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Ganglioside Increases Metastatic Potential and Susceptibility of Prostate Cancer to Gene Therapy *in vitro*

by

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ABSTRACT

Prostate cancer (CaP) is the 2nd most common cancer in North American men. Tumour management strategies are appropriate for early stage disease, but advanced disease has a poor prognosis and requires prompt treatment. Therefore, research into delay of tumour progression and efficacious treatment of aggressive cancer are of interest. Ganglioside was assessed for its role in altering markers of metastatic potential and susceptibility of CaP to adenovirus-mediated gene therapy. Healthy (RWPE-1) and malignant (DU-145, PC-3) prostate cells were cultured with or without mixed ganglioside. Differences in growth, ganglioside and integrin densities, and adenoviral infectivity were assessed between treatment and control groups. Ganglioside decreased ($p<0.01$) growth of PC-3 cells relative to untreated control. Ganglioside decreased ($p<0.01$) GD1a and increased ($p<0.04$) integrin densities in malignant prostate cells, suggesting ganglioside may increase metastatic potential of CaP. Ganglioside significantly increased adenovirus entry in PC-3 cells, thereby improving susceptibility of CaP to adenovirus-mediated gene therapy.

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LIST OF ABBREVIATIONS

ATCC	American Tissue Culture Collection
BFA	Brefeldin A
C/M	Chloroform/Methanol
CaP	Prostate Cancer
CAR	Coxsackie and Adenovirus Receptor
CMV	Cytomegalovirus Immediate Early
CPE	Cytopathic Effect
csELISA	Cell Suspension Enzyme-Linked Immunosorbent Assay
CTC	Circulating Tumour Cells
dd	Double-Distilled
DRE	Digital Rectal Examination
DNA	Deoxyribonucleic Acid
EBRT	External Beam Radiotherapy
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
g	Gravity
GC	Gas Chromatography
GD1a	α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]

	β-Gal-(1-4)-β-Glc-(1-1)-Cer
GD2	β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
GD3	α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
GFP	Green Fluorescence Protein
GM1	β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
GM3	α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
GT1b	α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
GT3	α-Neu5Ac-(2-8)-α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
HPTLC	High-Performance Thin Layer Chromatography
HR	Hormone-Refractory
hr	Hour
HS	Human Serum
HSA	Human Serum Albumin
IV	Intravenous
MFI	Mean Fluorescence Intensity
min	Minute
MOI	Multiplicity of Infection
MS	Mass Spectrometry
Neu5Ac	N-acetyl-neuraminic acid

GalNAc	N-acetylgalactosamine
OC	Osteocalcin
PBS	Phosphate-Buffered Saline
pfu	Plaque Forming Units
PSA	Prostate-Specific Antigen
PSADT	Prostate-Specific Antigen Doubling Time
PSMA	Prostate-Specific Membrane Antigen
RNA	Ribonucleic Acid
RP	Radical Prostatectomy
rpm	Revolutions per Minute
SFM	Serum-free Media
sIL-2R	Soluble Interleukin-2 Receptor
siRNA	Small Interfering Ribonucleic Acid
TRAIL	Tumour Necrosis Factor-Related Apoptosis-Inducting Ligand
v/v	Volume/Volume
w/v	Weight/Volume

1.0 LITERATURE REVIEW

1.1 PROSTATE CANCER OVERVIEW

CaP is the most prevalent cancer in men in North America (1, 2) and the second leading cause of cancer-related death (3). Screening for CaP is performed by combination of digital rectal exam (DRE) and prostate-specific antigen (PSA) test (4). A trivial DRE with or without elevated PSA may prompt transrectal ultrasound-guided (5) sextant needle prostate biopsy (6), which is ultimately needed to confirm CaP diagnosis (7). While the combination of DRE and PSA test has led to a surge in detection (8), autopsy data show that current detection protocol may be insufficient to diagnose the totality of clinically significant tumours (9).

Efficacy of a biomarker is a tradeoff between sensitivity and specificity. At the optimal cutoff value, PSA is only slightly superior to a coin flip in screening for CaP; therefore, this biomarker necessarily confers a large proportion of false positives and false negatives (10, 11). PSA performs poorly as a screening tool compared to recently discovered markers “early prostate cancer antigen-2” and “prostate cancer gene 3” (12, 13). High-grade cancers actually produce less PSA than low-grade cancers when corrected for the tumour volume (14). Advanced metastatic cancer may be present even at normal PSA (15). Therefore, the absolute level of PSA provides modest evidence pertaining to presence and extent of CaP.

Several PSA test modifications have proven useful as diagnostic and prognostic tools for CaP screening. PSA density, PSA standardized to prostate weight serves as a valuable adjunct in detection of clinically significant tumours (16, 17). PSA velocity (PSAV) and PSA doubling time (PSADT) give more useful prognostic information to a single PSA measurement (18, 19). The medical community is recommending that men enter the clinic at about age 45 to get a "starter" PSA value so that PSAV and PSADT can be determined earlier in life (20). Prostate-specific membrane antigen (PSMA) may aid in predicting tumour progression and/or recurrence after radical prostatectomy (RP) (21). CaP detection, progression, and response to treatment can be more accurately predicted when aided by one of several PSA test modifications.

Biopsy technique and tumour size/location may further complicate cancer detection. Slaughter's concept of field cancerization (22) proposes that a series of genetic changes occur in a tissue before becoming malignant (23). A false negative biopsy may be aided by assaying prostate tissue for a 3.4 kilobase pair deletion in the mitochondrial genome. One study found that malignant and proximal-to-malignant, but not benign prostate tissue presented with this deletion (24). Thus, negative biopsy can still obtain tissue that accurately diagnoses CaP. However, the most pressing clinical issue is to distinguish biologically relevant CaP from the clinically significant cases. Biologically relevant cases refer to disease that will inevitably lead to progression and death; these constitute about 10% of total diagnosed disease (25). Detection of biologically relevant cases may also be aided by an assay for circulating tumour cells (CTC) (26); CTC appear to

be inversely correlated with survival (27). However, no marker can definitively predict CaP progression; therefore, strategies to prevent development of advanced CaP should be prioritized.

1.2 PROSTATE TUMOUR CLASSIFICATION

In 1994, Epstein et al. set criteria to define clinical relevance of prostate tumours (Table 1A). Gleason score refers to a sum of two histopathologic scores related to appearance of biopsied prostate tissue; higher scores are indicative of poorly differentiated tissue (Table 1B). Cancers are also staged on clinical (Table 1C) and pathological (Table 1D) characteristics. Additionally, lymph node involvement (Table 1E) and distant metastasis (Table 1F) serve as important disease parameters to assess.

1.3 PROSTATE CANCER METASTASIS

Metastases refer to the presence of secondary tumours, beyond the original site of cancer. CaP preferentially metastasizes to the bone, seminal vesicles, and pelvic lymph nodes (28). Metastasis begins when a cell from the primary tumour locally invades surrounding extracellular matrix (ECM). After invading and crossing the basement membrane into the stroma, the cell then has direct access to blood and lymphatic vessels. The cell may migrate to a local blood vessel, intravasate, and circulate throughout the body. A cell that is able to recruit platelets as a protective surrounding forms a microthrombus and may have an enhanced chance of survival in the harsh conditions of the bloodstream (29). The microthrombus can attach to the lumen wall allowing the metastatic cell to proliferate until providing physical stress sufficient to disrupt the capillary

basement membrane and extravasate (30). The initially dormant micrometastasis may acquire the ability to proliferate once again, recruit a blood supply, and form a metastatic lesion (31, 32). It is thought that CaP metastasizes to bone because the bone releases a series of chemotactic factors into the blood that signal the cancerous prostate cell in circulation to attach and extravasate when in proximity to bone endothelium (33).

1.4 DAIRY AND PROSTATE CANCER

A recent meta-analysis assessed 45 observational studies and concluded that there is no association between CaP risk and dairy exposure (34). The pooled relative risk for CaP incidence and dairy exposure was 1.14 (1.00-1.29) for case-control studies and 1.11 (1.03-1.19) for cohort studies. Six prospective trials pertaining to dairy intake and CaP incidence have been published since the meta-analysis: one found no correlation between CaP risk and dairy consumption (35), two found positive correlations between CaP risk and total dairy consumption (36, 37), and three studies found positive correlations that were exclusive to low-fat dairy products and CaP risk (38-40). The true relationship, if any, between dairy exposure and CaP incidence is still unclear.

Considerable heterogeneity in the definition of dairy exists in studies that investigate the relationship between dairy intake and CaP incidence. Two studies which included buttermilk in the definition of dairy found strong positive trends for CaP risk with dairy consumption (41, 42). Both sweet and cultured bovine buttermilk products have much greater ganglioside content than whole milk (~10 times), and the ganglioside content of skim milk is much lower than that of whole

milk (~10 times) (43). The varying quantity of ganglioside across dairy products may contribute to some of the CaP risk-dairy consumption discrepancy. Since several studies have elucidated the biological role of gangliosides in metastasis (44-48), the role of dietary ganglioside in potentiating advanced CaP is of interest.

1.5 GANGLIOSIDE STRUCTURE, FUNCTION, AND LOCALIZATION

The cell membrane is composed of a variety of proteins, carbohydrates, and lipids (49). Among these constituents are a class of sialylated sphingolipids, gangliosides (50). Gangliosides consist of a hydrophilic region that protrudes from the cell surface and a hydrophobic portion anchored in the cell membrane (51). They are found primarily within lipid rafts of cell membranes (52). This area of the cell membrane is particularly rich in cholesterol and sphingolipids (53). Ganglioside composition of the lipid raft is known to influence numerous cell characteristics and processes including membrane fluidity (54), endocytosis (55, 56), secondary messenger signaling (57, 58), protein localization (59), and ligand binding (60).

1.5.1 GANGLIOSIDE SYNTHESIS

While particularly high in neural tissues (61), gangliosides are present in all tissues of the body. Ganglioside synthesis commences with ceramide synthesis in the endoplasmic reticulum (62). Ceramide is transported to the cytosolic Golgi face for the addition of glucose, then to the luminal side for the addition of galactose (63). From this point, sugar moities and sialic acids are added to form one of several gangliosides (Figure 1-1). These reactions are accomplished by sialyltransferases, galactosaminyltransferases, and galactosyltransferases on the

luminal face of the Golgi complex (64). The fatty acid component of the ceramide tail can vary in length as shown in A2780 ovarian carcinoma cells (65). Variability in sialic acid configuration, oligosaccharide size, and length of ceramide may have consequences that alter ganglioside localization and functionality (62, 66, 67).

1.5.2 GANGLIOSIDE UPTAKE

When provided in culture, gangliosides can be taken up by the cell. The concentration of ganglioside in aqueous culture medium affects micelle formation and subsequent uptake. Critical micellar concentration is defined as the concentration above which gangliosides will form micelles in aqueous solution (68). Gangliosides exist as monomers below the critical micellar concentration. Gangliosides may be taken up in micellar or monomeric forms: by adsorption, insertion, or receptor binding (Figure 1-2). The relative rate or amount of uptake may differ depending on method of uptake, but has not been determined.

The first work demonstrating ganglioside uptake into cell membrane was performed in mouse fibroblasts, which do not natively express GM1, but successfully incorporate this ganglioside when supplied in culture medium (69). GD3 (Figure 1-3) uptake has also been demonstrated in CaCo-2 intestinal epithelial cells (70). Cell surface GD3 density increases in M24 melanoma cells in a dose-dependent manner when provided in culture (71). Ganglioside uptake also occurs in several tissues *in vivo*. Provision of gangliosides GM3 and GD3 in the diet results in increased total ganglioside content in the intestinal cell membrane of weanling rats (72). GD3 content in rat retina is also increased upon addition of

GD3 to diet (73). Although demonstrated in many cell and tissue types, uptake of ganglioside in prostate has not been investigated.

1.5.3 EFFECT OF GD3 ON CELL GROWTH *IN VITRO*

GD3 is both an inducer of and a secondary messenger in apoptosis. GD3 can induce apoptosis directly by inhibition of nuclear factor-kappa B-survival pathway (74, 75). Alternatively, GD3 clusters in ganglioside-rich submicrodomains and recruits death receptors CD95/Fas/sIL-2R to the cell surface. Upon binding of apoptosis-inducing ligands to respective receptors, GD3 undergoes dynamic reallocation to mitochondria where it induces cytochrome c release, membrane depolarization, caspase induction, and cell death (76, 77). Direct mitochondrially-mediated GD3-induced cell death has been proposed as well which occurs without death receptor stimulation (78).

Mixed ganglioside treatment (including GD3) decreased cell growth in murine melanoma and Lewis lung carcinoma cells at supraphysiological concentrations, but had no effect or increased growth at lower doses (44). Demonstrations have implicated that GD3 has a cytotoxic effect on SKBR3 breast cancer (79) at a physiological concentration. The influence of GD3 on normal or malignant prostate cell growth has not yet been determined. Evidence presented from literature herein pertaining to ganglioside and tumour growth and metastasis is summarized in Table 1-2.

1.5.4 EFFECT OF GD3 ON CELL GROWTH *IN VIVO*

The effect of GD3 on tissue growth *in vivo* pertains predominantly to tumour growth. Mice were transplanted with either B16/BL6 melanoma or with

mouse MN5 fibrosarcoma cells in one experiment, then injected with a ganglioside mixture (including GD3) intraperitoneally on days 10-16 post-transplant. The ganglioside treated groups exhibited increased tumour growth versus the control group (44). Macrocyclic lactone brefeldin A (BFA) increases cellular GM3 and GD3, while inhibiting synthesis of more complex gangliosides (80, 81). Pre-treatment of HCT 116 colorectal carcinoma cells with BFA prior to injection in BALB/c nude mice inhibited tumour growth (82). Ganglioside influences tumour growth in a manner that likely depends on the model of cancer studied and the composition of ganglioside treatment. The particular role of GD3 is not clear since studies to examine it in isolation present with difficulties. Alteration of GD3 metabolism necessarily affects levels of other gangliosides which may compensate for or ablate the effects of GD3.

1.6 GANGLIOSIDE AND CANCER METASTASIS

Gangliosides play roles in several steps of metastasis including tumour cell migration, adhesion, and angiogenesis. Alessandri *et al.* report that providing a ganglioside mixture in culture leads to enhanced migratory potential of neoplastic cells (44). Siglecs are sialo-adhesion proteins that bind gangliosides GD2, GD3, and GT1b. Cell surface GD2, GD3, and GT1b density on renal carcinoma cells positively predict potential for metastasis to lungs, which express siglec7 (47). GD3 enhanced production of pro-angiogenic vascular endothelial growth factor in human glioma cells in culture (45). *In vitro* research has highlighted the role of ganglioside in metastasis.

The role of ganglioside in tumour metastasis has also been demonstrated *in vivo*. Silencing of GM3 synthase in highly metastatic 4T1 breast cancer cells inhibits cell migration and invasion *in vitro* (46). Formation of metastases was inhibited when GM3 synthase-silenced 4T1 cells were injected into BALB/c nude mice. An inhibitor of glucosylceramide synthase successfully depletes ganglioside in MEB4 murine melanoma cells (48). Mice injected with normal MEB4 cells formed more metastases than mice injected with ganglioside-depleted MEB4 cells. Ganglioside plays vital roles in promotion of cancer cell metastasis and therefore, may be exploited as therapeutic targets for prevention of cancer progression.

1.6.1 GANGLIOSIDE CONTENT IN NORMAL VERSUS CANCEROUS TISSUES

There are differences in amount and content/type of ganglioside expressed among healthy and cancerous tissues. Marquina *et al.* found total ganglioside content to be higher in breast tumour than healthy breast tissue with more GM3 and GD3 in tumours versus normal tissue (83). GM3 and GD3 were reported to be higher in brain tumours than healthy brain tissue (84). Both GM3 and GD3 gangliosides were also elevated in a sarcoma compared to a normal frontal cerebral cortex sample; however, total ganglioside content was lower in the gliosarcoma sample than in the normal frontal cerebral cortex sample (85). This finding is not necessarily a true reflection of the population, as a screen with more samples has not yet been reported. There are distinct differences in (total)

ganglioside content between tumours and healthy tissues that may highlight areas of intervention for cancer therapy.

The cell surface ganglioside profiles of healthy PrEC and malignant human prostate cell lines have been qualified and quantified using cell suspension ELISA (csELISA). Although GM1 is the most abundant ganglioside in PrEC, GD1a is the dominant ganglioside in malignant cell lines (86). Total cell ganglioside extract has been analyzed in prostate cell lines by HPTLC. The dominant ganglioside in PrEC is GM3 (87), not GM1 as found by csELISA. Malignant prostate cell lines are most abundant in GD1a, similar to findings from the cell surface assay (86). Gangliosides profiles have been determined in some prostate cell lines, but the results indicate discrepancies when different methodologies are used.

Glycosphingolipids are present in ER and nuclear membranes, but are heavily concentrated on the outer leaflet of plasma membranes (88). In malignant PC-3 and DU-145 prostate cells, cell surface and total cellular analyses revealed GD1a as the most abundant ganglioside. Although ganglioside GM1 was highest in PrEC when assayed at the cell surface, GM1 content was relatively low when total cell ganglioside was assayed. csELISA is a method for cell surface ganglioside quantification and is able to detect low picomolar concentrations of gangliosides using only $0.5x10^6$ cells (71). HPTLC assays ganglioside extract from $25x10^6$ cells and chromatograms stain very lightly (87). For this reason, HPTLC appears to be a less sensitive detection method and may present with greater inter-assay variability. Another explanation for the discrepancy may be

that anti-ganglioside antibody performance varies depending on the assay employed. Finally, this finding may challenge dogma that gangliosides are concentrated on the outer leaflet of the plasma membrane; there may be significant intracellular contribution of ganglioside in prostate cells.

1.6.2 MODIFIED GANGLIOSIDES IN CANCER

Cancer cells synthesize gangliosides that are not present in normal tissue and metabolize ganglioside into abnormal species when provided in culture. Provision of exogenous ganglioside to astrocytoma and melanoma cells in culture induces expression of novel cell surface gangliosides (89). Unusual gangliosides have been found in malignant, but not healthy breast tissue. Both HPTLC and GC/MS detected O-acetyl and N-glycolyl forms of GD3, GT3, and GM3 in breast carcinoma (83). Wilms renal tumours presented with N-glycolyl GM3, but non-tumoural fetal kidney did not stain positively for this ganglioside (90). Ravindranath *et al.* assessed ganglioside signatures in primary and metastatic melanomas within the same patient. Metastases presented with greater O-acetylated ganglioside than primary melanoma (91).

Elevations in anti-ganglioside antibodies have been assayed in soft tissue sarcoma (92), ovarian carcinoma (93) and CaP (86) patients. It has been suggested that this phenomenon is a response to increased shedding of ganglioside by tumours into the circulation (94). These circulating gangliosides may act as decoys, allowing tumours to minimize the chance of being recognized by the immune system. In an immune-competent individual, high levels of anti-ganglioside antibody may lead to increased recognition of tumours since total

ganglioside is higher in malignant tissue than non-malignant tissue (Section 1.6.1). Therefore, tumour cells may upregulate modification of gangliosides into atypical species so as to evade immune recognition and increase chance of survival.

1.7 POTENTIAL BIOMARKERS OF METASTASIS IN PROSTATE CANCER

1.7.1 GD3

A number of cancers aberrantly over-express GD3. GD3 synthetase activity and GD3 density is elevated in metastatic melanoma cell lines (95). Furthermore, the ratio of GM3:GD3 in melanoma tissue has been shown to be proportional to overall survival; patients with a higher ratio (low GD3) survive longer than those with a lower ratio (high GD3) (96). GD3 may also be used to predict response of tumours to therapy. Cell surface GD3 expression is inversely related to radiosensitivity of human melanoma cells (97). GD3 density is a marker that can provide information regarding tumour metastasis potential, stage, and response to therapy.

GD3 is functionally implicated in development of metastasis. F-11 neuroblastoma cells injected into mice form tumours and metastatic lesions. When GD3 synthase activity is knocked down in F-11 prior to injection, mice experience slower tumour growth and form fewer metastases (98). Furthermore, formation of metastasis is inhibited when BALB/c nude mice are injected with human melanoma cells in the presence of anti-GD3 antibody (99). Blocking GD3 exposure may prevent adherence and subsequent colonization of a metastasizing

cell at a secondary site. GD3-positive melanoma cells display greater adhesion to ECM than GD3-negative melanoma cells (100), which may influence a metastasizing cell to bind and colonize at a secondary site. The authors also reported that integrin β_1 co-localizes with cell surface GD3 and that β_1 is directly implicated in cell invasion. Given the structural similarity among gangliosides, other species may also co-localize with integrins and affect malignant properties of cells.

1.7.2 9-O-Acetyl GD3

When incubated with GD3 in culture, normal Chinese hamster ovarian cells appear to incorporate GD3 into the cell membrane before converting it to 9-O-acetyl GD3 (101). 9-O-acetyl GD3 has been found in malignancies including breast (83), melanoma (102), and those of neuroectodermal origin (103). Jurkat leukemia cells express 9-O-acetyl GD3 after 3 weeks of incubation with GD3 (104). It is unknown whether cancer cells have an enhanced capacity to convert GD3 to 9-O-acetyl GD3.

