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THE ROLE OF SEMINAL PLASMA

IN CERVICAL CARCINOMA

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Thesis presented for the Degree of
DOCTOR OF PHILOSOPHY
In the Division of Medical Biochemistry
UNIVERSITY OF CAPE TOWN

December, 2010

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DECLARATION

Except where due acknowledgements is made by reference, the studies undertaken herein are the unaided work of the author. No portion of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Jason R. Sutherland
December, 2010

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ACKNOWLEDGEMENTS

This thesis is the culmination of a research collaboration between the MRC/UCT Research Group for Receptor Biology and the MRC, Human Reproductive Sciences Unit (HRSU) at Edinburgh, Scotland. I am very privileged to have been given the opportunity to work in two beautiful countries with so many wonderful people.

First and foremost I would like to thank both my supervisors Professor Arieh Katz (Cape Town University, South Africa) and Professor Henry Jabbour (Medical Research Council, Edinburgh, Scotland), both of whom have been truly supportive in providing guidance and their experienced insights throughout the duration of this project and producing this thesis. Furthermore I would like to thank all the people who kindly gave consent for participating in this study as without the donation of these biological specimens, the results presented here within would not be possible.

In addition, the technical support I have received in the South African and Edinburgh laboratories when undertaking my research has been truly remarkable. Hiram Arendse who manages the animal facilities has been instrumental in orchestrating the organization of the mouse experiments and Sheila Wright and Sharon Battersby who endured numerous headaches trying to help me in the lab. Also a big shout out to Stuart Reid who always had some inspiring banter during the endless days of tedious experiments and to Kurt Sales for answering my never-ending list of questions. I would also like to thank my funders, the Medical Research Council units in both South Africa and Scotland.

Last, but certainly not least, I would like to thank my wife Angela who has stuck by me from the start of this journey, my son Jack and daughter Eva, whom with their cheeky grins have made the write-up bearable, my parents for their support, my sis and my bro and other family members and friends, you know who you are.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ACF	aberrant crypt foci
ADT	adenosine triphosphate
AGIF	adipogenesis inhibitory factor
AI	apoptotic index
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
ANOVA	analysis of variance
AOM	azoxymethane
AP1	activator protein-1
ATP	adenosine triphosphate
BCP	1-bromo-3-chloropropane
bFGF	basic fibroblast growth factor
BMPs	bone morphogenic proteins
Bp	base pairs
BSA	bovine serum albumin
CAM	cell-surface adhesion molecule
cAMP	adenosine 3',5'-cyclic monophospate
cDNA	complementary DNA
CDK	cyclin dependent kinase
cGMP	gunaosine 3',5'-cyclic monophosphate
CIN	cervical intraepithelial neoplasia
CM	conditioned media
COX	cyclooxygenase
CP	chronic prostatitis
cPGES	cystolic prostaglandin E synthase
CPPS	chronic pelvic pain syndrome
CRE	camp response element
DAB	3'3-diaminobenzidine
DAG	diacylglycerol
DC-SIGN	dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMEM	dulbecco's modified eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
dPBS	distilled phosphate buffered saline
EBM-2	endothelial basal medium
ECM	extracellular matrix
Ect-1	ectocervical cell line
EDTA	ethylenediamine tetraacetic acid
EGF	endothelial growth factor
EGFR	endothelial growth factor receptor
EGM2	endothelial growth medium

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EGR-1	early response growth factor-1
ERK	extracellular signal-related kinase
FAP	familial adenomatous polyposis
FCS	fetal calf serum
FGF	fibroblast growth factor
FIGO	international federation of obstetricians and gynaecologists
GPCR	G protein-coupled receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSK-3	glycogen synthase kinase-3
H&E	haemotoxylin and eosin
HCA-7	human colonic adenocarcinoma-7
hCG	human chorionic gonadtrophin
HiSIL	high grade squamous intra-epithelial lesions
HIV-1	human immunodeficiency virus-1
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
Hr	hour
HRP	horse –radish peroxidise
HT-29	colorectal carcinoma cell line
HUVECs	human umbilical vein endothelial cells
IGF-I	insulin growth factor I
IgG	immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IP_3	inositol (1,4,5)-trisphosphate
IVC	individually ventilated cages
Kb	base pairs
kDa	kilodalton
LIF	leukemia inhibitory factor
LPO	lipid peroxidation
LPS	lipopolysaccharides
LoSIL	low-grade squamous intra-epithelial lesions
Mg	milligram
µg	microgram
Ml	millilitre
µl	microlitre
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MEK	mitogen-activated protein kinase
MI	mitotic index
mins	minutes
MIP2-α	macrophage inflammatory protein 2-α
MIP2b	macrophage inflammatory protein 2b
MIP3a	macrophage inflammatory protein 3a
MMP	matrixmetalloproteinases
mPGES-1	membrane-bound prostaglandin E synthase-1
mPGES-2	membrane-bound prostaglandin E synthase-2
mRNA	messenger ribonucleic acid

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NF-κB	nuclear factor-kappa B
NK	natural killer cells
Nm	nanometers
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
NSCLC	non-small cell lung carcinoma
Os	opening of the cervix
OSE	ovarian surface epithelia
OVCAR-3	ovarian cancer cell line
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule
PG	prostaglandins
PGD_2	prostaglandin D_2
PGDS	prostaglandin D synthase
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFS	prostaglandin F synthase
PGG_2	prostaglandin G_2
PGH_2	hydroxy cyclic endoperoxide
PGI_2	prostacyclin
PGIS	prostacyclin I synthase
PGT	prostaglandin transporter
PIGF	placental growth factor
P13K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PLA_2	phospholipase A_2
PPAR	peroxisome proliferators-activated
PTGER1	prostanoid receptor EP1
PTGER2	prostanoid receptor EP2
PTGER3	prostanoid receptor EP3
PTGER4	prostanoid receptor EP4
PTGS	prostaglandin endoperoxide synthase
PTGS-1	prostaglandin endoperoxide synthase-1
PTGS-2	prostaglandin endoperoxide synthase-2
PTGS-3	prostaglandin endoperoxide synthase-3
PVDF	polyvinylidene difluoride
QRT-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
RT	reverse transcriptase
RTK	receptor tyrosine kinase
SDF-1	stromal cell-derived factor-1
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SIL	squamous intra-epithelial lesions

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SK-OV-3	ovarian cancer cell line
SLPI	secretory leukocyte protease inhibitor
SP	seminal plasma
SPARC	secreted protein acid and rich in cysteine
STAT3	signal transducers and activators of transcription
Taq	*thermus aquaticus*
TBS	tris-buffered saline
TGF	transforming growth factor
TGF-β	transforming growth factor-β
TGF-p	transforming growth factor-p
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor-α
Tris	tris (hydroxymethyl) amino methane
TXA_2	thromboxane A_2
TXS	thromboxane synthase
UTR	untranslated region
VEGF	vascular endothelial growth factor
VPF	vascular permeability factor
WHO	world health organisation
WT	wild type

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PROJECT SYNOPSIS

Cervical cancer is a worldwide public health problem with in excess of 370,000 new cases being reported each year. It is the leading cause of death from cancer among women in the developing world where 80% of cases occur. Human papilloma virus (HPV) has been identified as the main causative factor linked with the development and progression of cancer of the cervix, although other factors are known to exist and include genital warts, consenting to sex at an early age, smoking, long term use of contraceptive pills and multiple sexual partners. Despite the exact mechanisms underlying the disease not being well understood, there is a strong growing body of evidence to suggest that exposure of neoplastic cervical epithelium to seminal plasma (SP) may be of significant importance.

The initial aim of this research was to determine if SP treatment of normal cervical tissue could induce increased expression of prostaglandin endoperoxide synthase (PTGS) enzymes, prostaglandin receptors, pro-inflammatory mediators and pro-angiogenic factors known to be upregulated in cervical carcinomas. Quantitative real-time polymerase chain reaction (QRT-PCR) revealed that expression of PTGS enzymes -1 and -2, prostanoid receptors PTGER1, PTGER3 and PTGER4, pro-inflammatory cytokines IL6, IL-8 and IL-11, pro-angiogenic factor VEGF, growth related oncogene (Gro-α) and the intratumoral vascularisation marker CD31 were all significantly increased in response to seminal plasma treatment in normal cervical tissue in comparison to vehicle treated control group. This data highlights the potential of SP as a trigger for augmenting the local microenvironment that may in turn create favourable conditions for cervical tumour growth. It is established that HPV is the cause for developing cancer of the cervix, however it takes 10-15 years to develop, so SP poses as an additional insult that can cause premalignant lesion to grow and contributes to cervical cancer development.

In order to assess the signalling pathways which are involved in SP-activated transcription of these target genes, WT HeLa adenocarcinoma cells were employed as a model system and treated with SP in the presence/absence of an array of inhibitors or vehicle as control. The expression was determined by QRT-PCR analysis and

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demonstrated that SP treatment induced the over-expression of PTGS-1, PTGS-2, IL-6, IL-8, IL-11, Gro-α and VEGF. Specific inhibitors of MAPK kinase/ERK, EGFR and PTGS-2 were shown to inhibit the fold-induction that was induced of these target genes by SP and therefore suggest that these signalling pathways are central in the activation of pro-tumourigenic genes, although a role for NF-κB and to a lesser extent TGF-β is also evident. In order to determine whether the SP-mediated transactivation of target genes and signalling pathways culminated in an increased proliferation index in our model cell line system, QRT-PCR analysis and immunohistochemistry were employed and showed the expression of the proliferative marker Ki-67 to be significantly upregulated in WT HeLa cells following treatment with SP in comparison to the untreated control. Consistent with that, SP increased the cellular proliferation of WT HeLa adenocarcinoma cells as determined by using the CellTitre96 Aqueous One Solution proliferation assay. In addition, immunohistochemical staining of the proliferative marker Ki-67 was also found to be significantly elevated in squamous carcinoma when compared to normal cervical tissue which is indicative of increased cell proliferation and is consistent with the effects this thesis has shown SP exhibits on WT HeLa cells. These data display that SP can promote cellular proliferation of cervical epithelia by the activation of target genes involved in tumourigenesis, inflammation and angiogenesis through the activation of MAPK kinase/ERK, EGFR, PTGS enzymes, NF-κB and TGF-β.

To further investigate SP as a key promoter or even regulator of cervical tumourigenesis, a xenograft mouse model was established using MF-1 nude mice inoculated with HeLa adenocarcinoma cells and animals were then subjected to a treatment regime of 3 times weekly injections of SP (1:500) or PBS (control group) for a duration of ten weeks. SP treatment led to an increase in tumour size. At 10 weeks mice were sacrificed and all tumours were excised. Their analysis showed that tumours grown in mice administered with seminal plasma displayed a significant increase in weight, size and proliferation index, as determined by Ki-67, in comparison to tumours in control mice. Coincident with enhanced tumour growth, QRT-PCR analysis showed elevated expression of the inflammatory PTGS-1, PTGS-2 and the E-series prostanoid receptors PTGER1, PTGER2 and PTGER4 together with the potent pro-inflammatory

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cytokines interleukin (IL)-6 and IL-11 and the pro-angiogenic vascular endothelial growth factor (VEGF) in SP xenograft tumours compared with control. In addition, immunohistochemical staining for CD31 revealed significantly larger blood vessels in xenograft tumours in mice treated with seminal plasma. These data show for the first time that seminal plasma can promote cervical tumourigenesis *in vivo* via the induction of potent pro-inflammatory mediators and angiogenic factors to enhance the proliferative and angiogenic capacity of cervical cancer cells. SP induced the upregulation of the same genes in normal cervical tissue and WT HeLa adencarcinoma cells and thereby provides further evidence to support this data.

It is likely that SP does have a functional role in the development and progression of cervical cancer but further research is required to help elucidate the underlying mechanisms involved and therefore assist in implementing improved therapy for women with cervical carcinomas.

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Chapter 1

GENERAL INTRODUCTION

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1.1 Introduction

The interplay between inflammation and cancer has been recognized for some time. In the context of gynaecological malignancies, inflammation can contribute to the initiation and progression of disease via the release of local mediators, prostaglandins, interleukins, growth factors and cytokines (Coussens and Werb, 2002). This in turn facilitates immune cell recruitment, cell proliferation and angiogenesis to sustain tumour growth. It is well recognised that seminal plasma contains an abundance of growth factors, cytokines, protein kinases, signal transduction molecules, angiogenic factors and prostaglandins (Ness and Grainger, 2008; Robertson, 2005; Fung *et al.,* 2003). Therefore, the vast range of complexities contained within the ejaculate pool may help promote conditions which are favourable for the onset and progression of cervical cancer. This coincides with the increasing body of evidence which suggests that multiple sexual partners (Use, 1981; Ali *et al.,* 2010) may be pivotal in triggering the development of the disease. Human papilloma virus (HPV) is the precursor in nearly all cases of cervical cancer, however, it takes approximately 12-15 years before a persistent HPV infection manifests in cervical carcinoma (Muller *et al.,* 2006; Wallin *et al.,* 1999) and as a result this thesis will focus on testing the effect of SP on disease development and progression.

1.2 Epidemiology of Cervical Cancer

At present, there are more than 273,000 deaths worldwide from cervical cancer each year accounting for 9% of total cancer deaths in females (Ruiz-Godoy *et al.,* 2007). Cancer of the cervix remains the most prolific female cancer in developing countries with a notable decline in mortality rates in Western countries being ascribed to successful screening programmes and vaccine development (Ponten *et al.,* 1995; Harlan *et al.,* 1995; Kim and Goldie, 2008). The prevalence of cervical cancer in South Africa is reported as being one of the worst in the world, occurring on average in 29 out of every 100,000 women (Steckley *et al.,* 2003). This is regarded as being very high when compared to Italy, a country considered as having a low incidence rate where cervical cancer occurs in only 2.8 out of every 100,000 women (Aareleid *et al.,* 1998). However, the likelihood of a South African woman developing cancer of the

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cervix is somewhat altered when analysing sub-populations within the country. For example, cervical cancer is the most commonly occurring cancer in black and coloured South African females (31.2% and 22.9% respectively) whereas it is the fourth most common cancer amongst white South African females (2.7%) (Sitas *et al.,* 1997). Whilst trends in mortality rates in South Africa show a low incidence in the white population, it is thus clear that black and coloured females living in this country are at especially high risk of dying from cervical cancer due to inadequate screening programmes and lack of awareness about the disease (Bailie *et al.*, 1996).

1.3 Histology of the Uterine Cervix

The word 'cervix' means *neck* in Latin and is aptly named as it forms the neck of the uterus in the lower, narrow portion that protrudes into the vagina. The cervix is conical in shape, fibromuscular in origin and its opening is often referred to as the *os.* It is responsible for performing several functions that includes allowing the passage of spermatozoa into the genital tract, permitting menstrual blood flow during menstruation and acts as a defensive barrier, protecting the uterus against bacterial infection. An additional important feature of the cervix is highlighted during labour as it holds the fetus in place until birth and then has the ability to dilate allowing the fetus to pass from the uterus to the vagina. The cervix is covered by a thin layer of epithelial cells consisting of two distinct cell types called columnar epithelium and stratified squamous epithelium (Fichorova and Anderson, 1999). Columnar epithelium consists of a single layer of cells that line the endocervical canal and usually form a sterile passage into the upper genital tract. On the other hand, squamous stratified epithelium that line the vaginal wall and the exocervix is multilayered and is able to withstand mechanical or chemical insult such that the outer layers can be shed as to not expose the basement membrane, making these cells perfectly suited for maintaining the structural integrity of the cervix (Fichorova and Anderson, 1999). The junction between the squamous epithelium of the cervix and the columnar epithelium originating from the endocervical glands is referred to as the squamocolumnar junction (Figure 1.1).

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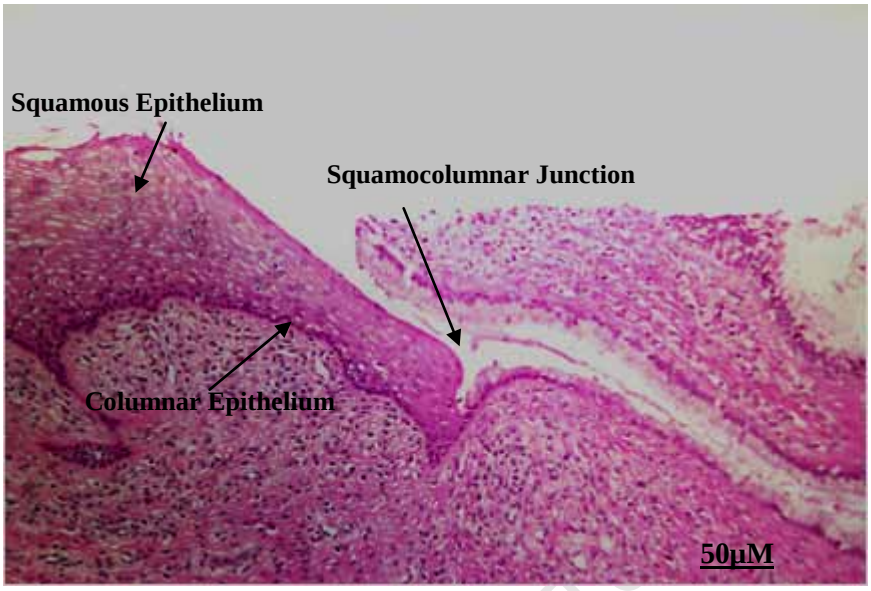

Figure 1.1: Histology of the uterine cervix taken from patient attending Groote Schuur Hospital, Cape Town, South Africa. Haemotoxylin and eosin (H&E) stained section (magnification x20).

During adolescence and pregnancy, the columnar epithelium is replaced by squamous epithelium to form a new squamocolumnar junction. The area between the new and old squamocolumnar junction is referred to as the transformation zone with several studies proposing that carcinogens act in this region to initiate cervical neoplasia (Martin-Hirsch *et al.*, 1997; Kristensen *et al.*, 1989).

1.4 Symptoms and Screening of Cervical Cancer

In the majority of cases, cervical cancer has no symptoms and when the signs are present, they often go undetected by the patient. The indicatory warnings that can arise during the early stages of cervical cancer include a continuous watery vaginal discharge which may be foul-smelling and/or abnormal vaginal bleeding between periods or after intercourse. Symptoms that manifest in later stages of the disease vary from loss of appetite and fatigue to pelvic pain, back pain and bone fractures.

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1.4.1 Pap Smear

The gold standard of cervical cancer detection is a test called the Pap smear. This technique was newly devised by Papanicolauo and Traut in 1943 and enables to detect precursor intraepithelial lesions of the cervix efficiently. This method revolutionarized the public health sector for being one of the most ever successful forms of cancer prevention.

The test itself is performed using either a spatula or a brush and requires the physician to collect approximately 500,000 cervical epithelial cells. These cells are then placed on a glass slide and checked under a microscope for abnormalities using selective dyes that are retained by the cells. However, the test should not be performed during menstruation as this can have fluctuating results. The determined age that a woman should first undergo a Pap smear varies from approximately 20-25 years old and continues every 2-3 years until approximately 60 years of age, but if abnormal lesions have been found to be present, check-up appointments should be made on a more frequent basis (Strander, 2009). In some populations there are reports documenting a 99% reduction in the incidence of cervical cancer, thus demonstrating the true value of the Pap smear (DeMay, 2007). Sadly, in today's society, the majority of women who do develop cervical cancer, are women who did not undergo regular Pap smear check-ups or women screened positively for the presence of dysplasic cells that did not return for a follow-up appointment (Misra *et al.,* 2004).

Unfortunately, one existing limitation of using the Pap smear to diagnose cervical cancer is its availability, or lack of it in non-westernised countries. Screening programmes in the USA have been highly successful with a reported 2-3 million abnormal Pap smears being recorded each year (http://www.emedicinehealth.com/pap_smear/article_em.htm). Although these figures are extremely encouraging, many countries in the developing world such as South Africa and other Sub-Saharan African countries do not have ample resources and cannot afford to implement an efficient screening programme and as a result, the incidence levels of the disease in these countries remain rather high in comparison. Therefore, further research is required to produce economically viable testing that is accurate and

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can be easily put into practice to reduce the overall incidence of cervical cancer worldwide.

While accurate diagnosis of cervical cancer is often made on account of performing a Pap smear or by visual detection of a lesion, this format of cytological screening fails to detect all precursor lesions and many results can turn out to be falsely negative in the presence of invasive cancer (Misra *et al.,* 2004). As a result, cone biopsy samples can be taken from suspicious lesions to evaluate the possibility of microinvasion and if already invaded, determine the scale. This is confirmed histopathologically and despite the great success with which the Pap smear has reduced the incidence of cervical cancer, cone biopsies are able to provide a more accurate assessment on the extent of damage any precursor lesion may have caused.

1.4.2 Histopathological Classification of Cervical Cancer

Cervical cancer is classified by histological diagnosis as either squamous cell carcinomas which arises from the ectocervix and accounts for approximately 60-80% of all cases, or adenocarcinoma which arises from the endocervical canal and accounts for 10-20% of cervical cancers (Blake, 1995; Leung *et al.*, 2004). A representative image displaying the position of the ectocervix and endocervix are illustrated in Figure 1.2 (Hladik and McElrath, 2008). Other types of cervical cancer exist such as adenosquamous carcinoma, a combination of both of the aforementioned, and adenoid basal cell carcinoma, but the incidence rate of these cervical cancers is very low (Scully *et al.*, 1994).

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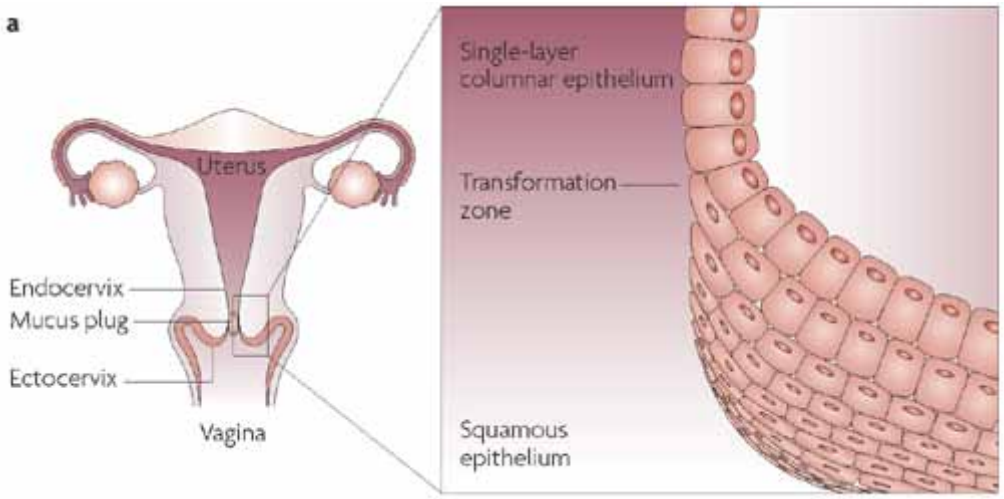

Figure 1.2: Schematic representation of the ectocervix, endocervix and the transformation zone where cervical neoplasia is initiated (diagram taken from Hladik and McElrath, 2008).

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A large degree of controversy still surrounds the histopathological classification of cervical cancer precursors as the World Health Organisation (WHO) classification system uses the terminology 'dysplasia' and 'carcinoma *in situ*' to refer to precursor lesions. During the 1960's, the term cervical intraepithelial neoplasia (CIN) was introduced by Richart and the nomenclature CIN grades I to III are still used today to describe pre-invasive epithelial lesions or various categories of dysplasia and carcinoma *in situ* (Richart, 1990; Wright, 2003). CIN grade I is equivalent to mild dysplasia in which undifferentiated cells occupy approximately the external/outer third portion of the epithelium layer. CIN grade II is equivalent to moderate dysplasia where undifferentiated cells replaces two thirds of the thickness of the normal epithelium, whereas, CIN grade III denotes severe dysplasia or carcinoma *in situ*. Severe dysplasia describes a condition in which undifferentiated cells replace all but one or two of the most superficial cell layers of the cervical epithelium (Wright, 2003), although, all varieties of dysplasia are pre-invasive, meaning that the basement membrane (stromal epithelial junction) does not become breeched. The diagnosis of carcinoma *in situ* is made when undifferentiated cells replace the entire surface of the epithelium.

Another classification system which was implemented to help allot cytological diagnosis is called the Bethesda System (Luff, 1992). This classification structure

categorises squamous intra-epithelial lesions (SIL) as either low grade (LoSIL) or high grade (HiSIL). Mild dysplasia or CIN I fall into the low grade category whereas moderate to severe dysplasia, or CIN II and CIN III are designated to the high grade group. Despite both classification systems being widely accepted, there is currently no general consensus as to which terminology should be used because it often takes years for pathologists and clinicians to make a full transition to a new classification system without referencing terminology from a previous classification system.

Cancer of the cervix is graded on biopsy by histopathological examination, confirming the presence of invasive carcinoma and depth of invasion. Carcinoma is defined according to the International Federation of Obstetricians and Gynaecologists (FIGO) (Table 1.1). The further advanced the staging of the disease, the worse the prognosis is for the patient.

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Stage	**Description**
Stage 0	Carcinoma *in situ,* intraepithelial carcinoma
Stage I	Carcinoma, confined to the cervix
Stage IA	Pre-clinical carcinomas of the cervix (identified microscopically)
Stage 1A1	Evident stromal invasion ≤ 3mm in depth and < 7mm in width
Stage IA2	Evident stromal invasion > 3mm but < 5mm in depth and < 7mm in width
Stage IB	Preclinical lesions > stage IA
Stage IB1	Clinical lesions ≤ 4cm.
Stage IB2	Clinical lesions > 4cm.
Stage II	Carcinoma extend beyond cervix
Stage IIA	Carcinoma extends beyond cervix into upper 2/3 of the vagina
Stage IIB	Carcinoma extends to paracervical tissue
Stage III	Carcinoma extends to lower portion of the vagina or pelvic sidewell
Stage IIIA	Carcinoma extends into lower 1/3 of the vagina
Stage IIIB	Carcinoma extends to pelvic sidewall and/or
Stage IV	Carcinoma extends beyond the pelvic region
Stage IVA	Carcinoma extends into adjacent organs such as the bladder
Stage IVB	Carcinoma metastasises and spreads to distant organds

Table 1.1: FIGO staging for cervical cancers

1.5 Causes of Cervical Cancer

1.5.1 Human Papillomavirus Virus (HPV) as a Cauasative Agent for the Development of Cervical Cancer

Current evidence has highlighted human papilloma virus (HPV) as the main causative agent in developing cervical cancer with virtually all cases of the disease being attributed to HPV infections (Lowy *et al.,* 2008). HPVs comprise a group of more than 150 highly diverse non-enveloped DNA viruses that possess the ability to infect cutaneous and mucosal epithelia which results in the formation of benign epithelial proliferations at the site of infection (Lowy *et al.,* 1994; de Villiers *et al.,* 2004). The majority of HPV infections often go clinically unnoticed as no symptoms are present, making it very difficult or near impossible for the individual to realise they have contracted HPV (Stanley *et al.,* 2007).

More than 40 known HPV types infect the genital tract and approximately 15 to 18 of these have been earmarked as being oncogenic with the potential to cause malignant tumours in the cervix (Munoz *et al.*, 2004; Schiffman *et al.,* 2005; Allan *et al.,* 2008). HPV-6 and HPV-11 are regarded as low-risk infections and are responsible for causing the majority of cases of genital warts, although, many other low-risk types do not display any pathological signs that would be indicative of infection (Lowy *et al.,* 2008). The highest risk HPV types, namely 16, 18, 45 and 56 are those which cause the most invasive cancers as a result of persistent, long term infection and as well as leading to the development of cervical cancer, these HPV types can also cause anal, vulvar and penile cancers (Bosch *et al.,* 1995; Munoz *et al.,* 2006). Other high-risk genital cancer causing types which are known to exist include 33, 35, 39, 51, 52, 58 and 59 (Parkin 2006). However, despite these HPV types not being considered as the highest risk, HPV-33 and -35 are commonly found in South African women whom have tested positive for cervical cancer (Allan *et al.,* 2008; Cooper *et al.,* 1991) whereas HPV-58 was the most strongly associated type with cancer of the cervix in Senegalese women (Xi *et al.,* 2003). This evidence shows that the hierarchy of the types of HPV causing cervical cancer differs greatly and this variation in distribution highlights the difficulty faced in generating a suitable vaccine that can be widely used within sub-Saharan Africa.

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1.5.2 HPV-16 and HPV-18 in Cervical Cancer

Although many types of HPVs exist, HPV-16 is the most frequently identified in connection with cervical cancer and accounts for approximately 50% of all cases, with HPV-18 second most common displaying an incidence in the region of 20% (Lowy *et al.,* 2008). Furthermore, high risk HPV types, such as HPV-16 and HPV-18 have been shown to express oncoproteins E6 and E7 which are known to have a strong involvement in stimulating cellular transformation and cell proliferation (Munger *et al.,* 1989; Werness *et al.,* 1990). HPV types 33 and 35 which are prevalent in confirmed cases of cervical cancer in South African women are also known to express oncoproteins E6 and E7 (Holm *et al.,* 2008), however, as HPVs' 16 and 18 are predominantly responsible for the majority of cases worldwide, research that has been undertaken to investigate E6 and E7 has tended to focus on E6 and E7 encoded by HPV types 16 and 18. Two characteristic features of most cervical cancers that contain either HPV-16 or HPV-18 is the presence of viral DNA which has integrated within the host genome and more often than not, only the viral E6 and E7 genes are expressed with the other viral genes being mutated or completely deleted (zur Hausen, 2002; Howley and Lowy, 2007). In addition, HPV-16 E6 and E7 has been shown to induce cervical cancer in transgenic mice and stimulated PTGS-2 transcription in CaSki and SiHa cervical cancer cell lines (Song *et al.,* 2000; Subbaramaiah and Dannenburg, 2007).

It is now routinely accepted that high risk HPVs play a pivotal role in inducing the development of cervical carcinoma, predominantly through the action of viral oncoproteins E5, E6 and E7 (Lowy *et al.,* 2007; Lagunas-Martinez *et al.,* 2010). However, recently published data has revealed that following HPV infection, these viral oncoproteins employ strategies to modulate apoptosis. The E2 viral protein induces apoptosis through activation of caspase-8, the E5 protein can impair CD95L, the ligand of which belongs to the tumour necrosis factor protein family and binding to its receptor leads to the induction of programmed cell death, thus suggesting that it may prevent apoptosis at early stages of viral infection whereas E6 inhibits apoptosis through the proteolytic inactivation of pro-apoptotic proteins such as p53 or

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procaspase-8 and by blocking their functional actions on apoptosis, all may become useful targets for therapeutic strategies in the future (Lagunas-Martinez *et al.*, 2010).

Vaccines have recently been developed as a preventative measure against HPV-16 and HPV-18 induced cervical cancer but this is unlikely to be beneficial to the individuals already infected with oncogenic HPV (Lowy and Schiller, 2006) or individuals in sub-Saharan Africa who are geographically disadvantaged and statistically more likely to develop a different strain of HPV. Despite the strong evidence stressing the correlation between certain types of HPV and cervical cancer, not all women who have HPV infections develop cervical carcinoma which poses the likelihood that other factors are involved that would distinguish why one person develops the disease and the other does not.

1.5.3 Seminal Plasma as a Risk Factor Contributing to the Development of Cervical Cancer

In 1992, a report released by the World Health Organisation (WHO) described normal human semen as having a volume of 2ml or greater, pH of 7.2 to 8.0, sperm concentration of $20x10^6$ spermatozoa/ml or more, sperm count of $40x10^6$ spermatozoa per ejaculate or more, and motility of 50% or more with forward progression of 25% or more with rapid progression within 60 minutes of ejaculate (World Health Organisation 2003). The primary function of human semen or seminal plasma (SP) is to transport spermatozoa to the uterus after sexual intercourse (Aumuller and Riva, 1992). The journey through the female reproductive tract is unfavourable for sperm cells as the natural environment of the vagina is very acidic, viscous and defended by immune cells. SP is secreted by the accessory organs in the male reproductive tract that include the gonads, prostate gland, ejaculatory ducts, seminal vesicles and bulbourethral glands and helps promote the survival of the spermatozoa by providing a nutritive and protective alkaline-based environment, thus preventing the DNA contained within the sperm from being susceptible to acidic denaturation.

