an increase in CD26 expression.)

While these results are preliminary (i.e. need to be fully replicated under different conditions and with further cell lines), they suggest that adenosine does not affect CD26 cell-surface expression or increase cell proliferation in normal fibroblasts. While further information would be required to confirm that fibroblasts from these two different sources behave differently, it is possible that adenosine is acting selectively to enhance functions of activated stromal cell types in the tumor microenvironment.

Appendix F. DMSO increases MMP-13 mRNA in HRT-18 cells.

In our experiments, most of the adenosine receptor agonists and antagonists were solubilized in DMSO. While the concentration of DMSO was controlled for, there appeared to be a noticeable effect of the solvent on MMP-13 mRNA.

HRT-18 cells were treated with different concentrations of DMSO for 72 hours. qRT-PCR was performed to look at MMP-13 mRNA.

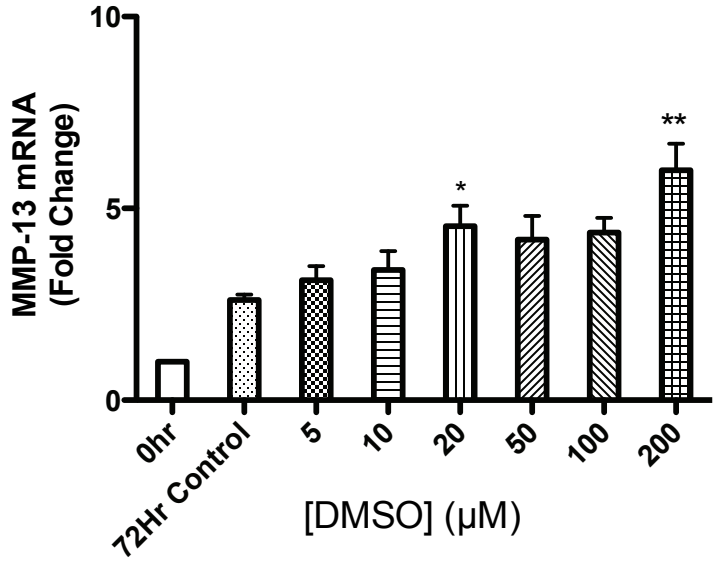


Figure F1. DMSO increases MMP-13 mRNA in HRT-18 cells. Data are represented as the mean +/- SEM (n=3).