9-O-acetyl GD3 is involved in resistance to apoptosis. Jurkat and Molt-4 melanoma cells incubated with 9-O-acetyl GD3 before exposure to shingosine or daunorubicin are resistant to apoptosis. However, cells incubated with GD3 before exposure to cell killing agents are sensitive to cell death (104). Introduction of a plasmid containing the gene for O-acetylesterase resulted in reduced cellular 9-O-acetyl GD3 and restored susceptibility of U118 glioma cells to apoptosis (105). These observations may entail altered drug uptake by cells containing 9-O-acetyl GD3, or protection from apoptosis by this ganglioside.

Tumour cells may upregulate production of 9-O-acetyl GD3 in effort to nullify the pro-apoptotic effect of GD3. The presence of 9-O-acetyl GD3 has not been documented in prostate and the capacity for prostate to convert GD3 to 9-O-acetyl GD3 is also unknown. Assaying tumour tissue for 9-O-Ac GD3 may be valuable prior to choosing course of therapy.

1.7.3 GD1a

GD1a is inversely related to metastatic potential of cancer cells. FBJ-LL osteosarcoma cells are relatively low in GD1a content, but have higher migratory capacity than GD1a-rich FBJ-S1 cells. In addition, incubation of FBJ-LL cells with GD1a significantly inhibited migratory potential of ostersarcoma cells in a transwell assay (106). Treatment of FBJ-LL with GD1a also decreases binding of cells to vitronectin, an ECM component that may serve as a site of anchorage for metastasizing cells. A line of FBJ-11 cells stably transfected to synthesize GD1a implanted in mice did not develop metastases whereas mice implanted with mock transfectants did (107).

The inverse relationship between GD1a density and metastatic capacity has also been extended to models beyond osteosarcoma. A reduction of GD1a was found in a highly metastatic variant of Eb lymphoma cells compared to the weakly metastatic (high-GD1a) parental line (108). Highly metastatic B77-AA6 cells were subcloned from poorly metastatic B77-3T3 murine fibroblasts. GD1a constituted a higher percentage of total cellular ganglioside in 3T3 than AA6 cells lending further support to the inverse relationship between cellular GD1a density and metastatic potential (109).

1.7.4 COXSACKIE AND ADENOVIRUS RECEPTOR

CAR is a tight junction protein expressed in many tissues (110). It is found to at least partially localize to the lipid-rich microdomain in PANC-1, ASPC-1 (111), and COS-7 cells (112). CAR expression was found to differ between normal, primary prostatic adenocarcinoma and metastatic prostate specimens by immunohistochemical staining (113). Eight-five percent of normal tissues stained very highly for CAR, but only 24% of low grade, organ-confined CaP tissue samples stained very highly. All metastatic tumour tissue samples stained very highly in the same study. It was suggested that primary CaP cells that are able to re-express CAR after migrating from the initial tumour site may have an advantage in forming secondary tumours (114).

Other evidence suggests a role for CAR as a tumour suppressor. B16 melanoma cells were transfected with control plasmid or with plasmid expressing CAR prior to injection in immunocompromised mice. Formation of lung metastases was significantly lower in mice injected with B16 cells stably expressing CAR (115). A similar experiment was performed by injecting nude mice with human U-118 MG glioma cells stably transfected to produce CAR. The CAR-transfectants had significantly lower tumour volume on days 14 and 21 post-transfection versus control transfectants (116). In an experiment conducted by Okegawa *et al.*, a series of PC-3 cells were generated that express CAR to varying degrees. Each of the PC-3 derivatives was injected subcutaneously to establish tumours in nude mice. Tumour growth was inversely related to CAR

expression in PC-3 cells (117). These data suggest that CAR modulation may be a potential therapeutic strategy to target in cancer formation and/or progression.

In contrast, CAR is strongly implicated in cell survival. Bruning *et al.* implanted preneoplastic mammary epithelial cells into mammary fat pads of mice and assessed tumourigenicity. Preneoplastic non-invasive tissue had low CAR expression, whereas tissues having successfully transitioned to invasive adenocarcinoma had high CAR expression at onset of growth (118). The investigators concluded that CAR expression provides preneoplastic cells with a survival advantage when tumour formation is initiating. This is supported by observations that elevated CAR-dense cells are positively selected for when exposed to chemotherapeutic agents in ovarian carcinoma cell lines (119). In addition, CAR expression was positively associated with reduced pro-apoptotic caspase activation and increased anti-apoptotic proteins bcl-2 and bcl-XL (118). CAR may play a role in transition to malignancy, tumour cell survival, or cell-cell attachment.

1.7.5 INTEGRINS $\alpha_v\beta_3$ AND $\alpha_v\beta_5$

Integrins modulate a series of interactions between cells and the extracellular environment (120) affecting cell attachment, invasion, and survival. Primary liver cancer cells transfected with siRNA for β_3 and β_5 integrins do not adhere to culture plates as well as β_3- and β_5-expressing controls adhere (121). This implies that colony formation of a tumour cell may be hindered when integrin expression is knocked down. Integrin $\alpha_v\beta_3$ particularly modulates CaP tumourigenicity. A study showed that immunocompromised mice injected with

PC-3 cells that express wildtype $\alpha_v\beta_3$ form bone metastases 100% of the time, but mice injected with PC-3 cells null for $\alpha_v\beta_3$ or with mutated $\alpha_v\beta_3$ only formed lesions 6% of the time (122). This study strongly implicates $\alpha_v\beta_3$ in adherence to bone matrix. PC-3 cells undergo greater apoptosis when incubated with antibodies that block $\alpha_v\beta_3$ and $\alpha_v\beta_5$ *in vitro* (123). Therefore, tumour cell survival may depend on these glycoproteins particularly for functional adhesion, since the same effect was not seen in cultures incubated with antibodies to other integrins. Integrins are strongly implicated in formation of CaP metastasis and cell survival; and therefore, represent a potential molecular target for intervention.

1.8 PROSTATE CANCER THERAPY

Progression-free survival after RP has significantly improved since the inception of the PSA test (8). While highly efficacious as a curative therapy in early stages of disease (124), RP presents with risks to quality of life parameters including urinary continence, bowel activity, erectile function; and risks associated with any major operation including heart problems, blood clots, and infection. Positive biopsy reasonably evokes patient anxiety, which may prompt request for unnecessarily aggressive treatment from the patient. In fact, 90% of patients want their CaP treated immediately upon diagnosis (21). However, many low-stage, low-grade, organ-confined cancers have a favourable prognosis under conservative management or "watchful waiting" (125). Although all cases should be detected, all need not be treated.

Other therapies exploit the testosterone-dependence of prostate tumours. Androgen ablation is intended to starve tissue of steroids (126). This can be

accomplished with 5α reductase inhibitor dutasteride (127) or luteinizing hormone-releasing hormone agonist, finasteride (128). Although the tumour may initially shrink, the hormone-refractory (HR) phenotype of disease may arise (129). Much promise has been shown in treatment of HR, metastatic CaP with abiraterone acetate. This drug is an inhibitor of CYP17, which catalyzes two steps involved in 5' dihydrotestosterone synthesis. Administration of abiraterone acetate lowered 5' dihydrotestosterone and PSA levels in a phase I trial of patients with HR CaP (130). Other routes of therapy chosen may include systemic docetaxel chemotherapy (131), external beam radiotherapy (EBRT) (132), or brachytherapy (133). Although a number of treatment strategies have been developed to combat CaP, advanced tumours are difficult to treat as many become resistant or unresponsive to therapy.

1.8.1 GENE THERAPY

Tumour-directed cancer gene therapy is a method by which a gene of therapeutic significance is delivered to cancerous tissue, where it exerts a cell killing effect. Other gene therapy strategies have also been developed in which other tissues can be targeted to interfere in tumour/metastatic development, or boost immune recognition of cancer cells. The particular gene(s) is/are strategically chosen based on knowledge of disease characteristics. DNA or RNA may be chosen as the genetic material. The gene may be directly transcribed and translated, or may be aimed at silencing or interfering with cellular DNA/RNA production. Genetic material is very susceptible to degradation. Thus, a delivery system or vector which is capable of delivering the intact genetic material to

diseased tissue while avoiding normal tissue is of critical importance to the effectiveness of cancer gene therapy.

1.8.2 ADENOVIRUS

Adenovirus is actively investigated as a gene therapy vector for cancer treatment (134). Adenovirus enters cells through clathrin-coated vesicles (135), or through the lipid raft (112). Virus entry into the cell is primarily facilitated by binding to CAR at the cell surface (136, 137). Internalization is usually made possible by binding of adenovirus to co-receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (138, 139). The adenovirus-receptor complex migrates to a region favourable for internalization, is taken up in an endosome (136), and enters the nucleus where its genes may be actively expressed (140).

The efficiency with which adenovirus enters cells differs among tissue types (141). This variability can be explained by altered levels of CAR among tissues (110). CaP is a promising prospect for adenovirus-mediated gene therapy due to high CAR expression in prostate relative to other tissues of the body (142). CAR density is elevated in metastatic CaP tissue, suggesting enhanced capacity for transduction by adenovirus (113). In addition, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are higher in cancerous prostate cells than in healthy prostate cells (143). Therefore, adenovirus-mediated gene therapy is a promising application for treatment of advanced CaP.

Since CAR predicts susceptibility to infection by adenovirus, increasing receptor density in primary tumour tissue is a plausible pursuit to enhance efficacy of experimental gene therapy. U-118 MG glioma cells stably transfected

to produce CAR are infected to a greater degree by adenovirus than mock or non-transfected cells (116). A mouse engineered to overexpress CAR shows enhanced infectivity by adenovirus (144). Pre-incubation of ovarian carcinoma cell lines SKOV3.ip1 and Hey with agents cisplatin, etoposide, topotecan, and trichostatin A led to increased CAR detection by FACS; and these changes positively correlated to DNA copy number of replication-deficient adenovirus after infection (119). Thus, increasing CAR level is a plausible target to improve adenovirus-vector gene therapy.

1.8.3 PROSTATE CANCER GENE THERAPY: EXPERIMENTAL STUDIES

Adenovirus can be utilized to restore functional tumour suppressor genes in cancer cells. Tumour suppressor gene DLC1 shows recurrent loss of function in CaP, and adenovirus-mediated restoration of DLC1 expression inhibited proliferation and invasive potential of PC-3 cells (145). Tissue specificity may be improved by utilizing the PSA promoter/enhancer region. Use of an adenoviral vector with a luciferase reporter under control of the PSA promoter/enhancer increased luciferase expression in PSA-positive LNCaP cells compared to PSA-negative PC-3 and non-prostate cell lines (146). Use of the PSMA promoter/enhancer in adenoviral vectors also improves tissue specific transgene expression. Ad-PSMA-EGFP was more active in androgen-dependent LNCaP and androgen-independent CL-1 CaP cells than in breast and lung cancer cell lines, assessed by flow cytometry (147). The group also showed that infection of cells with virus bearing CMV promoter (Ad-CMV-EGFP) yielded extensive expression

in all cell lines, assessed by fluorescence microscopy. *In vitro* studies have demonstrated that tissue specific promoter selection can guide vectors to selectively express genes in target tissues.

Studies performed *in vivo* also show that adenovirus can be fitted with a gene of therapeutic benefit for delivery to tumour tissue. Adenovirus-mediated delivery of tumour-suppressor gene REIC/Dkk-3 significantly inhibited metastasis in an orthotopic mouse CaP model using RM-9 cells (148). Replication-selectivity can be achieved by placing adenoviral E1A under the control of a cancer-specific promoter sequence in effort to express genes solely in tumour tissue. Sarkar *et al.* developed a model of CaP in nude mice by injection of modified DU-145 cells. Mice were then injected intratumourally with adenovirus carrying tumour suppressor gene melanoma differentiation associated gene -7 (also known as interleukin-24), in which E1A was under control of cancer-specific promoter progression elevated gene-3 (Ad.PEG-E1A-mda-7/IL-24). This virus is targeted to replicate and express the tumour suppressor genes only in cancerous tissues. Accordingly, local and distant tumours were completely eradicated by Ad.PEG-E1A-mda-7/IL-24, but not by control viruses (149).

In another mouse model of CaP, adenovirus-mediated gene therapy combined with EBRT improved tumour treatment endpoints over EBRT alone. Animal models of CaP were generated by inoculation of male mice with a subline of PSA-positive LNCaP cells. Freytag *et al.* generated a replication-competent Ad5-CD/TK for intratumoural injection. Gene-directed enzyme prodrug therapy was carried out by daily injection of 5-fluorocytosine and ganciclovir, which are

converted to toxic metabolites in infected tissues by the viral cytosine deaminase and thymidine kinase gene products respectively. Tumour volume and PSA were significantly decreased when treating mice with adenovirus and prodrugs in addition to EBRT (150).

1.8.4 PROSTATE CANCER GENE THERAPY: HUMAN STUDIES

Freytag *et al.* used their replication-competent Ad5-CD/TK virus in a human clinical trial where 16 patients with locally recurrent CaP were recruited. Patients were intraprostatically injected with Ad5-CD/TK and treated with prodrug for two weeks thereafter. 15 of 16 patients experienced a decline in serum PSA between pre- and post-treatment measures. PSA was measured up to one year after treatment; some patients continued to have declining PSA values while PSA values rose in other patients. This result could be stratified by presence of Ad5-CD/TK DNA in blood; PSA rebounded in patients where viral DNA was undetectable, but PSA stabilized or continued to decline in patients who had still detectable viral DNA (up to 4 months after administration) (151).

Shirakawa *et al.* recruited six CaP patients in a trial to test the effect of replication-deficient Ad5-OC-TK on tumour characteristics. The osteocalcin promoter is specifically activated in osteoblasts and CaP cells at the bone metastasis site (152, 153). Ad5-OC-TK is equipped with the osteocalcin promoter which drives expression of herpes simplex virus thymidine kinase. Ad5-OC-TK was injected into locally recurrent tumour or metastatic bone sites on days 1 and 8, and prodrug valacyclovir was orally administered everyday for 3 weeks. More than half of the patients experienced declines in PSA and all patients experienced

delays in time to PSA progression (154). This is a unique therapeutic strategy that co-targets the tumour cell and bone stroma, and is likely to receive more attention in future trials.

Success has also been achieved with intravenous injection of adenovirus. CG7870 is a replication-selective adenovirus, in which E1A is controlled by prostate-specific rat probasin promoter. Administration of CG7870 caused PSA declines between 25% and 49% in five of eight patients enrolled in a phase I trial for treatment of HR, metastatic CaP (155). Human clinical trials have demonstrated safety and efficacy of adenovirus-mediated gene therapy for CaP patients. Future trials will likely utilize adenovirus equipped with improved transductional and/or transcriptional targeting systems.

1.9 FIGURES AND TABLES

Table 1A Clinical relevance of prostate tumours

Clinical Relevance	Size (cm^3)	Confinement	Gleason
Insignificant	< 0.2	confined	< 7
Minimal	0.2 to 0.5	confined	< 7
Moderate	> 0.5 (or extracapsular)	confined or extracapsular	< 7
Advanced		extracapsular	≥ 7, metastatic, or positive margins

Clinical relevance of prostate tumours is classified by size, gland confinement, and Gleason grade criteria (16).

Table 1B Gleason grade characteristics of prostate biopsy tissue

Grade 1 - single, separate, closely packed acini
Grade 2 - single acini, more loosely arranged, less uniform
Grade 3 - single acini of variable size, and separation, cribriform and papillary patterns
Grade 4 - irregular masses of acini and fused epithelium, can show clear cells
Grade 5 - anaplastic carcinoma

Gleason grade is classified by histological features of prostate tumour tissue (156).

Table 1C Clinical classification of prostate tumours

TX: Primary tumour cannot be assessed
T0: No evidence of primary tumour
T1: Clinically inapparent tumour not palpable or visible by imaging
T1a: Tumour incidental histological finding in 5% or less of tissue resected
T1b: Tumour incidental histological finding in more than 5% of tissue resected
T1c: Tumour found in one or both lobes by needle biopsy, but not palpable or visible by imaging (elevated PSA)
T2: Tumour confined within the prostate
T2a: Tumour involves one half of one lobe or less
T2b: Tumour involves more than half of one lobe, but not both lobes
T2c: Tumour involves both lobes
T3: Tumour extends through the prostatic capsulea
T3a: Extracapsular extension (unilateral or bilateral)
T3b: Tumour invades the seminal vesicle(s)
T4: Tumour is fixed or invades adjacent structures other than the seminal vesicles, bladder neck, external sphincter, rectum, levator muscles and/or pelvic wall

Clinical classification of prostate tumour tissue is classified by palpability, gland confinement, lobe extension, and involvement of adjacent organs (156).

Table 1D Pathological classification of prostate tumours

pT2: Organ confined pT2a: Tumor involves one half of one lobe or less pT2b: Tumor involves more than half of one lobe, but not both lobes pT2c: Tumor involves both lobes
pT3: Extraprostatic extension pT3a: Extraprostatic extension pT3b: Seminal vesicle extension
pT4: Invasion of bladder, rectum

Pathological classification of prostate tumour tissue is classified by gland confinement, lobe extension, and extension to adjacent and distal organs (157).

Table 1E Classification of regional lymph node involvement attributable to CaP

NX: Regional lymph nodes cannot be assessed
N0: No regional lymph node metastasis
N1: Metastasis in regional lymph node or nodes

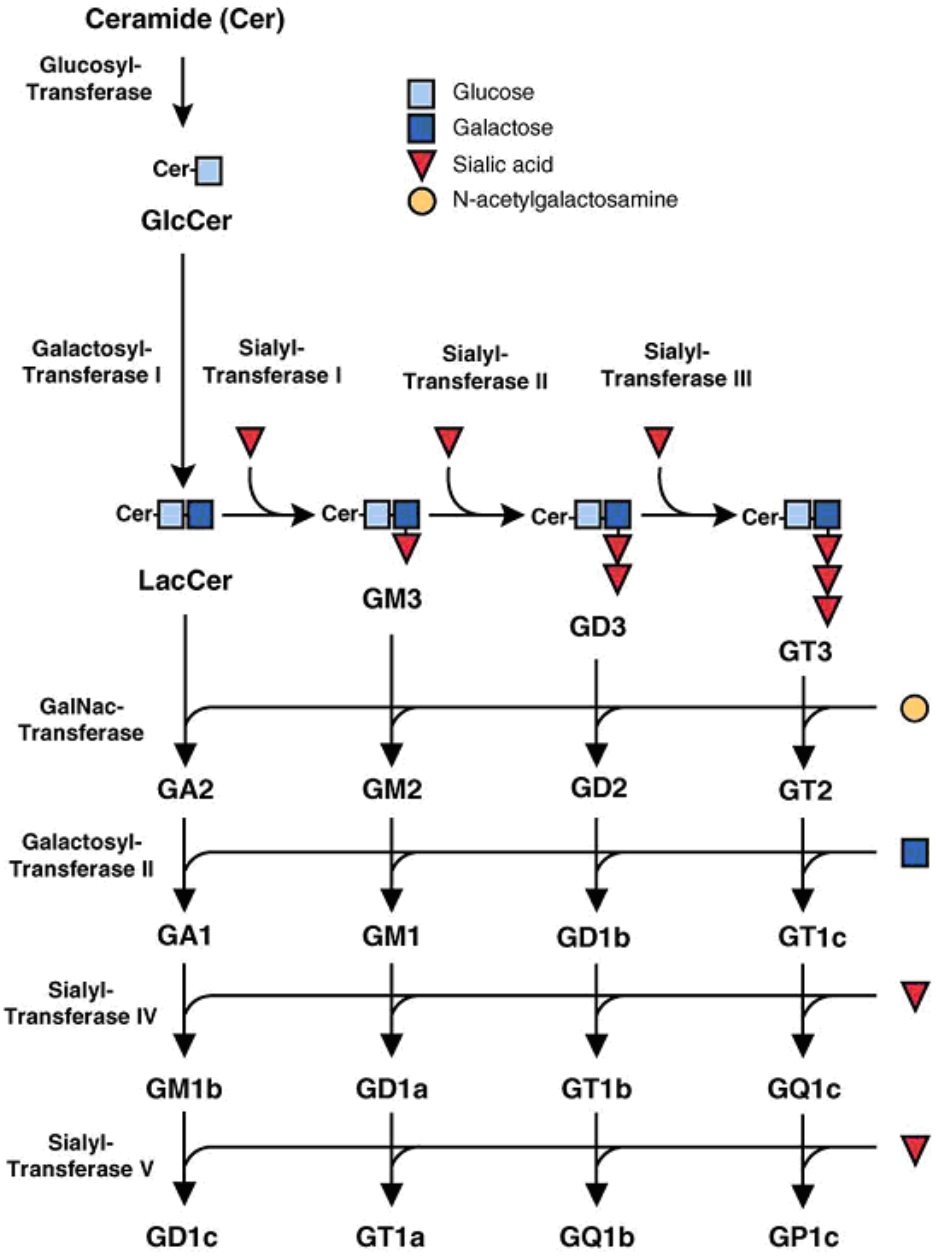
Regional lymph node involvement is classified by absence of presence of metastases in the lymph nodes (158).