Human seminal plasma is regarded as a complex liquid, comprised of a vast diversity of antigenically distinct proteins that include cytokines, angiogenic factors,

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prostaglandins, proteases, signal transduction molecules, protein kinases, peptides, glycopeptides, transporter proteins, structural molecules and immune response proteins (Ness and Grainger, 2008; Fung *et al.,* 2003). The components contained within SP have been implicated in the suppression of many immune functions, including lymphocyte activation and proliferation, natural killer (NK) cell activity, complement action and delayed hypersensitivity reactions as well as exhibiting agonistic effects on sperm-egg binding and promotion of successful implantation of the human embryo (Imade *et al.,* 1997; Chalabi *et al.,* 2002).

Research performed by Gutsche and colleagues (2003) ascertained that seminal plasma can influence the expression of genes in endometrial epithelial and stromal cells by mediating the synthesis of pro-inflammatory mediators such as IL-1β and IL-8 whereas work carried out by Sharkey and colleagues (2007) demonstrated that inflammatory cytokine genes were the predominantly activated gene family in Ect1 ectocervical cells in response to SP stimulation and included IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and monocyte chemotactic protein-1 (MCP-1) with the ectocervix being implicated as the primary site of responsiveness. Several chemokine genes were also induced by SP, namely Gro-α, Gro-β and Gro-γ which encode chemokines targeting monocytes, neutrophils and lymphocytes via CXCR1, CXCR2 and CCR6 receptor signalling pathways (Sharkey *et al.,* 2007). Other studies supporting the upregulation of inflammatory genes in the female reproductive tract following exposure to SP have shown elevated IL1A, IL1B, IL-6 and IL-8 expression to occur in both mouse and pig uterus after insemination with induced synthesis of IL-6 and leukemia inhibitory factor (LIF) in epithelial cells from the uterine endometrium (Robertson *et al.,* 1992, 1996; O'Leary *et al.,* 2004; Gutsche *et al.,* 2003).

Factors that exist within SP which have been attributed as having influential immunosuppressive properties include soluble necrosis factor p55, tumour necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), complement inhibitors and pro-inflammatory cytokine IL-6 (Robertson *et al.,* 2002; Gutsche *et al.,* 2003; Kelly, 1995). Furthermore, SP is known to contain very high concentrations of the

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polyamines spermine and spermidine (Curry *et al.*, 1980; Allen and Roberts, 1986) and is also comprised of antigen-specific lymphocytes (T cells). The T cells are principal immune cells responsible for initiating and directing the development of antibodies and can also mediate antigen-specific cytoxicity within the female reproductive tract (Paul and Seder, 1994; Alexander and Anderson, 1987), although, it is the highly expressed levels of prostaglandins which have attracted much interest of late.

Prostaglandins perform several actions that are relevant to the control of cytokine release, cell growth, differentiation and changes in vasculature and are present at a 10,000 fold higher concentration in SP than at the site of inflammation, with PGE_2 being one of the predominant types that is detected (Templeton *et al.*, 1978; Sales *et al.*, 2002b). Previous studies employing human tissue and cell lines have shown that in human cervical and endometrial cancers, seminal plasma promotes expression of tumourigenic and angiogenic genes which possess the capability to activate intracellular signalling and modify cellular function via their actions on their respective receptors such as PTGER2 and PTGER4 (Sales *et al.*, 2002b; Jabbour and Sales, 2004; Muller *et al.*, 2006; Battersby *et al.*, 2007). Therefore, the experimental evidence suggests that prostaglandins contained within SP (such as PGE_2) operate on the female reproductive apparatus, thereby adjusting molecular mechanisms to favour conditions of uncontrolled growth in cervical cancer (Sales *et al.*, 2002b; Muller *et al.*, 2006; Battersby *et al.*, 2007). Furthermore, SP induction of PTGS-2 activity and PGE_2 synthesis in cervical tissue would be expected to further amplify the immune regulatory effects of seminal plasma PGE_2 (Sharkey *et al.*, 2007).

SP is known to contain the protein transforming growth factor-β (TGF-β) in great abundance with concentrations being similar to that of colostrum (approximately 70 ng/ml) which is the most potent known biological source of TGF-β (Robertson *et al.*, 2002). The mammalian isoforms that exist (namely TGF-β1, TGF-β2 and TGF-β3) are encoded by individual genes with distinct promoters but share a 74% amino acid sequence homology and structural identity (Robertson *et al.*, 2002). TGF-β glycoproteins are comprised of over thirty distinct sequence-related growth and differentiation factors including activins and bone morphogenic proteins (BMPs)

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(Lawrence, 1996; Massague *et al.*, 2000). This cytokine is responsible for orchestrating cellular proliferation and differentiation and is deemed as a principal trigger for the induction of uterine inflammatory responses following mating in mice (Jakowlew, 2006; Tremellen *et al.*, 1998). Furthermore, at insemination when seminal TGF-β is deposited in the female tract, factors such as acidic vaginal pH and enzymes of male or female tract origin can participate in TGF-β activation and further promote its immune deviating properties (Robertson *et al.*, 2002).

Tumour cells often display elevated expression of TGF-ß and a supplementary response to exogenous TGF-ß (via exposure to SP), may provide some cervical epithelial cells with a growth advantage that ultimately leads to malignant expression (Jakowlew 2006). Studies employing athymic mice have shown that TGF-β1 can increase tumorigenecity of human breast cells (Arteaga *et al.*, 1993) and promote proliferation and transformation in mammary adenocarcinoma cells (Erdogan *et al.*, 2008), thus validating that exogenous TGF-β can increase tumor growth *in vivo*. PGE_2 and IL-8 that are also contained within SP can synergise with TGF-β in targeting female tract cells by inducing IL-1β, IL-6 and leukemia inhibitory factor (LIF) from endometrial epithelial cells (Gutsche *et al.*, 2003), while thrombospondin-1 and integrins αvβ6 and αvβ8 which reside on the epithelial cell surface in the female tract can bind with TGF-β to cause conformational cellular changes (Schultz-Cherry and Murphy-Ulrich 1993; Huang *et al.*, 1996; Bader *et al.*, 1998).

As mentioned previously in section 1.5.2., high-risk HPVs are closely associated with the development of cervical cancer and although infection is known to be sexually transmitted, the exact mechanism by which sexual contact promotes virus infection is not well understood (Perez-Andino *et al.*, 2009). Current evidence suggests that the transmission of HPV infection occurs through areas of epidermis which are vulnerable and thin, such as the transformation zone of the cervix or anus, or through micro-abrasions in the epithelium that are created during sexual activity (Frazer *et al.*, 2006). Once the virus enters the actively-dividing cells of the basal membrane, it ‘hijacks’ the cellular resources in order to replicate its own genetic material and express HPV proteins (Frazer *et al.*, 2006). However, there is also evidence to suggest that HPV can

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be transferred during oral sex as HPV has been detected in non-genital areas such as the mouth, oropharnyx and conjunctiva (Cason, 1996). These data are partly supported in work recently published by D'Souza and colleagues (2007) who demonstrated that HPV infected people whom were involved with more than five oral-sex partners had a 250% increased risk of developing HPV-related throat cancers, whereas people exhibiting HPV-16 and having more than five oral-sex partners were 750% more at risk of developing HPV-related throat cancers. One possible explanation is that because sperm cells need to be extremely motile for successful passage in reaching the uterus, they are somehow exploited by the HPVs to serve as vehicles that propagate virus dispersal (Perez-Andino *et al.*, 2009) and therefore have the potential to further exacerbate local pathologies not only in the cervix but also in the mouth, throat and anus.

In addition to the possible role SP may have as a vehicle for the spread of HPV, there is much evidence to suggest a direct role for SP in facilitating cervical tumourigienesis. Following exposure of the cervix to SP and infection by HPV, neoplastic cervical epithelial cells may be regulated by inflammatory factors present in SP. In addition, to prostaglandins and TGF-β, there are many other supplementary components contained within the ejaculate at sufficient concentrations to exhibit immunosuppressive properties. These include; tumor necrosis factor (TNF-α), VEGF, IL-1β, IL-6, leukemia inhibitory factor (LIF) and complement inhibitors (Liabakk *et al.*, 1993; Kelly *et al.*, 1995; Gutsche *et al.*, 2003). Thus it is likely that repeated exposure of the cervix to SP can promote the transmission of multiple isotopes of HPV as well as creating an inflammatory environment to promote cervical cancer.

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1.5.4 Other Risk Factors Contributing to the Development of Cervical Cancer

As high-risk HPVs are contracted sexually, having multiple sexual partners whereby the cervical neoplastic cells are exposed to a wide variety of SP would theoretically elevate the risk of persistent HPV infection or progression to invasive cancer (Castellsague *et al.*, 2002). However, other etiologic factors are also believed to be associated with contributing towards the emergence of HPV or cervical carcinoma and include; consenting to sex at an early age (<16 years), smoking, intact foreskin,

long term use of contraceptive pills, weakened immune system and human immunodeficiency virus (Mayeaux, 2007; Rent *et al.,* 1972; Palefsky and Holly, 2003; Appleby *et al.,* 2006; International Collaboration of Epidemiological Studies of Cervical Cancer 2007). Furthermore, it must be noted that women who refrain from sexual intercourse don't develop cervical cancer. A study performed by Ho and colleagues (1998) found HPV infection to be present in over 40% of young, sexually active college students and given that the onset of cervical cancer in this faction was somewhat uncommon in comparison to the incidence of viral infection, it is hypothesized that alternative biological pathways and additional causative factors other than HPV infection must be responsible for the transition of precursor lesions into the onset of cervical cancer (Ho *et al.,* 1998; Chan *et al.,* 2003; Schiffman and Brinton, 1995).

1.5.5 Current and Future Approaches to Combat Cervical Cancer

Cervical cytology screening remains the principle tool for detecting precursor intraepithelial lesions of the cervix, therefore allowing proficient administration of medical treatment and deterring from any possible onset of cervical cancer. Unfortunately, cytology screening programmes do not help woman who already have rapidly developing carcinoma of the cervix. Screening is also not readily available in developing countries and these happen to contain the populations which display the highest incidence rates, thus relying more heavily on the implementation of regular checks to help keep the prevalence of cervical cancer from escalating further (Sitas *et al.,* 1997). It is reported that 80% of cervical cancer cases and its related mortality occur in less resourceful countries (Parkin, 2006). Improvements in slide preparation and interpretation have also increased screening efficiency (Biscotti *et al.,* 2005), but regrettably screening is very costly. As a result, many new diagnostic approaches are being tested that are aimed at halting the onset of cervical carcinoma and improving the clinical outcome of patients unlucky to contract the disease.

At present, if HPV infection or any other causative anomalies has resulted in the development of invasive cervical cancer, radical hysterectomy and radiation therapy pose as the best advised forms of treatment. The success rates vary with a recurrence

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rate of approximately 10-20% reported in stage 1B-11A cervical carcinomas whereas patients with more advanced tumour can expect a relapse rate of anything as high as 70% and above (Podsypanina *et al.*, 2008). Chemotherapy is used less frequently and only tends to be employed when cancer growth has rapidly progressed and rendered surgery or radiation treatment as impractical options.

New intervention strategies being investigated to combat cervical cancer include the development of preventive HPV vaccines to halt commencement of the disease. The two commercial versions are called Cervavix which is manufactured by GlaxoSmithKline (GSK) and Gardasil which is produced by Merck. Cervavix contains the antigens for HPV-16 and HPV-18 which are derived from the L1 capsid protein from each respective HPV (Ruutu *et al.*, 2008), whereas Gardasil, also works against these HPVs, but in addition, protects against HPV-6 and HPV-11 (Paavonen *et al.*, 2007; Garland *et al.*, 2007). By vaccinating with these antigens, the recipient will then start producing neutralizing antibodies against the viral protein coat in order to oppose the respective virus. The vaccines contain no live virus or DNA and cannot infect the patient but both are fairly expensive and administered in three intramuscular injections over a 6-month period. Although the efficacy of both vaccines in disease prevention is unparalleled by any other vaccines, neither Cervavix nor Gardasil could provoke the regression of existing intraepithelial cervical lesions (Kirnbauer *et al.*, 1996). At present there is no commercial vaccine available that protects against HPV-33, HPV-35 and HPV-58 and because these virus types are of a high incidence rate in sub-Saharan Africa (Allan *et al.*, 2008), the introduction of Cervavix or Gardasil in that region would not be as effective.

Another form of cervical cancer prevention that has come to the fore in recent years is the identification of biological markers. The ability to highlight chemical indicators which are altered when cellular conditions are being primed for the development of cervical cancer would be hugely valuably, as essentially, the cancer could be halted before gaining the opportunity to initiate itself in the first place. Markers currently under the spotlight consist of p53, Ki-67 and CD31 as the expression of all three has been shown to be significantly upregulated in squamous cell carcinomas when

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compared to normal cervical samples (Silva-Filho *et al.*, 2004). Although the increased levels of these markers only serve as having a small prognostic benefit with the higher values being used to gauge how far the disease has progressed, it is hoped in the future that a simple and cheap test could be devised whereby the increase of a single gene or protein would allow quick, appropriate intervention. Another prognostic marker that has recently been investigated was shown in research performed by Leung and colleagues (2004) who tried forecasting the prognosis in cervical adenocarcinoma patients through evaluation of the mitotic index (MI) and the apoptotic index (AI), and comparing the ratio of the two indices (MI/AI) by means of examining cell morphology in adenocarinoma and normal cervical samples. The study revealed that the two indices closely correlated with tumour stage and may potentially provide an economically viable approach to predicting clinical outcome of patients with cervical adenocarcinoma (Leung *et al.*, 2004).

Despite the introduction of screening programmes, the development of preventative vaccines and increased use of biological markers as indicators of cervical cancer, there is still no intervention programme suitable to women in sub-Saharan Africa.

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1.6 Arachidonic Acid Metabolism

Arachidonic acid (AA) is an essential fatty acid that is derived from either the diet or by desaturation and elongation of dietary linoleic acid. The arachidonic acid cascade generates a family of bioactive lipids, collectively referred to as eicosanoids, and includes prostaglandins, thromboxanes and leukotrines which are responsible for modulating diverse physiological and pathophysiological responses in the female reproductive tract (Marnett, 1992).

The kinetics of the prostaglandin biosynthetic pathway is driven by the availability of free arachidonic acid. Following the activation of phospholipase A2 (PLA2), AA is released from plasma membrane phospholipids or dietary fatty acids, and is cyclized, oxygenated and reduced to the intermediary prostaglandin PGH_2 (Jabbour and Sales, 2004). This process is mediated by the rate limiting enzyme COX, also referred to as

prostaglandin-endoperoxide synthase (PTGS), with the addition of the 15-hydroperoxy group subsequently allowing the newly formed PGH_2 to serve as a substrate for terminal prostanoid synthase enzymes (Martnett, 1992) (Figure 1.3). These enzymes are named according to the prostaglandin they produce such that D_2 (PGD_2) is synthesized by prostaglandin-D-synthase (PGDS), prostaglandin E_2 (PGE_2) by prostaglandin-E-synthase (PGES), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) by prostaglandin-F-synthase (PGFS), prostacyclin by prostaglandin-I-synthase (PGIS) and thromboxane by thromboxane synthase (TXS) (Narumiya *et al.*, 1999). Following biosynthesis, prostanoids are transported out of the cell via a prostaglandin transporter (PGT) where they exert their biological function through G-protein coupled receptors (GPCRs), consequently establishing an autocrine/paracrine effect on target cells (Chan *et al.*, 1998).

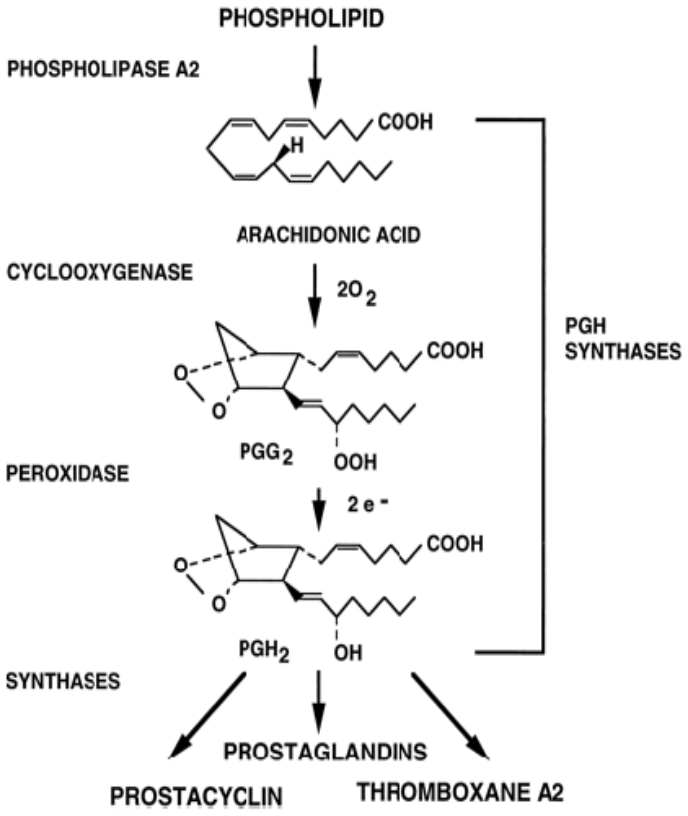

Figure 1.3: The arachidonic acid (AA) is hydrolyzed and released from the membrane triglycerides by phospholipase A_2 and is subsequently oxidized and reduced to PGH_2 by COX. PGH_2 then serves as a substrate for terminal prostanoid synthase enzymes to produce specific prostanoids (prostaglandins, prostacyclins and thromboxanes) (diagram taken from Narumiya *et al.*, 1999*).*

1.6.1 PTGS Enzymes

At present, there are three isoforms of PTGS which have been identified; PTGS-1, PTGS-2 and PTGS-3, termed according to their order of discovery (Morita 2002). PTGS-1 is ubiquitously expressed and believed to have a regulatory role by performing normal physiological functions, although more recently it has been shown to be upregulated in various carcinomas and have a central involvement in tumourigenesis (Sales *et al.,* 2002a; Narko *et al.,* 1997). PTGS-2 is regarded as an immediate early gene which can be rapidly induced by growth factors, oncogenes, carcinogens and tumour-promoting phorbol esters (Morita, 2002). A functional role for PTGS-3 in human physiology and pathophysiology remains to be established (Jabbour and Sales 2004).

1.6.1.1 PTGS-1

The human PTGS-1 or COX-1 gene was first cloned in 1998 and is approximately 22 kb in size (Tanabe and Tohnai, 2002). This isoform of the PTGS enzyme has a molecular mass of 70 kDa and is encoded by 2.8 kb mRNA transcribed from a gene located on chromosome 9. PTGS-1 is constitutively expressed in most cells but can be induced *in vitro* by VEGF, AA, cAMP and PGE_2 (Sales *et al.,* 2002a). The highest levels of PTGS-1 have been noted in vascular endothelial cells, platelets and collecting tubules of smooth muscle whereby the chief function of this enzyme is to manufacture PGs for homeostatic regulation (DuBois *et al.,* 1998). Several roles which demonstrate the indispensable involvement of PTGS-1 in maintaining homeostasis are; the mediation of prostaglandin dependent gastric protection as PTGS-1 is seen to be highly expressed in normal human gastric mucosa, regulation of kidney function through production of prostaglandins that help maintain the blood flow and also partaking in the regulation of blood pressure (Jackson *et al.*. 2000; Athirakul *et al.,* 2001).

Moreover, PTGS-1-derived prostaglandins are important for uterine cellular differentiation and proliferation, and for uterine edema and luminal closure (Langenbach *et al.,* 1995). Supplementary evidence of PTGS-1 having a part to play in the reproductive tract was displayed in data generated by Toth and colleagues (1996)

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whereby treatment of human amnion cells by human chorionic gonadtrophin (hCG) induced PTGS-1 mRNA and protein expression. This suggests that tightly regulated PTGS-1 could play a part in sustaining a healthy pregnancy.

1.6.1.2 PTGS-2

The human PTGS-2 gene is substantially smaller than PTGS-1, it is approximately 8 kb in size and located on chromosome 1 (Fletcher *et al.,* 1992). The PTGS-2 gene is transcribed as 2.8, 4.0 and 4.6 kb splice variants whilst the protein product exhibits a molecular mass of 70kDa, similar to the size of PTGS-1 (Tannabe and Tohnai, 2002). A unique difference between PTGS-1 and PTGS-2 is 18 amino acids inserted 6 residues upstream from the C terminus of PTGS-2 but an exact function for this additional structure is not yet established (Smith *et al.,* 2000). Unlike PTGS-1, PTGS-2 is undetectable in most normal tissue but can be rapidly induced by oncogenes, growth factors, cytokines, tumour promoters and during inflammatory states, including situations such as increased cell replication (DuBois *et al.,* 1994; Inoue *et al.,* 1995; Subbaramaiah *et al.,* 1996; Smith *et al.,* 2000). Moreover, PTGS-1 and PTGS-2 expressed in certain cell types, utilize AA that is generated by different phospholipases, demonstrating further that the two isoforms are involved in different signalling pathways (Langenbach *et al.,* 1999; Smith *et al.,* 1993).

Despite the tendency of PTGS-2 expression to only be induced under imposing circumstances such as infection and the inflammatory response, this gene does participate in performing regulatory biological processes. These include immunity, renal physiology, neurotransmission, bone reabsorption and pancreatic secretion (Rocca *et al.,* 1999; Cheng *et al.,* 1999; Breder *et al.,* 1995; Pilbeam *et al.,* 1997; Robertson, 1998). PTGS-2 has also been shown to be central in reproductive function as studies using mice that are deficient in PTGS-2 display multiple reproduction failures in ovulation, fertilization, implantation and decidualization, confirming that PGs produced by PTGS-2 are vital in these processes (Lim *et al.,* 1997). In the rat during pregnancy, both PTGS-1 and PTGS-2 are upregulated with a dramatic increase

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of 250-280% during parturition, further stressing the importance of PTGS enzyme activity (Dong *et al.*, 1996).

1.6.1.3 PTGS-3

PTGS-3 is a splice variant of PTGS-1, approximately 5.2 Kb in size and was discovered by Northern analysis of canine cerebral cortex RNA using a COX-1 cDNA probe. PTGS-3 retains intron 1 of the PTGS-1 transcript and it is possible that this decreases its enzymatic potential to generate PGE_2 (Chandrasekharan *et al.*, 2002). The mRNA expression of PTGS-3 is present in the celebral cortex and heart of humans and its activity is also well documented in canine brain homogenates through its inhibition by acetaminophen. PTGS-3 encodes proteins with relatively similar amino acid sequences to PTGS-1 as PTGS-1 only differs by having an extra 11 amino acids encoded by intron 1 (Kis *et al.*, 2005). Although the significant relevance of PTGS-3 remains to be determined, PTGS-3 is dramatically inhibited by acetaminophen (paracetamol), arguably the most widely used analgesic drug in the world and therefore clinical ramifications still remain a possibility for the involvement of PTGS-3 in prostaglandin-mediated fever and pain (Botting *et al.*, 2003).

1.6.2 The Role of PTGS Enzymes in Carcinogenesis

The over-expression of PTGS-1 is fast becoming regarded as a significant event in numerous pathologies despite normally being looked upon as a 'housekeeping gene' (Gupta *et al.*, 2003). Enhanced expression of PTGS-1 has been demonstrated in human breast tissue, human prostate carcinoma and mouse lung tumours of late, therefore suggesting that PTGS-1 enzymes and its products may function in promoting and maintaining the neoplastic state (Sales *et al.*, 2002a). Previous studies undertaken in our laboratory have shown that biosynthesis of PTGS-1 and PTGS-2 are upregulated in cancers of the cervix and endometrium and inhibitors of PTGS enzymes may form an option to treat carcinomas that produce overly high levels of PTGS enzymes (Jabbour and Sales, 2004; Subbaramaiah *et al.*, 1997).

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Another cancer which is demonstrating a close association with PTGS-1 is ovarian cancer. Data published from a study carried out by Lee and colleagues (1995) illustrated that serum levels of PTGS-1 rapidly declined in greater than 50% of patients following surgery to remove ovarian cancer, raising the possibility of PTGS-1 becoming a suitable marker for the monitoring and diagnosis of ovarian cancer patients in the future (Lee and Ng, 1995). Complementary findings released by Daikoku and colleagues (2005) showed the presence of PTGS-1 to be prominent in mouse ovarian surface epithelial (OSE) cells and when an inhibitor that is specific for PTGS-1 is applied (SC-560), a decrease in cellular proliferation and increase in apoptosis was observed.

Reports published from numerous *in vivo* studies have revealed more than a few interesting findings; overexpression of PTGS-1 in immortalized ECV endothelial cells can promote tumourigenesis in athymic nude mice and prostanoids derived from high levels of PTGS have been show to modulate the hepatic vascular tone of cirrhotic rat livers (Narko *et al.,* 1997; Graupera *et al.,* 2004). Furthermore, deficiency of PTGS-1 has been shown to reduce skin tumourigenesis in multistage skin model mice by 75% and also substantially reduce intestinal tumourigenesis in knockout mice with ablation of the PTGS-1 gene (Tiano *et al.,* 2002; Chuluda *et al.,* 2000). Yet another discovery to surface from these studies was that PTGS-2 is believed to compensate for the loss of PTGS-1 when it is genotypically removed and this may help explain why fertility and litter size are normal in PTGS-1 deficient mice (Reese *et al.,* 1999).

PTGS-2 has long been reputed for its induction in the inflammatory response but there is a rapidly increasing body of evidence which supports PTGS-2 as playing a major role in cancer development (Murakami and Kudo, 2004). Several cancers that have been presented with high expression levels of PTGS-2 include the colon, lung, breast, pancreatic and gastric (DuBois *et al.,* 1998; Wang *et al.,* 2007). Statistically, the elevated expression of PTGS-2 in human colon cancers is particularly incriminating with greater than 80% of sufferers displaying significantly increased levels of PTGS-2 when compared to normal adjacent tissue (Williams *et al.,* 1997).

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PTGS-2 regulates numerous effector molecules such as cytokines, oncogenes and growth factors, all of which are involved in cancer. PTGS-2 upregulation and subsequent increase of PGE_2 biosynthesis induces malignant change in epithelial cells through immunosuppression (DeWitt, 1991), inhibition of apoptosis (Tsujii and DuBois, 1995), increase in metastatic potential of epithelial cells (Tsujii *et al.,* 1997) and promotion of angiogenesis (Jones *et al.* 1999). In summary, the contribution of PTGS-2 in cancer development is thought to mainly occur through three processes; (i) development of malignancy, (ii) anti-apoptosis, and (iii) increased angiogenesis by induction of vascular endothelial growth factor as tumour growth requires the maintenance of a vascular network (Murakami and Kudo, 2004; Williams *et al.* 1999).

Sheng and colleagues (1997) reported a 90% inhibition of growth in HCA-7 tumour cells that positively expressed PTGS-2 following treatment with a PTGS-2 specific inhibitor while Tsuji and colleagues (1997) displayed that PTGS-2 can modulate production of angiogenic factors in colon cancer cells. Other authors have revealed that PTGS-2 is present in approximately 40% of human invasive breast cancers, (Singh *et al.,* 2007; Daniel *et al.,* 1999) and using the nonsteroidal anti-inflammatory drug (NSAID) celecoxib to treat breast cancer cell xenografts *in vivo* showed the rate of angiogenesis and growth of secondary bone tumours to be significantly suppressed (Klenke *et al.,* 2006). This raises further evidence of PTGS-2 playing a role in the growth of certain cancer cells due to its ability to act as a tumour promoter via stimulation of angiogenesis (Tsuji *et al.,* 1998).

Data published by our laboratory have demonstrated that seminal plasma can activate PTGS-2 in cervical and endometrial carcinoma cells (Sales *et al.,* 2002b; Battersby *et al.,* 2007). The consequence of PTGS-2 upregulation is elevated prostanoid receptor signalling which induces the activation of multiple effector signalling pathways that includes MAPK, EGFR and cAMP accumulation. This signalling is strongly involved in many cellular functions such as growth, differentiation and transformation as well as participating in promoting the release of the proangiogenic gene VEGF (Sales *et al.,* 2002b; Muller *et al.,* 2006; Battersby *et al.,* 2007). It is therefore possible to suggest

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that SP can promote cervical and endometrial tumourigenesis by inducing upregulation of PTGS-2 and the products it helps to synthesize.

In 1990, Folkmann hypothesized that tumour growth was dependent on angiogenesis (Folkman, 1990) and because any resulting increase in tumour mass must be preceded by an increase in vascular supply, the ability of tumour cells to induce angiogenesis is crucial (Tsujii *et al.,* 1998). Studies highlighting the role of PTGS-2 in various model systems and the ever increasing use of NSAIDs to inhibit PTGS enzymatic activity have provided ample evidence to confirm PTGS-2 as a strong component in promoting angiogenesis and other pro-tumourigenic outcomes (Tsujii *et al.,* 1998; Seed *et al.,* 1997).

The exact contributions of PTGS-1 and/or PTGS-2-derived products (including PGE_2) in mediating events associated with cervical neoplasia remain to be completely elucidated but upregulation of PTGS-2, induced by seminal plasma, certainly appears to be of central involvement (Sales *et al.,* 2002b; Muller *et al.,* 2006). Overall, PTGS-1 and PTGS-2 are seen to be pivotal in many events associated with the development of many cancers and characterizing the pathological consequence of each gene could be of vital importance in developing future therapeutic strategies. PTGS-1 and PTGS-2 even act in concert in some diseases, like in the instance of cholangiocarcinogenesis where malignant tumours form on the epitheilail cells lining the biliary ductsand aberrant PTGS-2 expression occurs in the early stages of the disease whilst PTGS-1 over expression is associated with the later stages and again illustrates how important it is to fully characterize the roles of each gene in determining the true effect in cervical carcinogenesis and other cancers (Chariyalertsak *et al.,* 2001).

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1.6.3 Inhibition of PTGS Enzymes

Probably the strongest indicator to highlight the important role of PTGS-1 and PTGS-2 in pathophysiological and disease states is the use of NSAIDs in a wide variety of clinical contexts (Herschman *et al.,* 1996). The observation that PTGS enzymes was the pharmaceutical target of NSAIDs was first reported by Vane in 1971 and the

majority of the current knowledge we now possess with regard to the functional physiological roles of PTGS-1, PTGS-2 and the PGs they produce stems from inhibitory studies performed with NSAIDs (Vane *et al.*, 1971).

The most familiarly known NSAIDs are aspirin, indomethacin, ibuprofen, piroxicam and sulindac. Aspirin is a competitive inhibitor and also covalently modifies the PGH synthase protein whereas indomethacin, ibuprofen, piroxicam and sulindac are competitive inhibitors that do not bind covalently to the protein (DeWitt *et al.,* 1990; Lands and Hanel 1983; Roth and Majerus 1975; Roth *et al.,* 1975; Rome *et al.,* 1976; Van Der Ouderaa *et al.,* 1980; Rome *et al.,* 1975). Aspirin has been shown to be extremely successful in combating cardiovascular disease by means of blocking the substrate binding site to which arachidonic acid attaches, thereby inhibiting cyclooxygenase activity (Marnett, 1992). The use of Aspirin can also significantly decrease the risk of colon cancer (Rosenberg *et al.*, 1991), although a study performed by Paganini-Hill and colleagues (1991) revealed that excess consumption of aspirin actually enhanced the incidence of colon cancer by up to 50% and is an example where the axiom "if a little works, a lot works better" does not apply (Marnett, 1992).

Another of the better known NSAIDs is called sulindac and administration of this anti-inflammatory is known to reduce the size and number of rectal polyps in individuals with familial polyposis (FAP) or who have undergone subtotal colectomy and ileoproctostomy (Waddell and Loughry, 1983). The same authors also reported sulindac-induced regression of tumours in affected individuals who had no previous surgical treatment (Waddell and Loughry, 1983).

The foundation on which NSAID therapy is based is attributed to inhibiting PTGS-1 which is predominantly expressed in the gastrointestinal tract, thus making it an easy to reach target for oral administration (Smith *et al.,* 2000). NSAIDs which selectively inhibit PTGS-2, such as NS-398, do not bare any significant inhibitory properties towards PTGS-1 but do considerably halt the production of inflammatory prostaglandins generated by PTGS-2 biosynthesis (Smith *et al.,* 2000).