Table 1F Classification of distant metastasis attributable to CaP

MX: Distant metastasis cannot be assessed
M0: No distant metastasis
M1: Distant metastasis
M1a: non-regional lymph node(s)
M1b: bone(s)
M1c: other site(s)

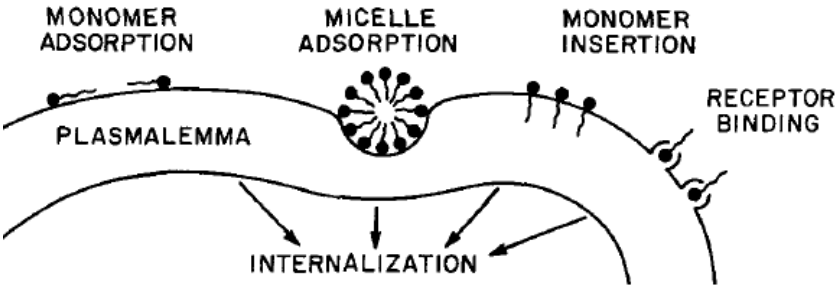
Metastasis of prostate tumour is classified by absence of presence of metastases at specific sites (157).

Figure 1-1 General scheme for ganglioside synthesis



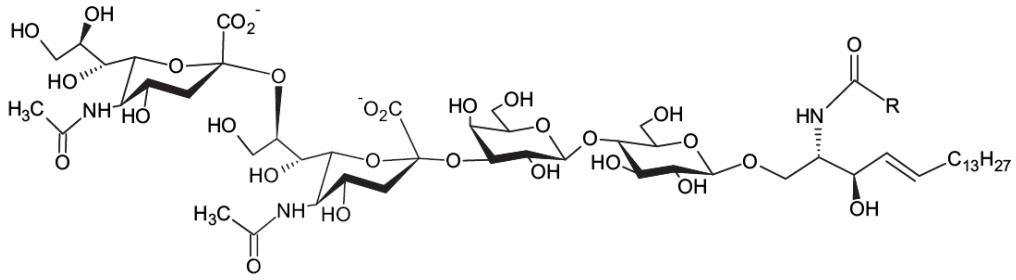
Ganglioside synthesis network (159). GM3 synthase (sialyl-transferase I) catalyzes the addition of sialic acid to lactosylceramide to form GM3. GD3 synthase (sialyl-transferase II) catalyzes the addition of sialic acid to GM3 to form GD3. Lac = lactosyl, Glc = glucosyl, GalNac = N-acetylgalactosamine

Figure 1-2 Modes of ganglioside uptake



Ganglioside is taken up by a variety of mechanisms *in vitro* (160). In aqueous medium, gangliosides may form micelles and be taken up as such. Micelles absorbed to the cell surface are serum-removable. Micelles or monomers attached to receptors at the plasma membrane are serum-resistant and trypsin-removable. Monomers that insert directly into the plasma membrane are trypsin-stable.

Figure 1-3 Structure of GD3



GD3 consists of ceramide, glucose, galactose, and two sialic acid residues (161).

Table 1-2 Summary of literature review papers pertaining to ganglioside and tumour growth and metastasis

Author (reference)	Year	Ganglioside	Model/Tumour	Measure	Effect
Ma R (79)	2004	GD3	SKBR3 breast cancer cells	growth	↓
Alessandri G (44)	1987	mixture	B16 melanoma xenograft	tumour growth	↑
			MN5 fibrosarcoma xenograft	tumour growth	↑
Nojiri H (82)	2002	brefeldin A: increases GM3, GD3 decreases all others	HCT 116 colorectal cancer	tumour growth	↓
Alessandri G (44)	1987	mixture	MCF-7 breast carcinoma	migration	↑
			MN5 fibrosarcoma cells	migration	↑
			A-375 melanoma cells	migration	↑
Koochekpour S (45)	1996	GD3	IPSB-18 human glioma cells	VEGF	↑
Gu Y (46)	2008	GM3 synthase silencing	4T1 breast cancer cells	migration	↓
				invasion	↓
			4T1 breast cancer xenograft	tumour growth	↓
Deng (48)	2000	depletion	MEB4 murine melanoma xenograft	tumour growth	↓
Ravindranath (91)	2008	O-acetyl species	1° vs. metastatic human melanoma	metastatic	↑
Rosenberg (95)	1988	GD3	1° vs. metastatic human melanoma	metastatic	↑
Ravindranath (96)	1991	GM3:GD3	human melanoma tissue	overall survival	α
Zeng (98)	2002	GD3 synthase knockdown	F-11 neuroblastoma xenograft	tumour growth	↓

Iliopoulos (99)	1989	anti-GD3 antibody	human melanoma cell xenograft	tumour growth	↓
Ohkawa (100)	2008	GD3 depletion	SK-MEL human melanoma cells	adhesion	↓
Hyuga (106)	1997	GD1a	FBJ-LL osteosarcoma	migration	↓
Hyuga (107)	1999	GD1a	FBJ-11 osteosarcoma xenograft	tumour growth	↓

1.10 LITERATURE CITED

1. Fitzpatrick JM. Is hormone ablation still the right choice for advanced prostate cancer? BJU Int 2007;100 Suppl 2:36-9.

2. Potvin K, Winquist E. Hormone-refractory prostate cancer: A primer for the primary care physician. Can J Urol 2008;15 Suppl 1:14,20; discussion 20.

3. Machiels JP, Mazzeo F, Clausse M, et al. Prospective randomized study comparing docetaxel, estramustine, and prednisone with docetaxel and prednisone in metastatic hormone-refractory prostate cancer. J Clin Oncol 2008;26(32):5261-8.

4. Okotie OT, Roehl KA, Han M, Loeb S, Gashti SN, Catalona WJ. Characteristics of prostate cancer detected by digital rectal examination only. Urology 2007;70(6):1117-20.

5. Hergan L, Kashefi C, Parsons JK. Local anesthetic reduces pain associated with transrectal ultrasound-guided prostate biopsy: A meta-analysis. Urology 2007;69(3):520-5.

6. Hodge KK, McNeal JE, Terris MK, Stamey TA. Random systematic versus directed ultrasound guided transrectal core biopsies of the prostate. J Urol 1989;142(1):71,4; discussion 74-5.

7. Singh H, Canto EI, Shariat SF, et al. Improved detection of clinically significant, curable prostate cancer with systematic 12-core biopsy. J Urol 2004;171(3):1089-92.

8. Desireddi NV, Roehl KA, Loeb S, et al. Improved stage and grade-specific progression-free survival rates after radical prostatectomy in the PSA era. Urology 2007;70(5):950-5.

9. Loeb S, Gonzalez CM, Roehl KA, et al. Pathological characteristics of prostate cancer detected through prostate specific antigen based screening. J Urol 2006;175(3 Pt 1):902-6.

10. Thompson IM, Ankerst DP, Chi C, et al. Operating characteristics of prostate-specific antigen in men with an initial PSA level of 3.0 ng/ml or lower. JAMA 2005;294(1):66-70.

11. Thompson IM, Pauler DK, Goodman PJ, et al. Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. N Engl J Med 2004;350(22):2239-46.

12. Leman ES, Cannon GW, Trock BJ, et al. EPCA-2: A highly specific serum marker for prostate cancer. Urology 2007;69(4):714-20.

13. de la Taille A. Progensa PCA3 test for prostate cancer detection. Expert Rev Mol Diagn 2007;7(5):491-7.

14. Partin AW, Carter HB, Chan DW, et al. Prostate specific antigen in the staging of localized prostate cancer: Influence of tumor differentiation, tumor volume and benign hyperplasia. J Urol 1990;143(4):747-52.

15. Ruiz-Martin I, Rodriguez-Sanchez CA, Ocana-Fernandez A, del Valle-Zapico J, Soto de Prado-Otero D, Cruz-Hernandez JJ. Metastatic prostate cancer with a normal prostate-specific antigen level. Clin Transl Oncol 2005;7(9):412-3.

16. Epstein JI, Walsh PC, Carmichael M, Brendler CB. Pathologic and clinical findings to predict tumor extent of nonpalpable (stage T1c) prostate cancer. JAMA 1994;271(5):368-74.

17. Radwan MH, Yan Y, Luly JR, et al. Prostate-specific antigen density predicts adverse pathology and increased risk of biochemical failure. Urology 2007;69(6):1121-7.

18. Daskivich TJ, Regan MM, Oh WK. Distinct prognostic role of prostate-specific antigen doubling time and velocity at emergence of androgen independence in patients treated with chemotherapy. Urology 2007;70(3):527-31.

19. Krejcarek SC, Chen MH, Renshaw AA, Loffredo M, Sussman B, D'Amico AV. Prediagnostic prostate-specific antigen velocity and probability of detecting high-grade prostate cancer. Urology 2007;69(3):515-9.

20. Vickers A. In: New prognostic markers: Pitfalls in the pathway from research to clinical practice. February 26, 2009; Orlando, Florida. Columbia, Maryland: ASCO; 2009. p. 12-3.

21. Perner S, Hofer MD, Kim R, et al. Prostate-specific membrane antigen expression as a predictor of prostate cancer progression. Hum Pathol 2007;38(5):696-701.

22. SLAUGHTER DP, SOUTHWICK HW, SMEJKAL W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. Cancer 1953;6(5):963-8.

23. Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH. A genetic explanation of slaughter's concept of field cancerization: Evidence and clinical implications. Cancer Res 2003;63(8):1727-30.

24. Maki J, Robinson K, Reguly B, et al. Mitochondrial genome deletion aids in the identification of false- and true-negative prostate needle core biopsy specimens. Am J Clin Pathol 2008;129(1):57-66.

25. Woolf SH. Defining clinically insignificant prostate cancer. JAMA 1996;276(1):28-9.

26. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450(7173):1235-9.

27. Morgan TM, Lange PH, Vessella RL. Detection and characterization of circulating and disseminated prostate cancer cells. Front Biosci 2007;12:3000-9.

28. Tantivejkul K, Kalikin LM, Pienta KJ. Dynamic process of prostate cancer metastasis to bone. J Cell Biochem 2004;91(4):706-17.

29. Weinberg RA. The biology of cancer. Zayatz E, editor. New York: Garland Science, Taylor & Francis Group, LLC; 2007.

30. Fidler IJ. The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. Nat Rev Cancer 2003;3(6):453-8.

31. Keller ET, Zhang J, Cooper CR, et al. Prostate carcinoma skeletal metastases: Cross-talk between tumor and bone. Cancer Metastasis Rev 2001;20(3-4):333-49.

32. Goel HL, Li J, Kogan S, Languino LR. Integrins in prostate cancer progression. Endocr Relat Cancer 2008;15(3):657-64.

33. Lai TH, Fong YC, Fu WM, Yang RS, Tang CH. Osteoblasts-derived BMP-2 enhances the motility of prostate cancer cells via activation of integrins. Prostate 2008;68(12):1341-53.

34. Huncharek M, Muscat J, Kupelnick B. Dairy products, dietary calcium and vitamin D intake as risk factors for prostate cancer: A meta-analysis of 26,769 cases from 45 observational studies. Nutr Cancer 2008;60(4):421-41.

35. Crowe FL, Key TJ, Appleby PN, et al. Dietary fat intake and risk of prostate cancer in the european prospective investigation into cancer and nutrition. Am J Clin Nutr 2008;87(5):1405-13.

36. Rohrmann S, Platz EA, Kavanaugh CJ, Thuita L, Hoffman SC, Helzlsouer KJ. Meat and dairy consumption and subsequent risk of prostate cancer in a US cohort study. Cancer Causes Control 2007;18(1):41-50.

37. Kurahashi N, Inoue M, Iwasaki M, Sasazuki S, Tsugane AS, Japan Public Health Center-Based Prospective Study Group. Dairy product, saturated fatty acid, and calcium intake and prostate cancer in a prospective cohort of japanese men. Cancer Epidemiol Biomarkers Prev 2008;17(4):930-7.

38. Ahn J, Albanes D, Peters U, et al. Dairy products, calcium intake, and risk of prostate cancer in the prostate, lung, colorectal, and ovarian cancer screening trial. Cancer Epidemiol Biomarkers Prev 2007;16(12):2623-30.

39. Park SY, Murphy SP, Wilkens LR, Stram DO, Henderson BE, Kolonel LN. Calcium, vitamin D, and dairy product intake and prostate cancer risk: The multiethnic cohort study. Am J Epidemiol 2007;166(11):1259-69.

40. Park Y, Mitrou PN, Kipnis V, Hollenbeck A, Schatzkin A, Leitzmann MF. Calcium, dairy foods, and risk of incident and fatal prostate cancer: The NIH-AARP diet and health study. Am J Epidemiol 2007;166(11):1270-9.

41. Tseng M, Breslow RA, Graubard BI, Ziegler RG. Dairy, calcium, and vitamin D intakes and prostate cancer risk in the national health and nutrition examination epidemiologic follow-up study cohort. Am J Clin Nutr 2005;81(5):1147-54.

42. Schuurman AG, van den Brandt PA, Dorant E, Goldbohm RA. Animal products, calcium and protein and prostate cancer risk in the netherlands cohort study. Br J Cancer 1999;80(7):1107-13.

43. Moore K, Ettinger A, Yokoyama M. Variation in ganglioside content of bovine dairy products. J Food Compos Anal 2000;13(5):783-90.

44. Alessandri G, Filippeschi S, Sinibaldi P, et al. Influence of gangliosides on primary and metastatic neoplastic growth in human and murine cells. Cancer Res 1987;47(16):4243-7.

45. Koochekpour S, Merzak A, Pilkington GJ. Vascular endothelial growth factor production is stimulated by gangliosides and TGF-beta isoforms in human glioma cells in vitro. Cancer Lett 1996;102(1-2):209-15.

46. Gu Y, Zhang J, Mi W, et al. Silencing of GM3 synthase suppresses lung metastasis of murine breast cancer cells. Breast Cancer Res 2008;10(1):R1.

47. Ito A, Handa K, Withers DA, Satoh M, Hakomori S. Binding specificity of siglec7 to disialogangliosides of renal cell carcinoma: Possible role of disialogangliosides in tumor progression. FEBS Lett 2001;504(1-2):82-6.

48. Deng W, Li R, Ladisch S. Influence of cellular ganglioside depletion on tumor formation. J Natl Cancer Inst 2000;92(11):912-7.

49. Edidin M. Lipids on the frontier: A century of cell-membrane bilayers. Nat Rev Mol Cell Biol 2003;4(5):414-8.

50. SVENNERHOLM L. The gangliosides. J Lipid Res 1964;5:145-55.

51. Sonnino S, Mauri L, Chigorno V, Prinetti A. Gangliosides as components of lipid membrane domains. Glycobiology 2007;17(1):1R-13R.

52. Vyas KA, Patel HV, Vyas AA, Schnaar RL. Segregation of gangliosides GM1 and GD3 on cell membranes, isolated membrane rafts, and defined supported lipid monolayers. Biol Chem 2001;382(2):241-50.

53. Gomez J, Sagues F, Reigada R. Actively maintained lipid nanodomains in biomembranes. Phys Rev E Stat Nonlin Soft Matter Phys 2008;77(2 Pt 1):021907.

54. von Euler G, Fuxe K, Bondy SC. Ganglioside GM1 prevents and reverses toluene-induced increases in membrane fluidity and calcium levels in rat brain synaptosomes. Brain Res 1990;508(2):210-4.

55. Pang H, Le PU, Nabi IR. Ganglioside GM1 levels are a determinant of the extent of caveolae/raft-dependent endocytosis of cholera toxin to the golgi apparatus. J Cell Sci 2004;117(Pt 8):1421-30.

56. Smith AE, Lilie H, Helenius A. Ganglioside-dependent cell attachment and endocytosis of murine polyomavirus-like particles. FEBS Lett 2003;555(2):199-203.

57. Wang XQ, Yan Q, Sun P, et al. Suppression of epidermal growth factor receptor signaling by protein kinase C-alpha activation requires CD82, caveolin-1, and ganglioside. Cancer Res 2007;67(20):9986-95.

58. Ohkubo S, Nakahata N. Role of lipid rafts in trimeric G protein-mediated signal transduction. Yakugaku Zasshi 2007;127(1):27-40.

59. Sun J, Shaper NL, Itonori S, Heffer-Lauc M, Sheikh KA, Schnaar RL. Myelin-associated glycoprotein (siglec-4) expression is progressively and selectively decreased in the brains of mice lacking complex gangliosides. Glycobiology 2004;14(9):851-7.

60. Nakano J, Yasui H, Lloyd KO, Muto M. Biologic roles of gangliosides G(M3) and G(D3) in the attachment of human melanoma cells to extracellular matrix proteins. J Investig Dermatol Symp Proc 1999;4(2):173-6.

61. Wang B, Brand-Miller J. The role and potential of sialic acid in human nutrition. Eur J Clin Nutr 2003;57(11):1351-69.

62. Muthing J, Maurer U, Weber-Schurholz S. Glycosphingolipids of skeletal muscle: II. modulation of Ca2(+)-flux in triad membranes by gangliosides. Carbohydr Res 1998;307(1-2):147-57.

63. Kolter T, Proia RL, Sandhoff K. Combinatorial ganglioside biosynthesis. J Biol Chem 2002;277(29):25859-62.

64. Birkle S, Zeng G, Gao L, Yu RK, Aubry J. Role of tumor-associated gangliosides in cancer progression. Biochimie 2003;85(3-4):455-63.

65. Prinetti A, Basso L, Appierto V, et al. Altered sphingolipid metabolism in N-(4-hydroxyphenyl)-retinamide-resistant A2780 human ovarian carcinoma cells. J Biol Chem 2003;278(8):5574-83.

66. Muthing J, Maurer U, Neumann U, Kniep B, Weber-Schurholz S. Glycosphingolipids of skeletal muscle: I. subcellular distribution of neutral glycosphingolipids and gangliosides in rabbit skeletal muscle. Carbohydr Res 1998;307(1-2):135-45.

67. Chiantia S, Ries J, Chwastek G, et al. Role of ceramide in membrane protein organization investigated by combined AFM and FCS. Biochim Biophys Acta 2008;1778(5):1356-64.

68. Formisano S, Johnson ML, Lee G, Aloj SM, Edelhoch H. Critical micelle concentrations of gangliosides. Biochemistry 1979;18(6):1119-24.

69. Moss J, Fishman PH, Manganiello VC, Vaughan M, Brady RO. Functional incorporation of ganglioside into intact cells: Induction of choleragen responsiveness. Proc Natl Acad Sci U S A 1976;73(4):1034-7.

70. Schnabl KL, Larcelet M, Thomson AB, Clandinin MT. Uptake and fate of ganglioside GD3 in human intestinal CaCo-2 cells. Am J Physiol Gastrointest Liver Physiol 2009.

71. Ravindranath MH, Bauer PM, Cornillez-Ty C, Garcia J, Morton DL. Quantitation of the density of cell surface carbohydrate antigens on cancer cells with a sensitive cell-suspension ELISA. J Immunol Methods 1996;197(1-2):51-67.

72. Park EJ, Suh M, Thomson B, Thomson AB, Ramanujam KS, Clandinin MT. Dietary ganglioside decreases cholesterol content, caveolin expression and inflammatory mediators in rat intestinal microdomains. Glycobiology 2005;15(10):935-42.

73. Park EJ, Suh M, Clandinin MT. Dietary ganglioside and long-chain polyunsaturated fatty acids increase ganglioside GD3 content and alter the phospholipid profile in neonatal rat retina. Invest Ophthalmol Vis Sci 2005;46(7):2571-5.

74. Tempera I, Buchetti B, Lococo E, et al. GD3 nuclear localization after apoptosis induction in HUT-78 cells. Biochem Biophys Res Commun 2008;368(3):495-500.

75. Colell A, Garcia-Ruiz C, Roman J, Ballesta A, Fernandez-Checa JC. Ganglioside GD3 enhances apoptosis by suppressing the nuclear factor-kappa B-dependent survival pathway. FASEB J 2001;15(6):1068-70.

76. Malisan F, Testi R. The ganglioside GD3 as the greek goddess hecate: Several faces turned towards as many directions. IUBMB Life 2005;57(7):477-82.

77. Garcia-Ruiz C, Colell A, Morales A, Calvo M, Enrich C, Fernandez-Checa JC. Trafficking of ganglioside GD3 to mitochondria by tumor necrosis factor-alpha. J Biol Chem 2002;277(39):36443-8.

78. Garcia-Ruiz C, Colell A, Paris R, Fernandez-Checa JC. Direct interaction of GD3 ganglioside with mitochondria generates reactive oxygen species followed by mitochondrial permeability transition, cytochrome c release, and caspase activation. FASEB J 2000;14(7):847-58.

79. Ma R, Koulov A, Moulton C, et al. Apoptosis of human breast carcinoma cells in the presence of disialosyl gangliosides: II. treatment of SKBR3 cells with GD3 and GD1b gangliosides. Glycoconj J 2004;20(5):319-30.

80. Young WW,Jr, Allende ML, Jaskiewicz E. Reevaluating the effect of brefeldin A (BFA) on ganglioside synthesis: The location of GM2 synthase cannot be deduced from the inhibition of GM2 synthesis by BFA. Glycobiology 1999;9(7):689-95.

81. Young WW,Jr, Lutz MS, Mills SE, Lechler-Osborn S. Use of brefeldin A to define sites of glycosphingolipid synthesis: GA2/GM2/GD2 synthase is trans to the brefeldin A block. Proc Natl Acad Sci U S A 1990;87(17):6838-42.