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At the cellular level, PTGS inhibitors have been shown to inhibit proliferation, induce apoptosis, inhibit angiogenesis, reduce carcinogen activation, and stimulate the immune system (Dannenburg *et al.,* 2001; Gupta and DuBois, 2001). Although reductions in prostanoid concentrations may underlie these observed activities, non-PTGS targets and mechanisms may be involved as well. For example, non-selective PTGS inhibitors have been shown to modulate levels of cGMP (guanosine 3`, 5`-cyclic monophosphate), NF-κB (nuclear factor-κB) activation, Bcl expression, and the binding of peroxisome proliferator-activated receptors (PPARs). Additional support for non-PTGS involvement is fuelled by the observation that the anti-neoplastic effectiveness *in vitro* has most often been described for concentrations of PTGS inhibitors which typically exceed those that may be clinically attainable (Hawk *et al.*, 2002).

Despite some conflicting data regarding the 'true' functioning of NSAIDs, they are still widely used today and understanding the mechanisms by which they are involved is made complicated by the multistage nature of the carcinogenic process (Marnett, 1992). Using aspirin and other NSAIDs to treat cardiovascular disease and colon cancer is now seen as a well-accepted therapeutic protocol (Reddy *et al.*, 1992; Marnett, 1992). Studies which have examined cellular responses towards exogenously added prostanoids and employed the use of NSAIDs demonstrated that prostanoids are likely to be of key significance in processes such as normal hemostasis, modulator of kidney function by controlling sodium excretion and water clearance, platelet aggregation, pain, respiratory function and fever generation (Smith and Marnett, 1991; DeWitt, 1991).

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1.6.4 Regulation of PTGS Enzymes

The PTGS-1 enzymes are expressed constitutively in the majority of cells and tissues with the highest levels of expression being noted in endothelium, monocytes, platelets, renal collecting tubules and seminal vesicles (Smith *et al.*, 2000). PTGS-1 is also believed to be developmentally regulated due to the fact that its expression increases in cells undergoing differentiation (Smith and DeWitt *et al.*, 1996). Transcriptional regulation of PTGS-1 has been challenging to study because

expression levels of this enzyme does not vary greatly in adult animals, however, relative success has been reported in utilizing endothelial cells which ubiquitously express PTGS-1 in high concentrations (DeWitt *et al.,* 1983). These studies revealed that PTGS-1 has a TATA-less promoter which contains multiple sites for transcription (Kraemer *et al.,* 1992; Xu *et al.,* 1997). Furthermore, PTGS-1 expression is deemed to be regulated by the Sp1 transcription factor at two binding sites with deletion of either site resulting in approximately 50% reduction in basal levels of PTGS-1 (Smith *et al.,* 2000).

Unlike PTGS-1, transcriptional activation of PTGS-2 is rapid and transient in response to a wide range of stimuli, including pathogens, cytokines, nitric oxide, irradiation, growth factors and various extracellular ligands (Tsatsanis *et al.,* 2006; Kang *et al.,* 2007). Cross-talk between protein kinase cascades and transcriptional activation of PTGS-2 is also well documented (Harper and Tyson-Capper, 2008), and taken as a whole suggests that the expression of PTGS-2 in many cell types appears to be sensitive to stimuli that regulate the unique physiological activities of each tissue (Smith *et al.,* 2000).

Although PTGS-2 is central to many biological processes, the activation of this enzyme is more commonly associated with its marked response toward growth factors and mediators of inflammation such as IL-1 and tumour necrosis factor-α (TNF-α) (Smith *et al.,* 2000). Signalling pathways concerned with the upregulation of PGTS-2 include NF-κB and C/EBP which are common to the inflammatory response (Poli, 1998; Ghosh *et al.,* 1998) and mitogen-activated protein kinase (MAPK) signalling cascades which are frequently operated by growth factors and oncogenes and include ERK1/2, JNK/SAPK, and p38/RK/MpK2 (Su and Karin, 1996). MAPK signalling cascades can also be activated when oncogenes v-ras and v-src (Su and Karin, 1996), inflammatory stimuli including IL-1β, TNF-α, LPS and phorbol ester TPA stimulate the expression of PTGS-2 (Subbaramaiah *et al.,* 1998; Wallach *et al.,* 1999) whereas the NF-κB signalling pathways commence their operation when PTGS-2 is stimulated by the likes of TNF-α, hypoxia, endothelin, IL-1β in osteoblastic cells and lipopolysaccharides (LPS) (Ghosh *et al.,* 1998; Yamamoto *et al.,* 1995).

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The transcriptional events of PTGS-2 are regulated at the 5-UTR (untranslated region) of the gene where binding sites are contained for numerous regulatory transcription factors that includes; two nuclear factor κB (NF -κB) motifs, two activator protein 1 (AP1) sites and two cAMP-response elements (CREs) among others (Appleby *et al.*, 1994). Transcription factors bind to these sites in a variety of combinations depending on cell type and also which regulatory pathway has been activated (Harper and Tyson-Capper, 2008). The regulation of PTGS-2 has also been reported to occur at a post-transcriptional level through changes in mRNA stability at the 3-UTR (untranslated region). This region of the PTGS-2 gene is complex and contains multiple copies of AU-rich elements that influence translational efficiency and mRNA degradation and stabilization of PTGS-2 (Dixon *et al.*, 2000).

Translation activation factors for PTGS-2 enzymes, mechanisms that regulate PTGS-2 expression and signalling pathways that are activated as a result of PTGS-2 upregulation are numerous and sometimes converge. Current data demonstrates the functional importance that translational events and mRNA alteration has in controlling PTGS-2 gene expression and its dysregulation can result in over-expression of PTGS-2 and the production of angiogenic factors detected in neoplasia (Dixon, 2004).

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1.6.5 PTGS Knockout Cells and Mouse Models

The use of transgenic mice deficient in PTGS genes has enabled researchers to fully investigate the individual physiological roles of PTGS-1 and PTGS-2 and the consequences on mice development when these genes are deficient. PTGS-2 was previously the more studied of the three prostaglandin synthase enzymes but PTGS-1 has been under the spot-light recently with much focus on its upregulation in various carcinomas and capacity to promote tumourigenesis (Sales *et al.*, 2002a; Hwang *et al.*, 1998; Narko *et al.*, 1997).

PTGS-1(-/-) null mice produced normal sized litters and lived normal length lifespans despite a reduction in PG levels of up to 99% (Langenbach *et al.*, 1999). By stark contrast, matings of PTGS-2(-/-) null mice resulted in various pathologies including a

60% pup survival rate, cardiac fibrosis was observed in 50% of animals, progressive renal disease was present and females were found to be infertile due to reduced ovulation, implantation and decidualization (Langenbach *et al.,* 1999; Dinchuk *et al.,* 1995). Attempts to generate a double knockout mouse, null for PTGS-1 and PTGS-2, have been recorded but were unsuccessful as all of the double null pups generated died shortly after birth despite appearing to have developed properly (Langenbach *et al.,* 1999). The phenotypical information gained from the PTGS-1 and PTGS-2 mouse studies has greatly aided our understanding in not only learning what regulatory tasks these genes perform in processes such as reproduction and the inflammatory response but also what mechanistic signalling roles they under-take in several carcinomas such as colon (Tsuji *et al.,* 1998), breast (Kundu and Fulton, 2002), endometrial (Jabbour and Sales, 2004) and cervical (Muller *et al.,* 2006).

In inflammation, PTGS-1 and PTGS-2 can both augment PG production, although the extent to which each isoform contributes will depend on inflammatory stimuli, the time after insult and relative levels of respective gene product in target tissue (Langenbach *et al.,* 1999). Under the umbrella of carcinogenesis, PTGS-2 has been linked with intestinal neoplasia (Oshima *et al.,* 1996) while absence of PTGS-1 and -2 can reduce skin tumourigenesis (Boutwell, 1974). High levels of PTGS-1 and PTGS-2 have been shown to exist in mouse lung tumours and due to their continued expression during neoplastic progression their function may also be able to help maintain the neoplastic state (Bauer *et al.,* 2000).

Over the years, numerous *in vivo* studies have been carried out to investigate the effects of PTGS-1 and PTGS-2 and it would be impossible to cover all the findings here within. In spite of this, the gathered evidence clearly outlines the many key functions with which PTGS-1 and PTGS-2 hold in a physiological and pathological manner.

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1.7 Prostagladins and Prostaglandin Receptors

1.7.1 Prostanoids and Prostanoid Receptors

Prostanoids are collectively referred to as eicosanoids and their prostaglandin associated activity was first observed in human seminal fluid in 1930 by Kurzrok and Leib (Coleman *et al.,* 1994). Prostanoids are generally categorised into two groups, termed either prostaglandins (PG) containing a cyclopentane ring or thromboxanes (TX) which contain a cyclohexane ring (Versteeg *et al.,* 1999). The prostaglandins are further subdivided and include the D, E, F, and I series whilst the thromboxanes are classified as TXA and TXB (Nariyuma *et al.,* 1999; Smith *et al.,* 2000).

The specific prostanoid cognate receptors belong to the superfamily of the G-protein coupled receptors (GPCRs), each exhibiting a distinctive seven hydrophobic transmembrane domain structure and constitute part of the family of the rhodopsin-type GPCRs (Hirata *et al.,* 1991; Narumiya *et al.,* 1999). There are eight receptors that are known to exist within this family and they are designated PTGER1, PTGER2, PTGER3 and PTGER4 receptors for PGE_2 and DP, FP, IP and TP receptors for PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA respectively (Coleman *et al.,* 1994; Nariyuma *et al.,* 1999; Breyer *et al.,* 2001). A schematic illustration of prostanoid synthesis, interaction with their specific prostanoid receptors and their corresponding second messengers is depicted in Figure 1.4.

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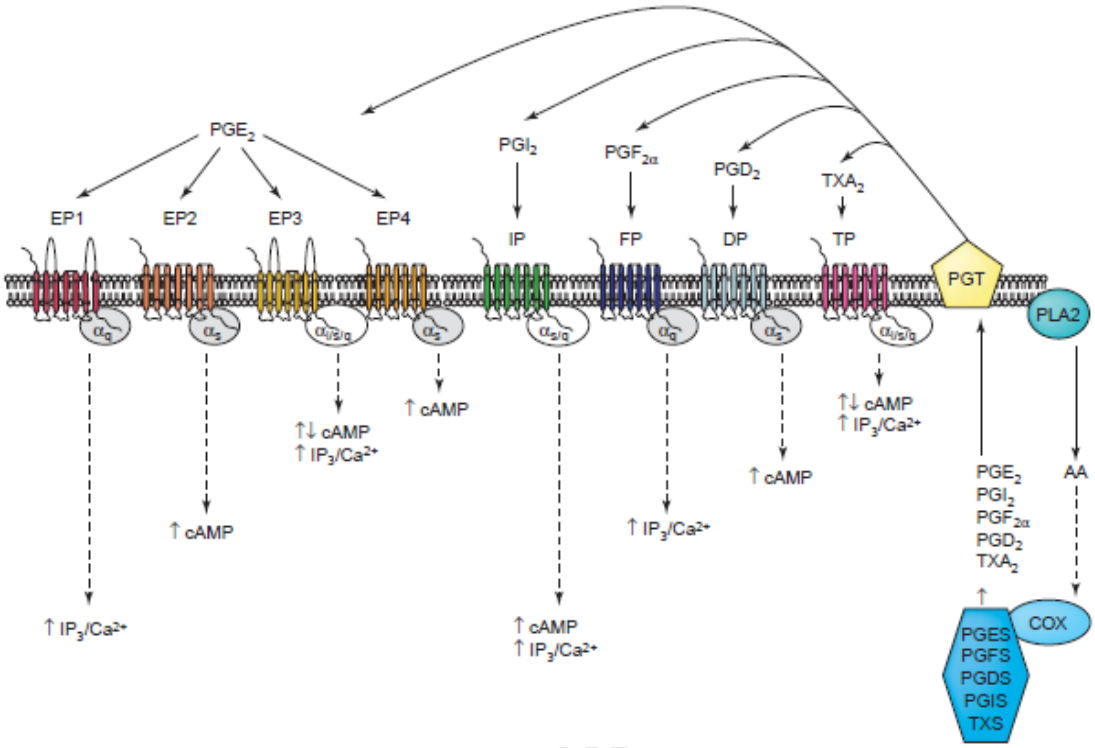

Figure 1.4: Schematic representation of prostanoids and their interactions with prostaniod receptors. Arachidonic acid (AA) is released from plasma membrane by phospholipase A_2 (PLA_2) and acts as a substrate for COX enzymes for production of prostanoids. Once prostanoids are formed they are transported out of the cytoplasm by prostaglandin transporter (PGT) and subsequently act on their cognate receptors to activate numerous secondary messengers (diagram taken from Jabbour and Sales, 2004).

Although these are the main operative pathways that the prostanoids are involved with, another class of receptors, peroxisome proliferators-activated receptors (PPAR) have also been shown to interact with prostaglandins. Prostanoids such as PGI_2 and PGJ_2 have been reported as ligands for PPARα and PPARδ and are believed to alter transcription of target genes concerned with lipid metabolism and homeostasis (Forman *et al.*, 1997; Sales and Jabbour, 2004).

Each of the individual prostanoid receptors display selective ligand binding specificity. These receptors share a 20-30% amino acid homology and have 65 conserved amino acids, 34 of which are identical across the prostanoid family (Bonvalet *et al.*, 1987). The similarities in structure that the prostanoids share and the wide range of biological

activities they perform in distinct tissues explains why the entirety of their physiological roles has not yet been fully characterised (DeWitt, 1991). However, the importance of prostanoid function is most appreciable when examining the worldwide use of non steroidal anti-inflammatory drugs in the pharmaceutical market place which has an estimated annual expenditure of 5-10 billion US dollars (Herschman, 1996).

In general, the prostaglandins are mainly involved in inflammatory and anaphylactic responses, the prostacyclins are active in the resolution of inflammation and the thromboxanes are mediators of vasoconstriction. The prostanoids are also known to be of fundamental in reproduction, having important roles in ovulation, implantation, foetal development, parturition and sperm migration (Smith and Marnett, 1991; DeWitt, 1991), further emphasizing their worth in physiological roles.

Another well documented characteristic of the prostanoid receptors is the existence of various splice variants. PTGER1, PTGER3, TP and FP are the members amongst the prostanoid family which boast splice variants with the alternative splicing having been shown to mostly occur in the intracellular loop of these receptors (Breyer *et al.,* 2001). The splicing is not thought to effect ligand-binding properties of the respective receptors but is believed to render changes in their signalling, their sensitivity to agonist-induced desensitization and their tendency towards constitutive activity (Breyer *et al.,* 2001; Jabbour and Sales, 2004).

Functional roles for the prostanoid receptors have been determined by studies in knockout mouse model systems, deficient for each of the receptors. One of the most interesting observations derived from these studies transpired from the PTGER2 receptor knockout mouse model which demonstrated that PTGER2 receptors are essential in female reproduction (Hizaki *et al.,* 1999; Kennedy *et al.,* 1999). Loss of PTGER2 receptor in mouse models because of gene ablation results in impaired ovulation and dramatic reduction in litter size (Hizaki *et al.,* 1999; Kennedy *et al.,* 1999). In a separate study, the FP receptor was also linked to female reproduction as mice not exhibiting this gene results in failure of parturition (Narumiya *et al.,* 1999).

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Other studies in receptor deficient mice show that a lack of PTGER1 receptor can result in fever generation and play a role in gastric ulcerogenic responses (Sugimoto *et al.,* 2001; Jackson *et al.,* 2001). Mice exhibiting disruption of the PTGER3 receptor exhibit alterations in urinary concentrating ability, highlighting PTGER3 antagonists could serve as antipyretic agents (Ushikubi *et al.,* 1998; Fleming *et al.,* 1998). TP-deficient mice showed an increased bleeding tendency (Thomas *et al.,* 1998) which was also observed in thrombotic patients with TP receptor abnormality (Hirata *et al.,* 1994).

A number of prostanoid receptors have also been reported to be involved in carcinogenesis. PTGER1 is believed to play a role in colon cancer (Watanabe *et al.,* 1999), PTGER2 receptors can induce the expression of VEGF in pancreatic cancer cells and endometrial adenocarcinoma cells via PGE_2 coupling (Sales *et al.,* 2004b; Eibl *et al.,* 2003), PTGER3 receptor deficient mice have reduced tumour-associated angiogenesis (Amano *et al.,* 2003) whereas PTGER4 and FP receptors have been reported in perivascular cells in endometrial carcinomas (Sales *et al.,* 2004a; Jabbour *et al.,* 2001) and in cervical adenocarcinoma cells in response to SP stimulation (Sales *et al.,* 2002b; Muller *et al.,* 2006).

1.7.2 Prostaglandin E_2 (PGE_2) and PTGER Receptors

Prostaglandin E_2 or PGE_2 as it is more commonly entitled is a major product of cyclooxygenase-initiated arachidonic metabolism and exerts its multiple biological actions by means of binding to PTGER receptors 1-4, each of which will be individually described later. The PGE_2 prostanoid is biosynthesised in cells from PGH_2 by the catalytic properties of prostaglandin E synthase (PGES) (Breyer *et al.,* 2001). Four different isoforms of PGES have been described; two membrane bound isoforms, mPGES-1 and mPGES-2, a cystolic isoform, cPGES and a glutathione S-transferase isoform, GST-μ, congregating together as the rate limiting enzymes in PGE_2 production (Murakami and Kudo, 2004; DeWitt, 1991). The preferred method of generating PGE_2 is by cPGES converting PTGS-1 derived PGH_2 resulting in immediate prostaglandin production (Narumiya *et al.,* 1999; Jabbour and Sales, 2004).

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The inducible membrane associated form, mPGES-1, is preferentially associated with PTGS-2 under conditions of limited AA supply (but can couple to PTGS-1 under conditions where AA is available) and is associated with delayed production of PGE_2 (Murakami *et al.,* 2000). mPGES-2 is capable of coupling with both PTGS-1 and PTGS-2 whereas the preferred binding of the GST-μ isoform is yet to be determined (Murakami and Kudo, 2004).

Following biosynthesis, PGE_2 is transported out of the cell by PGT where it elicits its effects on target cells via interaction with its cognate GPCRs as described in section 1.6. Once PGE_2 is bound to its respective receptors, intracellular signalling cascades become activated and the involvement of secondary messengers is dependent on the receptor sub-type to which PGE_2 is coupled (Jabbour and Sales, 2004). For example, interaction with the PTGER1 receptor promotes the production of intracellular calcium and inositol trisphosphate (IP_3) via $G_{\alpha q}$. Activation of receptors PTGER2 and PTGER4 induces increases in cyclic AMP via $G_{\alpha s}$ whereas PTGER3 receptor binding can result in triggering either a positive or negative cAMP response and elevated levels of IP_3 and Ca^{2+} depending on which splice variant and cell type is implicated (Sugimoto *et al.,* 1993; Narumiya *et al.,* 1999). This PGE_2 mediated trigger of chemical activators is an essential part of various biological events such as neuronal function, female reproduction, vascular hypertension, tumourigenesis, kidney function and inflammation (Kobayashi and Narumiya, 2002).

PGE_2 is the major type of prostaglandin present in seminal plasma (SP) and along with 19-hydroxyprostaglandin E (19-hydroxy PGE), which is also present in SP but at lower concentrations (Templeton *et al.,* 1978), these prostaglandins are considered to be the key effector molecules that provides SP with its immunosuppressive properties. Work carried out by our laboratory highlighted the biological effects of SP and PGE_2 by demonstrating that PTGS-2 and cAMP induced expression of the PTGER2 and PTGER4 receptors was central in elevated intracellular signalling in cervical adenocarcinoma cells as a result of SP and PGE_2 stimulation (Sales *et al.,* 2002b; Sales *et al.,* 2001). A study undertaken by another member of the group, Muller *et al.* (2006) revealed that SP and PGE_2 can modulate the expression of tumourigenic and

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angiogenic factors in cervical adenocarcinoma cells via the PTGER4 receptor, namely PTGS-2 and VEGF, whereas additional data published by Battersby and colleagues (2007) revealed that SP and PGE_2 can activate fibroblast growth factor 2 (FGF2) expression and PTGER2 receptor signalling in endometrial carcinoma cells. From examining all the converging data, it is therefore hypothesized that the PGE_2 contained within SP possesses the ability to promote endometrial and cervical tumourigenesis in sexually active women by means of an autocrine/paracrine manner through PGE_2-PTGER receptor specific interactions.

1.7.2.1 PTGER1 Receptor

The EP1 receptor, or PTGER1 as it is also named, was first cloned and characterized in 1993 by Funk, exhibiting a molecular mass of approximately 42 kDa (Funk *et al.,* 1993). This receptor was derived from human erythroleukemia (HEL) cells and encodes a 402 amino acid polypeptide. The distribution of PTGER1 mRNA expression is restricted to several organs, it is most highly expressed in the kidney, followed by gastric muscularis mucosae and then adrenal tissue (Watabe *et al.,* 1993; Okuda-Ashitaka *et al.,* 1996; Abramovitz *et al.,* 1995; Guan *et al.,* 1998) and along with PTGER2 is the least abundant of the PTGER receptors.

The discovery of PTGER1 is a fairly recent one and initially it was only believed to be involved with regulation of smooth muscle constriction in locations such as the gastrointestinal tract, the respiratory tract and the sphincter muscle (Lawrence *et al.,* 1992; Coleman and Kennedy, 1985; Dong *et al.,* 1986). Published data have revealed that activation of the PTGER1 receptor leads to signalling via inositol-1, 4, 5-trisphsophate (IP_3) generation and increased cellular Ca^{2+} (Breyer *et al.,* 2001), which may in part be responsible for the effects of PGE_2 on various tissue varieties (Okuda-Ashitaka *et al.,* 1996.). Other studies have shown PTGER1 to act as an inflammatory mediator by assisting in the mediation of pain perception and regulation of blood pressure as a result of its close association with PGE_2 (Funk *et al.,* 1993).

The binding of PGE_2 to PTGER receptors (including PTGER1) can be unfavourable because irregular control of this relationship is considered to play a major function in

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tumour progression (Wang *et al.,* 2007). A study using PTGER1 knockout mice and ONO-8713, a specific antagonist for the PTGER1 receptor, demonstrated a significant reduction in azoxymethane (AOM)-induced aberrant crypt foci (ACFs) (Watanabe *et al* 1999). The resulting inhibition of ACFs in male nude mice was approximately the same in the PTGER1 knockout group as the group being treated with ONO-8713, thus confirming PTGER1 receptor involvement in colon carcinogenesis.

Additional specific antagonists are known to exist for the PTGER1 receptor such as SC51089 and SC53122 and coupled with the successful generation of the EP1 (-/-) knockout mouse model, the distinct functional consequences of activating the PTGER1 receptor are being better understood (Funk *et al.,* 1993; Dong *et al.,* 1986). A study employing the use of a bile duct cancer cell line (CCLP1) displayed that treatment with a PTGER1 agonist called ONO-DI-004 resulted in an increase in tumour cell growth and invasion (Han and Wu, 2005). However, work carried out by Throat and colleagues (2007) investigated the role of PTGER1 in breast cancer cells and whilst nuclear PTGER1 expression correlated with the absence of nodal metastases and differentiation, there was no correlation with these parameters and cytoplasmic PTGER1 where it is known to play a role in differentiation in squamous cell carcinomas of the skin (Lee *et al.,* 2005), therefore suggesting that the distinct function of PTGER1 may be dependent on its localisation.

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1.7.2.2 PTGER2 Receptor

The correct nomenclature of the PTGER2 receptor was somewhat puzzling in the early days of its discovery. Bastien and colleagues first cloned the PTGER2 receptor in 1994 but this was later termed the PTGER4 receptor by Nishigaki *et al.* in 1995. The genuine PTGER2 receptor was derived from a human placenta library by Regan *et al.* (1994) and encodes a 358 amino acid polypeptide that surprisingly only shares a 30% identity with the other EP receptor subtypes (Regan, 1994). Despite the characterization of PTGER2 tissue distribution being only partially completed, the PTGER2 receptor is known to reside in abundant concentrations in the lung, spleen and uterus and to a lesser extent in the kidney (Breyer *et al.,* 2001). These locations mirror the findings in the mouse whereby PTGER2 mRNA levels were also found to

be expressed in these locations, in addition to the ileum and thymus (Honda *et al.*, 1993).

When originally characterised, PTGER2 was seen to be involved with the regulation of smooth muscle relaxation in cat trachea (Gardiner, 1986), however, this receptor is now closely associated with performing a multitude of tasks that include maintaining homeostasis, stimulation of secretion in the stomach and small intestine and regulation of bone reabsorption (Bastien *et al.*, 1994).

The functional role of PTGER2 has been divulged by the employment of several agonists that have a strong affinity for this receptor and the use of PTGER2 knockout mice. PTGER2 receptor agonist (CP-533536) has been shown to induce bone formation in canines (Paralkar *et al.*, 2003), whereas another PTGER2 receptor agonist, ONO-8815Ly was found to significantly inhibit uterine contractions in women (Steinwall *et al.*, 2004). In attempts to further unravel efficacy of PTGER2, a study undertaken by Sung and colleagues (2005) revealed that skin tumours taken from PTGER2 (-/-) mice contained more apoptotic cells when compared to the respective WT group. Other research carried out by Ansari and colleagues (2008) showed PTGER2 to be responsible for PTGS2 induction via PGE_2 production in mouse skin, thus proposing that the PTGER2 receptor plays a significant role in the pro-tumourigenic action of PGE2 in skin tumour development (Ansari *et al.*, 2008; Sung *et al.*, 2005).

Data published by Castellone and colleagues (2005) revealed that the activation of PTGER2 by PGE_2 in colorectal cancer cells was followed by downstream signalling that primarily occurred through Protein Kinase A (PKA) activation via the Wnt signalling pathway and suggests that cAMP mediates this process. This in turn leads to the activation of β-catenin transcription via the phosphorylation of glycogen synthase kinase-3 (GSK-3) by Akt. The involvement of cAMP was supported by findings generated by Ansari and colleagues (2008) who showed that PTGER2 signalling through PKA and cAMP response element binding (CREB) is responsible for PGE2 effects on cell proliferation and PTGS-2 induction in mouse skin carcinogenesis.

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Furthermore, PTGER2 receptor expression and the biosynthesis of PGE_2 have been shown to be significantly enhanced in endometrial cancers whereby upregulated levels of prostaglandin synthase enzymes, PGE_2 and PTGER2 result in increased autocrine/paracrine signalling and this subsequently promotes the release of the potent pro-angiogenic factor, VEGF (Battersby *et al.,* 2007; Jabbour *et al.,* 2001). Of late, Battersby and colleagues (2007) ascertained that seminal plasma can activate the PTGER2 receptor signalling pathways in endometrial cancer cells, the result of which was the induction of cAMP accumulation and phosphorylation of EGFR kinase and ERK. PTGER2 signalling activation has also been displayed in cervical adenocarcinoma cells through exposure to seminal plasma (Sales *et al.,* 2002b) and these data together highlight the potential of the PTGER2 receptor to modulate neoplastic cell function and cervical and endometrial tumourigenesis in response to seminal plasma exposure and activation.

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1.7.2.3 PTGER3 Receptor

The human PTGER3 receptor was first discovered by Yang and colleagues in 1994 and is the most ubiquitously expressed out of the four different EP receptor subtypes (Yang *et al.,* 1994). PTGER3 was cloned from a human kidney library and encodes a 367 amino acid polypeptide. This receptor was originally identified as a constrictor of smooth muscle (Coleman *et al.,* 1994) but mRNA expression has since been established in many tissues including kidney, uterus, adrenal gland, stomach, lung and brain (Breyer *et al.,* 2001; Coleman *et al.,* 1994; Sugimoto *et al.,* 2000).

A distinctive feature of PTGER3 amongst the prostanoid family is the multiplicity of splice variants that encode for this receptor and may explain why it has so many diverse biological functions (Breyer *et al.,* 2001). The differences in PTGER3 sequencing arises from alterations in the C-terminal tail of the receptor subtype and this resultantly leads to variations in receptor phosphorylation and desensitization and intracellular trafficking. Although the majority of PTGER3 splice variants inhibit cAMP via $G_{\alpha i}$ protein coupling, some variants of the receptor have been shown to participate in alternate signalling mechanisms that involve the binding of G_s proteins to

induce the release of Ca^{2+} and elevation of cAMP levels (Namba *et al.*, 1993; An *et al.*, 1994).

The numerous tasks with which the PTGER3 receptor is involved include; controlling the contraction in autonomic nerves by mediating the inhibition of neurotransmitter release, regulation of lipolysis in adipocytes (Strong *et al.*, 1992), regulation of water reabsorption in renal medulla (Sonnenburg and Smith, 1988), regulate a propaggretory effect in platelet cells (Jones and Wilson, 1990) and control the levels of acid secretion in gastric mucosal cells (Reeves *et al.*, 1988). The high abundance of PTGER3 present in gastric mucosa has been the target of NSAIDs which have been very successful in the treatment of gastric ulcers (Coleman *et al.*, 1994).

Specially bred knockout mice and receptor-specific agonists have also been useful in unravelling the roles of the PTGER3 receptor. A study carried out by Audoly and colleagues (2001) compared mice lacking the PTGER3 receptor with their WT counterparts and revealed that the PTGER3 receptor is involved in the mediation of vasoconstriction in the kidney and capable of buffering PGE_2-mediated renal vasodilation, therefore, PTGER3 receptor activation maybe central in regulating renal hemodynamics. Other studies that have used PTGER3 knockout mice showed that PTGER3 receptor signalling regulates tumour-associated lymphangoigenesis by upregulating expression of VEGF-C and its receptor VEGFR-3 in tumour stromal tissues (Kubo *et al.*, 2010) while Shoji and colleagues (2005) demonstrated that benign keratoacanthomas only developed in PTGER3 knockout mice in comparison to the wild-type (6/19 versus 0/24), thus showing a protective role against tumour development and therefore PTGER3 receptor signalling may have contrasting roles depending on tumour type. In addition, with sulprostone and SC-46275 being two agonists specific for the PTGER3 receptor that are capable of inducing its activation in several animal model cell lines, our knowledge of this particular PTGER subtype is being further enhanced all the time (Savage *et al.*, 1993, Breyer *et al.*, 2001). Results from a study carried out by Wang and Lau (2006) employed the use of sulprostone and SC-46275 and illustrated that PTGER3 was strongly involved in the release of histamine from mast cells which have a known role in the inflammatory response due

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to their potent capability to produce multiple pro-inflammatory mediators after activation (Brightling *et al.,* 2003).

Interestingly, seminal plasma, (known to contain PGE_2 in high concentrations) and PGE_2 itself were used to treat cervical adenocarcinoma cells *in vitro* and the results revealed that both forms of stimulus (SP and PGE_2) significantly induced the up-regulation of receptors PTGER1, PTGER2 and PTGER4 but not PTGER3 when compared to the respective non-treated control (Sales *et al.,* 2002b). All PTGER receptor subtypes are known to be closely engaged with PGE_2 signalling but it appears that PTGER3 does not play a noteworthy part in response to SP stimulation of cervical adenocarcinoma cells and that the signalling involved in cervical cancer is via PTGER2 as revealed by Sales and colleagues (2002b).

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1.7.2.4 PTGER4 Receptor

After being initially credited with discovering the PTGER2 receptor, Bastien *et al.* (1994) was later found to have actually cloned the PTGER4 receptor as subsequent molecular studies were to prove that what he originally unearthed did not posses any of the characteristics associated with the PTGER2 receptor and was therefore named PTGER4 accordingly (Bastien *et al.,* 1994; Nishigaki, 1995).

The PTGER4 receptor, like PTGER3, is expressed in most cell types with mRNA levels found in almost all mouse tissue examined (Narumiya *et al.,* 1999). The human PTGER4 receptor encodes a 488 amino acid polypeptide with an approximate mass of 53kDa and was first located in the piglet saphenous vein (Bastien *et al.,* 1994; Louttit *et al.,* 1992). In humans, the PTGER4 receptor is abundantly expressed in thymus, ileum, lung, spleen, adrenal and kidney tissues with it also having been shown to exhibit important vasodilator effects when activated in venous and arterial beds (Bastien *et al.,* 1994; Breyer *et al.,* 2001; Coleman *et al.,* 1994; Coleman *et al.,* 1994b).

Moreover, the PTGER4 receptor has gathered much interest recently, owing to the effect of its mediated activation by PGE_2 in carcinomas of the colon, breast, head and neck and cervix (Doherty *et al.,* 2009; Ma *et al.,* 2006; De Lorenzo *et al.,* 2005; Muller

et al., 2006). Several signalling pathways can be activated by the PTGER4 receptor and one such avenue in which PTGER4 activated signals are relayed is through protein kinase A-mediated cAMP. This particular pathway was exerted in head and neck squamous cell carcinoma (HNSCC) xenografts and treatment with ONO-AE3-208, a specific PTGER4 receptor antagonist caused a reduction in cAMP levels and PKA activity (De Lorenzo *et al.,* 2005). Additionally, ONO-AE3-208 caused a dose-dependent inhibition of HNSCC xenografts *in vivo* as determined by the 50% reduction in cellular proliferation measured by the proliferative marker Ki-67. The resulting decrease in tumour cell proliferation in HNSCC xenografts may be a consequence of the anti-angiogenic effects of the PTGER4 receptor antagonist and therefore, therapies that target PGE_2 synthesis or EP receptor antagonists may be useful in the treatment of certain malignancies, including HNSCC (De Lorenzo *et al.,* 2005).

The predominant pathways that PTGER4 utilizes in order to enhance expression of downstream target genes results in either activation of the Akt pathway or phosphorylation of ERK (Fujino *et al.*, 2003) and it was the later respective pathway that was deemed as being liable for promoting tumourigenic and angiogenic factors in cervical adenocarcinoma cells in response to seminal plasma stimulation (Muller *et al.,* 2006), thus showing that EP4 receptor upregulation could be a fundamental event in cervical cancer development and progression. ERK is a key signalling mechanism involved in the regulation of many processes such as cellular transformation and growth (Battersby *et al.,* 2007) and a previous study by Sales and colleagues (2004b) demonstrated that ERK could be phosphorlysed by increases in cAMP in a similar manner to PGE_2 via the PTGER2 receptor in EP2 sense cells (Hela cells transfected with the EP2 receptor). An earlier study undertaken by Sales and colleagues (2002b) showed that SP can increase cAMP via the PTGER2 and PTGER4 receptors and taken together proposes that SP can promote the growth of cervical adenocarcinoma cells via PTGER receptors 2 and 4 by increasing cAMP to activate ERK.

Overall, the upregulation of prostanoid receptors PTGER1, PTGER2, PTGER3 and PTGER4 and the subsequent expression of various cell signalling pathways they activate have been highlighted in a wide variety of biological processes. The PGE2

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receptor mice models, the use of agonists and antagonists and the manipulation of NSAIDs, has to a great extent, outlined the important clinical relevance of PTGER receptors in a host of pathophysiological roles. The expression of PTGER1, PTGER2 and PTGER4 receptors appears to be upregulated in response to seminal plasma in cervical carcinogenesis but the investigation to fully characterize these receptor subtypes is ongoing with a view that it could help generate new therapeutic options for the future.

1.7.3 Other Prostanoids and their Cognate Receptors

Several other receptors exist amongst the prostanoid family and include the PGF receptor, FP receptor, IP receptor, DP receptor and the TP receptor. These types of prostanoid receptors are also involved in a varied range of biological processes and since seminal plasma contains several prostaglandins, other prostaglandin receptors may be implicated in mediating the effects of SP.

1.7.3.1 Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the FP Receptor

The human FP receptor was originally cloned from uterus cDNA library by Abramovitz *et al.* in 1994 and encodes a 359 amino acid polypeptide (Abramovitz *et al.,* 1994). Prostaglandin $F_{2\alpha}$ ($PGF_2\alpha$) exerts its biological actions via the FP receptor and participates in various homeostatic functions that include smooth muscle contraction and relaxation, blood control pressure and regulation of inflammation and cellular proliferation (Watanabe *et al.,* 1994). Abramovitz and colleagues (1994) utilized oocytes to expressed the human FP receptor and revealed that receptor signalling induced an elevation in Ca^{2+} levels whereas other studies have demonstrated FP receptor signalling to initiate increases in IP3 via the PLC pathway and involvement in a Rho-mediated pathway (Watanabe *et al.,* 1994; Pierce *et al.,* 1994).

The FP receptor is a potent inducer of luteolysis and most abundantly expressed in the corpus luteum (Breyer *et al.,* 2001). The normal regulation of FP receptor output is requisite for a normal birth as disruptive control of this receptor results in unsuccessful parturition as an outcome of failing to decrease preterm progesterone levels (Sugimoto

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et al., 1997). Further roles for FP receptor signalling in reproduction were put forward in data published by Sales and colleagues (2004a) who investigated the role of $PGF_{2\alpha}$ and ensuing FP receptor activity in human endometrial adenocarcinoma cells and demonstrated that increased proliferation was a direct result of $PGF_{2\alpha}$ stimulation (Sales *et al.,* 2004a). Additional work carried out by Sales and colleagues (2005 and 2008a) ascertained that $PGF_{2\alpha}$ could modulate vascular function in endometrial adenocarcinomas by promoting the production of pro-angiogenic factor vascular endothelial growth factor (VEGF) whilst the FP receptor possesses the ability to alter adhesion, morphology and migration of endometrial adenocarcinoma cells respectively, therefore confirming an autocrine/paracrine regulation of neoplastic cell function by the $PGF_{2\alpha}$-FP interaction (Sales *et al.,* 2005; Sales *et al.,* 2008a).

1.7.3.2 Prostacyclin (PGI_2) and the IP Receptor

Prostacyclin (or PGI_2) synthesis is facilitated by prostacyclin synthase (PGIS) and exerts its physiological functions by binding to IP receptors (Wang *et al.,* 2007). PGI_2 production is central to several body functions that include antithrombosis, regulation of rennin release and is believed to play an important vasodilator role in the kidney (Breyer *et al.,* 2001; Bolger *et al.,* 1978; Bugge *et al.,* 1990).

The human IP receptor encodes a protein of 386 amino acid residues and is highly expressed in the heart, kidney, liver and lung tissues (Boie *et al.,* 1994; Nakagawa *et al.,* 1994). IP receptor mRNA expression is also present in the dorsal root ganglia and therefore thought to act as a messenger in pain perception (Breyer *et al.,* 2001). Upon activation, IP receptors couple to G_s proteins which trigger increases in cAMP and protein kinase A (Herbert *et al.,* 1998) and because IP receptor signalling is elevated during menstruation, it is therefore suggested that its increase is associated with painful menstrual cramps (Battersby *et al.,* 2004). Additional data recently published by Huang and colleagues (2007) have proposed a role for the IP receptor in embryo development and implantation in mice.

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1.7.3.3 Prostaglandin D_2 (PGD_2) and the DP Receptor

The most recently cloned member of the prostanoid receptors is the DP receptor and is probably the least well characterised (Breyer *et al.,* 2001). Prostaglandin D_2 (PGD_2) exerts its action through the DP receptor and this prostanoid is regarded as being involved with regulating body temperature, mediation of vasodilation and vasoconstriction, inhibition of platelet aggregation and also a proposed role in the sleep-wake cycle (Urade and Hayaishi 1999; Sri Kantha *et al.,* 1994; Giles *et al.,* 1989).

The human DP receptor encodes a 359 amino acid polypeptide and has also been cloned in the mouse and rat (Wright *et al.,* 1999, Hirata *et al.,* 1994). DP receptor mRNA is known to be expressed at low levels in most tissues although it is present in high quantities in leptomeninges, retina, and mucus-secreting cells of the gastrointestinal tract (Wright *et al.,* 1999; Oida *et al.,* 1997; Hirata *et al.,* 1994; Boie *et al.,* 1995). Activation of the DP receptor by PGD_2 results in accumulation of intracellular cAMP and mobilisation of Ca^{2+} and has been shown to be concerned in inflammatory diseases such as asthma (Breyer *et al.,* 2001; Murata *et al.,* 2008), although more recently, the upregulation of the DP receptor has been demonstrated to facilitate tumour growth in the lungs of nude mice by inciting angiogenesis (Murata *et al.,* 2008; Brown *et al.,* 1989).

1.7.3.4 Thromboxane A_2 (TXA_2) and the TP Receptor

The synthesis of Thromboxane A_2 (TXA_2) is catalyzed by thromboxane synthase TXS) and exerts its biological function through interactions with the TP receptor (Jabbour and Sales 2004). TXA_2 is required for platelet aggregation as well as smooth muscle contraction and proliferation (Breyer *et al.,* 2001). Increased synthesis of TXA_2 has been shown to be closely linked with cardiovascular diseases including acute myocardial ischemia, heart failure and renal diseases making TP receptor antagonists a potential therapeutic approach for these diseases (Oates *et al.,* 1988; Castellani *et al.,* 1997; Spurney *et al.,* 1992).

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The TP receptor was the first of the eicosanoid receptors to be cloned (Hirata *et al.* 1991). Two splice variants of this receptor exist, TPα, the original placental derived clone encoding a 343 amino acid polypeptide and TPβ, the subsequent spliced variant cloned from endothelium, encoding a 407 amino acid polypeptide, differing only in their C-terminus amino acid sequence (Breyer *et al.,* 2001). These TP receptors are highly expressed in tissue rich in vasculature such as the lung, kidney, heart and immune-related organs such as the thymus and spleen (Narumiya *et al.,* 1999; Namba *et al.,* 1992). Thromboxane receptors are typically characterised by signalling via G_q activation of Ca^{2+}/DAG signalling pathways, although, TP receptors have also been demonstrated to bind via G_{11}, G_{12}, G_{13} and GTP binding protein with transglutaminase G_h (Breyer *et al.,* 2001; Kinsella *et al.,* 1997; Becker *et al.,* 1999).

1.8 Pro-inflammatory Cytokines and Pro-angiogenic Factors Closely Associated in the Development and Progression of Cancer

1.8.1 Interleukins

The interleukin family are a group of cytokines that were initially seen to be expressed in leukocytes. Dr. Paetkau first coined the term ‘interleukin’ which was derived from ‘inter-’, meaning communication and ‘-leukin’ to denote that many of these proteins are produced by, and act on leukocytes (Paetkau, 1981). However, this assembly of signalling molecules are now known to be produced by a wide variety of body cells such as macrophages, monocytes, eosinophils, mast cells, endothelial cells, epithelial cells, natural killer cells and T cells and their function is mainly concerned with regulation of the immune system. At present, interleukins, IL-1 to IL-36 have been identified but for the purpose of this thesis, IL-6, IL-8 and IL-11 are going to be discussed in more detail because of their roles in tumour development and progression and their association with SP.

1.8.1.1 Interleukin-6 (IL-6)

IL-6 is a multifactorial cytokine and this is evident from all the different names it used to be called, each reflecting a different biological activity it controlled, until researchers realised that they had all cloned the same protein (Simpson *et al.,* 1997).

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Previous terms in which IL-6 was previously referred include interferon-β_2, B-cell stimulatory factor-2, hepatocyte stimulatory factor and cytotoxic T-cell differentiation factor (Weissenbach *et al.,* 1980; Hirano *et al.,* 1985; Gauldie *et al.,* 1987; Takai *et al.,* 1988).

IL-6 consists of 184 amino acids, is synthesized as a larger polypeptide precursor with a signal peptide of 28 amino acids and its gene is located at chromosome 7p21 and 5 in the human and mouse genomes respectively (Rose-John *et al.,* 1992; Sehgal *et al.,* 1986; Mock *et al.,* 1989). The biological activities of IL-6 are mediated by the IL-6 receptor-system and this comprises of two membrane proteins termed the signal transducing β subunit gp130, which also forms part of the receptor complex with IL-11 and leukemia inhibitory factor (LIF), and the ligand binding α-subunit receptor (IL-6R) (Simpson *et al.,* 1997; Taga *et al.,* 1989; Gearing *et al.,* 1992). The high-affinity binding of IL-6 is deemed to have resulted from its ability to interact simultaneously with both IL-6R and gp130 sites and the subsequent signal transduction allows the activation of tyrosine kinases (Murakami *et al.,* 1993).

While this cytokine is slightly redundant with normal day-to-day 'housekeeping' functions, it plays a central role in host defence due to its contribution in immune and hematopoietic activities (Simpson *et al.,* 1997). IL-6 is an important mediator of fever, is secreted by T cells and macrophages in response to trauma, can act as both a pro-inflammatory and anti-inflammatory cytokine and is elevated in response to muscle contraction.

The over-expression of IL-6 has been implicated in the pathology of a number of diseases such as rheumatoid arthiritis, psoriasis and postmenopausal osteoporosis (Simpson *et al.,* 1997). In addition, aberrant production of IL-6 has been reported in many cancers such as ovarian cell carcinoma, squamous cell carcinoma of the uterine cervix and renal cell carcinoma (Watson *et al.,* 1990; Eustace *et al.,* 1993; Takano *et al.,* 1996). IL-6 also acts in an autocrine growth fashion in myeloma, lung and breast cancers, it is closely associated with the transciptional activation of oncogenic STAT3 and is implicated as an important activator of Jagged-1/Notch signalling in breast

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tumours (Jenkins *et al.,* 2005; Aikra *et al.,* 1993; Grivennikov and Karin, 2008). Patients with advanced metastatic cancer are shown to have higher levels of IL-6 in their blood and along with its upregulated expression in cancer and other diseases, interest in developing anti-IL6 receptor therapeutic agents has significantly grown with tocilizumab having already been approved to treat rheumatoid arthiritis sufferers (Barton, 2005).

1.8.1.2 Interleukin-8 (IL-8)

IL-8 is one of several pro-inflammatory cytokines released by macrophages and epithelial cells during the inflammatory response to viral and bacterial infections (Roux *et al.,* 2000). This chemokine was first identified as a 72 amino acid neutrophil chemotactic polypeptide and along with other members of the CXC chemokine gene family, form a chemokine gene cluster located on chromosome 4q (Matsushima *et al.,* 1988; Modi *et al.,* 1990). There are two known receptors for IL-8 expressed on neutrophils and are called CXCR1 and CXCR2 (Murphy and Tiffany, 1991). Both of these chemokine receptors are members of the G-protein-coupled receptor family with the CXCR1 receptor being activated in response to binding of IL-8 and granulocyte chemotactic protein-2 whereas the CXCR2 receptor is activated by multiple CXC-chemokines, including the growth related oncogenes (Brat *et al.,* 2005).

Studies aimed at establishing the functional role of IL-8 have shown this chemokine to stimulate chemotaxis in basophils and T lymphocytes, induce neutrophils to release lysosomal enzymes and promote adherence of neutrophils to endothelial cells by increasing the expression of VCAM-1 and selectins (Gorman *et al.,* 1982; Walz *et al.,* 1987; Huber *et al.,* 1991). IL-8 can be induced in a wide range of cells and tissues by various reagents such as mitogens, IL-1 and TNF (Okamoto *et al.,* 1992). The upregulation of IL-8 signalling being shown to activate Akt which is strongly associated with modulating cell survival, angiogenesis, cell migration as well as being implicated in the growth of prostate cancer cell lines (Cheng *et al.,* 2008).

Additional research has demonstrated that IL-8 and Gro-α signalling increase the transcriptional activity of NF-κB in melanoma and prostate cancer cell lines, a

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response which appears to be mediated through activation of p38 and MAPK (Wang and Richmond 2001; Wilson *et al.,* 2006), whereas other published data has shown IL-8 expression and the subsequent transcriptional activation of MAPK and EGFR signalling to promote cell proliferation in ovarian and lung cancer cell lines (Venkatakrishnan *et al.,* 2000; Luppi *et al.,* 2007). Kim and colleagues (2010) recently revealed that PGE_2 forms a positive feedback loop with the PTGER3 receptor and this promotes enhanced IL-8 production via EGFR activation in lung cancer cells. SP contains PGE_2 in high concentrations and coupled with the finding by Sharkey and colleagues (2007) who demonstrated that IL-8 and IL-6 were upregulated in cervical epithelial cells in response to seminal plasma, this therefore implies that SP may potentially induce the inflammatory response in the cervix by enhancing the expression of IL-8 directly and indirectly through binding with the PGE_2 receptors.

1.8.1.3 Interleukin-11 (IL-11)

IL-11 is another member of the interleukin family which poses as a multifunctional cytokine. It was first identified in 1990 in a medium conditioned by the primate bone marrow-derived stromal cell line PU-34 in response to IL-1 and is also referred to as adipogenesis inhibitory factor (AGIF) and oprelvekin (Paul *et al.,* 1990; Kawashima *et al.,* 1991). The human IL-11 gene is located on chromosome 19, consists of 5 exons and 4 introns and encodes a 23 kDa protein (Mckinley *et al.,* 1992). As previously mentioned, IL-11 is a member of the IL-6 type cytokine family and its signal transduction is initiated upon binding of IL-11 to IL-11Rα and gp130 co-receptors and by phosphorylating intracellular tyrosine residues on gp130, it permits the JAK kinases to become activated (Heinrich *et al.,* 2003). A study carried out by Campbell and colleagues (2001) ascertained that IL-11 was upregulated in over 93% of malignant epithelial cells in primary ovarian carcinoma samples and this was found to correlate with the presence of both receptors (IL-11Rα and gp130). Although the exact function of the IL-11 receptor system in ovarian epithelial cell biology remains to be fully defined, the existence of both receptors appears to be significant.

Biological activities that are controlled by this novel cytokine include stem cell proliferation, hepatic acute phase responses, growth of certain lymphocytes and it is

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also a key regulator of multiple events in hematopoiesis, most notably the stimulation of megakaryocyte maturion (Kawashima *et al.,* 1992; Paul *et al.,* 1990). IL-11 is produced to strengthen long bones, performs various functions in a number of tissue types such as brain, gut and testis and is found to respond to agents such as IL-1, phorbol-esters and transforming growth factor-p (TGF-p) (Schendel and Turner, 1998).

In addition, IL-11 has been implicated in the induction of PTGS-2 in breast cancer cells, is associated with adenocarcinoma development as a potent inducer of proliferation in the setting of human gastric cancer via activation of STAT3 and ERK1/2 and its over-expression in prostate cancer cells suggests it may be useful as potential marker for prostate cancer progression (Singh *et al.,* 2006; Furuya *et al.,* 2005).

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1.8.2 Growth Related Oncogene (Gro)

Chemokines are known to play a central role in regulating physiological responses such as inflammation and wound healing as well as being implicated in tumour angiogenesis and metastases (Bobrovnikova-Marjon *et al.,* 2004). Gro is a member of the CXC chemokine family which is responsible for the recruitment of neutrophils to inflammatory sites with three distinct forms (α, β and γ) having been originally isolated from transformed fibroblasts, characterized and are often referred to as CXCL1, CXCL2 and CXCL3 respectively (Cuenca *et al.,* 1992; Wang *et al.,* 2006). All three of these ligands bind to the chemokine receptor CXCR2 with Gro-α (also identified as CXCL1 but termed as Gro-α throughout this thesis) having the highest affinity (Hammond *et al.,* 1996). The Gro-α, Gro-β and Gro-γ genes encode mRNAs approximately 1.1 kb in size and are all closely linked on human chromosome 4 (Lida and Grotendorst 1990; Gupta *et al.,* 2007).

1.8.2.1 Growth Related Oncogene-α (Gro-α)

Gro-α is expressed by macrophages, neutrophils and epithelial cells and has neutrophil chemoattractant activity (Lida and Grotendorst 1990; Becker *et al.,* 1994; Richmond *et al.,* 1988; Moser *et al.,* 1990). Normal regulatory functions of Gro-α include wound healing, a role in spinal cord development by inhibiting the migration

of oligodendrocyte precursors and acts as a chemotactic signal to cause the recruitment and activation of specific leukocytes within the ovulating follicle (Devalaraja *et al.*, 2000; Tsai *et al.*, 2002; Karstrom-Encrantz *et al.*, 1998). Although the notoriety of Gro-α stems from its involvement in processes such as inflammation, angiogenesis and tumourigenesis (Haghnegahdar *et al.*, 2000; Owen *et al.*, 1997), recent evidence by Omari and colleagues (2009) proposes it may offer a neuro-protective function after it was shown to successfully decrease the severity of multiple sclerosis in mice.

Upregulated Gro-α is expressed in a variety of tumours including breast, cervical and non-small cell lung cancer (Luboshits *et al.*, 1999; Niwa *et al.*, 2001; Arenberg, 1997). Gro-α has also been shown to promote cell transformation in immortalized melanocytes and along with Gro-β and Gro-γ, these chemokines can exert autocrine control over neoplastic proliferation in human melanomas (Richmond *et al.*, 1986). Gro-α is expressed in 70% of these tumours with the levels of Gro-β and Gro-γ being expressed to a much lesser extent (Luan *et al.*, 1997; Balentien *et al.*, 1991). Work undertaken by Wang and colleagues (2006) demonstrated that upregulated Gro-α expression was closely associated with elevated PGE_2 levels in human colorectal tumours and mouse intestinal adenomas and involves the activation of the EGFR/MAPK pathway. Additional findings from this study showed that conditioned media from PGE_2-treated colorectal cancer cells enhanced endothelial cell migration and tube formation and that treatment with an anti-Gro-α-neutralizing antibody reduced tumour growth with decreased microvessel formation (Wang *et al.*, 2006), thus suggesting that PGE_2 induced Gro-α expression is implicated in colorectal tumour growth and angiogenesis.

Other studies outlining the angiogenic potential of Gro-α includes the work of Caunt and colleagues (2006) who demonstrated that Gro-α is pivotal in thrombin-induced angiogenesis by markedly increasing vascular regulatory proteins such as MMP-1, MMP-2, VEGF, angiopoietin (Ang-2) and CD31 in human umbilical vein endothelial cells (HUVECs) whereas a study undertaken by Lane and colleagues (2002) illustrated that Gro-α and IL-8 mediate angiogenesis in Kaposi's sarcoma via the HIV-1 transactivator protein Tat and TNF-α. Furthermore, work carried out by Zhou and

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colleagues (2005) demonstrated that Gro-α increases tumourigenecity in glioma cells through the upregulation of MMP-2 and SPARC (secreted protein acid and rich in protein) and although the exact mechanisms are not known, the U251 glioma cell line being employed did not contain detectable levels of the CXCR2 receptor, leading to the possibility that Gro-α exerts its pro-tumourigenic effects via receptors other than CXCR2.

1.8.2.2 Growth Related Oncogene-β (Gro-β)

In addition to being called CXCL2, Gro-β is sometimes referred to as macrophage inflammatory protein 2-α (MIP2-α) and bares an amino acid sequence that is 90% identical to Gro-α. This chemokine is expressed by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells (Wolpe *et al.,* 1989; Lida and Grotendorst, 1990; Pelus *et al.,* 2006). Work undertaken by Wang and colleagues (2006) demonstrated that upregulation of Gro-α and Gro-β increases cellular proliferation in esophageal cancer cells and this was shown to be mediated via ERK1/2 signalling. Results from this study also revealed that elevated Gro-β expression promotes the transcription of early growth response factor-1 (EGR-1) which is known to regulate the expression of many genes involved in tumourigenesis including insulin-like growth factor-II and VEGF (Wang *et al.,* 2006; Bae *et al.,* 1999; Yan *et al.,* 2000).

Compared with Gro-α, less is known about the role of Gro-β in tumourigenesis. Cao and colleagues (1995) demonstrated that Gro-β suppressed the growth of lung tumours in immune-deficient mice by suppressing tumour vascularisation while results released by Owen and colleagues (1997) revealed that the expression of Gro-β in immortalized murine melanocytes enhanced colony formation in soft agar and tumourigenecity in nude mice with an associated induction of angiogenesis. Furthermore, a study carried out by Pulai and colleagues (2005) showed that Gro-β can augment inflammatory responses following its induction by fibronectin fragments generated from damaged extracellular matrix and taken together, experimental evidence therefore suggests that Gro-β mediates varied functions and that the biological responses to this cytokine are dependent on its cellular context.

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1.8.2.3 Growth Related Oncogene-γ (Gro-γ)

Like its other family member counterparts, Gro-γ mediates its effects via the CXCR2 chemokine receptor and is also termed macrophage inflammatory protein 2b (MIP2b) as well as CXCL3. This cytokine is primarily involved in controlling the migration and adhesion of monocytes (Smith *et al.,* 2005; Ahuja *et al.,* 1996) and shares an 86% similarity in amino acid homology with Gro-α (Haskill *et al.,* 1990). Gro-γ exhibits strong mitogenic and angiogenic properties as shown by it playing a significant role in mediating tumourigenesis (Luan *et al.,* 1997). For example, Gro-γ is highly expressed in human melanoma with tumours displaying a high degree of vascularity, however, when the tumours were depleted of Gro-γ, there was a marked reduction of tumour-derived angiogenesis and this correlated directly to inhibition of tumour growth (Luan *et al.,* 1997; Owen *et al.,* 1997). In addition, recently published work by Krogan-Sakin and colleagues (2009) showed that Gro-γ was secreted by stromal cells in response to treatment with conditioned media from immortalized non-transformed prostate epithelial cells and that this was predominantly mediated by IL-1. Gro-α, Gro-β and IL-8 were also found to be secreted and suggest that such interactions may contribute to prostatic inflammation and progression at early stages of prostate cancer formation (Krogan-Sakin *et al.,* 2009).

1.8.3 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is an important signalling protein that is involved in the formation of the circulatory system and the growth of blood vessels from pre-existing vasculature, processes called vasculogenesis and angiogenesis respectively (Risau, 1997). In addition, VEGF is known to mediate a vast array of biological processes in endothelial cells such as cell proliferation, migration, survival and differentiation (Cebe-Suarez *et al.,* 2006; Shibuya and Claesson-Welsh, 2005). VEGF was originally identified as vascular permeability factor (VPF) by Senger and colleagues (1983) and as vascular endothelial cell-specific growth factor by Leung and colleagues (1989). However, subsequent molecular cloning and expression studies revealed that these two proteins were essentially the same protein encoded by a single gene (VEGF gene) (Shibuya, 2001).

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In humans, the VEGF family consists of five members; VEGF-A, -B, -C, -D and placenta growth factor (PIGF). In structural terms, VEGF belongs to the platelet derived growth factor (PDGF) super-gene family with 8 cysteine residues being conserved at the same positions and 2 of these being used to generate intermolecular cross-linking S-S bonds (Wiesmann *et al.,* 1997). All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (the VEGFRs) and are termed VEGFR-1, -2 and -3, although VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF (Holmes *et al.,* 2007; Stuttfeld and Ballmer-Hofer, 2009). The general biochemical steps involved in the signal transduction of VEGF receptors entails ligand binding to the receptor, receptor dimerization, activation of tyrosine kinase, autophosphorylation of the receptor followed by binding and activation of adaptors at the autophosphorylation sites (Schlessinger, 2000).

VEGF is an important angiogenic factor in many human tumours and its over-expression has been observed in carcinoma of the ovary, cervix and breast amongst others (Cheng *et al.,* 1999). Several factors have been shown to participate in the regulation of the VEGF gene and include a variety of stimuli such as hypoxia inducible factors (HIF1α and HIF2α), growth factors, p53-mutation, estrogen, tumour promoters and transformation (Shibuya, 2001). The expression of PTGS-2, PGE_2 and TGF-β are often shown to be induced in human malignancies such as colorectal carcinoma and breast cancer and their upregulation in the same areas of the tumour as VEGF suggests their expression may be coordinated in cancer-induced angiogenesis (Fosslien, 2001).

Folkman (1971) reported that growth of solid tumours and their metastasis are dependent on angiogenesis and since VEGF is the major regulator of blood vessel formation, its expression in tumour growth is of the utmost importance. A study from our laboratory demonstrated that SP induced the upregulation of VEGF in HeLa adenocarcinoma cells expressing the PTGER4 receptor (Muller *et al.,* 2006). As a result, SP has the potential to promote changes in vasculature in dysplastic cells by directly inducing the over-expression of VEGF and indirectly via the upregulation of other pro-tumourigenic factors such as PGE_2 and PTGS-2 that can also enhance VEGF production.

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1.8.4 Proliferative Marker Ki-67

Ki-67 is strictly associated with human cell proliferation and the expression of this protein can be utilized to assess the growth fraction of a given cell population (Duchrow *et al.,* 1996). This protein was originally defined by the prototype monoclonal antibody Ki-67 which was generated by Gerdes and colleagues (1983) by immunizing mice with nuclei of the Hodgkin lymphoma cell line L428. The name Ki-67 is derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate (Scholzen and Gerdes, 2000). Two protein isoforms of Ki-67 are known to exist and exhibit molecular masses of 320 and 359 kD. The gene encoding the Ki-67 isoforms is localized on chromosome 10 and organized in 15 exons with the centre being formed by an extraordinary 6845 bp exon containing 16 successively repeated homologous segments of 366 bp known as 'Ki-67 repeats' (Duchrow *et al.,* 1996).

Ki-67 is regarded as an excellent proliferative marker because it is present during all active phases of the cell cycle (G_1, S, G_2 and mitosis), absent in resting cells (G_0) (Scholzen and Gerdes, 2000) and there is currently no convincing data to suggest that Ki-67 may not be expressed by any proliferating human cell type. Although the functional significance of Ki-67 remains unclear, the expression of this protein is an absolute requirement for progression through the cell-division cycle, thus making it a useful prognostic tool in tumour diagnostics (Scholzen and Gerdes, 2000). In cases of multiple myeloma, expression of Ki-67 was shown to correlate with disease progression (Drach *et al.,* 1992) and it can also be employed as a predictor of overall survival of patients with distant metastasis (Heslin *et al.,* 1998). Research performed by Wang and colleagues (2008) demonstrated that transfection with antisense Ki-67 cDNA in breast cancer cells MDA-MB-435s which highly express the Ki-67 protein resulted in a 70-80% reduction in proliferation and may offer itself as a potential option in anticancer therapy.

In addition to Ki-67 positively stained tumour cells bearing a close correlation in disease progression in carcinomas such as the breast and prostate (Scholzen and Gerdes, 2000), work carried out by Conesa-Zamora (2009) revealed that positive expression of Ki-67 was associated with severity of dysplasia in precursor lesions of

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cervical carcinoma and along with negative expression of p53, appeared to be linked with HPV-16 infection. As cancer of the cervix is multistage, several studies have used Ki-67 as a proliferative marker to investigate cervical tumour progression. These findings show the expression of this protein to correlate with bcl-2 levels and higher grades CIN2 and CIN3 in squamous cell carcinomas as well as acting as a gauge for apoptosis in adenocarcinoma in the uterine cervix and is of great value in determining how well a tumour responds to treatment (Looi *et al.,* 2008; Leung *et al.,* 2004; Scholzen and Gerdes, 2000).

As the presence of Ki-67 is essential for cellular proliferation, the generation of a suitable mouse phenotype is probably lethal and although a conditional approach using the cre/loxP system may be an option (Rajewsky *et al.,* 1996), the exact biological function of this protein remains to be fully understood. Nevertheless, the prognostic significance of Ki-67 is evident from the publication of much data correlating the expression of this protein with the clinical course of several cancers and patients likelihood of survival.

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1.8.5 Endothelial Marker CD31

CD31 is also termed as platelet endothelial cell adhesion molecule (PECAM-1) and its primary roles are the removal of aged neutrophils from the body and tissue repair. CD31 is widely distributed with it being located on endothelial cells, leukocytes, T and B cells, monocytes, granulocytes, platelets and 40% of bone marrow cells (McMichael *et al.,* 1987; Tang *et al.,* 1993). This adhesion molecule is a single-chain glycoprotein with a mass of approximately 130-140 kD (McMichael *et al.,* 1987) and the gene encoding CD31 is approximately 65 kb in length and localized to human chromosome 17. Alternative splicing of the tail appears to generate multiple isoforms of CD31 and this may regulate phosphorylation and affinity modulation (Kirschbaum *et al.,* 1994).

CD31 molecules have previously been identified to reside on human, rat and murine solid tumour cell lines, including colon adenocarcinoma, although the level of expression was shown to vary considerably amongst the different types investigated

(Tang *et al.,* 1993). Other research performed by Ding and colleagues (2005) demonstrated that there was a strong correlation between the expressions of VEGFR-3, CD31 and tumour metastases in Chinese males with prostate cancer and that this also correlated with increases in microvessel density (MVD).