82. Nojiri H, Yamana H, Shirouzu G, Suzuki T, Isono H. Glycotherapy for cancer: Remodeling of ganglioside pattern as an effective approach for cancer therapy. Cancer Detect Prev 2002;26(2):114-20.

83. Marquina G, Waki H, Fernandez LE, et al. Gangliosides expressed in human breast cancer. Cancer Res 1996;56(22):5165-71.

84. Pan XL, Izumi T, Yamada H, Akiyoshi K, Suenobu S, Yokoyama S. Ganglioside patterns in neuroepithelial tumors of childhood. Brain Dev 2000;22(3):196-8.

85. Vukelic Z, Kalanj-Bognar S, Froesch M, et al. Human gliosarcoma-associated ganglioside composition is complex and distinctive as evidenced by high-performance mass spectrometric determination and structural characterization. Glycobiology 2007;17(5):504-15.

86. Ravindranath MH, Muthugounder S, Presser N, Ye X, Brosman S, Morton DL. Endogenous immune response to gangliosides in patients with confined prostate cancer. Int J Cancer 2005;116(3):368-77.

87. Ravindranath MH, Muthugounder S, Presser N, et al. Gangliosides of organ-confined versus metastatic androgen-receptor-negative prostate cancer. Biochem Biophys Res Commun 2004;324(1):154-65.

88. Seyfried TN, Yu RK. Ganglioside GD3: Structure, cellular distribution, and possible function. Mol Cell Biochem 1985;68(1):3-10.

89. Furukawa K, Thampoe IJ, Yamaguchi H, Lloyd KO. The addition of exogenous gangliosides to cultured human cells results in the cell type-specific expression of novel surface antigens by a biosynthetic process. J Immunol 1989;142(3):848-54.

90. Scursoni AM, Galluzzo L, Camarero S, et al. Detection and characterization of N-glycolylated gangliosides in wilms tumor by immunohistochemistry. Pediatr Dev Pathol 2009:1.

91. Ravindranath MH, Muthugounder S, Presser N. Ganglioside signatures of primary and nodal metastatic melanoma cell lines from the same patient. Melanoma Res 2008;18(1):47-55.

92. Perez CA, Ravindranath MH, Soh D, Gonzales A, Ye W, Morton DL. Serum anti-ganglioside IgM antibodies in soft tissue sarcoma: Clinical prognostic implications. Cancer J 2002;8(5):384-94.

93. Ravindranath MH, Muthugounder S, Presser N, et al. Immunogenic gangliosides in human ovarian carcinoma. Biochem Biophys Res Commun 2007;353(2):251-8.

94. Chu KU, Ravindranath MH, Gonzales A, et al. Gangliosides as targets for immunotherapy for pancreatic adenocarcinoma. Cancer 2000;88(8):1828-36.

95. Rosenberg JM, Sander DJ, Derango RE, Cheresh DA. Enzymatic basis for increased expression of GD3 on human melanoma cells derived from metastatic lesions. JCLA 1988;2:91-100.

96. Ravindranath MH, Tsuchida T, Morton DL, Irie RF. Ganglioside GM3:GD3 ratio as an index for the management of melanoma. Cancer 1991;67(12):3029-35.

97. Thomas CP, Buronfosse A, Fertil B, Portoukalian J. Surface expression of GD3 disialogangliosides in human melanoma cells is correlated to both metastatic potential in vivo and radiosensitivity in vitro. C R Acad Sci III 1995;318(12):1233-8.

98. Zeng G, Gao L, Suetake K, Joshi RM, Yu RK. Variations in gene expression patterns correlated with phenotype of F-11 tumor cells whose expression of GD3-synthase is suppressed. Cancer Lett 2002;178(1):91-8.

99. Iliopoulos D, Ernst C, Steplewski Z, et al. Inhibition of metastases of a human melanoma xenograft by monoclonal antibody to the GD2/GD3 gangliosides. J Natl Cancer Inst 1989;81(6):440-4.

100. Ohkawa Y, Miyazaki S, Miyata M, Hamamura K, Furukawa K, Furukawa K. Essential roles of integrin-mediated signaling for the enhancement of malignant

properties of melanomas based on the expression of GD3. Biochem Biophys Res Commun 2008;373(1):14-9.

101. Chen HY, Challa AK, Varki A. 9-O-acetylation of exogenously added ganglioside GD3. the GD3 molecule induces its own O-acetylation machinery. J Biol Chem 2006;281(12):7825-33.

102. Ritter G, Boosfeld E, Markstein E, et al. Biochemical and serological characteristics of natural 9-O-acetyl GD3 from human melanoma and bovine buttermilk and chemically O-acetylated GD3. Cancer Res 1990;50(5):1403-10.

103. Kohla G, Stockfleth E, Schauer R. Gangliosides with O-acetylated sialic acids in tumors of neuroectodermal origin. Neurochem Res 2002;27(7-8):583-92.

104. Kniep B, Kniep E, Ozkucur N, et al. 9-O-acetyl GD3 protects tumor cells from apoptosis. Int J Cancer 2006;119(1):67-73.

105. Malisan F, Franchi L, Tomassini B, et al. Acetylation suppresses the proapoptotic activity of GD3 ganglioside. J Exp Med 2002;196(12):1535-41.

106. Hyuga S, Yamagata S, Tai T, Yamagata T. Inhibition of highly metastatic FBJ-LL cell migration by ganglioside GD1a highly expressed in poorly metastatic FBJ-S1 cells. Biochem Biophys Res Commun 1997;231(2):340-3.

107. Hyuga S, Yamagata S, Takatsu Y, et al. Suppression by ganglioside GD1A of migration capability, adhesion to vitronectin and metastatic potential of highly metastatic FBJ-LL cells. Int J Cancer 1999;83(5):685-91.

108. Murayama K, Levery SB, Schirrmacher V, Hakomori S. Qualitative differences in position of sialylation and surface expression of glycolipids between murine lymphomas with low metastatic (eb) and high metastatic (ESb)

potentials and isolation of a novel disialoganglioside (GD1 alpha) from eb cells. Cancer Res 1986;46(3):1395-402.

109. Calorini L, Fallani A, Tombaccini D, et al. Lipid characteristics of RSV-transformed Balb/c 3T3 cell lines with different spontaneous metastatic potentials. Lipids 1989;24(8):685-90.

110. Pandha HS, Stockwin LH, Eaton J, et al. Coxsackie B and adenovirus receptor, integrin and major histocompatibility complex class I expression in human prostate cancer cell lines: Implications for gene therapy strategies. Prostate Cancer Prostatic Dis 2003;6(1):6-11.

111. Triantafilou K, Triantafilou M. Lipid-raft-dependent coxsackievirus B4 internalization and rapid targeting to the golgi. Virology 2004;326(1):6-19.

112. Ashbourne Excoffon KJ, Moninger T, Zabner J. The coxsackie B virus and adenovirus receptor resides in a distinct membrane microdomain. J Virol 2003;77(4):2559-67.

113. Rauen KA, Sudilovsky D, Le JL, et al. Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: Potential relevance to gene therapy. Cancer Res 2002;62(13):3812-8.

114. Novak K. Metastasis: CAR problems. Nature Reviews Cancer 2002;2:555.

115. Yamashita M, Ino A, Kawabata K, Sakurai F, Mizuguchi H. Expression of coxsackie and adenovirus receptor reduces the lung metastatic potential of murine tumor cells. Int J Cancer 2007;121(8):1690-6.

116. Kim M, Sumerel LA, Belousova N, et al. The coxsackievirus and adenovirus receptor acts as a tumour suppressor in malignant glioma cells. Br J Cancer 2003;88(9):1411-6.

117. Okegawa T, Li Y, Pong RC, Bergelson JM, Zhou J, Hsieh JT. The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. Cancer Res 2000;60(18):5031-6.

118. Bruning A, Stickeler E, Diederich D, et al. Coxsackie and adenovirus receptor promotes adenocarcinoma cell survival and is expressionally activated after transition from preneoplastic precursor lesions to invasive adenocarcinomas. Clin Cancer Res 2005;11(12):4316-20.

119. Hemminki A, Kanerva A, Liu B, et al. Modulation of coxsackie-adenovirus receptor expression for increased adenoviral transgene expression. Cancer Res 2003;63(4):847-53.

120. Sun LC, Luo J, Mackey LV, Fuselier JA, Coy DH. A conjugate of camptothecin and a somatostatin analog against prostate cancer cell invasion via a possible signaling pathway involving PI3K/Akt, alphaVbeta3/alphaVbeta5 and MMP-2/-9. Cancer Lett 2007;246(1-2):157-66.

121. Niu JX, Zhang WJ, Ye LY, et al. The role of adhesion molecules, alpha v beta 3, alpha v beta 5 and their ligands in the tumor cell and endothelial cell adhesion. Eur J Cancer Prev 2007;16(6):517-27.

122. McCabe NP, De S, Vasanji A, Brainard J, Byzova TV. Prostate cancer specific integrin alphavbeta3 modulates bone metastatic growth and tissue remodeling. Oncogene 2007;26(42):6238-43.

123. Pidgeon GP, Tang K, Cai YL, Piasentin E, Honn KV. Overexpression of platelet-type 12-lipoxygenase promotes tumor cell survival by enhancing alpha(v)beta(3) and alpha(v)beta(5) integrin expression. Cancer Res 2003;63(14):4258-67.

124. Bill-Axelson A, Holmberg L, Ruutu M, et al. Radical prostatectomy versus watchful waiting in early prostate cancer. N Engl J Med 2005;352(19):1977-84.

125. Robinson D, Aus G, Bak J, et al. Long-term follow-up of conservatively managed incidental carcinoma of the prostate: A multivariate analysis of prognostic factors. Scand J Urol Nephrol 2007;41(2):103-9.

126. Scholz M, Lam R, Strum S, Jennrich R, Johnson H, Trilling T. Prostate-cancer-specific survival and clinical progression-free survival in men with prostate cancer treated intermittently with testosterone-inactivating pharmaceuticals. Urology 2007;70(3):506-10.

127. Shah SK, Trump DL, Sartor O, Tan W, Wilding GE, Mohler JL. Phase II study of dutasteride for recurrent prostate cancer during androgen deprivation therapy. J Urol 2009;181(2):621-6.

128. Rubin MA, Allory Y, Molinie V, et al. Effects of long-term finasteride treatment on prostate cancer morphology and clinical outcome. Urology 2005;66(5):930-4.

129. Girling JS, Whitaker HC, Mills IG, Neal DE. Pathogenesis of prostate cancer and hormone refractory prostate cancer. Indian J Urol 2007;23(1):35-42.

130. Attard G, Reid AH, Yap TA, et al. Phase I clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms that castration-resistant prostate cancer commonly remains hormone driven. J Clin Oncol 2008;26(28):4563-71.

131. Berthold DR, Sternberg CN, Tannock IF. Management of advanced prostate cancer after first-line chemotherapy. J Clin Oncol 2005;23(32):8247-52.

132. Kuban DA, el-Mahdi AM, Schellhammer P. The significance of post-irradiation prostate biopsy with long-term follow-up. Int J Radiat Oncol Biol Phys 1992;24(3):409-14.

133. Namiki S, Satoh T, Baba S, et al. Quality of life after brachytherapy or radical prostatectomy for localized prostate cancer: A prospective longitudinal study. Urology 2006;68(6):1230-6.

134. Lai CM, Lai YK, Rakoczy PE. Adenovirus and adeno-associated virus vectors. DNA Cell Biol 2002;21(12):895-913.

135. Paccaud JP, Reith W, Johansson B, Magnusson KE, Mach B, Carpentier JL. Clathrin-coated pit-mediated receptor internalization. role of internalization signals and receptor mobility. J Biol Chem 1993;268(31):23191-6.

136. Meier O, Greber UF. Adenovirus endocytosis. J Gene Med 2004;6 Suppl 1:S152-63.

137. Bergelson JM, Krithivas A, Celi L, et al. The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. J Virol 1998;72(1):415-9.

138. Zhang Y, Bergelson JM. Adenovirus receptors. J Virol 2005;79(19):12125-31.

139. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 1993;73(2):309-19.

140. Harel A, Forbes DJ. Welcome to the nucleus: CAN I take your coat? Nat Cell Biol 2001;3(12):E267-9.

141. Tallone T, Malin S, Samuelsson A, et al. A mouse model for adenovirus gene delivery. Proc Natl Acad Sci U S A 2001;98(14):7910-5.

142. Tomko RP, Xu R, Philipson L. HCAR and MCAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc Natl Acad Sci U S A 1997;94(7):3352-6.

143. Bureyko T, Hurdle H, Metcalfe JB, Clandinin MT, Mazurak VC. Reduced growth and integrin expression of prostate cells cultured with lycopene, vitamin E and fish oil in vitro. Br J Nutr 2008:1-8.

144. Schmidt MR, Piekos B, Cabatingan MS, Woodland RT. Expression of a human coxsackie/adenovirus receptor transgene permits adenovirus infection of primary lymphocytes. J Immunol 2000;165(7):4112-9.

145. Guan M, Tripathi V, Zhou X, Popescu NC. Adenovirus-mediated restoration of expression of the tumor suppressor gene DLC1 inhibits the proliferation and tumorigenicity of aggressive, androgen-independent human prostate cancer cell lines: Prospects for gene therapy. Cancer Gene Ther 2008;15(6):371-81.

146. Shi CX, Hitt M, Ng P, Graham FL. Superior tissue-specific expression from tyrosinase and prostate-specific antigen promoters/enhancers in helper-dependent

compared with first-generation adenoviral vectors. Hum Gene Ther 2002;13(2):211-24.

147. Zeng H, Wei Q, Huang R, et al. Recombinant adenovirus mediated prostate-specific enzyme pro-drug gene therapy regulated by prostate-specific membrane antigen (PSMA) enhancer/promoter. J Androl 2007;28(6):827-35.

148. Edamura K, Nasu Y, Takaishi M, et al. Adenovirus-mediated REIC/Dkk-3 gene transfer inhibits tumor growth and metastasis in an orthotopic prostate cancer model. Cancer Gene Ther 2007;14(9):765-72.

149. Sarkar D, Lebedeva IV, Su ZZ, et al. Eradication of therapy-resistant human prostate tumors using a cancer terminator virus. Cancer Res 2007;67(11):5434-42.

150. Freytag SO, Paielli D, Wing M, et al. Efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in combination with radiation therapy in an orthotopic mouse prostate cancer model. Int J Radiat Oncol Biol Phys 2002;54(3):873-85.

151. Freytag SO, Khil M, Stricker H, et al. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. Cancer Res 2002;62(17):4968-76.

152. Desbois C, Hogue DA, Karsenty G. The mouse osteocalcin gene cluster contains three genes with two separate spatial and temporal patterns of expression. J Biol Chem 1994;269(2):1183-90.

153. Matsubara S, Wada Y, Gardner TA, et al. A conditional replication-competent adenoviral vector, ad-OC-E1a, to cotarget prostate cancer and bone

stroma in an experimental model of androgen-independent prostate cancer bone metastasis. Cancer Res 2001;61(16):6012-9.

154. Shirakawa T, Terao S, Hinata N, et al. Long-term outcome of phase I/II clinical trial of ad-OC-TK/VAL gene therapy for hormone-refractory metastatic prostate cancer. Hum Gene Ther 2007;18(12):1225-32.

155. Small EJ, Carducci MA, Burke JM, et al. A phase I trial of intravenous CG7870, a replication-selective, prostate-specific antigen-targeted oncolytic adenovirus, for the treatment of hormone-refractory, metastatic prostate cancer. Mol Ther 2006;14(1):107-17.

156. Montironi R, Mazzucchelli R, Kwast T. Morphological assessment of radical prostatectomy specimens. A protocol with clinical relevance. Virchows Arch 2003;442(3):211-7.

157. Montironi R, van der Kwast T, Boccon-Gibod L, Bono AV, Boccon-Gibod L. Handling and pathology reporting of radical prostatectomy specimens. Eur Urol 2003;44(6):626-36.

158. TNM classification of malignant tumours: International union against cancer. 6th ed. Sobin L and Wittekind C, editors. New York: Wiley-Liss; 2002.

159. Malisan F, Testi R. GD3 ganglioside and apoptosis. Biochim Biophys Acta 2002;1585(2-3):179-87.

160. Saqr HE, Pearl DK, Yates AJ. A review and predictive models of ganglioside uptake by biological membranes. J Neurochem 1993;61(2):395-411.

161. Sorensen LK. A liquid chromatography/tandem mass spectrometric approach for the determination of gangliosides GD3 and GM3 in bovine milk and infant formulae. Rapid Commun Mass Spectrom 2006;20(24):3625-33.

2.0 STUDY RATIONALE

2.1 RATIONALE

CaP is the second leading cause of cancer-related death in men (1). Thus, it is a pressing issue to be able to distinguish and treat biologically-relevant CaP. Biologically-relevant cases are a subset of clinically detected cases; it refers to disease that will inevitably lead to progression and death. A recent meta-analysis assessed 45 observational studies and concluded that there is no association between CaP risk and dairy exposure (2), however; the definition of "dairy" varies considerably among studies and may obscure the true relationship between dairy exposure and CaP risk (Section 1.4). In fact, a few studies published after the meta-analysis found positive correlations between dairy exposure and CaP incidence (3, 4). While the true relationship between dairy exposure and incidence of CaP is still in question, future study should be directed particularly toward determining specific dietary factors associated with biologically-relevant CaP.

Another consideration to make is that a component of dairy might be specifically related to CaP risk, not dairy products as a whole. Gangliosides GD3 and GD1a have been linked to cancer progression in several studies (Sections 1.7.1, 1.7.3). Ganglioside is widespread in dairy and its concentration varies ~100-fold among dairy products (5). Much of the discrepancy between dairy exposure and CaP risk may be attributable to the large variability in ganglioside content of dairy. Therefore, future studies pertaining to diet and CaP incidence may reach more definitive conclusions when food intake data is stratified for

ganglioside concentration. These considerations may ultimately help to clarify the true relationship between incidence of (advanced) CaP and dairy exposure.

The first objective of this study examines the effects of a physiologically-relevant concentration of ganglioside on growth and markers of metastasis in CaP. Results from this study could direct future experimentation that may (i) encourage design of epidemiological studies to account for ganglioside content of foods, as it pertains to CaP incidence; (ii) inform dietary advice that can be given to CaP patients in effort to minimize the chance of developing aggressive disease; and consequently, (iii) help CaP patients to preserve quality of life by reducing the demand for RP.

Approximately 10% of gene therapy clinical trials in the United States have focused on CaP treatment (6). Adenovirus-mediated gene therapy is a promising treatment for CaP due to high levels of the primary (CAR) and secondary ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) adenovirus receptors in prostate tissue (Section 1.8.2). Gene therapy is still an experimental treatment and may require a combination of agents to realize its full safety and efficacy before routine employment. Polyunsaturated fatty acids have been recognized for their capacity to increase efficacy of cancer treatments including radio- and chemotherapy (7, 8). Utility of ganglioside in relation to potentiating efficacy of cancer therapy is a novel pursuit. The second objective of this study explores the potential for ganglioside to improve safety and efficacy of experimental CaP gene therapy.

2.2 HYPOTHESES AND OBJECTIVES

The overall hypothesis of this thesis research is that ganglioside increases metastatic potential of prostate cancer; but also increases susceptibility of prostate cancer to adenovirus-mediated gene therapy. This hypothesis is explored in two studies.

1. Effects of Ganglioside on Growth and Cell Surface Ganglioside Densities in Prostate Cancer *in vitro*

Hypothesis 1: Mixtures of ganglioside (92% GD3) decrease growth and increase metastatic potential of CaP.

Objectives:

Using an *in vitro* model of CaP, to determine whether supplemental ganglioside:

i. decreases cell growth, estimated by trypan blue exclusion using a haemacytometer
ii. increases cell surface GD3 density, assayed by ELISA;
iii. decreases cell surface GD1a density, determined using ELISA.

2. Effects of Ganglioside on Cell Surface Adenovirus Receptor Densities and Adenovirus Transduction in Prostate Cancer *in vitro*

Hypothesis 2: Mixtures of ganglioside (92% GD3) increase cell surface adenovirus receptor density and consequently, susceptibility of CaP to infection by adenovirus.

Objectives:

Using an *in vitro* model of CaP, to determine whether supplemental ganglioside:

i. increases cell surface CAR density, measured by flow cytometry;

ii. increases cell surface $\alpha_v\beta_3$ and $\alpha_v\beta_5$ densities, assayed by flow cytometry;

iii. increases adenovirus entry, using flow cytometry to assess degree of infectivity by GFP-expressing adenovirus.

2.3 CHAPTER LAYOUT

Chapter 3 examines the effects of ganglioside treatment on normal and malignant prostate cell growth, and on specific cell surface ganglioside densities (Hypothesis 1: i, ii, iii). This chapter also elaborates on the differences in cell culture conditions between this research and that of other investigators. A version of this chapter has been published. Miklavcic J, Hitt MM, Mazurak, VC, Clandinin MT 2009. 100th Annual AOCS Annual Meeting & Expo. Abstract no. 29738.

Chapter 4 examines the effects of ganglioside treatment on densities of cell surface adenovirus receptors and entry of adenovirus in normal and malignant prostate cells (Hypothesis 2: i, ii, iii). A version of this chapter has been published. Miklavcic J, Hitt MM, Mazurak, VC, Clandinin MT 2009. ASCO 2009 GUCS. Abstract no. 64.

Chapter 5 is a general summary and discussion regarding the impact of ganglioside on growth, cell surface mediators of metastasis, and susceptibility to infection by adenovirus in normal and malignant prostate epithelial cells.

2.4 LITERATURE CITED

1. Machiels JP, Mazzeo F, Clausse M, et al. Prospective randomized study comparing docetaxel, estramustine, and prednisone with docetaxel and prednisone in metastatic hormone-refractory prostate cancer. J Clin Oncol 2008;26(32):5261-8.

2. Huncharek M, Muscat J, Kupelnick B. Dairy products, dietary calcium and vitamin D intake as risk factors for prostate cancer: A meta-analysis of 26,769 cases from 45 observational studies. Nutr Cancer 2008;60(4):421-41.

3. Rohrmann S, Platz EA, Kavanaugh CJ, Thuita L, Hoffman SC, Helzlsouer KJ. Meat and dairy consumption and subsequent risk of prostate cancer in a US cohort study. Cancer Causes Control 2007;18(1):41-50.

4. Kurahashi N, Inoue M, Iwasaki M, Sasazuki S, Tsugane AS, Japan Public Health Center-Based Prospective Study Group. Dairy product, saturated fatty acid, and calcium intake and prostate cancer in a prospective cohort of japanese men. Cancer Epidemiol Biomarkers Prev 2008;17(4):930-7.

5. Moore K, Ettinger A, Yokoyama M. Variation in ganglioside content of bovine dairy products. J Food Compos Anal 2000;13(5):783-90.

6. Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2007--an update. J Gene Med 2007;9(10):833-42.

7. Conklin KA. Dietary polyunsaturated fatty acids: Impact on cancer chemotherapy and radiation. Altern Med Rev 2002;7(1):4-21.

8. Baracos VE, Mazurak VC, Ma DW. n-3 polyunsaturated fatty acids throughout the cancer trajectory: Influence on disease incidence, progression, response to therapy and cancer-associated cachexia. Nutr Res Rev 2004;17(2):177-92.

3.0 EFFECTS OF GANGLIOSIDE ON GROWTH AND CELL SURFACE GANGLIOSIDE DENSITIES IN PROSTATE CANCER *IN VITRO*

3.1 INTRODUCTION

Several human cell lines have been established representing various stages of CaP. Cultured cells are typically grown in nutrient media supplemented with fetal bovine serum (FBS). The albumin component of serum inhibits ganglioside uptake *in vitro* (1, 2); therefore, serum-free media (SFM) is commonly used when incubating cultures with ganglioside. Interpretations from these studies may be obscured since use of serum-deficient media deviates from optimal culture conditions. Albumin concentration is approximately two times higher in human serum (HS) than FBS (3). We developed a model that employs 1% (v/v) HS as opposed to the typical 10% (v/v) FBS; equating to approximately one-fifth the amount of albumin. This model minimizes the inhibitory effect of albumin and other serum components (4) on ganglioside uptake and minimizes misinterpretation that may result from using serum-deficient media.

Prostate Cell Culture Model

Use of HS in place of FBS bears additional advantages for prostate cell culture. FBS is devoid of many nutrients and growth-regulating hormones found in HS derived from males. HS provides a source of testosterone, which is absent in FBS. Normal and malignant prostate epithelial cell growth is influenced by testosterone in nutrient medium (5, 6). Evidence suggests that HR CaP is not dependent on testosterone for growth, but still responds to presence of androgens

(7). The high sympathetic innervation of prostate and preponderance of α_1 and β_2 adrenergic receptors suggests necessity of testosterone for growth and maturation of the gland (8). The lipid profiles of HS and FBS also differ. Species of fatty acids vary greatly among phospholipid, triacylglycerol, and cholesterol ester fractions between FBS and HS. There is a strong trend indicating higher n-6/n-3 fatty acid ratio and higher quantities of total saturated, monounsaturated, and polyunsaturated fats in HS compared to FBS (9, 10). In order to better represent *in vivo* conditions, HS is arguably a more appropriate serum source for use in culture of human prostate cells *in vitro*.

Ganglioside Uptake, Metabolism, and Effect on Cell Growth

Ganglioside has been investigated for its influence mainly in melanoma and osteosarcoma, but not in CaP. The role of GD3 as both an inducer and mediator of apoptosis has been extensively reviewed (11) (Section 1.5.3). The effect of GD3 on growth of CaP cells has not yet been determined. GD3 uptake has been described in many biological models (Section 1.5.1), but has not been investigated in CaP. This research is also among the first to explore whether CaP cells natively express 9-O-acetyl GD3 and whether treatment with GD3 can induce its appearance (Section 1.5.2). GD1a is synthesized in a parallel ganglioside metabolism pathway, and has been reported to be particularly abundant in DU-145 and PC-3 cells (Section 1.5.3). The effect of GD3 on cell surface GD1a density remains to be established. This research also highlights differences in ganglioside uptake and metabolism between models of healthy and malignant prostate.

3.2 HYPOTHESIS AND OBJECTIVES

It is hypothesized that ganglioside decreases growth and increases metastatic potential of CaP. This hypothesis was explored in metastatic *in vitro* models of CaP. Cell counts were performed to test whether supplemental ganglioside decreases growth of CaP cells. GD3 and GD1a were assayed by ELISA to determine whether treatment increases metastatic potential of CaP cells, as it pertains to binding of a cell at a secondary site.

3.3 MATERIALS AND METHODS

Growth Conditions

RWPE-1, DU-145, and PC-3 cell lines were obtained from the American Type Culture Collection (ATCC). Cell line characteristics are outlined in Table 3-1. Cultures were maintained in Costar 3516 six-well tissue culture treated plates. RWPE-1 cells were cultured in Keratinocyte-SFM (Invitrogen Corp.) containing L-glutamine, supplemented with bovine pituitary extract (193 μL/100 mL) and human recombinant epidermal growth factor (0.591 μL/100 mL). Medium was replaced two-three times/week and cells were subcultivated (1:6) every seven days. DU-145 cells were cultured in Minimum Essential Medium (Sigma) containing Earle's Salts, L-glutamine, non-essential amino acids; and supplemented with $NaHCO_3$ (2.2 g/L), and sodium pyruvate (1.0 mM). Medium was replaced three times/week and cells were subcultivated (1:10) every five days. PC-3 cells were cultured in Ham's F-12 Nutrient Mixture (Invitrogen Corp.) containing L-glutamine and supplemented with $NaHCO_3$ (1.18 g/L). Medium was replaced three times/week and cells were subcultivated (1:5) every 7 days. All cell

cultures were supplemented with 1% (v/v) pooled HS and 1% (v/v) antibiotic/antimycotic (Invitrogen Corp.): penicillin (10,000 units/mL), streptomycin (10,000 μg/mL), amphotericin B (25 μg/mL). Cells were grown in standard culture incubation conditions of 37ºC and 5% atmospheric CO_2. To passage cells, monolayers were rinsed once with phosphate-buffered saline (PBS) before being dislodged by cell lifter into fresh medium and pipetted to disperse clumps.

Human Serum

Ethics approval was obtained from the University of Alberta Faculty of Agricultural, Life, and Environmental Sciences Human Research Ethics Board (Biomedical Panel) to draw blood from six healthy males aged 18-35 having no history of cancer, autoimmunity, or other disease. Subjects using steroidal medications, hormone therapies, or having had surgery within 3 months were excluded. Blood (100 mL) was drawn from the subcubital vein into tubes and spun (365 *g* for 30 min at 37ºC) to obtain serum fractions. Serum samples from all subjects were pooled, aliquoted, and frozen. Serum was thawed immediately prior to use.

Ganglioside Extraction

Ganglioside was extracted from zeta dairy lipid powder (Fonterra, Cambridge NZ) by modified Folch method (12, 13). Powder (0.50 g) was added to 30 mL of chloroform/methanol (C/M) (2:1 v/v), vortexed (30 sec), and shaken (>2 hr). 0.025% (w/v) $CaCl_2$/double-distilled (dd) H_2O was added to samples before inversing several times. Samples were spun (1,000 rpm for 10 min at room

temperature) and the upper layer was withdrawn and filtered through a Sep-Pak Classic C18 cartridge. Cartridges were rinsed with 10 mL ddH_2O before eluting ganglioside with 2 mL of methanol, followed by 10 mL of C/M (2:1 v/v). C/M was removed under N_2 gas before ganglioside was redissolved in 500 μL C/M (2:1 v/v) and stored at 4ºC before quantifying. Ganglioside extract is composed of 4% GM3, 92% GD3, and 4% unknown (Table 3-2).

Quantification

Samples were redissolved in C/M (2:1 v/v) after drying under N_2 gas. Aliquots (10 μL) were taken in duplicate and dried under N_2 gas before adding 500 μL ddH_2O and vortexing. Resorcinol-HCl (500 μL) was added to test tubes; then capped, vortexed, and heated (8 min at 160ºC). After cooling to room temperature, 500 μL of butylacetate/butanol (85:15 v/v) was added to test tubes and vortexed. The upper layer was withdrawn and read by a spectrophotometer (8452A, Hewlett Packard) at 580 nm. Ganglioside quantitification was determined by relating absorbance values to an authentic N-acetyl neuraminic acid (Neu5Ac) standard curve. Samples were dried under nitrogen gas and suspended in appropriate cell culture medium to the desired concentration before filter sterilization (0.22 μm).

Cell Growth Assay

Cells were grown to 60% confluence before replacing medium with fresh medium containing 0, 10, 20, or 30 μg/mL of ganglioside. After 48 hr, cells were rinsed once with PBS and harvested with 0.25% trypsin-EDTA for 5-10 min before inactivation with FBS. Cell counts were estimated by trypan blue exclusion

using a haemacytometer. High viability (>95%) was obtained for each experiment. Cell counts in ganglioside-supplemented groups were computed as a percentage relative to cell counts in the non-supplemented group.

Cell Surface Ganglioside Measures

Cells were grown to 60% confluence before adding fresh medium with or without 10 μg/mL of ganglioside. After 24 hr, respective control and treatment media were replaced. After another 24 hr, cells were rinsed once with PBS and harvested by cell lifter since trypsinization affects ganglioside detection (14). Cells (0.2 – 0.5 $x10^6$) were added to individual wells in Costar 3894 96-well tissue culture treated v-bottom plates and incubated with either 1:100 mouse anti-9-O-acetyl GD3 monoclonal antibody (clone 7H2, Abcam Inc.), 1:2000 mouse anti-GD3 monoclonal antibody (clone MB3.6, Millipore Corp.), or 1:2000 mouse anti-GD1a monoclonal antibody (clone GD1a-1, Millipore Corp.) in 180 μL of PBS-4% human serum albumin (HSA) and put on a water bath shaker (60 min at <10ºC). Negative controls were incubated with PBS-4% HSA alone, I_GG_3 or I_GG_1 isotype control mouse antibodies (clones B10, 15H6, SouthernBiotech Corp.). Cells were spun (>1730 *g* for 2 min at 4ºC) and rinsed three times in PBS-4% HSA after primary antibody incubation. Cells were then incubated with 1:5000 peroxidase-conjugated goat anti-mouse antibody (115-036-062, Jackson ImmunoResearch Laboratories Inc.) in PBS-4% HSA (150 μL) and put on a water bath shaker (60 min at <10ºC). Cells were washed three times as before, then transferred to a new plate and incubated with of *o*-phenylenediamine dihydrochloride peroxidise substrate (50 μL) for 40 min in the dark. Cells were

spun (>1730 *g* for 5 min at 4ºC) before supernatant was added to Costar 3598 96-well flat-bottom plates containing of 6 N H_2SO_4 (60 µL) in each well. Finally, dual absorbance was read at 490-650 nm using a microtitre plate reader (SPECTRAmax 190, Molecular Devices).

Statistics

Each experiment ('n') was conducted 4-6 times in samples obtained from consecutive cell passages. Samples were assayed in duplicate or triplicate, in which each observation was obtained from an individual microtiter plate well. A t-test for proportion means was conducted to determine whether ganglioside treatment altered cell growth relative to untreated control. Means for cell surface ganglioside absorbance were computed for treatment and control groups and compared using Student's paired t-test.

3.4 RESULTS

Cell Growth

There was no difference in cell viability between treatment and control groups. Ganglioside did not alter cell growth in RWPE-1 or DU-145 cells at concentrations of 10, 20, or 30 µg/mL. At 30 µg/mL, a 30% reduction ($p<0.01$) in the number of PC-3 cells was observed (Figure 3-1). Results of Figure 3-1 are summarized in Table 3-3.

Cell Surface Ganglioside

Cell surface 9-O-acetyl GD3 was not detected in control or treatment groups in any of the cell lines (Figure 3-2). Cell surface GD3 density increased ($p<0.03$) by 12% in RWPE-1 cells with treatment, but did not significantly

change in DU-145 or PC-3 cells (Figure 3-3). Cell surface GD1a density was reduced by half in DU-145 ($p<0.01$) and PC-3 cells ($p<0.01$) in the treatment groups, but did not change in RWPE-1 cells (Figure 3-4).

3.5 DISCUSSION

The effect of ganglioside was tested on growth of healthy and malignant prostate cells *in vitro*. Growth was expected to decrease as ganglioside has demonstrated an apoptotic effect in numerous cancer cells (15-17). Although no effect was shown at lower doses (10 and 20 μg/mL), mixed ganglioside treatment (92% GD3) decreased the number of viable, adherent PC-3 cells at the highest dose (30 μg/mL) tested. A similar finding was shown in B16/BL6 melanoma and 3LL lung carcinoma cells; low dose (~same concentration used in this study) did not affect (or increased) cell growth, but very high dose (~10 times higher concentration than used in this study) inhibited growth (18). Although the mechanism was not investigated, this effect is believed to have occurred via apoptosis. A peculiar finding in the present research, however, was that GD3 failed to reduce growth of RWPE-1 and DU-145 cells.

GD3 is a proven facilitator of apoptosis, but some tumour types employ methods to subvert agents of cell death. Some cell/tumour types exhibit the capacity to nullify the pro-apoptotic effect of GD3 via 9-O-acetylation (19). RWPE-1 and DU-145 were expected to stain positively for 9-O-acetyl GD3, as growth in these cell lines was unaffected by treatment. Interestingly, cell surface 9-O-acetyl GD3 was not detected in any cell line tested, and treatment was insufficient to induce its appearance. Therefore, conversion of GD3 to 9-O-acetyl

GD3 is not the reason for no growth change in RWPE-1 and DU-145 cells. However, Kniep *et al.* report appearance of 9-O-acetyl GD3 in Jurkat-R cells after 3 weeks of culturing with GD3 (20). In the present experiment, dose or time exposures may have been insufficient to induce acetylation. Other GD3 metabolites (N-glycolyl GD3, de-N-acetyl GD3) have been documented in cancer (21, 22). Conversion to a GD3 metabolite or downstream ganglioside like GD2 or GT3 may explain why GD3 did not decrease growth in RWPE-1 or DU-145 cells, but was not assessed in the present study. Furthermore, 9-O-acetyl GD3 density was assayed only at the cell surface. Intra- or total cellular 9-O-acetyl GD3 may be functionally implicated in resistance to apoptosis. PC-3 cells may be sensitive to GD3-induced apoptosis, and may be unable to counteract (via 9-O-acetylation) the pro-apoptotic influence of GD3. Therefore, GD3 may be exploited as a potential anti-CaP agent at a concentration slightly higher than physiologically-relevant.

Many cells and tissues have been shown to take up ganglioside when provided *in vitro* (12, 14) and *in vivo* (23). Ganglioside uptake in prostate cell lines has not been documented. When provided in culture, ganglioside treatment increased cell surface GD3 density in RWPE-1, but not malignant prostate cells. Elevated GD3 density in RWPE-1 cells is believed to have occurred via GD3 uptake, and not increased ganglioside synthesis. It is unknown whether ganglioside is taken up in a dose-dependent manner in RWPE-1 cells, or whether a higher dose would have resulted in greater GD3 density in malignant cell lines. Changes in cell surface GD3 density were explored, but changes in GD3

composition/structure were not investigated. Treatment may have reasonably induced a change in ganglioside composition, as ganglioside from diet has more variation in structure than endogenous ganglioside (24, 25). This study suggests differential ganglioside uptake between healthy and malignant prostate cells, but further investigation in more models of CaP is required to confirm this suggestion.

Conversion of GD3 to another ganglioside may explain failure to detect increased GD3 in malignant prostate cell lines. As previously indicated, treatment did not induce appearance of 9-O-acetyl GD3. However, conversion of GD3 to GD3 metabolites or downstream gangliosides was not assessed. Gangliosides are known to co-localize with other components of cell membrane (26-28). Thus, ganglioside uptake and organization in the cell membrane may affect display of the ganglioside antibody-recognition site and subsequent detection. For example, trypsinization has been shown to increase display/detection of GD2 (14). This may occur via digestion of proteins than block GD2 exposure at the cell surface. On the other hand, GD3 taken up by malignant cells may be shed before being assayed 48 hr after incubation. Evidence suggests that ganglioside shedding may be implicated in tumour cell growth (29-31). It has been shown that ganglioside uptake can occur in as little as five min (32). Therefore, an acute increase in GD3 in CaP cells may be blunted by shedding of GD3 over 48 hr. Though, some cell lines simply do not noticeably incorporate ganglioside into the cell membrane when provided in culture. This study suggests that RWPE-1, but not malignant

prostate cells experience significantly increased cell surface GD3 density after treatment with a physiologically-relevant concentration of ganglioside.

GD1a is the most abundant cell surface ganglioside in DU-145 and PC-3 cells (33). In the present study, treatment with ganglioside mixture decreased GD1a in malignant prostate, but not in RWPE-1 cells. This is among the first work to explore the effect of simple ganglioside on level/density of a complex ganglioside. Since GD3 is positively (27, 34) and GD1a is negatively related to metastatic potential of tumour cells (35, 36), the net effect of treatment may promote metastasis. The results of this study highlight distinct differences in uptake of ganglioside between healthy and malignant prostate cells, as well as the effect of GD3 on downstream ganglioside GD1a. The findings indicate that a physiologically-relevant concentration of ganglioside may increase the metastatic potential of CaP.

3.6 FIGURES AND TABLES

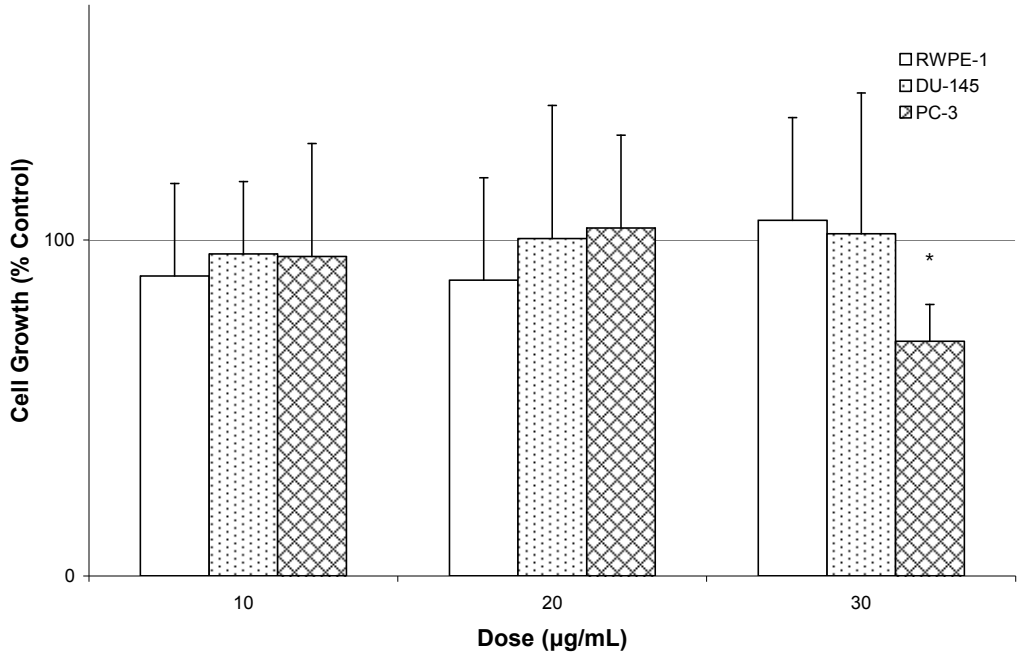
Table 3-1 Cell lines employed in current study

Cell Line	Type	Androgen Responsiveness	PSA	Origin
RWPE-1	Normal	Yes	+	Normal Human Prostate Cells
DU145	Tumour	No	-	Human Brain Metastasis
PC 3	Tumour	No	-	Human Bone Metastasis

Table 3-2 Ganglioside composition of zeta dairy lipid powder

Ganglioside	Relative %
GM3	3.86
GD3	92.2
Unknown	3.92

Figure 3-1 Ganglioside decreases growth of PC-3 cells *in vitro*



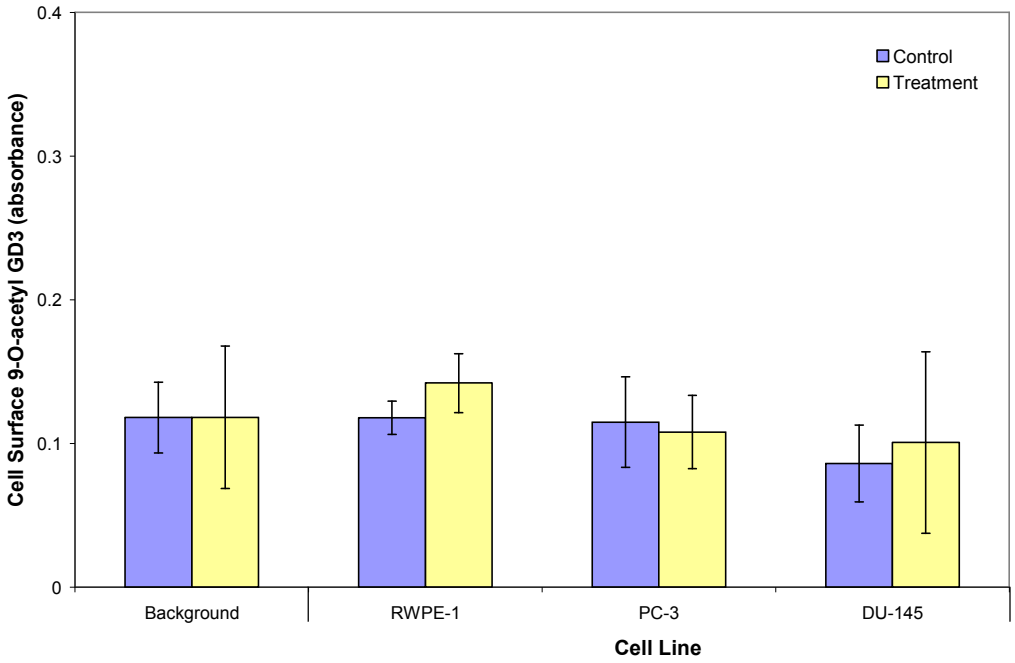
Cell cultures were incubated with ganglioside for 48 hr. Adherent cells were harvested and cell counts were estimated by trypan blue exclusion using a haemacytometer. Treatment group counts were calculated as a percentage of control group counts ($n \geq 5$). Asterisk indicates significant ($p < 0.05$) difference from 100%.