Further studies have employed CD31 to serve as an endothelial marker for angiogenesis with Mazibrada and colleagues (2008) demonstrating that expression of CD31 correlated with MVD and disease progression in clinical samples of cervical dysplasia and cervical carcinogenesis when compared to normal cervix, while Darai and colleagues (1998) showed that immunohistochemical expression of CD31, as determined by MVD, was significantly higher in malignant tumours than in borderline tumours and benign tumours of the ovary and did not correlate with levels of VEGF. In human tumours, angiogenesis is evaluated by MVD in carcinomas of the breast, lung, prostate, head and neck and rectum as well as in malignant melanoma and taken together suggests that using CD31 as a marker of angiogenesis can distinguish the extent of disease progression and patients survival chances and this applies to cancer of the cervix. (Horak *et al.,* 1992; Yamazaki *et al.,* 1994; Wakui *et al.,* 1992; Petruzzelli *et al.,* 1993; Saclarides *et al.,* 1994; Barnhill *et al.,* 1992).

1.9 Physiological Processes Closely Associated with the Development and Progression of Cancer

1.9.1 Inflammation

A functional relationship between inflammation and cancer has been long established with Rudolf Virchow hypothesizing as early as 1863 that the beginnings of cancer originated from chronic sites of inflammation (Coussens and Werb, 2002). It is now estimated that approximately 15% of global cancer is attributable to chronic inflammation (Balkwill and Mantovani, 2001). Further proof of inflammation playing an important role in cancer development is the use of NSAIDs which have been shown to prevent spontaneous tumour development in people with familial adenomatous polyposis (FAP) and more recently, the use of NSAID inhibitors which target PTGS-2 (a major mediator of inflammation) have been entrusted to reduce the risk of

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developing colorectal carcinogenesis and prostate cancer (Oshima *et al.* 1995; Moustakas *et al.*, 2002; Cheng *et al.*, 2007).

The tumour microenvironment, which is partly orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, although, whether inflammation alone is sufficient to cause tumour-initiating changes remains to be proven (Dvorak, 1986). For cancer to develop and progress unabated, it requires the acquisition of six fundamental properties that includes; self-sufficient proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, unlimited replicative potential, self-maintenance of vascularization and finally, in order to create conditions for malignancy, tissue invasion and metastasis need to occur (Balkwill and Mantovani, 2001). Nevertheless, to fully appreciate the role of inflammation in the evolution of cancer, a firm understanding of how inflammation helps co-ordinate physiological and pathological responses such as tissue repair and regeneration is required (Dvorak, 1986).

When tissue injury occurs, the normal reaction from the human body is to mend itself and the first step taken towards achieving this goal is accomplished by releasing a magnitude of chemical signals at the damaged site such as members of the interleukin family including IL-1 and IL-8, histamine, prostaglandins, IFN-γ,VEGF, nitric oxide (NO), platelet derived growth factor (PDGF) and monocyte chemotactic protein (MCP), thus enlisitng a vast range of effector cells to partake in healing the effected tissue (Balkwill and Mantovani, 2001; Coussens and Werb, 2002) . This mode of action signifies the beginning of the host's acute inflammatory response and once activated by chemotactic recruitment, effector cells which include; leukocytes, macrophages, neutrophils, monocytes, eosinophils and lymphocytes migrate to the damaged area, where each performs their individual restorative function (Grivennikov and Karin, 2009; Balkwill and Mantovani, 2001). The effector cells mediate their uniform regulation of wound healing via the release of a multifactorial array of cytokines, cytoxic mediators, matrix metalloproteinases (MMPs) and mediators of cell killing such as TNF-α and interferons (Kuper *et al.*, 2000; Wahl and Kleinman, 1998). Neutrophils are earliest to arrive at the scene which occurs via chemotaxis and their

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main function is to engulf and remove the foreign material by phagocytosis. Macrophages that are derived from monocytes follow shortly after and provide an abundance of growth factors and cytokines to aid with repair. Mast cells are also crucial in the inflammatory process due to the release of pro-inflammatory mediators that help govern the overall response, and in doing so, are of great importance to the host.

However, irregular production of inflammatory mediators, such as IL-1, IL-6 and IL-23 can activate oncogenic transcription factors NF-κB or STAT3 which are intrinsic to inducing expression of anti-apoptopic genes and have been found to exist in over 50% of all cancers (Grivennikov and Karin, 2009; Grivennikov and Karin, 2010; Yu *et al.*, 2007; Karin 2006; Levy and Darnell, 2002). Furthermore, another inflammatory mediator that requires tight regulation is that of tumour necrosis factor-α (TNF-α). It is accountable for controlling inflammatory cell populations but sometimes this cytokine is not governed properly, usually resulting in the development of chronic diseases that include acute chronic pancreatitis, chronic renal failure and obstructive chronic pulmonary disease (COPD), as well as several cancers such as chronic lymphocytic leukaemia (Moustakas *et al.*, 2002; Kiyici *et al.*, 2009; Descamps-Latscha *et al.*, 1995; Keatings *et al.*, 1996; Balkwill and Mantovani, 2001). Other cytokines that are heavily involved in the inflammatory response includes IL-6 which is secreted by T-cells and macrophages to stimulate the immune response in reply to trauma injuries such as burns, IL-8 which is responsible for inducing neutrophils to leave bloodstream and enter into surrounding tissue and CXCL12, the production of which helps co-ordinate cell trafficking that is important for maintaining homeostasis (Barbieri *et al.*, 2010). Although the primary function of these cytokine messengers is to aid with the inflammatory response, their aberrant expression can have effects on recruitment of immunosuppressive cells, cellular proliferation and ability to encourage cell migration, all of which are cancer-favouring traits, thereby stimulating tumour development.

The main concept to be noted from examining inflammation and cancer in combination is that during the 'normal' inflammatory response, as in tissue repair, the process is self-limiting, in other words, it has an end, no means of continuing once the

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stimulus is removed. Whereas, if any of the converging factors which regulate inflammation become dysfunctional, abnormalities can occur and this appears to be what happens during neoplastic progression (Coussens and Werb, 2002). Further highlighting the strong associations between inflammation and cancer development arises from the fact that many molecules and pathways are linked, playing dual roles in homeostasis, wound healing and tumourigenesis (Balkwill and Mantovani, 2001). For example, the Wnt/β-catenin pathway is involved in maintaining the steady-state proliferative compartment and tumourigenesis of tissues, whilst PTGS-1 and PTGS-2, whom play a critical role in synthesizing prostaglandins to mediate tissue repair in the alimentary tract, are also heavily involved in tumour development (Dicarlo *et al.*, 2001; Coussens *et al.*, 1999; Coussens *et al.*, 2000).

PTGS-1 is constitutively expressed in most tissues and was initially perceived as synthesizing low levels of prostaglandins in the maintenance of physiological functions (Smith *et al.*, 1996), whereas PTGS-2 on the other hand is highly inducible in response to pro-inflammatory stimuli, cytokines, mitogens and reputed for its role in activating inflammatory prostaglandins such as PGE_2 (Rajakariar *et al.*, 2006). The observation that many NSAIDs operate on the basis of selectively inhibiting the actions of PTGS-2 further stresses the significant role of PTGS-2 in inflammation, although, several studies have now implicated PTGS-1 in tumour associated inflammation with it being shown to promote cell differentiation in the human monocytic leukaemia cell line (THP-1) in response to phobel esters and is over-expressed in cervical adenocarcinoma with its products appearing to act in an autocrine/paracrine role in regulating the expression of PTGS-2, PGE_2 and its receptors (Rajakariar *et al.*, 2006; Sales *et al.*, 2001). PGE_2 levels that are increased by the upregulation of PTGS-1/-2 enzymes can further augment the inflammatory response through influencing the capacity of human T cells to produce cytokines such as IL-12 and IFN-γ via PTGER receptors (Boniface *et al.*, 2009) and have been shown to promote the upregulation of Gro-α, a chemokine known to play an important role in regulating inflammation (Wang *et al.*, 2006).

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Seminal plasma is known to contain potent anti-inflammatory properties in order to provide a survival advantage to the spermatozoa within the hostile female tract and is demonstrated by SP and its component prostaglandins being able to stimulate the release of the anti-inflammatory cytokine IL-10 (Denison *et al.,* 1999a). However, contrasting data has shown pro-inflammatory leukoctosis to occur post-coitus in humans and animals with data generated from a study performed on mares revealing that a significant increase in inflammation was observed in the uterine canals as a direct result of inseminating with low doses of seminal plasma (Portus *et al.,* 2005). It is therefore possible that SP initiates anti-inflammatory and pro-inflammatory responses in the female reproductive tract in order to firstly facilitate its survival and then to aid with its removal (Denison *et al.,* 1999a).

Many of the effects that seminal plasma exhibits are not fully understood, although evidence is gathering to support a possible role in cervical tumourigenesis. PTGS-1 and PTGS-2 have been frequently linked with inflammation and cancer progression, as have similar roles been suggested for IL-6 and TGF-β1 (Grivennikov and Karin, 2009; Moustakas, 2002). SP has previously been shown to upregualte PTGS-1, PTGS-2, IL-1β, IL-6, IL-8 and TGF-β1 in several cancers including cervical carcinoma and it is therefore possible that by mediating inflammation in the cervical canal, SP could trigger tumour-initiating factors to favour tumourigenesis and cancer progression (Jabbour and Sales, 2004; Gutsche *et al.,* 2003; Sharkey *et al.,* 2007; Robertson *et al.,* 2002). Research performed by Khadra and colleagues (2006) investigated levels of cytokines in SP in patients with chronic prostatitis (CP) and chronic pelvic pain syndrome (CPPS) and demonstrated that patients with these chronic conditions constantly exhibited elevated levels of IL-8 in their SP, thus suggesting that IL-8 maybe a contributing pro-inflammatory component in the pathogenesis of these conditions as well as other reproductive malignancies.

1.9.2 Angiogenesis

Angiogenesis is a multistep physiological process that involves the formation of new blood vessels from pre-existing ones and its regular operation is essential for normal growth and development (Racia *et al.,* 2009). For angiogenesis to perform

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correctly, angiogenic growth factors firstly act upon cognate receptors on vascular endothelial cells present in pre-existing blood vessels and following their activation, the endothelial cells release protease enzymes which degrade the basement membrane, thus permitting their escape. Once the endothelial cells have departed from their original vessel walls, they then migrate by means of chemotactic stimuli, proliferate into the surrounding extracellular matrix, forming solid branches that connect to neighbouring tissue. Development of these new branches allows new vessels to grow across gaps in the vasculature and displays why angiogenesis is also important as a repair mechanism (Burri *et al.,* 2004). However, when angiogenesis occurs excessively or deficiently, several different pathological conditions can arise that include; ischaemia, blindness, tumour growth and tumour progression (Racia *et al.,* 2009).

In order for a tumour to grow, it requires a supply of nutrients and oxygen and this demand increases as the tumour develops, often generating needs that outweigh the current supply. The logical way around this problem is to increase vascularisation and the cancer cells undertake this task by inducing many factors that are strongly involved in the angiogenic process such as vascular growth factor (VEGF), basic fibroblast factor (bFGF), endothelin-1 and platelet derived growth factor (PDGF) (Tsujii *et al.,* 1998). Recruitment of these pro-angiogenic factors induces angiogenesis and the more these factors are expressed, the more aggressive a cancer can become, having developed sufficient vasculature to support a quicker progression of the disease.

PTGS-2 is very important in many cancers as it strongly induces VEGF which is a common angiogenic factor that directs tumour-induced angiogenesis (Detmar, 2000). Various case studies have revealed that expression of PTGS-2 was found to be significantly elevated in 90% of lung cancers (18/20), 70% of colon adenocarcinomas (14/20) and 55% of breast tumours (11/20) compared to non-tumour tissue from the respective origin (Soslow *et al.,* 2000). High levels of PTGS-2 are indicative of poor prognosis as patients who develop cancers with overly expressed levels of PTGS-2, such as cervical carcinomas, tend to be associated with a shorter survival (Gaffney *et al.,* 2001). A mouse model of angiogenesis revealed that administration of the PTGS-2

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inhibitor, NS-398, blocked angiogenesis, and further stresses the importance of PTGS-2 involvement in the progressing of cancers (Hernandez *et al.*, 2001).

Upregulation of VEGF is one of the driving forces controlling angiogenesis and expression of this growth factor has been demonstrated to be significantly increased in several cancer types, including; ovarian, breast and renal (Garzetti *et al.*, 1999; Yoshiji *et al.*, 1996; Paradis *et al.*, 2000). Colorectal carcinoma is another human malignancy that exhibits upregulation of VEGF along with prostaglandins and PTGS-2 and because their expression is localised to the same area of the tumour, it suggests that their coordinated efforts are involved in cancer-induced angiogenesis (Fosslien, 2000; Musunaga, 2000).

Besides VEGF, other factors closely associated with the induction of changes in vasculature are the angioproteins Ang1 and Ang2. Studies in knock-out mice show that these protein growth factors are required for the formation of mature blood vessels and this is achieved by binding to their cognate tyrosine receptors (Tie-1 and Tie-2) in order to trigger cell signalling cascades (Thurston, 2003). MMPs are another major contributor to angiogenesis as they help degrade proteins whose function it is to maintain solid vessel walls. Following degradation, nearby endothelial cells migrate into the interstitial matrix and results in sprouting angiogenesis. This is supported in findings generated by Haas and colleagues (2000) who demonstrated that inhibiting MMPs in rat skeletal muscle prevented the formation of new blood capillaries. Furthermore, work carried out by Nikolopoulos and colleagues (2004) in mice showed tumour angiogenesis to be dependent on α6β4 signalling with endothelial cell migration and invasion being induced by promoting the nuclear translocation of ERK and NF-κB. The integral nature of angiogenesis and cancer has led to the development of angiogenesis based tumour therapy with Avistan, a monoclonal antibody directed against an isoform of VEGF, being recently approved to treat colorectal cancer in combination with chemotherapy.

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1.9.3 Homeostasis and Apoptosis

Homeostasis is the ability of the human body to maintain a stable condition by upholding the internal environment in a state of equilibrium. A wide variety of practices are carried out by the body to preserve a constant status and these range from the utilization of insulin and glucagon in regulation of blood glucose levels to sweating or shivering in order to decrease or increase body temperature (Bhagavan, 2002).

Another mechanism that needs to be tightly safeguarded in preserving a homeostatic environment is cell number constancy, achieved through cellular proliferation and cell death. Cells must be replaced when they malfunction or become diseased, but proliferation must be offset by cell death (Thompson, 1995). The process of self-programmed cell death or apoptosis occurs when a cell is damaged beyond repair or infected with a virus and therefore apoptosis functions to remove the damaged/infected cell to prevent the abnormalities from spreading. Apoptosis is inhibited or completely absent during tumourigenesis, therefore allowing the cells to continually divide unabated and develop into a tumour (Hanahan and Weinberg, 2000). There are numerous apoptosis-regulating genes which have been identified to date and these include; p16, p21, p27, p53 and E2F genes, caspase genes and the anti-apoptotic bcl-2 gene family (Opalka *et al.,* 2002). The most prominent apoptotic-regulator is the p53 tumour suppressor gene, with inactivation of its encoded p53 protein being reported in 50% of human cancers (Harris, 1996). Moreover, downregulation of p53 has been closely correlated with overexpression of PTGS-2 in gastric, head and neck tumours, but no such relationship was established in an endometrial cancer study (Shun *et al.,* 2003; Gall *et al.,* 2003). In addition, various model systems which use overexpressing PTGS-2 epithelial cell lines have displayed that using selective inhibitors for PTGS-2 coincided with a restoration of apoptosis (Erickson *et al.,* 1999).

1.9.4 Mitogenesis and Cellular Proliferation

Mitogenesis is the induction of mitosis in a cell, the manner by which all cells divide. In other words, it is the process that encompasses cellular proliferation, normally resulting in two new nuclei, each of which contains a complete copy of the

parental chromosomes (De Souza and Osami, 2007). Numerous cell types exhibit life spans that are limited and the course of action that mitosis takes permits the cells to be renewed on a regular basis. A prime example being the growth of a foetus into a baby, then progression to childhood and subsequent adulthood, giving emphasis to the fact that without the constant growth and renewal of cells, life would cease to exist. Under normal physiological conditions, mitogenic growth factors and adhesion to the extracellular matrix (ECM) are requisite by the cells in order to proliferate (Bottazzi and Assoian, 1997). Such molecules that allow the cells to propagate successfully include; epidermal growth factor (EGF), insulin growth factor I (IGF-I), receptor tyrosine kinases (RTKs), cyclin dependent kinases (CDKs), p21 and p53 (Bottazzi and Assoian, 1997; Steller *et al.,* 1995). However, when cancer occurs, many tumour cells attain the capability to produce their own growth factors to which they respond, enabling the cancer to self-govern itself by heightening the rate of cellular proliferation through increasing mitogenesis.

EGF is an important mediator of regulated cellular proliferation, although deficient control of this growth factor is often mediated by exposure to excessive quantities of TGF-β which is present in high concentrations in seminal plasma and closely linked with malignant tumour progression (Kerbel, 1993, Robertson *et al.,* 2002). Furthermore, the enhanced expression of EGF has been noted in colonic and cervical cancer cell lines, HCA-7 and HT-3 respectively, subsequently resulting in increased levels of VEGF which is reflected by amplified rates of cellular proliferation (Merchant *et al.,* 2005; Steller *et al.,* 1993; Mathur and Mathur, 2004). Another common enzyme that is inducible to various stimuli, including mitogenic factors, is PTGS-2, whose upregualtion has been noted in several cancer forms. A study employing rat intestinal cell lines and mouse colon cancer cell lines demonstrated that normal cell proliferation and growth of malignant cells was inhibited as a direct result of PTGS-2 inhibitors causing a dose-dependent response in mitogeneis (Erickson *et al.,* 1999). It can therefore be concluded that growth factors appear to have a role in the development and expression of malignant transformation because tumour promoters and mitogenic hormones share common pathways in eliciting mitogenesis in certain cell types (Rozengurt, 1986).

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1.9.5 Tissue Invasion and Metastasis

Metastasis is a characteristic trademark of many cancers and the 'term' is used to describe the spread of the disease from one organ to another organ or body part. The most common places where metastasis originates from are the lungs, liver, and the brain, with cancer cells moving away from these organs to other locations in the body via the bloodstream or the lymphatic system (Demetrious and Demetrious, 2008; Paye *et al.,* 1998; Barnholtz-Sloan *et al.,* 2004). Cervical cancer repeatedly metastasizes to the retroperitoneal lymph nodes as a result of the rich lymphatic network utilized by the cervix. In extreme circumstances, cervical carcinoma has also been shown to metastasize to the breast (Kumar *et al.,* 1999) and the brain (Ikeda *et al.,* 1998), but these events are very uncommon and rarely do cases present themselves with these findings.

In order for cancer cells to invade surrounding tissue, they must first become mobile, which is achieved through changes in cell-surface adhesion molecules (CAMs) (Sales and Jabbour, 2003). Alterations in E-cadherin function are the most widely observed in CAMs and model systems that overexpress E-cadherin have demonstrated a reduction in metastasis (Christofori and Semb, 1999). However, in a large number of solid tumours, E-cadherin is downregulated and closely correlates with enhanced invasion of neoplastically transformed cells (Mayer *et al.,* 1993). Studies using overexpressing PTGS-2 cells have displayed that a loss of cell surface adhesion molecules and ensuing decrease in E-cadherin is a direct result from increased expression of PTGS-2 (Tsujii and DuBois, 1995). This in turn suggests that PTGS-2 upregulation may promote cell invasion and metastasis by downregulating CAMs (Tsujii and DuBois, 1995). Furthermore, upregulation of the prostanoid EP receptors has been shown to incite migration of tumour cells *in vitro* (Fulton *et al.,* 2006) and because this process is mediated by PGE_2 (contained in high concentrations within SP), and SP has the ability to increase production of PTGS-2 and subsequently PGE_2 (Sales *et al.,* 2002b), SP may potentially aid in metastasizing cancer of the cervix.

Once mobile, tumour cells invade parts of the body where space and nutrients are not in limited supply and this is accomplished by degradation of the extracellular matrix

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by matrix metalloproteinases (MMPs). Carcinomas of the cervix have previously displayed upregulated expression of matrix metalloproteinases MMP-2 and MMP-9 compared with normal cervix (Moser *et al.*, 1999) and although the overall biological effect of SP on the neoplastic cervical epithelium of sexually active women is still not known, SP has been shown to encourage the release of matrix metalloproteinases which can result in enhancement of metastasis (Jermemias and Witkin, 1999). In addition, TGF-β is contained within SP in high concentrations and can also regulate extracellular matrix composition and degradation that are important in tissue invasion and metastasis (Jakowlew, 2006). Therefore, neoplastic epithelial cells undergoing repeated exposure to SP in sexually active women may augment expression of MMPs and extracellular matrix, thus amplifying the progression of cervical cancer. Besides being strongly associated in cervical cancer, metastatic disease is very frequently the cause of death in colon cancer sufferers and inhibition of this process may therefore be of significant benefit to the patient (Marnett, 1992).

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1.10 General Aims and Objectives of the Thesis

Cervical cancer is the second leading cause of cancer death in women worldwide with approximately 500,000 women developing the disease every year (Minaguchi, 2010). Although HPV is a prerequisite for developing cancer of the cervix, it takes on average 10-15 years for carcinoma to develop (Munoz *et al.,* 1992; Bosch *et al.,* 1995) and as a result there is accumulating evidence to suggest a role of other causative factors that contribute to the onset and progression of cervical cancer, including exposure of neoplastic epithelial cells to seminal plasma (SP) (Wallin *et al.,* 1999; Muller *et al.,* 2006; Beral *et al.,* 1994). More studies are required to elucidate the exact mechanisms involved in the development and progression of cervical cancer and to assess if SP is fundamental in the chain of events. The general objective of this research was to determine a possible functional role for SP in cervical cell neoplasias. This was done by addressing the following specific aims:

(i) *Determine whether seminal plasma can induce upregulation of pro-inflammatory and pro-angiogenic mediators in normal cervical tissue.*

(ii) *Determine the effect of seminal plasma on target gene regulation and cell signalling by employing WT HeLa cells as a model system.*

(iii) *Determine the effect of seminal plasma on growth of WT HeLa xenografts in MF-1 nude mice.*

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Chapter 2

GENERAL MATERIALS AND METHODS

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2 General Methods and Materials

2.1 Materials and Reagents

All chemicals used were molecular biology grade, and were obtained from Sigma Chemical Company (Cape Town, South Africa)(SA) or (Dorset, United Kingdom)(UK) or IBI (Cambridge, UK) unless otherwise stated.

Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 was purchased from Whitehead Scientific (Whitehead Scientific, Cape Town, SA) and Gibco (Gibco, Life Technologies, Paisely, UK). Foetal Bovine Serum (FBS) and penicillin-streptomycin were purchased from Highveld Biological (Highveld Biological, Johannesburg, SA) and PAA Laboratories (PAA Laboratories, Middlesex, UK). Phosphate buffered saline (PBS) and trypsin-EDTA were purchased from BioWhittaker (BioWhittaker, Berkshire, UK). HeLa-S3 cells were purchased from BioWhittaker (BioWhittaker, Berkshire, UK).

RNA later was purchased from Southern Cross Biotechnology (Southern Cross Biotechnology, Cape Town, SA) and Ambion (Applied Biosystems, Warrington, UK). Trizol™ was purchased from Highveld Biological (Highveld Biological, Johannesburg, SA) and Invitrogen (Invitrogen, Paisley, UK) and phase lock tubes obtained from Flowgen (Flowgen, Bioscience, Nottingham, UK). 1-bromo-3-chloropropane (BCP) and isopropanol were purchased from Sigma (Sigma Chemical Company, Nottingham, UK) and the TaqMan GeneAMP RNA PCR kit was purchased from Perkin Elmer, PE Biosystems (Perkin Elmer, PE Biosystems, Warrington, UK).

Percoll® was purchased from Sigma (Sigma Chemical Company, Nottingham, UK). Celltitre96® AQueos One Solution Proliferation Reagent was purchased from Promega (Promega Corp, WI, USA). Chemical inhibitors AG-1478 (100µM stock in DMSO), PD-98059 (50mM stock in DMSO) and NS-398 (10mM stock in DMSO) were all purchased from Calbiochem (Merck Chemicals, Nottingham, UK). SC-560 (10mM stock in DMSO) was purchased from Caymen Chemical (Caymen Chemical, Michigan, USA). TGF-β1 inhibitor (50µg/ml stock in 10mM citric acid) was

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purchased from Peprotech (Peprotech, New Jersey, USA) and finally, NF-κB inhibitor (5mg/ml stock in H_2O) was purchased from Enzo Life Sciences (Enzo Life Sciences, Exeter, UK).

Pre-cast 4-12% Bis-Tris gels and SeeBlue® Plus2 pre-stained marker were purchased from Novex (Novex, UK). Polyvinylidene diflouride (PVDF) membrane was obtained from Millipore (Millipore, Watford, UK). Whatman No.3 paper was purchased from Whatman (Whatman, UK). Odyssey blocking buffer was purchased from Li-Cor BioScience (Li-Cor BioScience, Cambridge, UK).

Purchases made for immunohistochemistry included 3.3'-diaminobenzidine (DAB), obtained from Dako (Dako, Cambrigeshire, UK) and Pertex which was obtained Cellpath (Cellpath, Hemel Hampstead, UK). Primary antibodies Ki-67 and CD31 were purchased from Vector Laboratories (Vector Laboratories, Peterborough, UK) and Abcam (Abcam, Cambridge, UK respectively whereas secondary antibodies goat anti-rabbit and swine anti-rabbit were purchased from Sigma (Sigma Chemical Co, Nottingham, UK).

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2.2 Tissue Collection and Processing

Ethical approval for collection of normal and malignant cervical tissue was obtained from University of Cape Town Human Research Ethics Committee of the Faculty of Health Sciences under ethics number (REC/REF: 152/99) and written informed consent was obtained from all patients prior to collection.

2.2.1 Cervical Tissue

Normal cervical tissue was a mixture of ectocervical and endocervical specimens collected from women undergoing complete or partial hysterectomy for non-malignant conditions at Groote Schuur Hospital, Cape Town, South Africa. Immediately after surgery, tissue was divided into 3 portions, one portion was stored in RNA Later (Ambion) at -70°C for RNA extraction, another portion was fixed in 4% neutral buffered formalin and subsequently wax embedded for histological analysis

and the third portion was cultured in serum free medium for seminal plasma stimulation. All samples were confirmed histologically by a pathologist based on the H&E-stained slides that were produced from the collected tissue (Bancroft and Stevens, 1982).

2.2.2 Cervical Carcinoma Tissue

Cervical carcinoma specimens were obtained at the time of surgery/biopsy from patients who were attending the Gynecologic Oncology Clinic at Groote Schuur Hospital, Cape Town, South Africa and who had previously been diagnosed with invasive carcinoma of the cervix. Punch biopsies were taken from the lesion by an experienced gynaecologist with a special interest in oncology. At collection, tissue was fixed in 4% neutral buffered formalin and wax embedded for histological analysis. The biopsy samples were very small and were only of sufficient size to perform histological analysis which was deemed the priority. Cervical biopsies were assessed by a pathologist and designated as either squamous or adenocarcinoma tumours by means of histological confirmation on H&E-stained tissue sections (Bancroft and Stevens, 1982).

2.3 Semen Donors and Preparation

Semen was obtained from 10 healthy male volunteers. The collected ejaculates were pooled in batches of 3 or 4 samples and incubated at room temperature for 30 min to liquefy prior to overlaying on a 100-50% percoll gradient using Percoll® (Sigma Chemical Company, Nottingham, UK). Seminal plasma (SP) was isolated from the pooled ejaculate by percoll density gradient centrifugation at 500 *g* for 20min. The seminal plasma was aliquoted and stored at -80˚C until required. Prior to treatment of cervical tissue and the WT HeLa cell line, seminal plasma was thawed on ice and 1 aliquot of each batch was further pooled prior to beingdiluted in serum-free culture medium to use at concentrations of 1:500 and 1:100 respectively. At dilutions of 1:50 seminal fluid has been reported to exert no effect on WT HeLa cell viability (Jeremias *et al.*, 1997). Approval for the study was obtained from the University of Cape Town Human Research Ethics Committee, and informed consent was obtained from all patients prior to sample collection, ethics approval no. (REC/REF 152/99).

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2.4 Animals

2.4.1 Housing

MF-1 nude mice were obtained from the University of the Witwatersrand, Johannesburg, South Africa. The mice were used between 6-18 weeks (wks) of age and housed in the BSL2 Animal Unit, University of Cape Town, South Africa. The mice were maintained in individually ventilated cages (IVC) under sterile conditions. These cages were operated under negative air pressure in a controlled environment of approximately -10 to -20 Pascal for bio-containment. Room temperature was controlled electronically and maintained within a range of 20-23°C and light was supplied by cool white fluorescent tubes. Food and water were available *ad libitum.* All experimental procedures were performed in a separate procedure room in safety cabinets. Animal care and experimental protocols were approved by the Animal Ethics committee of the University of Cape Town, approval no. (REC/REF: 006/044).

2.4.2 WT HeLa Cell Xenografts in MF-1 Nude Mice

A suspension of 2 x 10^6 Wild type HeLa cells in a total volume of 0.2 ml PBS was injected subcutaneously into the right dorsal flank of MF-1 nude mice. The mice were assigned into two treatment groups, Control (n=8) and SP (n=6). The inoculated MF-1 nude mice were injected three times weekly with 100µl PBS or SP (1:500) (diluted in PBS) via intra-peritoneal injection for ten weeks. During the treatment period, body weights (g) were recorded weekly and tumours measured (mm^3) as per the method by Tomayko and Reynolds (1988) which employs the formula (1/2 x length x width x height). At the end of the study, animals were sacrificed by CO_2 exsanguinations, tumours were excised and weighed. A proportion of the tumour was fixed in 4% paraformaldehyde for wax-embedding and immunohistochemistry and the remainder used for RNA extraction.

2.5 Cell Culture and Cellular Investigations

2.5.1 Culture Conditions and Passaging

The HeLa-S3 cell line, referred to as wild type (WT), are derived from cervical epithelial cells that were originally taken from a cervical adenocarcinoma and were

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used throughout this study. WT HeLa cells were routinely maintained in Dulbecco's Modified Eagles Medium (DMEM) nutrient mixture F-12 with Glutamax-1 and pyridoxine supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin (500 IU/ml penicillin and 500 µg/ml streptomycin; complete medium) at 37°C and 5% CO_2 (v/v). All experiments and cell treatments were performed on WT HeLa cells using culture medium without fetal bovine serum (serum-free conditions).

For passaging, cells were grown in T162 flasks and maintained in humidified conditions at 37°C with 5% CO_2 (v/v). Cells were passaged twice weekly. For passaging, cells were washed three times with PBS to remove complete medium followed by addition of 3ml 0.05% trypsin-EDTA. Cells were incubated at 37°C for approximately 5 minutes with regular agitation until detached from the culture flask. The trypsin was then neutralised with the addition of 7ml complete medium and 3mls of this cell suspension was added to 35ml complete medium and reseeded in a new T162 flask. For experiments cells were counted using a haemocytometer then plated into culture dishes at the densities indicated in Table 2.1.

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Culture Vessel	Cell Density / Well
96 well plate	5×10^3
12 well plate	1×10^5
60 mm dish	5×10^5
2-well chamber slides	5×10^4

Table 2.1: Cell densities in different culture vessels used

2.5.2 Cervical Tissue Explants Culture Conditions

Normal cervical tissue was finely chopped in DMEM nutrient mixture F-12 with Glutamax-1 and pyridoxine medium (serum free medium) and maintained overnight in humidified conditions at 37°C with 5% carbon dioxide. For treatments, approximately 50-100mg of chopped tissue was placed in a well of a 6 well plate and 1 ml serum free medium containing the treatment was added.

2.5.3 Cell and Tissue Treatments

A previous study by Sales and colleagues (2002b) determined working concentrations of SP to be 1:50 to treat cervical adenocarcinomas whereas a ratio of 1:500 was used by Muller and colleagues (2006) to treat HeLa cells expressing the PTGER4 receptor. The decision by Muller and colleagues (2006) to use SP at a dilution of 1:500 was because this equated to the working concentrations of 300nM PGE_2 that was also being used to treat cells, thus providing comparable results. It was therefore decided that an SP concentration of 1:500 be employed to treat normal cervical tissue and was incubated for the periods 2, 4, 6, 8, 12 and 24 hr in an attempt to illicit a noteworthy response.

In order to determine effect of SP stimulation on gene expression, WT cells were treated for 2, 4, 6, 8, 12, 24 and 48 hours with vehicle or SP (1:100). The results revealed that a treatment period of 6 hr and a SP concentration of 1:100 were the optimal treatment conditions. To investigate signalling pathways regulated by SP, WT HeLa cells were treated for 6 hours with vehicle or a SP concentration of 1:100 and a panel of chemical inhibitors (section 2.5.4), each one separately. All treatments were carried out with a control of cells treated with inhibitor alone and each experiment repeated four times.

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2.5.4 Chemical Inhibitors

The chemical inhibitors used within this study are listed in Table 2.2. The optimal concentration of all chemical inhibitors was determined empirically by titration using the manufacturer's guidelines as described by previous studies performed by our laboratory and other research groups (Sales *et al.*, 2008b; Lampiasi *et al.,* 2005; Lash *et al.,* 2005; Lin *et al.,* 1995; Vlahos *et al.,* 1994; Nasrallah *et al.,* 2001). The mechanisms of action of these inhibitors are described below.

Inhibitor	Molecule Inhibited	Working Conc.	Supplier
AG-1478	EGFR	200 nM	Calbiochem (Nottingham, UK)
PD-98059	MEK	50 µM	Calbiochem (Nottingham, UK)
NS-398	PTGS-1	100 nM	Calbiochem (Nottingham, UK)
SC-560	PTGS-2	10 µM	Caymen Chemical (Michigan, USA)
NF-κB Inhibitor	NF-κB	50 µg/ml	Enzo Life Sciences (Exeter, UK)
TGF-β1Inhibitor	TGF-β1	10 ng/ml	Peprotech (New Jersey, USA)

Table 2.2: Chemical inhibitors and working concentrations used

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AG-1478 inhibits epidermal growth factor receptor (EGFR) and it performs this task by acting as an analogue of adenosine triphosphate (ATP). In order for the EGFR to cause activation, it must bind to both ATP and substrate and it cannot do this as the addition of AG-1478 displaces the ATP binding (Gan *et al.*, 2007).

PD-98059 inhibits the mitogen activated protein kinase (MEK) phosphorylation of the extracellular-regulated kinases (ERK) 1/2 cascade. PD-98059 exerts its blocking properties by reversibly inhibiting MEK by the way of preventing MEK phosphorylation at a site distinctive to the active site (Dudley *et al.*, 1995).

SC-560 is a member of the diaryl heterocycle class of PTGS inhibitors and is specific for PTGS-1 while NS-398 is a specific inhibitor for PTGS-2. PTGS-1 and PTGS-2 have high affinities for PGE_2 and both are found to exert their respective inhibitory properties by suppressing synthesis of this substrate (Barnett *et al.*, 1994; Banu *et al.*, 2007).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex which is involved in the regulation of a large number of cellular processes such as the imflammatory response, cellular growth and apoptosis (Gilmore 2006). The transcription factors contained within the NF-κB complex include p50, p52, RelA, c-Rel and RelB (Hoffman *et al.,* 2006) and the NF-κB inhibitor impedes translocation of the NF-κB family into the nucleus (Lin *et al.,* 1995), thereby hindering its biological activity.

Three mammalian isoforms of TGF-β are known to exist, although TGF-β1 is the most abundant as it is expressed in almost every cell type. TGF-β1 is a multi-tasking cytokine that participates in cell proliferation and growth differentiation and has been widely studied in close association with cancer progression and metastases (Lash *et al.,* 2005). The different TGF-β receptor types can be distinguished by their structural and functional properties. Both receptor types I and II have a high affinity for TGF-β1 and low affinity for TGF-β2 while receptor type III has a high affinity for both TGF-β1 and TGF-β2 (Cheifetz *et al.,* 1988). The TGF-β1 inhibitor works as a receptor antagonist by preventing binding to the cognate receptor.

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2.5.5 Cell Proliferation Assay

WT HeLa cells were seeded in 96 well plates at 3000 cells/well (counted using a haemocytometer) and allowed to propagate overnight before serum starvation for 24hrs. Thereafter, cells were treated for durations of 24, 48 and 72hr as described in the results section in the presence/absence of inhibitors mentioned in section 2.5.4. Following treatment, proliferation was determined using the CellTitre96 Aqueous One Solution Proliferation Reagent (Promega Corp, Madison, WI) as per the manufacturers guidelines. The reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and this compound is bioreduced by cells into a formazan salt product that is soluble in tissue culture medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge and Tan 1993) and the quantity of the formazan product, as measured by the

absorbance at 490nm, is directly proportional to the number of living cells in culture. Absorbances were recorded, calculated and fold difference determined against the untreated control with each experiment being repeated three times.

2.6 RNA

2.6.1 Total RNA Isolation from Human Cervical Tissue

Total RNA was extracted from human cervical tissue using Trizol™ according to the manufacturer's guidelines. Volumes were adjusted accordingly, allowing approximately 1ml Trizol per 50-100mg of tissue. Normal cervical tissue which was stimulated by SP (1:500) or PBS (control) for the indicated times was homogenised twice in Trizol using a benchtop Polytron® PT1600E economy homogeniser (Capitol Scientific Inc, Austin, USA) for 2mins at 20,000 rpm according to the manufacturer's protocol. Once completely dissociated, samples were placed in Phase Lock tubes and a volume of 200µl bromochloropropane (BCP) was added. After shaking vigorously for 20 seconds, the samples were allowed to stand at room temperature for 15mins before centrifuging at 14,000 *g* for 15min at 4°C. Following centrifugation, the RNA remains in the upper aqueous phase above the gel layer of the Phase lock tube and DNA and proteins are contained in the organic phase below. Thereafter, the upper aqueous phase containing the RNA was transferred to a fresh RNAse-free eppendorf tube to which 500µl isopropanol was added. The tubes were incubated at room temperature for 10mins and then centrifuged at 14,000 *g* for 15min at 4°C to precipitate the RNA. Following centrifugation, the supernatant was removed and the RNA pellet was washed in 1ml 75% ethanol by further centrifugation as 14,000 *g* for 5min at 4°C. The remaining RNA pellet was then air dried and resuspended in approximately 30-50µl of 0.1% v/v diethylpyrocarbonate (DEPC) treated water.

2.6.2 Total RNA Isolation from Nude Mice Xenografts

All animals were killed after 10 weeks of treatment at which point, tumours were immediately excised and transferred to 2ml microfuge tubes containing 1ml RNA later. Total RNA was extracted from MF-1 nude mice xenografts using Trizol™ (Invitrogen) according to the manufacturer's guidelines. Volumes were adjusted

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accordingly, allowing approximately 1ml Trizol per 50-100mg of tissue. All samples were then homogenised twice in Trizol using a Tissuelyser II and RNA extracted as described in section 2.6.1.

2.6.3 Total RNA Extraction from WT HeLa Cells

Total RNA was extracted from WT HeLa cells using Trizol™ according to the manufacturer's guidelines. A volume of 1ml of Trizol™ was added to experimental wells (60mm dish) to lyse cells and samples were subsequently placed in Phase Lock tubes. RNA extraction was then undertaken as described in section 2.6.1.

2.6.4 Determination of RNA Concentration

The concentration and quality of RNA derived from human cervical tissue, nude mice xenografts and WT HeLa cells was determined by spectrophotometry. The concentration of RNA was determined by measuring the absorbance at 260nm and 280nm using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). The concentration was calculated using the value obtained at 260nm, given that an optical density of 1.0 is equal to 40µg/ml for RNA. The quality of RNA was determined by dividing the 260nm absorbance reading by the 280nm absorbance reading; a ratio of 1.6 to 1.9 was taken to be of sufficient quality containing minimal protein contamination. Purified RNA was stored at -70°C for later use.

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2.6.5 cDNA Synthesis

Reverse transcription for the generation of single strand cDNA was carried out on the isolated RNA. For each reverse transcription reaction, 1µg of the RNA was reversed transcribed using a TaqMan GeneAMP RNA PCR kit. After incubating the samples for 10min at room temperature, the polymerase chain reaction (PCR) was undertaken with the addition of the reagents mentioned below in table 2.3 and contained PCR reaction buffer (1x), $MgCl_2$ (5.5mM), dNTPs (2mM), random hexamers (2.5µM), RNAse inhibitor (0.4U/µl), Multiscribe™ reverse transcriptase (1.25U/µl) and RNAse-free water to a final volume of 10µl. PCR was performed for 1 cycle under the following conditions; 1hr 42°C, 5min 99°C and 5min at 5°C and a tube

without the addition of reverse transcriptase was included to control for any genomic DNA contamination.

Reagents	Stock Conc.	Final Conc.
RT-PCR Reaction Buffer	10x	1x
Magnesium Chloride	25mM	5.5mM
Deoxynucleoside Triphosphate (dNTPs)	10mM	2mM
Random Hexamers	50µM	2.5µM
RNAse Inhibitor	20U/µl	0.4U/µl
Multiscribe™ Reverse Transcriptase	50U/µl	1.25U/µl
RNAse Free Water	-	-

Table 2.3: Reverse transcription reaction mix; reagents and concentrations. (All reagents supplied by Perkin Elmer, PE Applied Biosystems, Warrington, UK).

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2.6.6 Taqman Quantitative Real Time PCR (QRT-PCR)

Taqman quantitative real-time PCR was performed to assess the relative expression of target genes in a sample by using specific primers, probes and PCR reagents. The reaction design is based on the principle that Taqman probes contain a quencher dye (TAMRA) attached to the 3' end and a fluorophore (FAM) attached to the 5' end, whilst the 18S probe uses a VIC fluorophore in order to distinguish from the target gene. Following the annealing between the primer and target gene, Taq polymerase extends the primer 5' to 3' which results in cleavage of FAM from the DNA probe. This therefore leads to a separation of the fluorophore and the quencher and a subsequent increase in which fluorescence is measured (Figure 2.1). Taqman probes are designed to hybridize to an internal region of the target gene, thus, the increases in fluorescence that are generated are proportional to the amount of probe cleaved at seprtation of FAM and the DNA probe. 18S is used as an internal control because it is constitutively expressed, remaining constant across all samples being analysed.

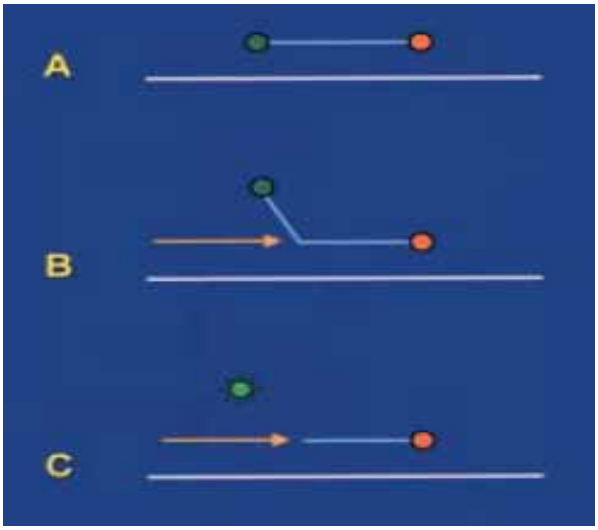

Figure 2.1: Principle of the TaqMan reaction. The 5' nuclease activity of Taq DNA polymerase is exploited to cleave a TaqMan probe during polymerase chain reaction (PCR). **(A)** TaqMan probe is shown annealed to the target DNA. The probe contains the FAM reporter dye at the 5' end of the probe (green circle) and a TAMRA quencher dye at the 3' end of the probe (red circle). **(B)** During PCR, a complementary strand of DNA is synthesised, and the 5' exonuclease activity of Taq DNA polymerase excises the reporter dye. **(C)** Fluorescence of the reporter dye (indicated by bright green starburst effect of reporter dye) occurs as a result of separation of the reporter dye from the quencher dye (Uhl *et al.*, 2002).

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RNA samples were extracted from human cervical tissue, MF-1 nude mice xenografts and HeLa cells as described in section 2.5.1, 2.5.2 and 2.5.3 respectively and reverse transcribed as detailed in section 2.5.4. A reaction mix was made using the reagents described in table 2.4 and contained Taqman buffer (5.5mM $MgCl_2$, 200µM dATP, 200µM dCTP, 200µM dGTP and 400µM dUTP), AmpliTaq Gold DNA polymerase (0.025U/µl), ribosomal 18S forward and reverse primers and probe (all at 50nM). Forward and reverse primers for genes of interest were also added to the reaction mix, were used at 250nM and these sequences are described in table 2.5. A volume of 48µl of reaction mix was aliquoted into separate tubes for each cDNA sample and 2µl/replicate of cDNA was added. Following mixing, 23µl of the reaction mix from each sample, including a no reverse transcriptase control and a calibrator (positive control) were added to Fast optical 96-well MicroAMP PCR plates (PE Applied Biosystems, Warrington, UK). The PCR plate was sealed with MicroAMP optical adhesion film (PE Applied Biosystems, Warrington, UK) and the cDNA expression

was measured using an ABI Prism 7900 HT Fast Real Time PCR machine (PE Applied Biosystems, Warrington, UK).

Reagents	**Stock Conc.**	**Final Conc./Vol.**
Taqman Buffer (containing 5.5mM $MgCl_{2,}$ 200µm of each dNTPs, 0.025U/µl of AmpliTaq Gold DNA polymerase)	2x	1x
Ribosomal 18S Primer (forward and reverse probe)	25µM	50nM each
Target Gene (forward and reverse primers)	25µM	300nM each
Target Gene Probe	5µM	100nM each
cDNA	-	2µl/50µl

Table 2.4: Quantitative real time (QRT-PCR) reagents and concentrations; (All reagents supplied by Perkin Elmer, PE Applied Biosystems, Warrington, UK).

Data were analysed by the comparative ct method and processed using Sequence Detector Version v1.63 (PE Applied Biosystems, Warrington, UK). The expression of each target gene was normalised for each sample using their respective 18S rRNA and were expressed using the relative mRNA expression or as a fold induction calculated as a fold increase of SP-treated samples above control-treated samples. In addition, primers and probes for 18S were also included in the reaction mix as an internal control.

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Target Gene	Sequence of Primers and Probe (5'-3')	
PTGS-1	Forward primer	TGTTCGGTGTCCAGTTCCAATA
	Reverse primer	ACCTTGAAGGAGTCAGGCATGAG
	Probe (FAM)	CGCAACCGCATTGCCATGGAGT
PTGS-2	Forward primer	CCTTCCTCCTGTGCCTGATG
	Reverse primer	ACAATCTCATTTGAATCAGGAAGCT
	Probe (FAM)	TGCCCGACTCCCTTGGGTGTCA
PTGER1	Forward primer	AGATGGTGGGCCAGCTTGT
	Reverse primer	GCCACCAACACCAGCATTG
	Probe (FAM)	CAGCAGATGCACGACACCACCATG
PTGER2	Forward primer	GACCGCTTACCTGCAGCTGTAC
	Reverse primer	TGAAGTTGCAGGCGAGCA
	Probe (FAM)	CCACCCTGCTGCTGCTTCTCATTGTCT
PTGER3	Forward primer	GACGGCCATTCAGCTTATGG
	Reverse primer	TTGAAGATCATTTTCAACATCATTATCA
	Probe (FAM)	CTGTCGGTCTGCTGGTCTCCGCTC
PTGER4	Forward primer	ACGCCGCCTACTCCTACATG
	Reverse primer	AGAGGACGGTGGCGAGAAT
	Probe (FAM)	ACGCGGGCTTCAGCTCCTTCCT
IL-6	Forward primer	GCCGCCCCACACAGACA
	Reverse primer	CCGTCGAGGATGTACCGAAT
	Probe (FAM)	CCACTCACCTCTTCAGAACGAATTGACAAAC
IL-8	Forward primer	CTGGCCGTGGCTCTCTT
	Reverse primer	TTAGCACTCCTTGGCAAAACTG
	Probe (FAM)	CCTTCCTGATTTCTGCAGCTCTGTGTGAA
IL-11	Forward primer	CCCAGTTACCCAAGCATCCA
	Reverse primer	AGACAGAGAACAGGGAATTAAATGTGT
	Probe (FAM)	CCCCAGCTCTCAGACAAATCGCCC
VEGF	Forward primer	TACCTCCACCATGCCAAGTG
	Reverse primer	TAGCTGCGCTGATAGACATCCA
	Probe (FAM)	ACTTCGTGATGATTCTGCCCTCCTCCTT
GRO-α	Forward primer	GTTTTCAAATGTTCTCCAGTCATTATG
	Reverse primer	CCGCCAGCCTCTATCACAGT
	Probe (FAM)	TTCTGAGGAGCCTGCAACATGCCA
Ki-67	Forward primer	GGACTTGCACGACTAA
	Reverse primer	CCGTACGTCAATTGAC
	Probe (FAM)	TTCGAACTGATCAT
18S	Forward primer	CGGCTACCACATCCAAGGAA
	Reverse primer	GCTGGAATTACCGCGGCT
	Probe (FAM)	TGCTGGCACCAGACTTGCCCTC

Table 2.5: Sequences of Taqman primers and probes for Quantitative Real Time (QRT-PCR).

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2.7 Protein

2.7.1 Protein Extraction from WT HeLa Cells

WT HeLa adenocarcinoma cells were seeded at a density of $1x10^5$ cells per 60mm diameter dish and allowed to adhere for 24hrs before serum-starvation overnight. Following serum-starvation, WT HeLa cells were treated for time intervals in the presence/absence of various inhibitors as described in the results section. Following completion of treatment, cells were rinsed with ice-cold phosphate-buffered saline, placed on ice and lysed by the addition of a protein lysis buffer (150mM NaCl, 10% Glycerol, 0.6% (v/v) NP-40, 50mM Tris/HCl pH 7.4 and 10mM EDTA) and a protease inhibitor cocktail tablet. Proteins were then extracted by scraping off the cells using a plate scraper which were transferred to eppendorfs tubes and centrifuged at 14,000 *g* for 15min at 4°C to pellet and insoluble material. Thereafter, the clarified lysate samples were transferred to a new eppendorf tube for protein quantification and western blotting.

2.7.2 Protein Concentration Determination

All protein concentrations were quantified using the standard BIO-RAD Dc assay (BIO-RAD) as directed by the manufacturer's instructions. Bovine serum albumin (BSA) was used as protein standard and a concentration range from 0µg/ml to 400µg/ml in distilled water was established. 20µl of BSA protein standards or samples were loaded into a 96-well plate (in duplicate) followed by the addition of 25µl of working reagent (comprising Reagent S™ and Reagent A™ in 1:50 ratio). To this, 100µl of Reagent B™ was added to each well and the plate incubated for 15min at room temperature. Thereafter, the absorbance was measured at 690nm using a Labsystems Multiskan® EX plate reader. The standard curve and the average concentration of each protein sample was determined using Assay Zap (Biosoft, Cambridge, UK).

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2.7.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using pre-cast 4%-12% Bis-Tris gels (Novex, Invitrogen). Gels were positioned in the running tank containing running buffer (25mM Tris-HCl, 0.2M glycine, 0.1% SDS) and a total of 40µg protein (as determined in 2.7.2) was resuspended in a total volume of 25µl loading buffer (125mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol and 0.05% bromophenol blue), boiled for 5min at 95°C and then loaded into individual wells of the gel. A separate well containing 10µl of SeeBlue™ pre-stained molecular weight marker (Novex, Invitrogen) was set-up to verify protein size. Gels were run at a constant current of 50mA for approximately 90min prior to immunoblotting.

2.7.4 Western Blotting

Following electrophoresis, the gel was transferred to a protein-free tray and equilibrated with transfer buffer (25mM Tris/HCl, 0.192M glycine and 20% methanol) for 10min alongside Whatman no. 3 paper (Whatman, UK). Millipore Immobilon™-FL PVDF membrane (Millipore, Watford, UK) was pre-cut to the same dimensions of the gel and was also equilibrated in transfer buffer but only subsequent to being soaked in methanol for 1min and rinsed briefly in distilled water. The blot was assembled by overlaying three pieces of equilibrated Whatman paper with the Millipore Immobilon™ FL PVDF membrane followed by the gel and three layers of Whatman paper to from a sandwich. Any air bubbles that might be present were removed by rolling a clean glass pipette over each surface. Protein was transferred at a constant voltage of 14V using a semi-dry blotter (BIO-RAD) for approximately 90min. Following transfer, the membrane was immersed in Odyssey blocking buffer (Li-Cor Bioscience, Cambridge, UK) for 1hr at room temperature. Next, the membrane was incubated with the relevant primary antibody at 4°C overnight with gentle shaking. In the morning, the membrane was washed with 10ml PBS-Tween (PBS with 0.1% Tween 20) and this process was repeated three times. After washing, the membrane was incubated with the appropriate secondary fluorescence antibody for 1hr at room temperature. Following incubation, the membrane is subjected to another replicate of washes in PBS-Tween prior to viewing the proteins using the Odyssey Infrared imaging system (Li-Cor Bioscience, Cambridge, UK). The secondary antibody is

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detected at wavelengths of 680nm and 800nm and each experiment repeated four times.

2.8 Immunohistochemical (IHC) Staining

2.8.1 IHC of Cervical Sections

Levels of the proliferation marker Ki-67 in cervical sections comprising of normal tissue (n=5), squamous carcinoma (n=5) and adenocarcinoma (n=5) was assessed using cervical tissue specimens collected as described in sections 2.2.1 and 2.2.2 respectively. The tissue portion set aside for histological examination was fixed in 4% formalin and then subsequently placed in disposable embedding moulds (Polysciences) for paraffin wax-embedding. Glass slides being used for immunohistochemical staining were washed in a 0.25% solution of 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone, followed by an acetone wash and then a rinse in double distilled water and dried. A hand operated '820' Spencer Microtone and a D-profile knife were used to section the paraffin-wax embedded tissue to a thickness of 5µM, following which, the sections were floated on water, transferred to the coated slides and dried overnight before use.

Slides were dewaxed in xylene for approximately 10min, followed by rehydration through graded ethanol (100%, 95%, 80% and 70%) for 30secs each and then washed in water. Thereafter, antigen retrieval was performed by boiling the slides in a pressure cooker containing sodium citrate buffer (0.01M, pH 6.0) for 5min and left to stand for a further 20min. Endogenous peroxidise activity was then blocked by washing slides in 3% hydrogen peroxidase (H_2O_2) in methanol for 30min followed by a brief wash in tap water and two washes in Tris buffered saline (TBS; 50mM Tris-HCl, 150mM NaCl pH 7.4) for 5min each. Sections were subsequently blocked in normal swine serum (species secondary antibody was raised in) diluted in TBS for 30min at room temperature. The tissue sections were then incubated with the specific primary antibody for Ki-67 (mouse monoclonal antibody) (1:200) diluted in 2 parts serum, 4 parts TBS: 0.5% BSA at 4°C overnight. Control sections were included with non-immune IgG.

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The following day, tissue sections underwent additional washing in TBS for 5min, and the process repeated three times. Next, tissue sections were subjected to secondary antibody and Horseradish Peroxidase detection. Firstly, the slides were incubated with biotinylated goat anti-mouse secondary antibody at a dilution of 1:200 for 1hr at room temperature. After incubation, excess secondary antibody was removed from the slides by three additional washes in TBS for 5min each and subsequently the streptavidin-horseradish peroxidase complex (Dako, UK) was added for 30min at room temperature, diluted at a concentration of 1:1000 in TBS. Bound antibody for Ki-67 was visualised by incubating the tissue sections with a solution of 225µM 3.3'-diaminobenzidine (DAB) (Dako, UK) in 0.05M Tris/HCl, pH 7.6 containing 0.01% hydrogen peroxide. After the colour reaction had developed, the reaction was immediately stopped by washing the slide in distilled water. The tissue sections were then counterstained with haemotoxylin and eosin, differentiated in acid-alcohol and blued in Scotts tap water. Finally, sections were rehydrated, cleared in xylene and coverslipped with pertex (Cellpath, Hemel Hampstead, UK). The number of positively stained cells for Ki-67 was quantified using standard stereological techniques.

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2.8.2 IHC of MF-1 Nude Mice Xenografts

IHC was used to assess the levels of Ki-67 (Vector Laboratories, Peterborough, UK) and CD31 (Abcam, Cambridge, UK) in MF-1 nude mice xenografts arising from inoculation with WT HeLa adenocarcinoma cells. Tumours were excised from nude mice upon termination as described is section 2.4.2 and later wax embedded and mounted onto coated slides using standard protocols as previously described (section 2.8.1).

For investigating levels of Ki-67 expression, tissue sections were then subsequently subjected to the exact procedures as detailed in section 2.8.1. For CD31 expression, tissue sections also underwent the process as mentioned in section 2.8.1, except for the differences in primary and secondary antibodies. Instead of applying Ki-67, the sections were incubated with the specific primary antibody for CD31 (polyclonal rabbit anti-mouse/human antibody) (1:250), and was again diluted in 2 parts serum, 4 parts TBS: 0.5% BSA at 4°C overnight. The secondary antibody used in conjunction

with CD31 was biotinylated swine anti-rabbit and was also applied with a working concentration of 1:250. Control sections were included with non-immune IgG. The size of microvessels density as determined by CD31 was quantified using standard stereological techniques. Primary and secondary antibodies and the dilutions they were used at for DAB immunohistochemistry are indicated in Table 2.6.

Primary Antibody / Supplier	Species / Dilution used	Antigen Retrieval	Block	Secondary Antibody / Supplier
Ki-67 (Vector Laboratories, Peterborough, UK)	Rabbit (1:200)	Citrate buffer	Goat	Goat anti-rabbit (Sigma Chemicals, Nottingham, UK)
CD31 (Abcam (ab28364), Cambridge, UK)	Rabbit (1:250)	Citrate buffer	Swine	Swine anti-rabbit (Sigma Chemicals, Nottingham, UK)

Table 2.6: List of primary and secondary antibodies used for DAB immunohistochemistry.

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2.8.3 IHC of WT HeLa Cells

WT HeLa adenocarcinoma cells were seeded at a density of 5 x 10^4 in 2 well-chamber slides, and allowed to adhere for 24hrs before serum-starvation overnight. Following serum-starvation, WT HeLa cells were treated with SP (1:100) or PBS control for 24, 48 or 72hrs in an incubator with 5% CO_2 at 37°C. Once the respective treatment times had elapsed, the media was removed from the slides and the cells were washed in distilled phosphate buffered saline (dPBS). Following dPBS washing, the cells were fixed with 4% paraformaldehyde (diluted in PBS) for 10min. After incubation, the 4% formaldehyde was removed and the cells were further fixated in 100% ice cold methanol and stored at -20°C for 10min. Thereafter, the cells were subjected to two washes in TBS (50mM Tris/HCl, 150mM NaCl, pH 7.4) for 5min each. Following TBS washing, the cells were blocked using 5% normal swine serum diluted in TBS for 30min at room temperature. Cells were then subsequently incubated with the specific primary antibody for Ki-67 (1:200) diluted in 2 parts serum, 4 parts TBS: 0.5% BSA at 4°C overnight.

The following day the cells underwent procedures for additional washing and secondary antibody Horseradish Peroxidase detection as descrbied in section 2.8.1 and subsequently washed and mounted in Permafluor for microscope analysis. The number of positively stained cells for Ki-67 was quantified using standard stereological techniques.

2.8.4 IHC Imaging

DAB immunohistochemistry performed on cervical tissue samples, tumours excised from MF-1 nude mice and WT HeLa cells was visualised on a PROVIS microscope (Olympus Optical, London, UK) and images obtained using Canon EOS image capture software (Canon, Woodhatch, Surrey, UK).

2.9 Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significant differences were determined by unpaired student's t-test or one-way analysis of variance (ANOVA) using Prism 5.0 software (Graphpad Software Inc., San Diego, CA) (*$p<0.05$; **$p<0.005$; ***$p<0.0001$).

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Chapter 3

SP UPREGULATES THE EXPRESSION OF PTGS ENZYMES, PROSTAGLANDIN RECEPTORS, PRO-INFLAMMATORY CYTOKINES AND PRO-ANGIOGENIC FACTORS IN NORMAL CERVICAL TISSUE

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3.1 Introduction

Cervical cancer is regarded as a major health problem worldwide and is the second most common cancer in South African females, with only breast cancer displaying a higher incidence rate (Vorobiof *et al.,* 2001). Although, HPV is regarded as the main causative agent of cervical carcinomas, other contributing factors are coming to the fore. On average, it takes approximately 12-15 years before a persistent HPV infection manifests in cervical carcinoma (Muller *et al.,* 2006; Wallin *et al.,* 1999) and recent studies now suggest seminal plasma to be a potential key player in cervical neoplastic transformation (Robertson *et al.,* 2002; Sales *et al.,* 2002b).

SP has been shown to upregulate numerous growth factors implicated in tumour development such as PTGS-2, VEGF and cAMP signalling (Sales *et al.,* 2002b; Muller *et al.,* 2006). Although the expression of these pro-tumourigenic elements has been well documented in cervical adenocarcinoma cells, the effects of SP on normal cervical tissue is limited. However, data produced by Sharkey and colleagues (2007) demonstrated that normal ectocervical cells were particularly sensitive to seminal plasma activation, the response of which was the production of pro-inflammatory cytokines including; IL-6, IL-8, monocyte chemotactic protein (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF). The exact mechanisms that seminal plasma activates to elicit this response is not yet fully understood but it is envisaged that the abundance of chemical activators contained within the ejaculate pool may trigger the over-expression of cervical cytokine synthesis and therefore create conditions that are favourable toward the onset and progression of cancer in normal cervical tissue in sexually active women.

Dysregulation of PTGS enzymes and prostanoid receptor production is known to affect colorectal and pancreatic tumourigenesis by means of promoting tumour maintenance and progression, encouraging metastatic spread and perhaps participating in tumour initiation (Greenhough *et al.,* 2009; Chu *et al.,* 2003). The PTGS-PTGER pathway has also been shown to be closely associated with cervical cancer as revealed by several *in vitro* studies which have displayed that induction of PTGS enzymes coincidently up-regulate prostaglandin receptor expression and signalling (Muller *et al.,* 2006; Jabbour

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and Sales, 2004; Sales and Jabbour, 2003). Evidence linking the upregulation of PTGS-2 and cervical cancer was further highlighted in a study carried out by Subbaramaiah and Dannenberg (2007) who demonstrated that HPV-16 E6 and E7 oncoproteins, which are involved in cellular transformation (Muger *et al.,* 1989), induced PTGS-2 transcription in cervical cancer cell lines (CaSki and SiHa) by activating the EGFR/MAPK kinase pathway. Cervical cancer development is a multistage process which is initially triggered by HPV then followed by other subsequent changes to the localised epithelia. Therefore, not only does the presence of HPV E6 and E7 oncoproteins appear to increase PTGS-2 levels and stimulate cell proliferation but SP could theoretically enhance tumour growth by acting as 'secondary hit' in causing further impairment to the cervix through augmenting the manufacture of PTGS enzymes and prostanoid biosynthesis.

The relationship between inflammation and cancer has long been appreciated with sustained cell proliferation in an environment rich in inflammatory cells, growth factors and DNA-damaging agents being known to potentiate and/or promote neoplastic risk (Coussens and Werb, 2002). However, the accumulation of genetic lesions in cells is essential to initiate the development of cancer and explains why chronic infection in immunocompetent hosts in the form of HPV leads to cervical carcinoma (Rakoff-Nahoum, 2006; Kinzler and Vogelstein, 1996) with exposure to SP possibly causing further insult to injury. Inflammatory effectors such as prostaglandins are known to regulate the tumour microenvironment by inducing pro-inflammatory cytokines and potent pro-angiogenic factors in order to mobilize immune cells and promote angiogenesis and vascular permeability (Tsujii *et al.* 1998; Takehara *et al.,* 2006; Toomey *et al.,* 2009). Pro-inflammatory cytokines whose over-expression have previously been displayed in reproductive tract cancers includes IL-6, IL-8 and IL-11 (Eustace *et al.,* 1993; Hirano *et al.,* 1987; Xu *et al.,* 1999; Yap *et al.,* 2010) and it is therefore postulated that their upregulation may also be induced in the cervix in response to SP treatment.