Table 3-3 Summary of cell growth data

Dose (μg/mL)	RWPE-1	p-value	DU-145	p-value	PC-3	p-value
10	89.3	NS	95.9	NS	95.1	NS
20	88.0	NS	100	NS	104	NS
30	106	NS	102	NS	69.8	<0.01

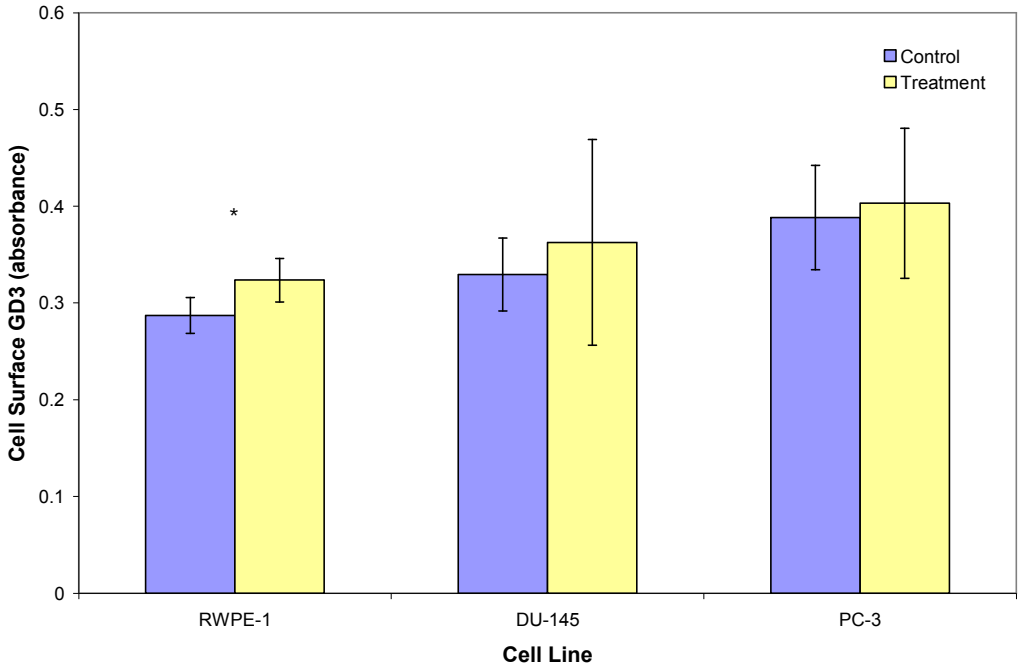
Cell cultures were incubated with ganglioside for 48 hr. Adherent cells were harvested and cell counts were estimated by trypan blue exclusion using a haemacytometer. Values are reported as treatment group counts calculated as percentage of control group counts ($n \geq 5$). p-values denote significant difference from 100%, NS=not significant.

Figure 3-2 9-O-acetyl GD3 is undetectable in prostate cells *in vitro*



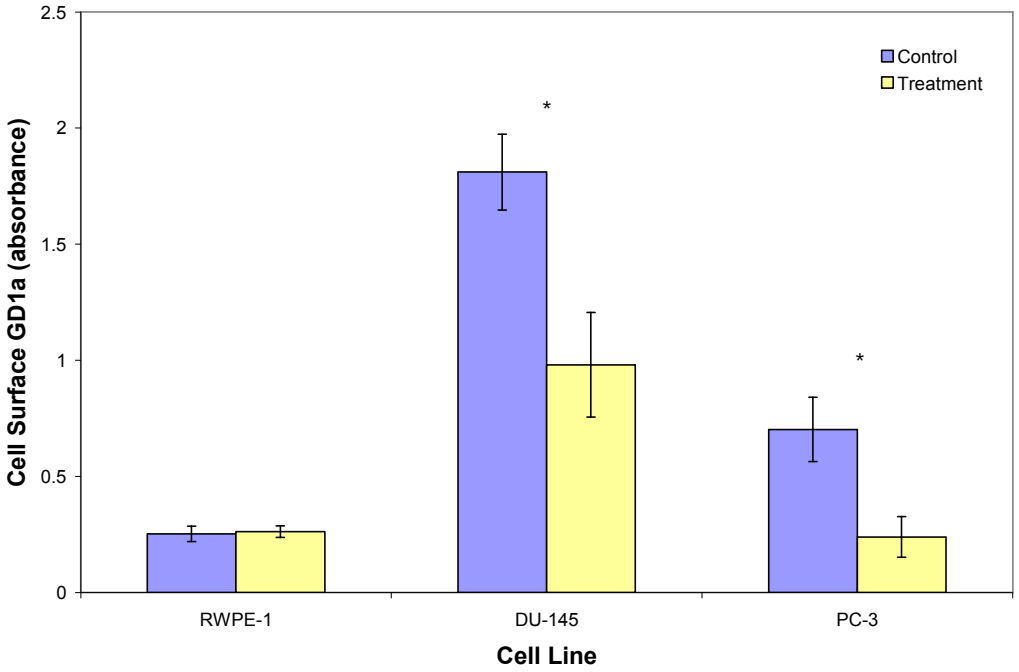
Cell cultures were incubated with 10 μg/mL ganglioside for 48 hr. Cells were harvested and stained for 9-O-acetyl GD3 prior to analysis by csELISA (n≥4).

Figure 3-3 Ganglioside increases cell surface GD3 density in RWPE-1 but not malignant prostate cells *in vitro*



Cell cultures were incubated with 10 μg/mL ganglioside for 48 hr. Cells were harvested and stained for GD3 prior to analysis by csELISA (n=5). Asterisk indicates significant ($p<0.05$) difference between treated and untreated control group.

Figure 3-4 Ganglioside decreases cell surface GD1a density in malignant prostate but not RWPE-1 cells *in vitro*



Cell cultures were incubated with 10 μg/mL ganglioside for 48 hr. Cells were harvested and stained for GD1a prior to analysis by csELISA ($n \geq 4$). Asterisk indicates significant ($p < 0.05$) difference between treated and untreated control group.

3.7 LITERATURE CITED

1. Leskawa KC, Erwin RE, Leon A, Toffano G, Hogan EL. Incorporation of exogenous ganglioside GM1 into neuroblastoma membranes: Inhibition by calcium ion and dependence upon membrane protein. Neurochem Res 1989;14(6):547-54.

2. Tomasi M, Roda LG, Ausiello C, et al. Interaction of GMI ganglioside with bovine serum albumin: Formation and isolation of multiple complexes. Eur J Biochem 1980;111(2):315-24.

3. Tognon G, Frapolli R, Zaffaroni M, et al. Fetal bovine serum, but not human serum, inhibits the in vitro cytotoxicity of ET-743 (yondelis, trabectedin), an example of potential problems for extrapolation of active drug concentrations from in vitro studies. Cancer Chemother Pharmacol 2004;53(1):89-90.

4. Facci L, Leon A, Toffano G, Sonnino S, Ghidoni R, Tettamanti G. Promotion of neuritogenesis in mouse neuroblastoma cells by exogenous gangliosides. relationship between the effect and the cell association of ganglioside GM1. J Neurochem 1984;42(2):299-305.

5. Vihko P, Herrala A, Harkonen P, et al. Control of cell proliferation by steroids: The role of 17HSDs. Mol Cell Endocrinol 2006;248(1-2):141-8.

6. Soronen P, Laiti M, Torn S, et al. Sex steroid hormone metabolism and prostate cancer. J Steroid Biochem Mol Biol 2004;92(4):281-6.

7. Libertini SJ, Tepper CG, Rodriguez V, Asmuth DM, Kung HJ, Mudryj M. Evidence for calpain-mediated androgen receptor cleavage as a mechanism for androgen independence. Cancer Res 2007;67(19):9001-5.

8. McVary KT, McKenna KE, Lee C. Prostate innervation. Prostate Suppl 1998;8:2-13.

9. Pratt VC, Watanabe S, Bruera E, et al. Plasma and neutrophil fatty acid composition in advanced cancer patients and response to fish oil supplementation. Br J Cancer 2002;87(12):1370-8.

10. Hurdle HL. Examination of *in vitro* prostate cancer models supplemented with lycopene, vitamine E and fish oil. 2006.

11. Malisan F, Testi R. GD3 ganglioside and apoptosis. Biochim Biophys Acta 2002;1585(2-3):179-87.

12. Schnabl KL, Larcelet M, Thomson AB, Clandinin MT. Uptake and fate of ganglioside GD3 in human intestinal CaCo-2 cells. Am J Physiol Gastrointest Liver Physiol 2009.

13. FOLCH J, LEES M, SLOANE STANLEY GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226(1):497-509.

14. Ravindranath MH, Bauer PM, Cornillez-Ty C, Garcia J, Morton DL. Quantitation of the density of cell surface carbohydrate antigens on cancer cells with a sensitive cell-suspension ELISA. J Immunol Methods 1996;197(1-2):51-67.

15. Ma R, Koulov A, Moulton C, et al. Apoptosis of human breast carcinoma cells in the presence of disialosyl gangliosides: II. treatment of SKBR3 cells with GD3 and GD1b gangliosides. Glycoconj J 2004;20(5):319-30.

16. Tempera I, Buchetti B, Lococo E, et al. GD3 nuclear localization after apoptosis induction in HUT-78 cells. Biochem Biophys Res Commun 2008;368(3):495-500.

17. Omran OM, Saqr HE, Yates AJ. Molecular mechanisms of GD3-induced apoptosis in U-1242 MG glioma cells. Neurochem Res 2006;31(10):1171-80.

18. Alessandri G, Filippeschi S, Sinibaldi P, et al. Influence of gangliosides on primary and metastatic neoplastic growth in human and murine cells. Cancer Res 1987;47(16):4243-7.

19. Malisan F, Franchi L, Tomassini B, et al. Acetylation suppresses the proapoptotic activity of GD3 ganglioside. J Exp Med 2002;196(12):1535-41.

20. Kniep B, Kniep E, Ozkucur N, et al. 9-O-acetyl GD3 protects tumor cells from apoptosis. Int J Cancer 2006;119(1):67-73.

21. Furukawa K, Thampoe IJ, Yamaguchi H, Lloyd KO. The addition of exogenous gangliosides to cultured human cells results in the cell type-specific expression of novel surface antigens by a biosynthetic process. J Immunol 1989;142(3):848-54.

22. Popa I, Pons A, Mariller C, et al. Purification and structural characterization of de-N-acetylated form of GD3 ganglioside present in human melanoma tumors. Glycobiology 2007;17(4):367-73.

23. Park EJ, Suh M, Clandinin MT. Dietary ganglioside and long-chain polyunsaturated fatty acids increase ganglioside GD3 content and alter the phospholipid profile in neonatal rat retina. Invest Ophthalmol Vis Sci 2005;46(7):2571-5.

24. Prinetti A, Basso L, Appierto V, et al. Altered sphingolipid metabolism in N-(4-hydroxyphenyl)-retinamide-resistant A2780 human ovarian carcinoma cells. J Biol Chem 2003;278(8):5574-83.

25. Sorensen LK. A liquid chromatography/tandem mass spectrometric approach for the determination of gangliosides GD3 and GM3 in bovine milk and infant formulae. Rapid Commun Mass Spectrom 2006;20(24):3625-33.

26. Cheresh DA, Pytela R, Pierschbacher MD, Klier FG, Ruoslahti E, Reisfeld RA. An arg-gly-asp-directed receptor on the surface of human melanoma cells exists in an divalent cation-dependent functional complex with the disialoganglioside GD2. J Cell Biol 1987;105(3):1163-73.

27. Ohkawa Y, Miyazaki S, Miyata M, Hamamura K, Furukawa K, Furukawa K. Essential roles of integrin-mediated signaling for the enhancement of malignant properties of melanomas based on the expression of GD3. Biochem Biophys Res Commun 2008;373(1):14-9.

28. Sun J, Shaper NL, Itonori S, Heffer-Lauc M, Sheikh KA, Schnaar RL. Myelin-associated glycoprotein (siglec-4) expression is progressively and selectively decreased in the brains of mice lacking complex gangliosides. Glycobiology 2004;14(9):851-7.

29. Ladisch S, Kitada S, Hays EF. Gangliosides shed by tumor cells enhance tumor formation in mice. J Clin Invest 1987;79(6):1879-82.

30. Valentino LA, Ladisch S. Localization of shed human tumor gangliosides: Association with serum lipoproteins. Cancer Res 1992;52(4):810-4.

31. Li RX, Ladisch S. Shedding of human neuroblastoma gangliosides. Biochim Biophys Acta 1991;1083(1):57-64.

32. Radsak K, Schwarzmann G, Wiegandt H. Studies on the cell association of exogenously added sialo-glycolipids. Hoppe Seylers Z Physiol Chem 1982;363(3):263-72.

33. Ravindranath MH, Muthugounder S, Presser N, Ye X, Brosman S, Morton DL. Endogenous immune response to gangliosides in patients with confined prostate cancer. Int J Cancer 2005;116(3):368-77.

34. Ravindranath MH, Tsuchida T, Morton DL, Irie RF. Ganglioside GM3:GD3 ratio as an index for the management of melanoma. Cancer 1991;67(12):3029-35.

35. Hyuga S, Yamagata S, Tai T, Yamagata T. Inhibition of highly metastatic FBJ-LL cell migration by ganglioside GD1a highly expressed in poorly metastatic FBJ-S1 cells. Biochem Biophys Res Commun 1997;231(2):340-3.

36. Hyuga S, Yamagata S, Takatsu Y, et al. Suppression by ganglioside GD1A of migration capability, adhesion to vitronectin and metastatic potential of highly metastatic FBJ-LL cells. Int J Cancer 1999;83(5):685-91.

4.0 EFFECTS OF GANGLIOSIDE ON CELL SURFACE ADENOVIRUS RECEPTOR DENSITIES AND ADENOVIRUS TRANSDUCTION IN PROSTATE CANCER *IN VITRO*

4.1 INTRODUCTION

CAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ have dual implication in CaP. They are functional as modulators of experimental gene therapy and may mediate steps in the cascade of metastasis. There is contradictory evidence pertaining to the role of CAR in facilitating metastasis. It is believed to act as a suppressor of metastasis, but is also implicated in cancer cell survival (Section 1.5.4). Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are believed to facilitate adhesion of prostate cells to ECM (1), thereby promoting colonization at a site of metastasis (Section 1.5.5). Integrin-mediated adhesion may be indirectly influenced by ganglioside. In the cell membrane, gangliosides are known to co-localize with proteins which bear specific amino acid sequences (2). GD3 has been shown to cluster with β_1 integrin and affect malignant properties controlled by integrin-mediated signalling (3). Thus, the influence of ganglioside on density of CAR, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ in CaP cells is of interest.

Implications for Gene Therapy

Adenovirus serves as a vector that can be utilized for gene therapy (Section 1.8.1, 1.8.2). CAR is the primary adenovirus receptor and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ function as secondary adenovirus receptors (Section 1.6.1). Adenovirus receptor density predicts susceptibility of tissue to infection by adenovirus. Metastatic prostate tumours are particularly CAR-dense (4) and adenovirus co-

receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are higher in cancerous prostate than in healthy prostate cells (5). Therefore, adenovirus-mediated gene therapy is a promising candidate for treatment of advanced CaP. While still in experimental stages, compounds able to favourably modulate adenovirus receptor density may help transition gene therapy from principle to practice.

4.2 HYPOTHESIS AND OBJECTIVES

It is hypothesized that ganglioside increases susceptibility of CaP to infection by adenovirus, via mediation of cell surface adenovirus receptor density. This hypothesis was explored in an *in vitro* model of CaP. Flow cytometry was performed to test whether supplemental ganglioside increased adenovirus receptor density and entry of adenovirus into CaP cells.

4.3 MATERIALS AND METHODS

Cell Surface Protein Measures

Cell growth conditions were identical to those presented in Section 3.2. Cells were grown to 60% confluence before adding fresh medium with or without 10 μg/mL mixed ganglioside. After 24 hr, respective control and treatment media were replaced. After another 24 hr, cells were rinsed once with PBS and harvested by cell lifter. Cells ($1.0 – 1.1 \times 10^6$) were added to individual wells in Costar 3894 96-well tissue culture treated v-bottom plates and incubated with either 1:100 mouse anti-CAR monoclonal antibody (clone RmcB, Millipore Corp.), 1:100 mouse anti-$\alpha_v\beta_3$ monoclonal antibody (clone 23C6, R&D Systems Inc.), or 1:100 mouse anti-$\alpha_v\beta_5$ monoclonal antibody (clone P5H9, R&D Systems Inc.) in 100 μL of PBS-4% HSA (30 min at 4ºC). Negative controls were incubated with PBS-4%

HSA alone, or isotype control I_GG_1 mouse antibody (clone 15H6, SouthernBiotech Corp.). Cells were spun (430 *g* for 2 min at 4ºC) and washed three times in PBS-4% HSA after primary antibody incubation. Cells were then incubated with 1:100 fluorescein-conjugated goat anti-mouse polyclonal antibody (12-506, Millipore Corp.) in 100 μL PBS-4% HSA (30 min at 4ºC). Cells were washed three times as before, then transferred to Falcon tubes in 1% (w/v) paraformaldehyde-PBS (300 μL) and stored in the dark overnight (4ºC). Mean fluorescence intensities (MFI) of samples were obtained on Becton Dickinson FACScan at 492 nm excitation and 528 nm emission wavelengths. 10,000 events were analyzed and gated to exclude dead and clumped cells.

Adenovirus Vector Propagation

HEK293 and A549 cells were obtained from ATCC. AdBM116GFP (a generous gift from Dr. Frank Graham, McMaster University) was derived from plasmid pBM116GFP. It is a replication-deficient virus in which the CMV promoter controls GFP expression. Stock virus (0.5 mL) was diluted in PBS++ (3.5 mL). Infections were carried out each time by 1 mL of dilution laid over 150 mm plates of HEK293 cells at 90% confluence. Plates were incubated for 30 min (37ºC in 5% CO_2). After incubation, MEMF11-5% horse serum was added to dishes. Upon completion of cytopathic effect (CPE) at 48 hr, cells were scraped into spent medium, pooled, and spun (1000 *g* for 15 min). Supernatant was removed and the cell pellet was resuspended in 4 mL PBS++-10% glycerol (v/v). Crude virus stock was frozen (-78ºC) and thawed three times. Crude stock (2 mL) was diluted in PBS++ (38 mL) and infections were carried out as before. After

incubation, MEMF11-5% horse serum was added to dishes. CPE was complete after 48 hr and cells were scraped into spent medium, combined, and centrifuged (1730 *g* for 20 min). Supernatants were removed and the cell pellets were resuspended in a total of 15 mL 0.1M Tris-HCl (pH 8.0). Crude lysate was frozen (-78ºC).