Interleukin-6 (IL-6) acts as both a pro-inflammatory cytokine and anti-inflammatory cytokine but patients with advanced metastatic cancer are normally found to have

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higher than normal levels of this cytokine in their blood (Salgado *et al.,* 2002). IL-6 has also been shown to exhibit anti-apoptotic effects and as this cytokine is known to exist in SP, it is possible that IL-6 may help facilitate oncogenesis in human cervical cancer from being produced as part of the pro-inflammatory response as well as being contained within the ejaculate pool (Wei *et al.,* 2001; Sharkey *et al.,* 2007). IL-8 is a chemoattractant chemokine whose primary function is the induction of chemotaxis in target cells as well as being involved in angiogenesis, mitogenesis and inflammation by means of stimulating the recruitment of inflammatory cells (Vlahopoulos *et al.,* 1999). In addition, IL-8 is regarded as one of the dominant proteins contained within SP that is capable of modulating cervical and uterine inflammation (Robertson, 2005). IL-11 is a key regulator of multiple events in hematopoiesis besides being known to affect cell growth, differentiation and proliferation (Paul *et al.,* 1990; Du and Williams, 1994). The cell proliferation and differentiation that IL-11 participates in, mainly occurs in bone cells, and explains why its upregulation is strongly involved in breast cancer metastasis to the bone (Hanavadi *et al.,* 2006). A study performed by Singh *et al.,* (2006) employing PTGS-2 transfected MDA-231 cells found that treatment with PTGS-2 inhibitors reduced the production of IL-11, thus confirming the involvement of PTGS-2 in IL-11 induction in breast cancer cells.

Furthermore, angiogenesis is regarded as a crucial process involved in normal cell growth and differentiation and can lead to tumour growth if the means by which this procedure is controlled are not correctly regulated (Racia *et al.,* 2009). Potent pro-angiogenic factors which may contribute to cervical tumourigenesis are Gro-α and VEGF. Gro-α is classified as a growth related oncogene that contributes to the recruitment of inflammatory cells whereas VEGF is a multifactorial cytokine that stimulates angiogenic acitivity by increasing vascular permeability and acting as a endothelial cell mitogen (Becker *et al.,* 1994; Ferrara *et al.,* 1995). Upregulation of Gro-α and VEGF and several other angiogenic factors have been reported in an array of cancers including pancreatic, lung and cervix (Li *et al.,* 2009; Cai *et al.,* 2009; Chopra *et al.,* 1998).

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Another important requirement for a tumour to grow unabated are changes in existing vasculature and these alterations subsequently allow a sufficient supply of nutrients and oxygen to meet the increasing demand of the tumour. Modifications in vasculature can be determined by measuring CD31, a glycoprotein that is expressed on endothelial cells and expression of this cell marker is frequently used to measure angiogenesis in various cancers, including cervical carcinoma (Tjalma *et al.,* 1997). Two recent studies have revealed findings of similar nature by showing that tumuor angiogenesis in cervical carcinoma cells as evaluated by CD31 microvascular density analysis (MVD) was significantly upregulated in women with early cervical cancer (Randall *et al.,* 2009) and secondly, CD31 expression was found to be progressively increased along the continuum from normal cervical epithelium specimens to squamous cell carcinoma ($p<0.0001$) (Mazibrada *et al.,* 2009), thus proposing that CD31 may have a prognostic value in determining stage of tumour progression in cancer of the cervix.

Despite HPV infection being regarded as the instigating stage in cervical cancer development, SP-induced increases in PTGS enzymes, prostanoid biosynthesis, pro-inflammatory cytokines and pro-angiogenic factors such as VEGF and Gro-α may accelerate progression or onset of the disease by means of exacerbating local inflammatory responses.

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3.2 Aim of the Study

Previous studies have demonstrated that cervical carcinomas exhibit an enhanced expression of PTGS -1 and -2 enzymes, prostaglandin receptors (PTGER1-PTGER4), pro-inflammatory cytokines and pro-angiogenic factors when compared to normal cervical tissue. The aim of this study was to determine if SP-treatment could promote cervical cancer development in normal cervical tissue by upregulating genes that are expressed in this cancer and contribute to its growth by investigating the following;

(i) The effect of SP treatment on transcription of genes encoding PTGS-1, PTGS-2 and prostaglandin subtype receptors PTGER1, PTGER2, PTGER3 and PTGER4.

(ii) The effect of SP treatment on transcription of genes encoding pro-inflammatory cytokines.

(iii) The effect of SP treatment on transcription of genes encoding for pro-angiogenic factors.

(iv) Establish whether seminal plasma induces any change in tissue vasculature that could sustain tumour growth and development.

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3.3 Materials and Methods

3.3.1 Cervical Tissue Collection and RNA Extraction

Normal cervical tissue was collected from women undergoing complete or partial hysterectomy for benign gynaecological malignancies (n=5) as described in section 2.2.1. All specimens were histologically confirmed by a pathologist using histological staging. Total RNA was extracted from normal cervical tissue as described in section 2.6.1 and the concentration and purity of the RNA was determined by measuring the absorbance at 260nm and 280nm using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

3.3.2 cDNA Synthesis and Quantitative Real-Time PCR

Quantitative real-time PCR (QRT-PCR) was performed to investigate the relative expression of PTGS-1, PTGS-2, prostaglandin receptor subtypes (PTGER1-PTGER4), IL-6, IL-8, IL-11, Gro-α, VEGF and CD31 in normal human cervical tissue stimulated with SP (1:500). Approximately 1µg of total RNA was reverse transcribed in the presence of PCR reaction buffer (1x), $MgCl_2$ (5.5mM), dNTPs (2mM), random hexamers (2.5µM), RNAse inhibitor (0.4U/µl) and Multiscribe™ reverse transcriptase (1.25U/µl) (PE Applied Biosystems, Warrington, UK) as described in section 2.6.5. Thereafter, QRT-PCR was carried out as outlined in section 2.6.6 using specific primers and probes listed in table 2.5 (250nM each). The reaction mix for QRT-PCR contained Taqman buffer (5.5mM $MgCl_2$, 200µM dATP, 200µM dCTP, 200µM dGTP and 400µM dUTP), AmpliTaq Gold DNA polymerase (0.025U/µl), ribosomal 18S forward and reverse primers and probe (all at 50nM), forward and reverse primers for genes of interest (all at 250nM) and 2µl of cDNA.

3.3.3 Statistical Analysis

All data are presented as mean ± SEM. Statistical significant differences were determined by ANOVA using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) (*, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$).

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3.4 Results

3.4.1 Expression of PTGS-1 and PTGS-2 in Normal Cervical Tissue Treated with SP

SP has been shown to induce the over-expression of PTGS-2 in HeLa cervical adenocarcinoma cells (Muller *et al.*, 2006), while the elevated expression of PTGS-1 and PTGS-2 have been reported in several cancers of the reproductive tract including; ovarian, endometrial and carcinoma of the cervix (Lee *et al.*, 1995; Battersby *et al.*, 2007; Sales *et al.*, 2002a). As a result, it was decided to assess whether SP could upregulate PTGS-1 and PTGS-2 in normal cervical tissue. Quantitative RT-PCR was used to determine the effect of SP (1:500) on the mRNA expression of PTGS-1 and PTGS-2 in normal cervical tissue treated for 2, 4, 6, 8, 12 and 24 hrs. As the values for normal cervical tissue treated with SP for 2, 4 and 6 hrs were similar and the values for 8 and 12 hrs were similar, it was decided to present the data together to give treatment periods of 2-6, 8-12 and 24 hrs which were maintained throughout this chapter. As shown in figure 3.1, SP treatment of normal cervical tissue for 24 hrs significantly elevated the expression of PTGS-1 and PTGS-2 mRNA compared to vehicle treated control samples (6.5 ± 1.8 and 3.9 ± 1.3 fold increase respectively; Figure 3.1A; $P<0.0001$ and Figure 3.1B; $P<0.0001$), while, the expression of PTGS-1 and PTGS-2 in response to treatment of SP (1:500) was not statistically significant at either 2-6 or 8-12 hrs.

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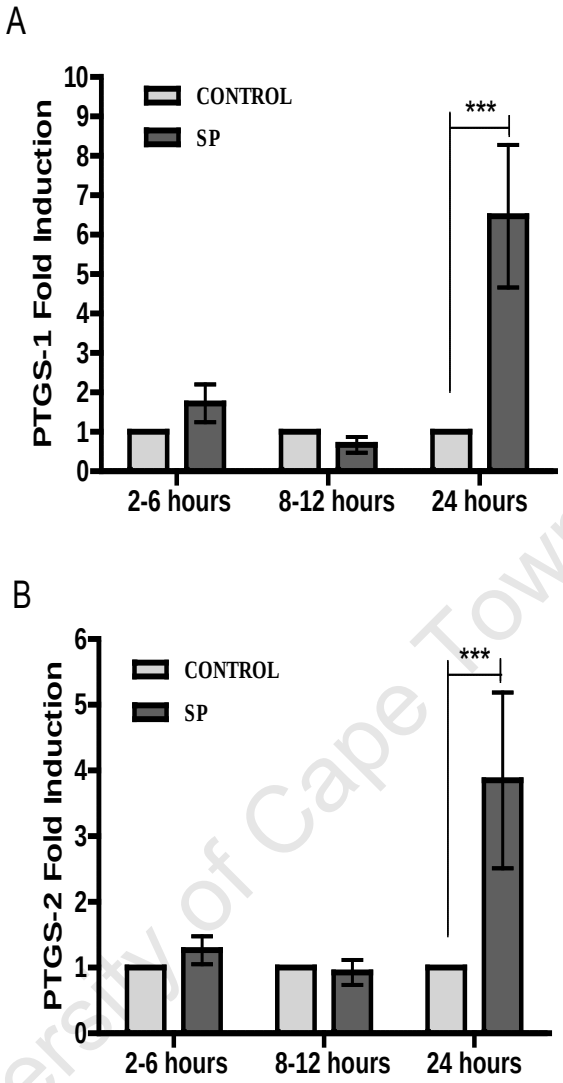

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Figure 3.1 (A) PTGS-1 and **(B)** PTGS-2 mRNA expression in normal cervical tissue (n=5) treated by SP (1:500) for 2-6, 8-12 and 24 hrs. Quantitative RT-PCR determination revealed PTGS-1 and PTGS-2 to be significantly increased after treatment for 24 hrs when compared to the control, ($P<0.0001$*** and $P<0.0001$***) respectively. Data are represented as mean ± SEM from three independent experiments.

3.4.2 Expression of PGE2 Prostanoid Receptors in Normal Cervical Tissue Treated with SP

Since SP upregulated the expression of PTGS-1 and PTGS-2, it was decided to examine if SP treatment also induced changes in the expression levels of PGE_2 receptors. Quantitative RT-PCR was employed to investigate the mRNA expression levels of prostanoid receptors PTGER1, PTGER2, PTGER3 and PTGER4 in normal cervical tissue in response to stimulation by SP (1:500) for 2, 4, 6, 8, 12 and 24 hrs. Treatment of normal cervical tissue with SP induced the over-expression of PTGER1, PTGER3 and PTGER4 only after 24hrs (2.1 ± 1.2-, 2.7 ± 0.9-, 2.3 ± 1.0-fold induction respectively; Figure 3.2A and 3.2D; $P<0.05$, Figure 3.2C $P<0.005$). However, there was no significant effect on expression of PTGER2 (Figure 3.2B). The upregulation of prostaglandin signalling has previously been shown to be closely associated with enhanced tumour growth (Jabbour *et al.*, 2001) and this result suggests a possible role for SP in promoting tumourigenesis in the cervix by inducing over-expression of prostaglandin receptors PTGER1, 3 and 4.

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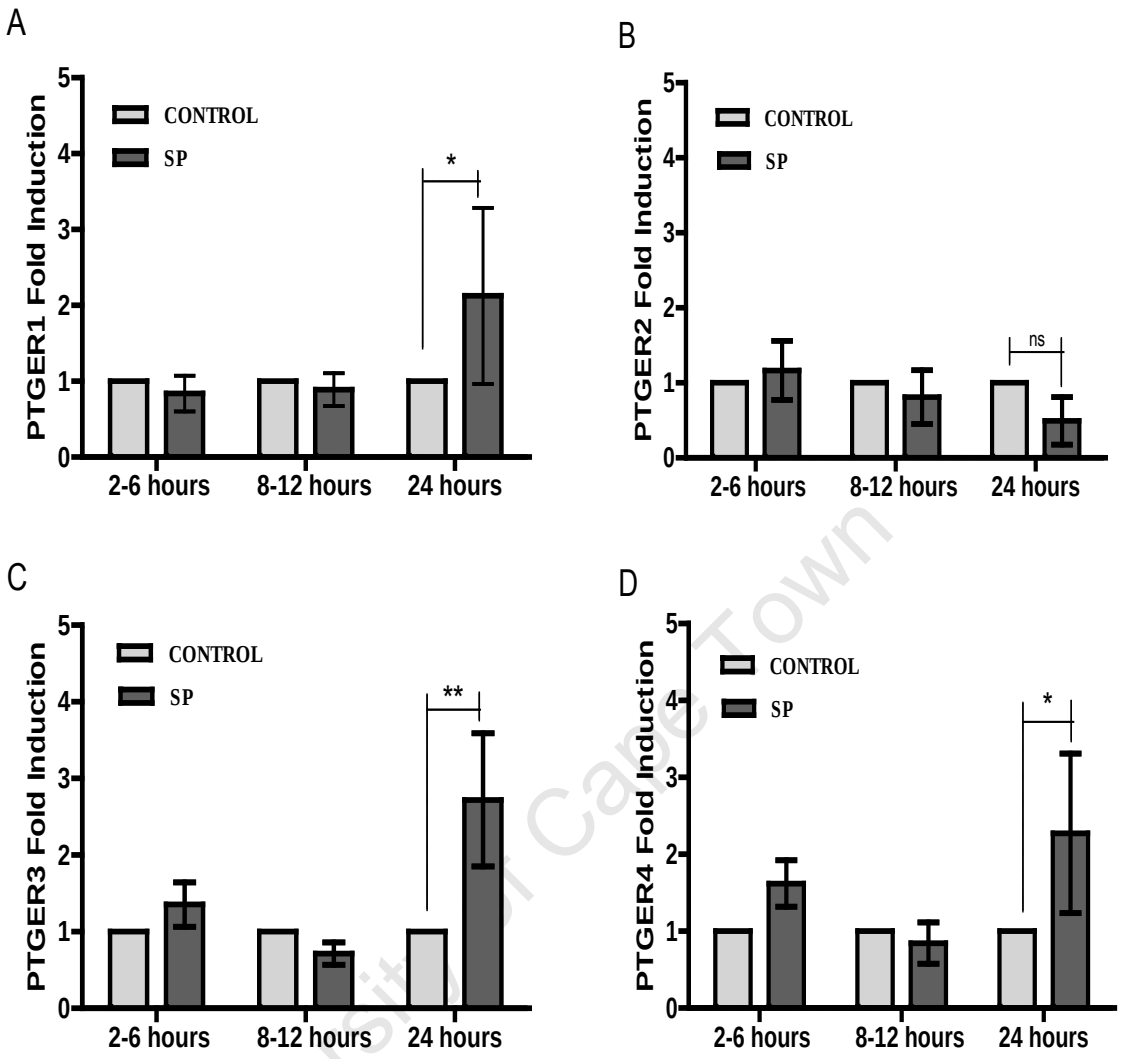

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Figure 3.2 The mRNA expression of the 4 different subtypes of the PTGER receptors were investigated and assessed by quantitative RT-PCR. Namely PTGER1, PTGER3 and PTGER4 were increased in normal cervical tissue (n=5) in response to SP (1:500) treatment for 24 hrs **(A)** PTGER1 ($P<0.05$*) **(C)** PTGER3 ($P<0.005$**) and **(D)** PTGER4 ($P<0.05$*). **(B)** SP (1:500) had no significant effect on the mRNA expression of the PTGER2 receptor ($P<0.5$). Data are represented as mean ± SEM from three independent experiments.

3.4.3 Expression of Pro-inflammatory Cytokines IL-6, IL-8 and IL-11 in Normal Cervical Tissue Treated with SP

Having established that SP induces the over-expression of PTGS enzymes -1 and -2 and prostaglandin receptor signalling, it was decided to investigate the effect of SP on the expression of potent pro-inflammatory cytokines with known roles in tumour development. Normal cervical tissue was treated with SP (1:500) for time periods of 2, 4, 6, 8, 12 and 24 hrs and mRNA expression levels measured using quantitative RT-PCR analysis. The results showed a significant increase in over-expression of interleukins IL-6, IL-8 and IL-11 only after 24 hrs when treated with SP in comparison to the control (3.8 ± 0.8-, 5.5 ± 2.3-, 3.1 ± 1.0-fold increase respectively; Figure 3.3A; $P<0.005$, Figure 3.3B; $P<0.005$ and Figure 3.3C; $P<0.05$). The expression of IL-6, IL-8 and IL-11 in response to treatment of SP (1:500) was slightly increased at the earlier treatment periods of 2-6 and 8-12 hrs but was not deemed to be statistically significant. This suggests that SP may be central in regulating the tumour microenvironment by encouraging the upregulation of pro-inflammatory cytokines.

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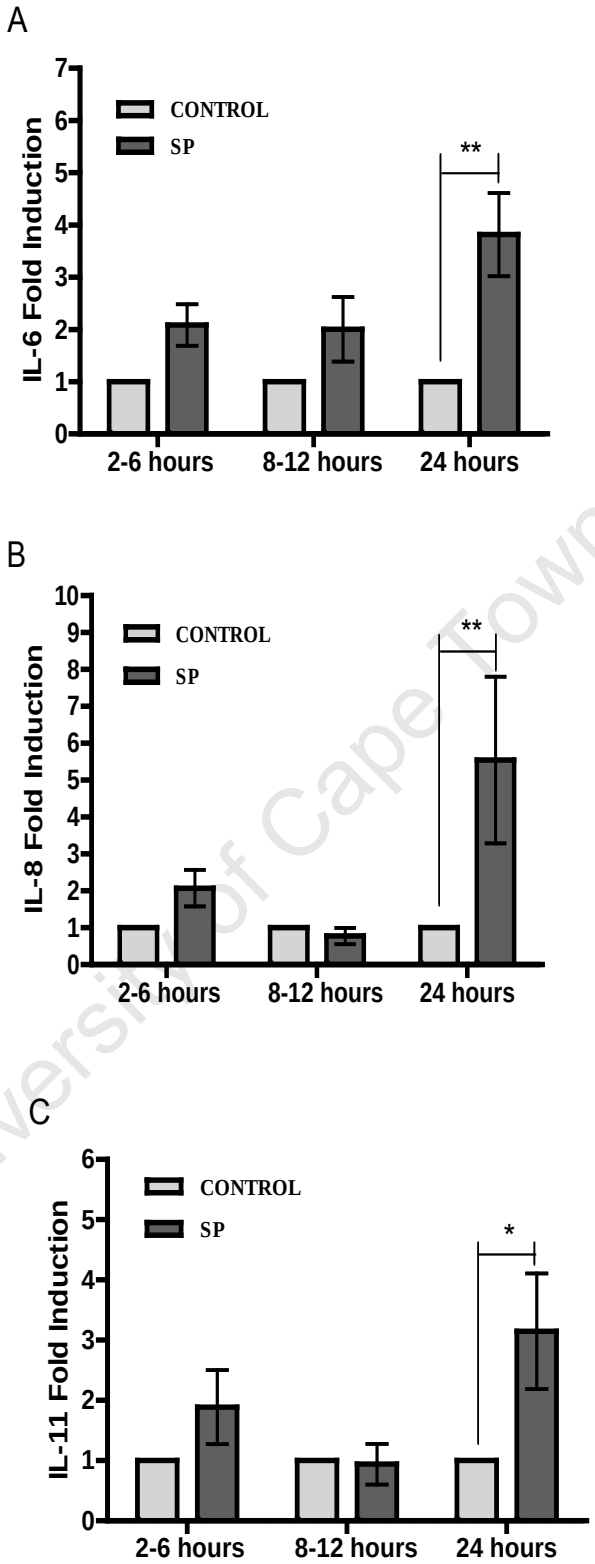

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Figure 3.3 Quantitative RT-PCR analysis showed mRNA levels of **(A)** IL-6 **(B)** IL-8 **(C)** IL-11 to be significantly over expressed in normal cervical tissue (n=5) treated with SP (1:500) for 24 hrs in comparison to the control ($P<0.005$**, $P<0.005$** and $P<0.05$* respectively. Data are represented as mean ± SEM from three independent experiments.

3.4.4 Expression of Pro-angiogenic factors Gro-α and VEGF in Normal Cervical Tissue Treated with SP

Angiogenesis plays a vital role in sustaining tumour growth and development and it was therefore decided to investigate whether pro-angiogenic factors were upregulated as a result of seminal plasma treatment. Quantitative RT-PCR was used to determine the mRNA expression levels of Gro-α and VEGF in normal cervical tissue (n=5) in response to SP (1:500) treatment for 2, 4, 6, 8, 12 and 24 hr time periods. (A) Gro-α was shown to be significantly over expressed in normal cervical tissue (n=5) treated with SP (1:500) for 24hrs when compared to the control group (6.2 ± 1.5-fold increase) (Figure3.4A; P<0.0001) as was (B) VEGF (6.1 ± 2.6-fold increase) (Figure3.4B; P<0.0001), while, the expression of Gro-α and VEGF in response to treatment of SP (1:500) was not statistically significant at either 2-6 or 8-12 hrs.

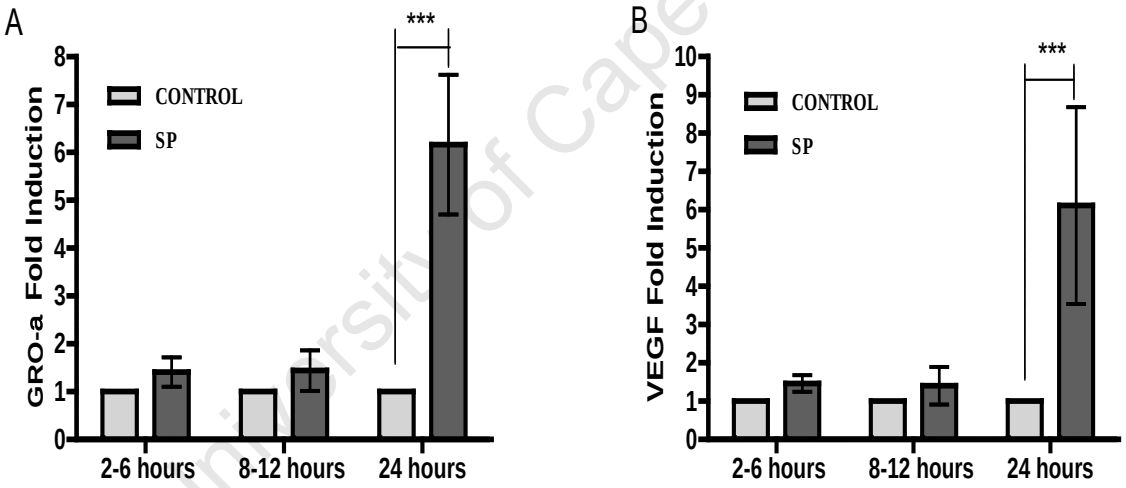

Figure 3.4 Quantitative RT-PCR analysis revealed the mRNA expression of **(A)** Gro-α and **(B)** VEGF to be significantly upregulated in normal cervical tissue (n=5) treated with SP (1:500) for 24 hrs in comparison to the control (both P<0.0001***). Data are represented as mean ± SEM from three independent experiments.

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3.4.5 Expression of CD31 in Normal Cervical Tissue Treated with SP

Having identified that normal cervical tissue treated with SP had elevated Gro-α and VEGF expression, next to be investigated was whether these normal tissue samples also displayed alteration in their vasculature in response to SP stimulation. This was done by determining whether SP upregulates transcription of CD31. Quantitative RT-PCR analysis showed a significant increase in expression of CD31 in normal cervical tissue when treated with SP for 24 hours in comparison to the control treatment group (1.6 ± 0.3 fold increase; Figure 3.5; $P<0.05$). There were no significant differences between control and SP-treated normal cervical tissue for the 2-6 hr and 8-12 hr treatment periods.

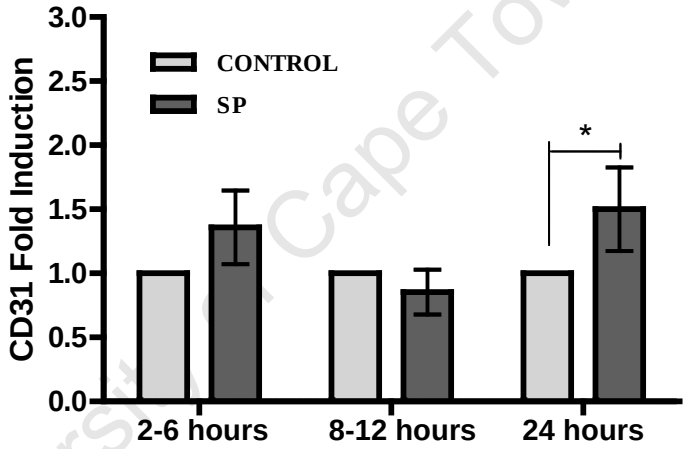

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Figure 3.5 (A) CD31 mRNA expression was measured by quantitative RT-PCR and is significantly increased in normal cervical tissue (n=5) following treatment with SP (1:500) for 24 hours as compared to the control ($P<0.05$*). Data are represented as mean ± SEM from three independent experiments.

3.5 Discussion

PTGS enzymes, prostaglandins and prostaglandin receptors have been long recognised as key molecules in reproductive biology but more recently their upregulation has been closely associated with several cancers of the reproductive tract including ovarian, endometrial and cervical carcinomas (Kino *et al.,* 2005; Jabbour *et al.,* 2006; Muller *et al.,* 2006). Our laboratory has previously demonstrated elevated expression levels of PTGS-2, PGE_2 and VEGF in cervical adenocarcinoma HeLa cells in response to SP treatment with the effects that were exerted shown to be regulated via receptors PTGER-2 and PTGER-4 (Sales *et al.,* 2002b; Muller *et al.,* 2006). Moreover, the activation of PTGS-2 and PGE_2 receptor expression and the subsequent cell signalling in response to SP, was shown to trigger a multitude of phenotypic changes in cervical tissue which includes the promotion of angiogenesis and vascular function (Sales *et al.,* 2002b). This therefore suggests that SP can play an important role in modulating neoplastic cell function and cervical tumourigenesis in sexually active women in an autocrine/paracrine manner as an upshot of initiating signalling cascades that involve PTGS enzymes, prostaglandins, their cognate receptors and activator molecules such as VEGF.

Despite the mounting evidence that correlates the progression of cervical pathologies with SP exposure (Gutsche *et al.,* 2003: Sales 2002b; Muller *et al.,* 2006), the data concerned with seminal plasma treatment of normal human cervical tissue is very limited and the main objective of this study was to ascertain whether the upregulated expression of PTGS enzymes, prostaglandins, their receptors and the array of cytokines witnessed in cervical cancer could also be induced in normal cervical tissue as a result of SP stimulation. PTGS-1 and PTGS-2 were both significantly upregulated in normal cervical tissue treated with SP for 24 hr relative to untreated normal cervical tissue as the control. PTGS-1 expression levels have previously been shown to be enhanced in cervical adenocarcinoma cells (Sales *et al.,* 2001), except in this study, the increase occurred in normal cervical tissue as a direct result of SP stimulation. Furthermore, PTGS-2 expression levels were also statistically elevated in normal cervical tissue as an impact of SP treatment with similar findings also being displayed in cervical adenocarcinoma samples (Sales *et al.,* 2002b) and HeLa cells expressing

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the PTGER4 receptor (Muller *et al.,* 2006). This in turn suggests that by provoking the production of PTGS enzymes -1 and -2, SP could cause increased autocrine and paracrine signalling of PG to establish favourable conditions in the cervix that permits the onset of tumour development and progression.

In addition, expression levels of prostanoid receptors (PTGER1, PTGER3 and PTGER4) were statistically elevated in normal cervical tissue treated with SP for 24 hr in comparison to the PBS-treated control for the respective time period. PTGER1 receptor expression has been shown to be increased in cervical adenocarcinoma cells in response to SP stimulation (Sales *et al.,* 2002b), although, additional results from the same study demonstrated that receptors PTGER2 and PTGER4 were more predominantly elevated. This finding is consistent with earlier data published by Sales and colleagues (2001) and supports the interpretation that SP may play a role inducing the upregulation of PTGER2 and PTGER4 seen in cervical carcinoma. However, newly formed data released by Bai and colleagues (2010) demonstrated that hepatocellular carcinoma cells treated with PGE_2 resulted in the upregulation of survivin (an inhibitor-of-apoptosis) and this was found to be mediated by PTGER1. It is therefore possible to suggest that the PGE_2 content of SP may also possess the capability of inducing this anti-apoptotic effect in cervical cancer cells. The increased expression of PTGER1 receptor signalling that was observed following 24 hr of SP treatment may be involved with permitting cervical cells to proliferate uncontrollably, thereby contributing to the establishment of conditions favouring cancer growth in the cervix. Nevertheless, as the exact mechanisms by which SP elicits it effects on normal cervical tissue remain to be fully elucidated, it may or may not be the PGE_2 component of SP that is responsible here.

PTGER3 receptor expression was also established as being statistically upregulated by SP in normal cervical tissue, although, its expression was not found to be significantly increased by SP in adenocarcinoma HeLa cells as reported by Sales and colleagues (2002b). It is possible that the PTGER3 receptor is principally involved in the initiation of cervical cancer development and becomes redundant once the disease is established but further research is required to clarify the exact role of PGE_2 receptors

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in the transition of normal cervical tissue becoming cancerous. PTGER3 receptor activity has been linked with skin carcinoma (Shoji *et al.,* 2005) and colon cancer development (Shoji *et al.,* 2004) and is implicated in performing a protective role in PTGS-2-induced mammary tumours whereby it is down-regulated and PTGER receptors 1, 2 and 4 are strongly upregulated but why this occurs is unclear (Chang *et al.,* 2004).

In this study, SP also induced the up-regulation of PTGER4 receptor signalling and this mirrors findings of Muller *et al.,* (2006) whereby this receptor was significantly increased in cervical adenocarcinoma HeLa cells in response to SP, further supporting that upregulation of PTGER4 is closely associated with the promotion of early carcinogenesis and metastatic behaviour (Fulton *et al.,* 2006).

The result of PTGER2 receptor expression not being significantly induced in normal cervical tissue by SP treatment is inconsistent because upregulation of PTGER2 was previously found to be enhanced in carcinomas of the cervix (Sales *et al.,* 2001). To reason this point, the finding of upregulated PTGER2 receptor expression by Sales and colleagues (2001) was determined in adenocarcinoma HeLa cells, was not induced by SP treatment and like PTGER3, may play different roles at different stages of the disease with the effects of SP exposure in sexually active women potentially influencing the way that cervical epithelial cells are regulated during cancer development. Published data have demonstrated PTGS-2 derived PGE_2 to be central in tumour growth in cancers such as the lung, with the PTGER2 receptor being shown to occupy an essential role in mediating the pro-tumourigenic effects of PTGS-2/PGE_2 in intestinal polyposis and oesophageal adenocarcinoma (Williams *et al.,* 2000; Sonoshita *et al.,* 2001; Jiminez *et al.,* 2010). Furthermore, HPV oncogenes E6 and E7 are known to induce PTGS-2 and coupled with the fact that PTGS-2 derived PGE_2 promotes tumour growth via PTGER expression, it is possible that this mechanism is being operated upon in the cervix. In addition, Battersby and colleagues (2007) revealed expression of the PTGER2 receptor to be upregulated in endometrial carcinoma following incubation with SP. Although the evidence provided shows that SP can induce PTGER2, it does not possess the capability of stimulating the over-expression

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of this receptor in normal cervical tissue and demonstrates that the effect of seminal plasma is cell type dependent. Taken together, increased expression of PTGS-1/-2 and PTGER receptors suggests that increased autocrine/paracrine signalling can lead to changes in normal cervical epithelia that typify those taking place in malignant cells.