Adenovirus Purification

Crude lysate was frozen (-78ºC) and thawed three times. 5% sodium deoxycholate (1.5 mL), 2M $MgCl_2$ (150 µL), and DNase I (75 uL) were added to each 15 mL of lysate. The vial was incubated (30 min at 37ºC) with mixing every 10 min. The sample was clarified (3000 *g* for 15 min at 5ºC) and overlaid on CsCl gradient (1.5d/1.35d/1.25d, 0.5mL/3.0mL/3.0mL) in SW41TI ultraclear centrifuge tubes. Tubes were topped with 0.1M Tris-HCl (pH 8.0) to within 2 mm of tube top. Tubes were spun (35,000 rpm for one hr at 10ºC) and the viral band was collected with needle (18 gauge) and syringe (3 mL) at the 1.25d/1.35d CsCl interface. The collected virus was added to SW60TI ultraclear centrifuge tube, parafilmed, and inverted 3 times. The tube was spun (35,000 rpm for 16 hr at 4ºC) and the viral band was collected from the middle of the tube (0.5 mL). The virus was injected with a needle (18 gauge) into a Slide-A-Lyzer cassette (10,000 mwco), pre-wet in 10 mM Tris-HCl (pH 8.0). Residual air was withdrawn from the cassette before the being dialyzed in 500 mL of 10 mM Tris-HCl (pH 8.0) at 4ºC. Dialysis buffer was replaced twice within 48 hr. After 48 hr, virus was withdrawn from the cassette with a fine needle. Sterile glycerol was added to a final volume of 10% and the virus was aliquoted (100 µL) and frozen (-78ºC).

Adenovirus Plaque Assay

Stock virus at dilutions between 10^{-8} and 10^{-11} (0.2 mL) were laid over 60 mm plates of confluent HEK293 cells, in duplicate. Plates were incubated (40 min at 37ºC in 5% CO_2) and rocked once after 15 min. After incubation, plates were overlaid with 10 mL MEMF11 (with 10% horse serum)-agarose (44ºC) and incubated for 11 days before plaques were counted.

Test for Adenovirus Contamination

Three 150 mm dishes of A549 cells were grown to confluence in MEMα-10% FBS and infected with 10^6, 10^7, or 10^8 plaque forming units (pfu) of AdBM116GFP. MEMα-10% horse serum was added, and dishes were incubated (37ºC in 5% CO_2). After 7 days, cells were harvested by scraping into spent medium and glycerol was added to 10% of the final volume. Cells were frozen (-78ºC) and thawed three times. 1.0 mL lysates were used to infect plates of confluent A549 cells, as before. Media was replaced after 5, 10, and 15 days of infection. On day 21, infected dishes were compared to control (uninfected) dishes for signs of CPE.

Cell Infectivity Assay

Cells were grown to 60% confluence in 24-well plates before adding fresh medium with or without 10 ug/mL mixed ganglioside. After 24 hr, respective control and treatment media were replaced. After another 24 hr, a control well was counted and cells were rinsed twice with PBS and overlaid with 0, 10, 20, 50, or 100 pfu/cell AdBM116GFP in PBS++ (50 µL). Dishes were incubated (37ºC in 5% CO_2) with gentle rocking every 10 min. After 30 min, fresh media, without

ganglioside, was added to wells and plates were returned to the incubator. After 24 hr, media was removed and cells were rinsed twice with PBS. Cells were harvested by 0.25% trypsin-EDTA before inactivation with FBS. Samples were added to Falcon tubes and spun (430 *g* for 5 min at 4ºC). Supernatant was removed and samples were suspended in 1% (w/v) paraformaldehyde-PBS (400 μL) and stored in dark overnight (4ºC). MFI of samples were obtained on Becton Dickinson G5 FACSort at 492 nm excitation and 528 nm emission wavelengths. Uninfected cells served as background controls for infected samples. 10,000 events were analyzed and gated to exclude dead and clumped cells.

Statistics

Each experiment ('n') was conducted 3-5 times in samples obtained from consecutive cell passages. Samples were assayed in duplicate or triplicate, in which each observation was obtained from an individual microtiter plate well. Means for MFI were computed for treatment and control groups and compared using Student's paired t-test.

4.4 RESULTS

Cell Surface Adenovirus Receptors

Ganglioside treatment significantly reduced ($p<0.02$) CAR density by 8% in DU-145 cells, but had no effect on CAR density in RWPE-1 cells (Figure 4-1). Treatment increased ($p<0.01$) CAR density by 18% in PC-3 cells (Figure 4-1). A 27% increase in cell surface $\alpha_v\beta_3$ density was observed in the DU-145 treatment group ($p<0.04$); no differences were observed in other cell lines (Figure 4-2). Although treatment did not alter cell surface $\alpha_v\beta_5$ density in RWPE-1 cells, 30%

($p<0.01$) and 53% ($p<0.01$) increases were detected in DU-145 and PC-3 cells respectively (Figure 4-3).

Adenovirus Infection

Ganglioside treatment of RWPE-1 cells did not affect GFP expression following AdBM116GFP infection at the three lowest multiplicities of infection (MOI). At 100 pfu/cell, GFP detection in RWPE-1 treatment group was 20% ($p<0.01$) lower than in control group (Figure 4-4). Ganglioside treatment resulted in decreased GFP expression in DU-145 cells at each MOI tested. GFP detection was 15% ($p<0.01$), 13% ($p<0.02$), 21% ($p<0.01$), and 21% ($p<0.06$) lower at 10, 20, 50, and 100 pfu/cell respectively (Figure 4-5). Ganglioside treatment resulted in increased adenoviral GFP expression in PC-3 cells at each MOI tested. GFP detection was 27% ($p<0.02$), 47% ($p<0.01$), 24% ($p>0.02$), and 9% ($p<0.22$) higher at 10, 20, 50, and 100 pfu/cell respectively (Figure 4-6). Adenovirus transduction data (Figures 4-4, 4-5, 4-6) are summarized in Table 4-1.

4.5 DISCUSSION

Cell surface CAR density was analyzed after culturing prostate cells with a physiologically-relevant concentration of mixed ganglioside. CAR density was unchanged in RWPE-1, slightly decreased in DU-145, and marginally increased in PC-3 cells with treatment. This effect is not likely mediated by transcription due to contrasting results among cell lines. Cells that are CAR-dense/rich show resistance to apoptosis by ultraviolet radiation and serum starving (6). That is, CAR-negative cells are more susceptible to death/apoptosis. Ganglioside treatment also decreased PC-3 cell growth (Section 3.4), possibly via apoptosis.

Thus, ganglioside treatment may select for the CAR-rich population of PC-3 cells and kill those with low CAR density. Ganglioside may modify metastatic tumours to make them more susceptible to adenovirus infection by eliminating cells with little or no CAR, which has positive implications for adenovirus-mediated CaP gene therapy.

Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ function as secondary adenovirus receptors (Section 1.8.2). Cell surface $\alpha_v\beta_3$ and $\alpha_v\beta_5$ densities were analyzed after culturing prostate cells with a physiologically-relevant concentration of mixed ganglioside. Treatment increased integrin densities in malignant, but not RWPE-1 cells. This finding extends the suggestion that ganglioside uptake/metabolism differs between healthy and malignant prostate (Section 3.5). Ganglioside may also exert effects on targets in malignant, but not healthy prostate. Integrin densities poorly predicted susceptibility to infection by adenovirus in this and in other studies (7). It is not known however, whether CAR functions additively or synergistically with integrins to control adenovirus entry. On the other hand, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are strongly implicated in tumour cell survival and metastatic potential of CaP. When incubated with antibodies that block $\alpha_v\beta_3$ or $\alpha_v\beta_5$, PC-3 cells undergo greater apoptosis (8). Mice injected intratibially with $\alpha_v\beta_3$–null or $\alpha_v\beta_3$-mutant PC-3 cells form fewer metastases than those injected with PC-3 cells expressing wildtype $\alpha_v\beta_3$ (9). Thus, these findings suggest that a physiologically-relevant concentration of ganglioside may promote CaP metastasis.

The potential for treatment to increase susceptibility of CaP to adenovirus-mediated gene therapy was explored. Prostate cells were pre-treated with a

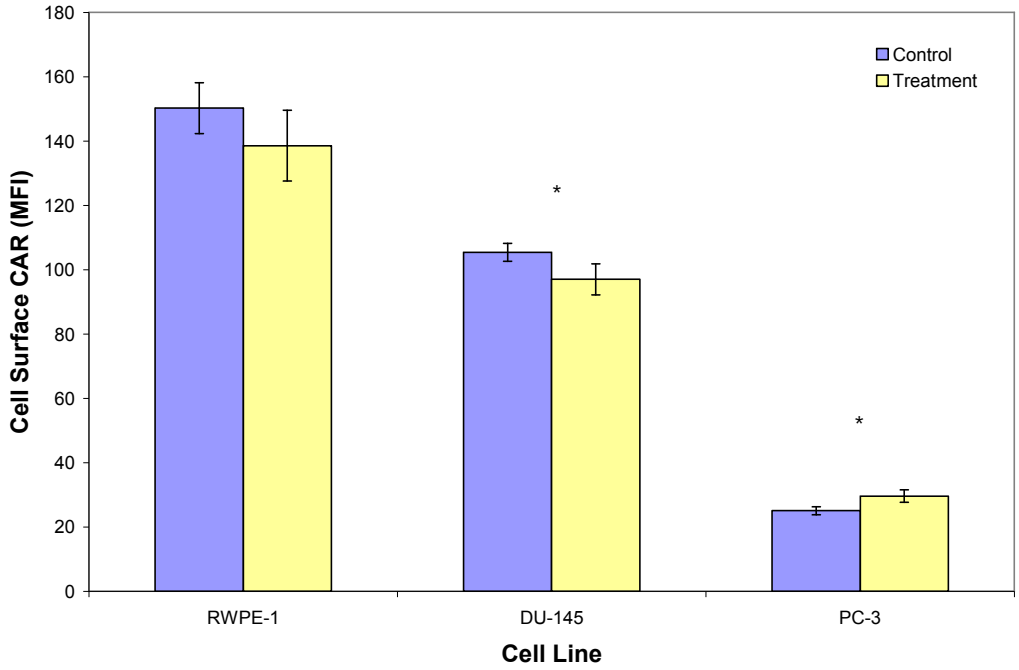
ganglioside mixture prior to infection by adenovirus. GFP encoded by adenovirus and controlled by the CMV promoter was assayed to assess viral transduction. Adenovirus delivery did not increase in RWPE-1 cells, and even decreased at the highest MOI after pre-treatment with ganglioside; which corresponded to the non-significant decrease in CAR density after treatment. Some gene therapy strategies hinge on preferential delivery of genetic material to diseased rather than healthy tissue; therefore, ganglioside may be used as a treatment to achieve this function. While the issue of safety was addressed in RWPE-1 cells, adenovirus particles can be toxic to the liver *in vivo*. This experiment could be repeated in a model of human liver cells to determine whether ganglioside affects CAR density and subsequent toxicity potential. Entry significantly decreased in DU-145 cells at each MOI after pre-treatment; again, correlating to the significant decrease in CAR density after treatment. Finally, ganglioside pre-treatment resulted in significantly increased adenovirus entry into PC-3 cells. The results entail a positive correlation between CAR density and adenovirus entry in each cell line tested. The finding in PC-3 cells lends support to the utility of ganglioside in improving transduction of adenovirus into malignant prostate for gene therapy.

Detection of adenovirus entry by assaying GFP (expressed by adenovirus) presents with complications. Another assay for viral DNA copy number post-infection can more directly inform the degree of adenovirus transduction. The effect of ganglioside treatment on the CMV promoter has not been established. If an interaction/influence between the promoter and the treatment exists, the interpretations drawn from this study may be obscured. However, this effect is

unlikely as infective potential can be explained by the strong correlation between CAR density and GFP expression within cell lines. A treatment-promoter complex interaction is also unlikely since ganglioside had opposing effects on GFP detection/adenovirus entry in DU-145 and PC-3 cells. It stands to reason that if an interaction exists, ganglioside pre-treatment would have a uniform effect on GFP expression in each cell line after infection. The potential relationship could be definitively excluded by exposing cells transfected with the plasmid from which the experimental virus was derived (pBM116GFP) to ganglioside and assaying differences in reporter protein between supplemented and unsupplemented groups. Treatment increased adenovirus entry into PC-3, but not RWPE-1 cells; therefore, ganglioside supplementation may constitute a viable adjunct to gene therapy in future experimental studies.

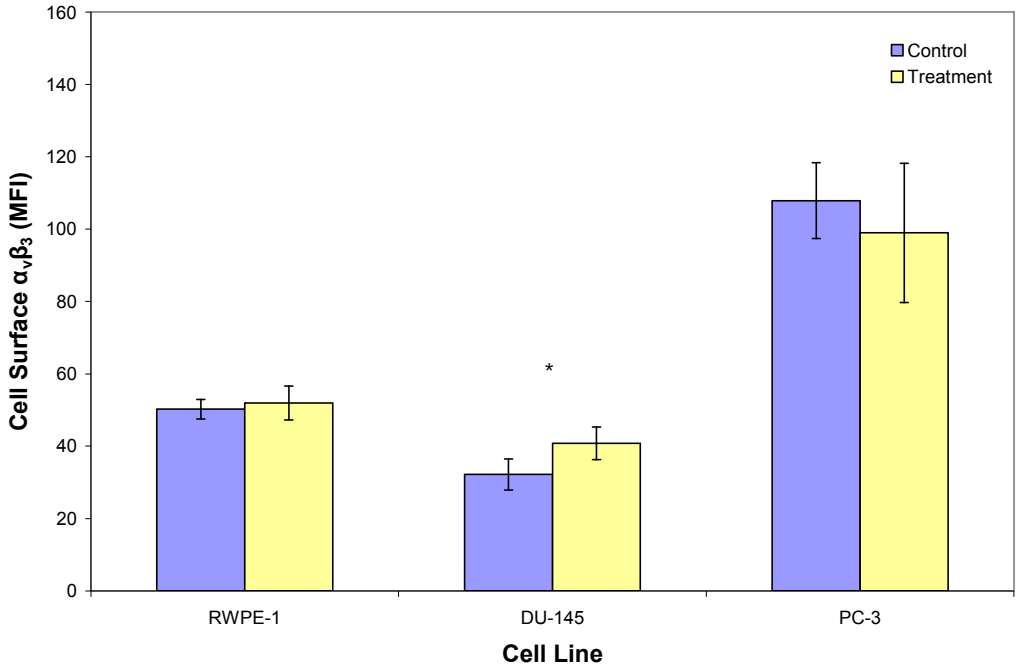
4.6 FIGURES AND TABLES

Figure 4-1 Effect of ganglioside on cell surface CAR density differs in each prostate cell line *in vitro*



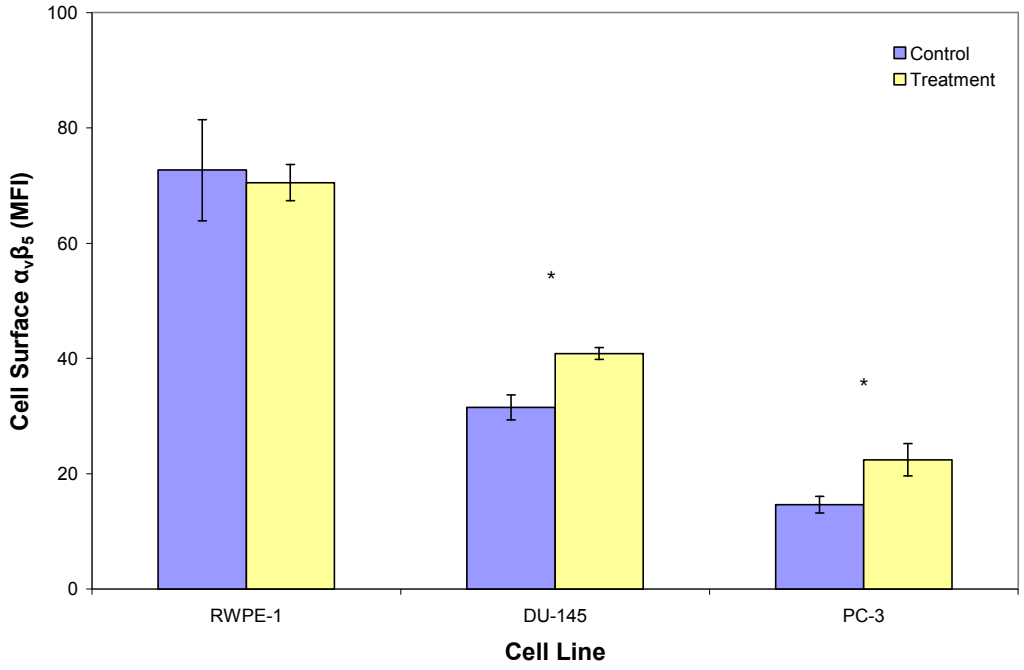
Cell cultures were incubated with 10 μg/mL mixed ganglioside for 48 hr. Cells were harvested and stained for CAR prior to analysis by flow cytometry ($n \geq 4$). Asterisk indicates significant ($p < 0.05$) difference between treated and untreated control group.

Figure 4-2 Ganglioside increases cell surface $\alpha_v\beta_3$ density in DU-145 cells *in vitro*



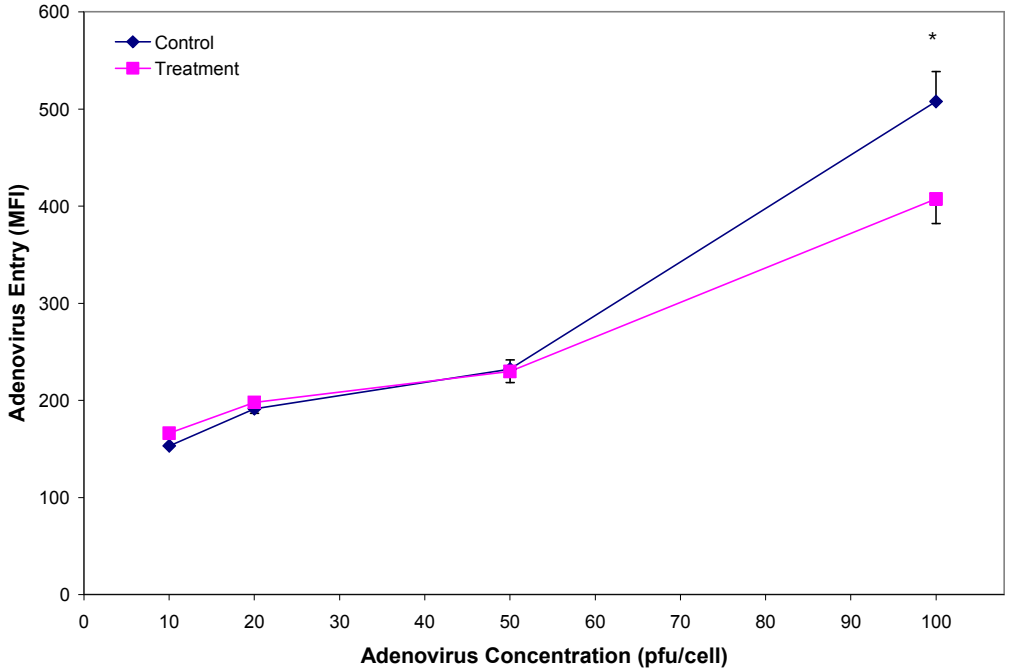
Cell cultures were incubated with 10 μg/mL mixed ganglioside for 48 hr. Cells were harvested and stained for $\alpha_v\beta_3$ prior to analysis by flow cytometry ($n \geq 3$). Asterisk indicates significant ($p<0.05$) difference between treated and untreated control group.

Figure 4-3 Ganglioside increases cell surface $\alpha_v\beta_5$ density in malignant prostate but not RWPE-1 cells *in vitro*



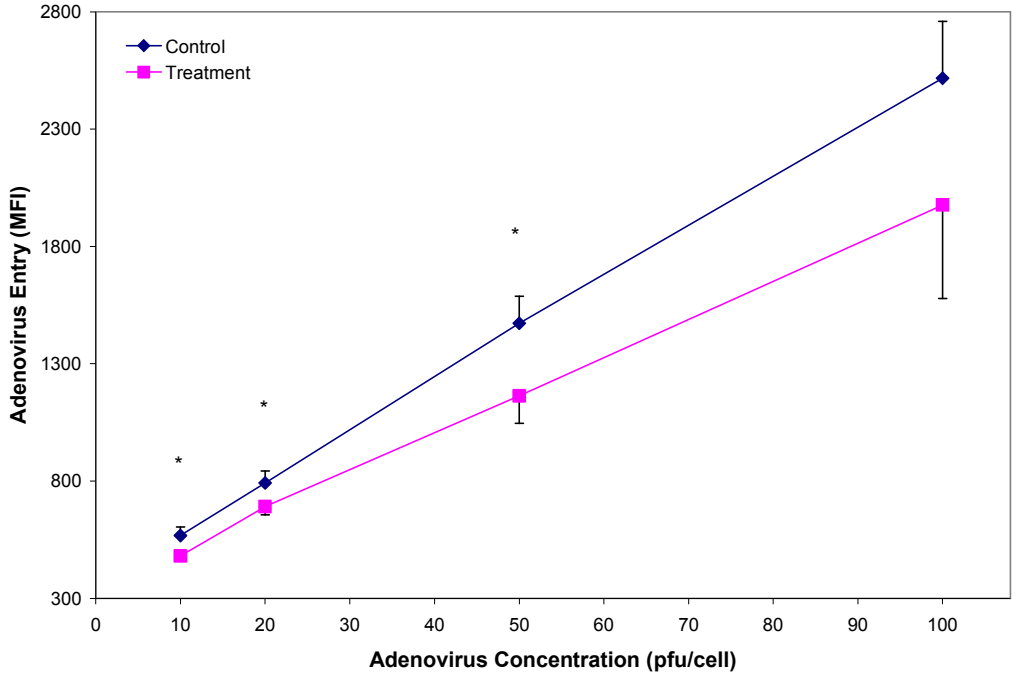
Cell cultures were incubated with 10 μg/mL mixed ganglioside for 48 hr. Cells were harvested and stained for $\alpha_v\beta_5$ prior to analysis by flow cytometry ($n \geq 3$). Asterisk indicates significant ($p<0.05$) difference between treated and untreated control group.

Figure 4-4 Ganglioside does not increase entry of adenovirus into RWPE-1 cells *in vitro*



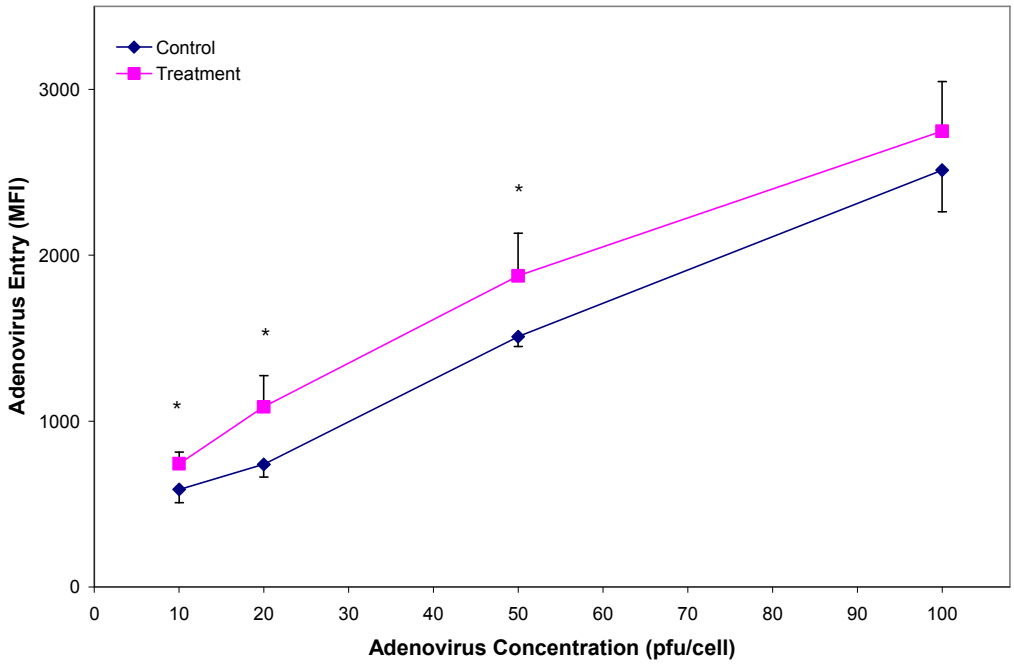
Cell cultures were incubated with 10 µg/mL mixed ganglioside for 48 hr before infection with adenovirus. Cells were harvested 24 hr after infection and analyzed for GFP by flow cytometry (n=5). Asterisk indicates significant ($p<0.05$) difference between treatment and control group.

Figure 4-5 Ganglioside decreases adenovirus entry into DU-145 cells *in vitro*



Cell cultures were incubated with 10 μg/mL mixed ganglioside for 48 hr before infection with adenovirus. Cells were harvested 24 hr after infection and analyzed for GFP by flow cytometry (n=4). Asterisk indicates significant ($p<0.05$) difference between treatment and control group.

Figure 4-6 Ganglioside increases adenovirus entry into PC-3 cells *in vitro*



Cell cultures were incubated with 10 μg/mL ganglioside for 48 hr before infection with adenovirus. Cells were harvested 24 hr after infection and analyzed for GFP by flow cytometry (n=5). Asterisk indicates significant ($p<0.05$) difference between treatment and control group.

Table 4-1 Summary of adenovirus transduction data

Adenovirus Concentration (pfu/cell)	RWPE-1		p-value	DU-145		p-value	PC-3		p-value
	Control	Treatment		Control	Treatment		Control	Treatment	
10	152	166	NS	567	480	<0.01	586	743	<0.02
20	191	197	NS	792	691	<0.02	738	1090	<0.01
50	232	229	NS	1470	1160	<0.01	1510	1880	<0.02
100	507	407	<0.01	2520	1980	<0.06	2510	2750	NS

Cell cultures were incubated with 10 μg/mL ganglioside for 48 hr before infection with AdBM116GFP. Cells were harvested 24 hr after infection and analyzed for GFP by flow cytometry (n=5). Values are reported as mean fluorescence intensity. p-values denote differences between treated and untreated control group within cell line, NS=not significant.

4.7 LITERATURE CITED

1. Sun LC, Luo J, Mackey LV, Fuselier JA, Coy DH. A conjugate of camptothecin and a somatostatin analog against prostate cancer cell invasion via a possible signaling pathway involving PI3K/Akt, alphaVbeta3/alphaVbeta5 and MMP-2/-9. Cancer Lett 2007;246(1-2):157-66.

2. Cheresh DA, Pytela R, Pierschbacher MD, Klier FG, Ruoslahti E, Reisfeld RA. An arg-gly-asp-directed receptor on the surface of human melanoma cells exists in an divalent cation-dependent functional complex with the disialoganglioside GD2. J Cell Biol 1987;105(3):1163-73.

3. Ohkawa Y, Miyazaki S, Miyata M, Hamamura K, Furukawa K, Furukawa K. Essential roles of integrin-mediated signaling for the enhancement of malignant properties of melanomas based on the expression of GD3. Biochem Biophys Res Commun 2008;373(1):14-9.

4. Rauen KA, Sudilovsky D, Le JL, et al. Expression of the coxsackie adenovirus receptor in normal prostate and in primary and metastatic prostate carcinoma: Potential relevance to gene therapy. Cancer Res 2002;62(13):3812-8.

5. Bureyko T, Hurdle H, Metcalfe JB, Clandinin MT, Mazurak VC. Reduced growth and integrin expression of prostate cells cultured with lycopene, vitamin E and fish oil in vitro. Br J Nutr 2008:1-8.

6. Bruning A, Stickeler E, Diederich D, et al. Coxsackie and adenovirus receptor promotes adenocarcinoma cell survival and is expressionally activated after transition from preneoplastic precursor lesions to invasive adenocarcinomas. Clin Cancer Res 2005;11(12):4316-20.

7. Pandha HS, Stockwin LH, Eaton J, et al. Coxsackie B and adenovirus receptor, integrin and major histocompatibility complex class I expression in human prostate cancer cell lines: Implications for gene therapy strategies. Prostate Cancer Prostatic Dis 2003;6(1):6-11.

8. Pidgeon GP, Tang K, Cai YL, Piasentin E, Honn KV. Overexpression of platelet-type 12-lipoxygenase promotes tumor cell survival by enhancing alpha(v)beta(3) and alpha(v)beta(5) integrin expression. Cancer Res 2003;63(14):4258-67.

9. McCabe NP, De S, Vasanji A, Brainard J, Byzova TV. Prostate cancer specific integrin alphavbeta3 modulates bone metastatic growth and tissue remodeling. Oncogene 2007;26(42):6238-43.

5.0 GENERAL SUMMARY AND DISCUSSION

5.1 SUMMARY OF RESULTS

The overall hypothesis of this thesis research is that ganglioside increases metastatic potential of CaP, and increases susceptibility of CaP to adenovirus-mediated gene therapy. This hypothesis is explored in two studies.

The hypothesis driving Chapter 3 (Effects of Ganglioside on Growth and Cell Surface Ganglioside Densities in Prostate Cancer *in vitro*) was that supplemental ganglioside decreases growth; and increases metastatic potential in an *in vitro* model of CaP.

Specifically, it was investigated whether the mixture of ganglioside (92% GD3) affected:

i. cell growth;

ii. cell surface density of pro-metastatic marker, GD3 (Section 1.7.1);

iii. cell surface density of anti-metastatic marker, GD1a (Section 1.7.3).

Results of the first objective supported the hypothesis as treatment reduced PC-3 cell growth. There was no effect on growth in other cell lines. Results from the second objective neither supported, nor opposed the hypothesis; treatment did not change GD3 density in CaP cells. The hypothesis was supported by results obtained from the third objective; GD1a density decreased in each CaP cell line with treatment. Overall, the hypothesis was supported as ganglioside treatment was shown to decrease growth and increase metastatic potential based on decreased density of anti-metastatic marker GD1a in a model of CaP.

The hypothesis underlying Chapter 4 (Effects of Ganglioside on Cell Surface Adenovirus Receptor Densities and Adenovirus Transduction in Prostate Cancer *in vitro*) was that supplemental ganglioside increases cell surface adenovirus receptor density and consequently, susceptibility of CaP to infection by adenovirus in an *in vitro* model of CaP.

Specifically, it was determined whether the mixture of ganglioside (92% GD3) affected:

i. cell surface density of high affinity virus receptor, CAR (1) (Section 1.8.2);

ii. cell surface densities of low affinity virus receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (2) (Section 1.8.2);

iii. adenovirus entry.

Results of the first objective supported the hypothesis in one of the malignant cell lines used (PC-3); CAR density increased as a result of treatment. However, CAR density decreased in the other model of malignancy (DU-145) and did not change in a model of healthy prostate. The hypothesis was supported by results from the second objective; treatment increased integrins in CaP cells. Results from the third objective supported the hypothesis in a parallel manner to the first; adenovirus entry increased in one malignant cell line (PC-3), decreased in the other (DU-145) and did not change in a model of healthy prostate. Overall, the hypothesis was supported as ganglioside treatment was shown to increase adenovirus transduction mediated by CAR in at least one model of CaP.

The overall hypothesis was supported by evidence from the current study. Supplemental ganglioside may increase metastatic potential of CaP. This treatment may also improve susceptibility of CaP to adenovirus-mediated gene therapy.

5.2 GENERAL DISCUSSION

Future epidemiological studies should aim to describe the relationship between dairy intake and advanced CaP. Since dairy products are considerably heterogeneous in energy, macro- and micronutrient, and ganglioside quantities, a more thorough analysis relating food composition to incidence or characteristics of CaP is justified. Two studies which included buttermilk in the definition of dairy found positive correlations between CaP risk and dairy exposure (3, 4). Buttermilk is considerably rich in ganglioside. The results obtained in the current study suggest that ganglioside, a common component of dairy; decreases GD1a density and therefore, may increase metastatic potential of CaP. However, metastasis is a complex process mediated by a plethora of molecules. The effect of ganglioside on the sum of these mediators or on functional steps in metastasis would better implicate this glycolipid in advanced cancer risk.

This study assessed growth and cell surface markers in adherent prostate cells only. This was done for relevance to infectivity assays, in which only adherent cells are exposed to adenovirus. Future investigation should examine total (floating and adherent) cells for effect of treatment on growth and cell surface markers. It is recommended that future studies continue to employ use of polypropylene culture plates, as cell attachment to polystyrene is influenced by

ganglioside content (5). This is important since treatment may alter ganglioside composition and binding of cells to culture plate accordingly, thereby obscuring interpretation of results.

Future investigation should explore the influence of ganglioside in more models of CaP *in vitro*, including LNCaP and CWR22Rv1. This would help to elucidate whether the results are achievable in CaP as opposed to the specific cell lines used in this study. From the data obtained in this *in vitro* study, it would be difficult to advise whether ganglioside intake or supplementation should be recommended for CaP patients. The treatment mixture has opposing effects on tumour properties; ganglioside resulted in growth decrease, but metastatic potential increase in a model of advanced CaP. It would be beneficial to decrease growth of tumours, but the potential of promoting metastasis may be too great to risk. Future studies in animal models would help to clarify the risk-benefit analysis. A couple of shortcomings are evident in this process. There is currently an insufficient number of relevant animal models that accurately predict outcomes in human clinical trials (6). Additionally, there is a relatively low abundance of malignant prostate models *in vitro*, compared to other tissues such as breast. Research into establishing a larger library of CaP models is recommended to alleviate this issue. Alternatively, cell migration and invasion assays can be performed to determine whether the effects of ganglioside treatment on markers of metastasis have functional consequences in CaP cells.

It would be valuable to determine whether ganglioside composition can be changed via treatment since a number of functional processes would be affected.

The structure of ganglioside ceramide tail alters the immunosuppressive capability of cancer cells (7). Ceramides also function as secondary messengers in cell death (8). Cancers may alter ceramide metabolism as a method to escape host immune detection and cell death. That is, tumours may produce variants of ceramide species (9, 10) that aid in evasion of antibody-mediated immune recognition and disrupt messenger signalling in apoptosis. This can lead to decreased sensitivity of cancer cells to several therapies including chemo-, radio-, and hormonal (11-13). Therefore, alteration ganglioside composition or ceramide metabolism is a plausible area of investigation.

Ganglioside (GD3) is mainly lipid raft-sequestered. A recent study has shown that caveolin-1 overexpression results in homogenous cell membrane distribution of GD3 in human melanoma cells (14). Surprisingly, malignant properties of these cells are subsequently curtailed. It would be interesting to determine whether exogenous ganglioside treatment results in altered GD3 distribution. It is feasible that this would occur since localization is determined by ceramide composition, and because composition varies greatly in food products (15, 16).

The role of ganglioside in metastasis has been investigated in a number of animal studies. These *in vivo* studies have experimentally transfected plasmids to modify ganglioside metabolism in cancer cells prior to injection in animals. Future studies may be designed to determine the dietary influence of ganglioside in metastasis. Tumour xenografts can be established from unmodified cancer cells, followed by treatment with dairy or ganglioside-enriched diet in comparison

to standard chow diet control groups. Number of animals that develop tumours, number of tumours, size of tumour, and time to death are a few measures that can be taken to inform whether ganglioside, as a component of diet, plays a functional role in development of metastasis.

Since only about 10% of prostate tumours progress to biologically-relevant disease (17), it would be advantageous to determine which tumours were going to progress and what measures could be taken to prevent tumour progression. Many men with CaP die of causes unrelated to their disease (18), but; one of the frontline treatments, RP is concomitant with disturbances to quality of life. Surgery may be avoidable with successful drug/diet development designed to combat prostate tumour progression in a man with early stage CaP. In doing so, he would preserve quality of life and might enjoy a long life that will not come to an end as a result of CaP.

Increased transduction of PC-3 cells with adenovirus following ganglioside treatment is a promising finding. Decreased transduction in DU-145 cells necessitates future investigation in more models of malignant prostate, as mentioned before. The data is limited to suggesting that ganglioside increases viral entry into PC-3, not all CaP cells. It is believed that adenovirus designed for oncolytic effect or gene-directed enzyme prodrug therapy would enter PC-3 cells to a greater extent after ganglioside treatment. Treatment is assumed not to interfere with any function of the virus in between entry and expression in the nucleus. Much investigation has been dedicated to designing targeted adenovirus based on cancer cell-specific markers. For example, the HER2 receptor of breast

cancer has been exploited for this function (19). A CaP-specific surface marker has not been identified, so CAR level modification is still one of the more plausible therapeutic targets.

This is among the first work to explore the utility of ganglioside in adjunct to gene therapy. Ganglioside had not been investigated for a potential additive or synergistic anti-tumour effect with conventional cancer therapies. Similarly, polyunsaturated fatty acids have been exploited for improved efficacy with chemo- and radiotherapies (20, 21), but have not been investigated for an effect that may potentiate gene therapy. Tumours develop complex mechanisms by which to evade cell death. A trend toward developing treatment cocktails catered to optimally combat each tumour is developing. Combination of particular fatty acid species with cancer treatments has been promising thus far and future investigation is encouraging.

It has been established in much literature that ganglioside metabolism is different between healthy and malignant tissue (Sections 1.6.1, 1.6.2). This is among the first work to demonstrate that ganglioside uptake, and effects on cell surface ganglioside and integrin densities may differ between the tissues as well. This will hopefully encourage comparisons between healthy and malignant cell models in future explorations of ganglioside uptake and metabolism. Using a model of healthy prostate was a particular strength for adenovirus infection assays. It was found that ganglioside increases entry into malignant, but not normal prostate. This was not an intended objective to establish, but a rather serendipitous finding. The influence of a single compound on CaP characteristics

may be lost in the complex combination of nutrients in the diet. Since ganglioside is not an essential dietary nutrient, it would be difficult to say whether its presence in the diet would cause any significant effect above and beyond ganglioside that is endogenously synthesized. This work successfully demonstrates that dietary compounds may have a number of effects on cancer characteristics. These effects may be negated or potentiated by other components of the diet, but an understanding of mechanism of action can propel designs for future studies aimed at intervening malignant processes.

5.3 LITERATURE CITED

1. Meier O, Greber UF. Adenovirus endocytosis. J Gene Med 2004;6 Suppl 1:S152-63.

2. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 1993;73(2):309-19.

3. Tseng M, Breslow RA, Graubard BI, Ziegler RG. Dairy, calcium, and vitamin D intakes and prostate cancer risk in the national health and nutrition examination epidemiologic follow-up study cohort. Am J Clin Nutr 2005;81(5):1147-54.

4. Schuurman AG, van den Brandt PA, Dorant E, Goldbohm RA. Animal products, calcium and protein and prostate cancer risk in the netherlands cohort study. Br J Cancer 1999;80(7):1107-13.

5. Ravindranath MH, Ravindranath RM, Morton DL, Graves MC. Factors affecting the fine specificity and sensitivity of serum antiganglioside antibodies in ELISA. J Immunol Methods 1994;169(2):257-72.

6. Pienta KJ, Abate-Shen C, Agus DB, et al. The current state of preclinical prostate cancer animal models. Prostate 2008;68(6):629-39.

7. Ladisch S, Li R, Olson E. Ceramide structure predicts tumor ganglioside immunosuppressive activity. Proc Natl Acad Sci U S A 1994;91(5):1974-8.

8. Cai Z, Bettaieb A, Mahdani NE, et al. Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF7 cells to tumor necrosis factor-alpha-mediated cytotoxicity. J Biol Chem 1997;272(11):6918-26.

9. Senkal CE, Ponnusamy S, Rossi MJ, et al. Role of human longevity assurance gene 1 and C18-ceramide in chemotherapy-induced cell death in human head and neck squamous cell carcinomas. Mol Cancer Ther 2007;6(2):712-22.

10. Schiffmann S, Sandner J, Birod K, et al. Ceramide synthases and ceramide levels are increased in breast cancer tissue. Carcinogenesis 2009;30(5):745-52.

11. Wang H, Charles AG, Frankel AJ, Cabot MC. Increasing intracellular ceramide: An approach that enhances the cytotoxic response in prostate cancer cells. Urology 2003;61(5):1047-52.

12. Park HW, Song JY, Kim KS, et al. Enhancement of radiosensitivity by combined ceramide and dimethylsphingosine treatment in lung cancer cells. Exp Mol Med 2004;36(5):411-9.

13. Eto M, Bennouna J, Hunter OC, et al. C16 ceramide accumulates following androgen ablation in LNCaP prostate cancer cells. Prostate 2003;57(1):66-79.

14. Nakashima H, Hamamura K, Houjou T, et al. Overexpression of caveolin-1 in a human melanoma cell line results in dispersion of ganglioside GD3 from lipid rafts and alteration of leading edges, leading to attenuation of malignant properties. Cancer Sci 2007;98(4):512-20.

15. Prinetti A, Basso L, Appierto V, et al. Altered sphingolipid metabolism in N-(4-hydroxyphenyl)-retinamide-resistant A2780 human ovarian carcinoma cells. J Biol Chem 2003;278(8):5574-83.

16. Sorensen LK. A liquid chromatography/tandem mass spectrometric approach for the determination of gangliosides GD3 and GM3 in bovine milk and infant formulae. Rapid Commun Mass Spectrom 2006;20(24):3625-33.

17. Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2007--an update. J Gene Med 2007;9(10):833-42.

18. Newschaffer CJ, Otani K, McDonald MK, Penberthy LT. Causes of death in elderly prostate cancer patients and in a comparison nonprostate cancer cohort. J Natl Cancer Inst 2000;92(8):613-21.

19. Magnusson MK, Henning P, Myhre S, et al. Adenovirus 5 vector genetically re-targeted by an affibody molecule with specificity for tumor antigen HER2/neu. Cancer Gene Ther 2007;14(5):468-79.

20. Conklin KA. Dietary polyunsaturated fatty acids: Impact on cancer chemotherapy and radiation. Altern Med Rev 2002;7(1):4-21.

21. Baracos VE, Mazurak VC, Ma DW. n-3 polyunsaturated fatty acids throughout the cancer trajectory: Influence on disease incidence, progression, response to therapy and cancer-associated cachexia. Nutr Res Rev 2004;17(2):177-92.