Other findings from this study reveal that the expression of the pro-inflammatory mediator IL-6 is significantly upregulated following 24 hr treatment with SP and this may be a direct result from SP exposure or a subsequent outcome from SP increasing the circulatory levels of PTGS enzymes and prostaglandin receptor signalling. Work undertaken by Hinson and colleagues (1996) illustrated that increased PGE_2 synthesis causes the induction of IL-6 in a murine model of inflammation and therefore, IL-6 may act as a growth factor in gynaecological tumours because elevated production of this cytokine has been shown to exist in ovarian cell carcinoma and squamous cell carcinoma of the uterine cervix (Hirano *et al.*, 1987; Watson *et al.*, 1990; Eustace *et al.*, 1993). In addition, IL-6 upregulation has also been shown to be a host response to a foreign pathogen in mice as it is required for resistance against the bacterium *Streptococcus pneumoniae* (van der Poll *et al.*, 1997). The production of IL-6 may therefore be a consequence of normal cervical tissue cells responding to SP as a foreign body or due to activational factors within SP such as PGE_2, 19-hydroxy PGE_2, TGF-β and TNF-α that are capable of inducing the expression of IL-6 as well as other inflammatory mediators such as IL-8 and IL-10 (Dension *et al.*, 1999a; Robertson 1992). As many blood vessels also produce IL-6, it could be envisaged that SP not only directly stimulates IL-6 production from interacting with normal cervical tissue but also as a secondary effect through stimulating pro-angiogenic factors such as VEGF and Gro-α which resultantly increases the vasculature by recruiting more blood vessels for angiogenesis which sequentially promotes further levels of IL-6.

Several studies have illustrated that over-expression of IL-8 is associated with playing an important role in tumour progression and metastasis in a variety of human cancers such as lung, breast and prostate (Yuan *et al.*, 2005; Green *et al.*, 1997; Singh and Lokeshwar, 2009). Research published by Fujimoto and colleagues (2000) demonstrated that IL-8 expression levels were significantly increased during the

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advancement of cervical cancer from stage I through IV, may act as an angiogenic switch in advancement of later stages of uterine cervical cancers and could therefore operate as a prognostic indicator. Furthermore, IL-8 and IL-6 concentrations have been noted in seminal plasma and results published by Eggert-Kruse and colleagues (2001) showed that the concentration of IL-8 in particular was inversely related to semen quality in terms of sperm motility and migratory ability. The increased expression of IL-8 in normal cervical tissue in response to SP treatment after 24hr was revealed in this study and suggests that upregulation of IL-8 may promote angiogenesis and further recruitment of inflammatory cells in cervical carcinomas. However, if normal cervical tissue in sexually active women is exposed to supplementary IL-8 via subfertile seminal plasma, the women in question may further augment the cervical microenvironment and therefore implement conditions that support cervical carcinogenesis.

Interleukin-11 (IL-11) is another pro-inflammatory mediator that was found to be upregulated in normal cervical tissue in response to SP following 24hr of treatment. Information regarding the implications of IL-11 in the development and progression of cervical cancer is relatively sparse. Nevertheless, the induction of IL-11 has been observed in carcinoma of the breast and endometrium as a result of PTGS-2 over-expression (Singh *et al.*, 2006; Yap *et al.*, 2010) and been shown to be significantly elevated in inflammation-associated gastrointestinal cancer as a result of persistent STAT3 activation (Putoczki and Ernst, 2010). Furthermore, IL-11 is reported as being expressed in SP with levels increasing in males exhibiting infertility problems and urogenital infections and shows an inverse correlation with sperm quality (Matalliotakis *et al.*, 1998). Fichorova and Anderson (1999) demonstrated that the immune-deviating effects of seminal cytokines are amplified in the female tract by locally synthesized TGF-β and PGE_2 as well as IL-4, IL-6 and IL-10 secreted from endocervical and endometrial cells. This suggests that cervical epithelia may potentially be exposed to increased levels of IL-11 via heightened expression of PTGS-2 and from SP tumour-promoting cytokines such as IL-6, IL-8 and IL-11 becoming enhanced in reproductive tract.

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This study also showed upregulation of vascular endothelial growth factor (VEGF) and Gro-α expression in normal cervical tissue treated with seminal plasma for 24 hr compared to the control. VEGF is well renowned for its part in stimulating the growth of new blood vessels and restores the oxygen supply to tissues when blood circulation is insufficient (Holmes *et al.,* 2007), whereas Gro-α is primarily expressed by epithelial cells and has several distinct roles including that of innate immunity, wound healing and the inflammatory response (Devalaraja *et al.,* 2000; Haghnegahdar *et al.,* 2000). When factors such as VEGF and Gro-α become dysregulated so that they are chronically expressed, angiogenesis and tumourigenesis can follow (Cheng *et al.,* 1999; Fimmel *et al.,* 2007; Owen *et al.,* 1997; Tsai *et al.,* 2002). High VEGF expression levels have been closely associated with poor prognosis in breast cancer (Liang *et al.,* 2006) and are also known to exist in carcinoma of the cervix (Cheng *et al.,* 1999; Muller *et al.,* 2006). Studies involving Gro-α have demonstrated this chemokine to act as a growth factor in promoting the development of the pancreatic cancer cell line Capan-1 (Takamori *et al.,* 2000) and significantly increases tumourigenicity towards glioma cells, even implicating that targeting Gro-α may be a useful therapeutic tool to control brain tumour biology (Zhou *et al.,* 2005). Moreover, SP can promote VEGF in cervical adenocarcinoma cells via the PTGER4 receptor (Muller *et al.,* 2006) whilst Gro-α concentrations within SP are believed to increase with infertility and also stimulate further cytokine recruitment in endometrial cell cultures (Rajasekaran *et al.,* 1996; Ochsenkuhn *et al.,* 2008).

Finally, CD31 expression, normally used to investigate alterations in vasculature as a marker of angiogenesis (Mazibrada *et al.,* 2008), was shown to be significantly upregulated in normal cervical tissue as a result of SP treatment for 24 hr when compared to the respective untreated sample. Earlier results confirmed pro-angiogenic factors VEGF and Gro-α to be increased and the fact that CD31 expression was also found to be elevated, provides other evidence that VEGF and Gro-α act by modifying the vasculature of normal endothelial cells via exposure to SP.

Taken together, the findings in this study evidently display that SP can induce a multitude of pro-tumourigenic factors such as PTGS-1, PTGS-2, IL-6, IL-8, IL-11,

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VEGF and Gro-α in normal cervical tissue and judging by the observation that PTGER receptor expression is significantly increased, there is a high possibility that this enhanced production of growth factors, pro-inflammatory cytokines and angiogenic traits is regulated, or at least in part, by the prostanoid receptors. Furthermore, sexually active women who engage in intercourse with multiple partners may be at an additional risk as there is a higher chance of an encounter with a partner who has infertility problems and seminal plasma which originates from individuals with fertility issues tends to contain elevated expression levels of IL-6, IL-8 and Gro-α, causing further irregularities to the cervical microenvironment that benefits cancer growth. As a result, a correlation between cervical cancer and sexual partners with infertility problems should be explored in the future.

In summary, these results demonstrate that SP is a potent immune deviating agent and exposure could be pivotal in activating mechanisms responsible for the transition of cervical epithelial cells being normal to becoming 'cancer prone' via the induction of increased inflammatory responses but more research is required to determine the exact nature of these events and the multi-factorial nature of cervical cancer development makes this task even more difficult.

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Chapter 4

WT HELA CELLS AS A MODEL SYSTEM TO STUDY EFFECTS OF SEMINAL PLASMA ON EXPRESSION OF PROINFLAMMATORY AND ANGIOGENIC GENES AND ON CELLULAR PROLIFERATION

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4.1 Introduction

In the previous chapter, findings have ascertained that exposure to seminal plasma can induce transcription of genes in normal human cervical tissue, that result in, increasing cytokine production, augmenting the expression/production of growth factors and enhancing prostaglandin levels. These characteristics are all classic hallmarks of inflammation and favours the development and progression of cervical carcinoma by promoting pro-tumourigenic events such as cellular transformation, local tumour cell growth, invasion through the ECM and vascular basement membrane, recruitment of effector cells such as T and natural killer cells and creating a pro-angiogenic environment to encourage blood vessel formation (Vicari and Caux, 2002). The transcription of PTGS-1 and PTGS-2, PTGER receptor signalling, pro-inflammatory mediators IL-6, IL-8 and IL-11, pro-angiogenic factors Gro-α and VEGF and marked expression of CD31 have been closely associated with an array of cancers such as cervical carcinoma (Smith *et al.,* 2000; Herschman 1995; Fossilen 2001). The heightened levels of these contributory components are brought about by SP as a consequence of upregulating PTGS-1/2 and prostaglandin signalling via its cognate receptors and as a direct response of the cytokines contained within SP itself, such as IL-6, IL-8, TNF-α, TGF-β, MMPs and PGE_2. When infected with HPV, the E6 and E7 proteins are able to cause cervical epithelial cells to become immortalized and down-regulates tumour suppressors such as p53 in order to transform cells into neoplastic ones (Woodworth *et al.,* 1989; Werness *et al.,* 1990; Malik, 2005). Dysregulation of the HPV E6/E7 proteins also has the potential to increase cell proliferation and upregulate expression of PTGS-2 which in turn further augments the inflammatory response (Subbaramaiah and Dannenberg, 2007). It is therefore hypothesized that SP can activate inflammatory events which can drive cancer in neoplastically transformed cervical cells in sexually active women infected with HPV.

PTGS-1 is constitutively generated and although the first concern of this gene is to regulate normal physiological function by means of prostaglandin production, (Eberhart *et al.,* 1994), increased expression of PTGS-1 is now being associated as having a role in tumourigenesis and when PTGS-1 is absent in knockout mice, intertestinal neoplasia and skin papilloma formation are significantly reduced

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(Langenbach *et al.*, 1999; Chulada *et al* 1998; Jang *et al.*, 1997). Although augmentation of PTGS-1 can have negative effects, as confirmed by its over-expression being observed in cervical carcinomas (Sales *et al.*, 2002a), it is the upregulation of PTGS-2 that is more commonly associated with cancer development. High levels of PTGS-2 expression have been demonstrated to exist in many cancer types, including the lungs, breast and cervical (Soslow *et al.*, 2000; Kulkarni *et al.*, 2001). As a result, chemical inhibitors that are specific for PTGS-1 and PTGS-2 may have a significant role in arresting the development of cervical carcinoma.

Data released from our laboratory has demonstrated SP to activate increases in PTGS-2 and PGE_2 receptor expression in cervical adenocarcinoma cells (Sales *et al.*, 2002b) and to promote expression of VEGF in adenocarcinoma HeLa cells via the PTGER4 receptor (Muller *et al.*, 2006) as well as upregulating fibroblast growth factor-2 (FGF-2) in endometrial adenocarcinoma (Battersby *et al.*, 2007). However, the complex mechanisms by which SP manages to provoke increases in growth factor production, augment angiogenesis and enhance the inflammatory response are not fully understood. Muller and colleagues (2006) did reveal that SP induces rapid phosphorylation of ERK1/2 in HeLa cells over-expressing the PTGER4 receptor whereas Battersby and colleagues (2007) demonstrated that SP can promote cAMP accumulation via the PTGER2 receptor in endometrial adenocarcinoma cells, both of which could be impeded by inhibiting EGFR signalling, thus suggesting that this pathway may be central with regard to SP activation. While these findings highlight the signalling pathways that SP is capable of inducing and that SP can upregulate VEGF, the pro-inflammatory genes and pro-angiogenic genes that are involved remain to be identified as do the signalling mechanisms that they operate.

Besides focusing on PTGS-1 and PTGS-2, researchers have paid particular attention to IL-6 when investigating the pathogenesis of cervical cancer. A study performed by Wei and colleagues (2003) revealed that human cervical cancer C33A cells expressing IL-6 promotes *in vivo* tumour growth by VEGF-dependent angiogenesis and this has been shown to occur via STAT3 signalling. Another pro-inflammatory mediator whose over-expression has been found to play a pivotal role in tumour angiogenesis is IL-8.

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Studies have shown that inhibition of IL-8 reduces tumourigenesis of human non-small lung cancer in SCID mice (Arenberg *et al.,* 1996) while Fujimoto and colleagues (2000) reported a strong correlation between microvessel counts and IL-8 levels in cervical cancer. In addition, the over-expression of IL-11 may be the main trigger for inflammation-associated gastric cancer (Ernst *et al.,* 2008) and levels of this cytokine have also been shown to be increased locally by PGE_2 in human endometrial stromal cells (Dimitriadis *et al.,* 2005). Although the underlying mechanisms which sustain cervical cancer development remain elusive, increased levels of IL-6, IL-8 and IL-11 have been shown to promote tumour growth and it is anticipated that identifying products involved in the production of these cytokines could possibly help in elucidating pathways that SP is mediating during cervical cancer growth.

As well as being over-expressed in normal cervical tissue in response to SP, high levels of pro-angiogenic factors Gro-α and VEGF have previously been shown to be upregulated in carcinomas of the cervix (Chopra *et al.,* 1998). They help promote the progression of cancer by assisting in improving the vasculature that supplies the developing cancer cells with essential oxygen and nutrients. Microvascular regulatory processes that occur in the cervix, such as cell proliferation, immune response and protein kinase activity are governed by VEGF signalling (Mowa *et al.,* 2008) and because SP can induce VEGF expression, it may regulate cervical epithelial proliferation. Unfortunately, studies aimed at determining the exact role that SP performs in gynaelogical malignancies are limited and more research is required in this area to help isolate the pathways and cell signalling that is involved. The huge variety of different compounds contained within seminal plasma makes this task even more difficult.

In the previous chapter, it was shown that PTGS enzymes -1 and -2, PTGER receptor signalling, pro-inflammatory genes, IL-6, IL-8 and IL-11, and pro-angiogenic genes Gro-α and VEGF are upregulated in normal cervical tissue in response to SP stimulation. In this chapter we employ WT HeLa cells which are $HPV16^+$ adenocarcinoma cell line as a model system in order to determine signalling mechanisms involved in inducing the transcription of these genes.

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The variety of signalling components that exist within SP is vast, containing many different activator molecules that have the potential to operate on signalling pathways involved with crucial cellular functions such as the inflammatory response and growth (Robertson *et al.,* 2002; Gutsche *et al.,* 2003). These molecule types include cytokines, TGF-β, PGE_2, members of the interleukin family such as IL-8 and TNF-α and the upregulation of these signalling factors is closely associated with cancer development (Sharkey *et al.,* 2007; Robertson *et al.,* 2002).

The various inhibitors being used herewithin have been chosen because of their ability to inhibit the signalling molecules contained within SP and the signalling pathways they operate, thus blocking the pro-tumourigenic effects this can elicit. Inhibitors for PTGS-1/-2 were employed as their elevated expression has been shown to promote tumour cell proliferation, reduce apoptosis and induce angiogenesis in cervical adenocarcinomas and colon cancer cells as well as their alliances with PGE_2 and PTGER signalling (Sales *et al.,* 2001; Sales *et al.,* 2002a; Tsuji *et al.,* 1988). Studies performed in our laboratory by Muller and colleagues (2006), and Battersby and colleagues (2007), both showed that treatment with SP and PGE_2 promoted signalling via ERK1/2 and EGFR in cervical adenocarcimona cells expressing the PTGER4 receptor and in endometrial carcinoma cells respectively. Results from Muller and colleagues (2006) also revealed that phosphorylation of ERK by SP and PGE_2 treatment was in an EGFR- and Raf- dependent manner because ERK1/2 activity was completely inhibited by a chemical inhibitor specific for EGFR (AG1478). The MAPK kinase pathway with which these signalling molecules exist is a key mechanism in cell growth, differentiation and transformation and appears to be activated by SP (Lewis *et al.,* 1998), thus providing adequate reasoning for choosing inhibitors specific for EGFR, and ERK/MAPK to test the effects of SP.

TGF-β is a principal stimulatory agent contained in SP that has the potential to exert autocrine and endocrine effects (Robertson *et al.,* 2002). The signalling properties of TGF-β are closely associated with the onset and progression of cancer as demonstrated by the generation of anti-TGF-β cancer therapy and the observation that high levels of this growth factor is found to correlate with poor prognosis (Saunier and Akhurst

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2006). TGF-β has been shown to induce Smad signalling of pancreatic cancer cells via ERK2 activation (Ellenrieder *et al.,* 2000) and can upregulate VEGF and MMP-2 in metastatic pancreatic cell lines (Uchima *et al.,* 2007). In addition, TGF-β can synergise with IL-8 to promote over-expression of IL-1β, IL-6 and LIF from endometrial epithelial cells (Gutsche *et al.,* 2003), thus providing further evidence of its pro-tumourigenic potential.

The NF-κB signalling mechanism is another pathway implicated in cancer development as shown by its activation via protein kinase A in head and neck squamous cell carcinoma (Arun *et al.,* 2009) and its increased expression with PTGS-2 in colorectal carcinomas (Dolcet *et al.,* 2005). Furthermore, IL-1 has been shown to upregulate the functional HIF-1α protein through a classical inflammatory signalling pathway in A549 cells involving NF-κB and PTGS-2 which in turn promotes VEGF, a potent pro-angiogenic factor reguired for tumour growth (Jung *et al.,* 2003). NF-κB signalling is also induced in cells from *Ikk* beta knockout mice by IL-1 and TNF-α (a compound contained within SP in abundance) (May and Ghosh, 1999).

The complex nature of SP, the multiple signalling pathways within the female reproductive tract with which SP can interact and multistage process of cervical cancer development hinders the elucidation of the explicit mechanisms underlying this disease. The inflammatory potential of SP may further complicate the understanding of cervical cancer development in sexually active women because its presence may influence in helping establish pro-inflammatory conditions which are favourable to promote the onset of cervical cancer. As a result, there is a great need to ascertain the signalling pathways involved and whether the effects of SP further exacerbate this process.

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4.2 Aim of the Study

The previous chapter established that SP exposure induces expression of PTGS enzymes, PGE_2 receptor subtypes (PTGER1, PTGER3 and PTGER4), pro-inflammatory cytokines, pro-angiogenic factors and an increase in the angiogenic

marker CD31 in normal cervical tissue. These genes together can contribute to the development of cervical cancer and therefore suggest that SP can exacerbate cervical cancer development. However, the signalling pathways leading to the activation of these genes in the cervix are not known and here we plan to use WT HeLa cells as a model system to identify signalling pathways required for their transcriptional activation. In addition, employing the WT HeLa cells as a model system we plan to examine whether SP can promote cellular proliferation which is a classic hallmark of cancer development.

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4.3 Materials and Methods

4.3.1 Treatment of Cells and RNA Extraction

WT HeLa cells were seeded in culture vessels as described in table 2.1 depending on the assay being performed. Once cells were confluent, they were then serum starved for 24hr prior to treatment. The following day, cells were treated with SP (1:100) in the presence/absence of various inhibitors as mentioned in section 2.5.3 and thereafter underwent RNA extraction as mentioned in section 2.6.3. The concentration and purity of the RNA was determined by measuring the absorbance at 260nm and 280nm using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

4.3.2 cDNA Synthesis and Quantitative Real-Time PCR

Quantitative real-time PCR (QRT-PCR) was performed to investigate the relative mRNA expression of PTGS-1, PTGS-2, IL-6, IL-8, IL-11, Gro-α, VEGF and Ki-67 in WT HeLa cells stimulated with SP (1:100), in the presence/absence of inhibitors listed in table 2.2. Approximately 1µg of total RNA was reverse transcribed in the presence of PCR reaction buffer (1x), $MgCl_2$ (5.5mM), dNTPs (2mM), random hexamers (2.5µM), RNAse inhibitor (0.4U/µl) and Multiscribe™ reverse transcriptase (1.25U/µl) (PE Applied Biosystems, Warrington, UK) as described in section 2.6.5. Thereafter, QRT-PCR was carried out as outlined in section 2.6.6 using specific primers and probes listed in table 2.5 (250nM each). The reaction mix for QRT-PCR contained Taqman buffer (5.5mM $MgCl_2$, 200µM dATP, 200µM dCTP, 200µM dGTP and 400µM dUTP), AmpliTaq Gold DNA polymerase (0.025U/µl), ribosomal 18S forward and reverse primers and probe (all at 50nM), forward and reverse primers for genes of interest (all at 250nM) and 2µl of cDNA.

4.3.3 SDS-PAGE and Western Blot Analysis

WT HeLa cells were seeded at $1x10^5$ cells per 60mm diameter dish and allowed to propogate for 24hrs before serum-starvation overnight. Following serum-starvation, WT HeLa cells were treated with SP (1:100) in the absence/presence of AG-1478 (200nM), NS-398 (100nM), SC-560 (10µM), PD-98059 (50µM), or TGF-β

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inhibitor (10ng/ml) for time intervals of 0, 2, 5, 10, 20, 30 and 60min. Cells being treated in the presence of inhibitors were pre-incubated with the respective inhibitor for 1 hour prior to SP treatment. Thereafter, cells were harvested and extracted as described in section 2.7.1 and protein quantified according to section 2.7.2. A total of 40µg of cell extract was resuspended in a total volume of 25µl loading buffer (Appendix A) and boiled for 5min at 95°C. Samples and SeeBlue™ pre-stained protein marker (Novex, Invitrogen) were then fractionated by electrophoresis (50mA, 90min) on a pre-cast 4%-12% Bis-Tris gel (NOVEX, Invitrogen). Thereafter, the gel was transferred onto Immobilon™-FL PVDF membrane (Millipore, Watford, UK) in a transfer buffer (Appendix A) using a semi-dry blotter at a constant voltage of 14V for approximately 90min. Following transfer, immunoblots were blocked in Odyssey Blocking buffer™ (LI-COR Biosciences, Cambridge, UK) before overnight incubation with primary phospho-p42/44 and p42/44 antibodies (diluted 1:1000 in Odyssey blocking buffer) at 4°C. The following day, blots were washed and incubated with the goat anti-mouse IRDyeTM 800 (1:10,000) (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) and goat anti-rabbit Alexafluor 680 (1:5000) (Invitrogen) for 60 minutes at room temperature. Immunoreactive proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). ERK1/2 phosphorylation was calculated by dividing the value obtained from the phosphorylated ERK1/2 channel (700nm) by the value obtained from total ERK1/2 channel (800nm) and expressed as fold above vehicle controls.

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4.3.4 Cell Proliferation Assay

WT HeLa cells were seeded in 96 well plates at 3000 cells/well (counted using a haemocytometer) and allowed to propagate overnight before serum starvation for 24hrs. Thereafter, cells were treated for durations of 24, 48 and 72hr with SP (1:100) in the presence/absence of inhibitors of PD-98059 (MAPK kinase inhibitor), AG-1478 (EGFR tyrosine kinase inhibitor), NS-398 (PTGS-2 inhibitor), SC-560 (PTGS-1 inhibitor) and NF-κB (NF-κB inhibitor). Inhibitors were applied 1hr prior to treatment. Following cessation of the respective treatment periods, proliferation was determined using the CellTitre96 Aqueous One Solution Proliferation Reagent (Promega Corp, Madison, WI) by means of spectrophotometry detection. This was carried out by

adding 20ul of Proliferation Reagent per well and then subsequently incubating the 96-well plate at 37°C for 2hr in a humidified 5%, CO_2 atmosphere. After incubation, absorbances were recorded at 490nm, calculated and fold difference determined against the respective control.

4.3.5 Immunohistochemistry (IHC)

Immunohistochemical staining for the proliferation marker Ki-67 was carried out using cervical sections that comprised of normal tissue (n=5), squamous carcinoma (n=5) and adenocarcinoma (n=5) using methods mentioned in section 2.8.1 whereas Ki-67 staining of WT HeLa cells was undertaken according to details stated in section 2.8.3. Thereafter, all slides containing either cervical tissue samples, or WT HeLa cells treated with SP (1:100), were mounted in Permafluor for microscope analysis and the number of positively stained cells for Ki-67 was quantified using standard stereological techniques.

4.3.6 Statistical Analysis

All data are presented as mean ± SEM. Statistical significant differences were determined by unpaired student's *t*-test or ANOVA using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) (*, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$).

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4.4 Results

4.4.1 SP Titration *in vitro*

In the previous chapter it was shown that treating normal human cervical tissue with SP (1:500) increased the production of PTGS-1, PTGS-2, PTGER signalling, pro-inflammatory mediators, pro-angiogenic factors and expression of CD-31. It was therefore decided to use WT HeLa cells to establish whether these genes are upregulated in this cell line, thus making it suitable to serve as a model system, and also identify the signalling pathways required for the activation of these genes as well as to determine the effect of SP on cell proliferation. Previously, our group has shown that SP induces increased production of PTGS-2 and VEGF at a concentration of 1:500 in HeLa cells over-expressing the PTGER4 receptor (Muller *et al.,* 2006). However, the concentration of SP required to stimulate these target genes in WT Hela cells was not tested. Therefore, it was decided to perform a titration experiment and measure the SP induced transcription of IL-6, IL-8 and Gro-α using a range of dilutions of SP at time points of 2-24hr in order to determine the optimum SP dilution and treatment period that would be required for conducting experiments with chemical inhibitors specific for the different signalling molecules. SP dilutions of (1:250), (1:100) and (1:50) were used to treat WT HeLa cells for 2, 4, 6, 8, 12 and 24 hrs and the relative mRNA levels of IL-6, IL-8 and Gro-α were measured in SP treated cells and in control cells treated with media without SP. Quantitative RT-PCR previously showed a statistically increased production of IL-6, IL-8 and Gro-α in normal human cervical tissue treated with SP. Quantitative RT-PCR revealed IL-6, IL-8 and Gro-α to be upregulated at the majority of time points tested with inductions being observed at 2hr, reaching a maximal response at 6 hr and then declining until 24 hr post SP treatment. Furthermore, the resulting fold inductions of IL-6, IL-8 and Gro-α for each SP dilution at 6hr revealed that 1:50 induced a greater response than 1:100 (9.15- vs 4.54-, 162.5- vs 28.8- and 46.4- vs 9.44- fold increase respectively), while the SP dilution of 1:250 induced a very weak response (1.83-, 4.06 and 2.44- fold-increase; Figure 4.1A, 4.1B and 4.1C respectively). Since SP at 1:100 directed a significant induction of these genes it was then decided to use this dilution in the experiments employing chemical inhibitors as an approach to identify the signalling pathways required for transcription of the target genes involved in inflammation and angiogenesis.

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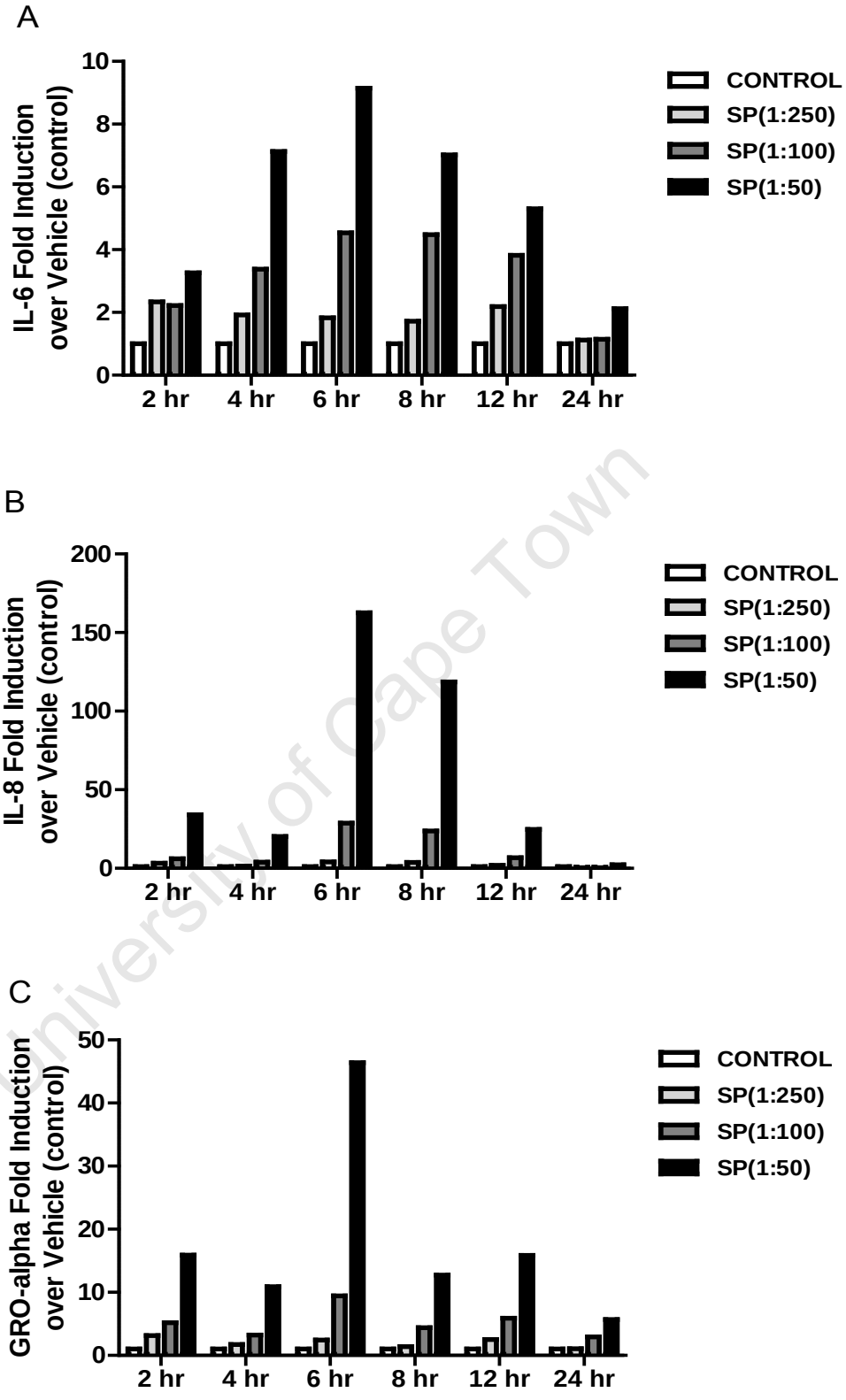

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Figure 4.1: Quantitative RT-PCR analysis revealed the mRNA expression of **(A)** IL-6, **(B)** IL-8 and **(C)** Gro-α levels in WT HeLa cells in the presence of SP at dilutions of (1:250, light grey bars), (1:100, dark grey bars) or (1:50, black bars) in comparison to the control group (media alone, open bars) for 2, 4, 6, 8, 12 and 24hr,(n=1).

4.4.2 Activation of ERK by SP in WT HeLa Cells

In the previous section we have shown that a SP dilution of 1:100 is sufficient to induce a marked increase in the induction of the genes IL-6, IL-8 and Gro-α. Following that experiment we decided to ascertain whether SP treatment could prompt the activation of extra-cellular regulated kinase (ERK), an early signalling event that is strongly correlated to cancer, involved in the activation of many genes and is also shown to be induced downstream to PG receptors (Handra-Luca *et al.,* 2003). SP and PGE_2 have previously been shown to induce phosphorylation of ERK via the PTGER4 receptor (Muller *et al.,* 2006), whereas Battersby and colleagues demonstrated that SP activated ERK signalling in endometrial carcinoma cells via the PTGER2 receptor. In these experiments we are trying to find out by the use of specific inhibitors which molecules in SP play a role in the transcriptional activation of ERK, whether the role they play is direct or indirect, does the TGF-β present in SP have a role and also whether SP-induced PTGS-1 and PTGS-2 has a role. The activation of ERK was measured by treating WT HeLa cells with SP in the presence/absence of various inhibitors and performing Western blotting which revealed ERK phosphorylation in WT HeLa cells treated with SP (1:100) significantly increased after 2, 5 and 10 min when compared to the untreated (0 min) (5.26 ± 1.31-, 6.00 ± 1.32- and 4.57 ± 0.82-fold increase, respectively; Figure 4.2) (all $P<0.05$) and thereafter ERK phosphorylation declined and was hardly observed at 20, 30 or 60min. As a control, WT HeLa cells were pre-treated with PD-98059 (MAPK/ERK inhibitor), 1hr prior to SP (1:100) stimulation and as expected PD-98059 abolished the phosphorylation of ERK for the same treatment periods 2, 5 and 10 min as shown by not being significantly different from the untreated (0 min) (1.16 ± 0.15, 1.37 ± 0.25 and 1.32 ± 0.23 respectively; Figure 4.2).

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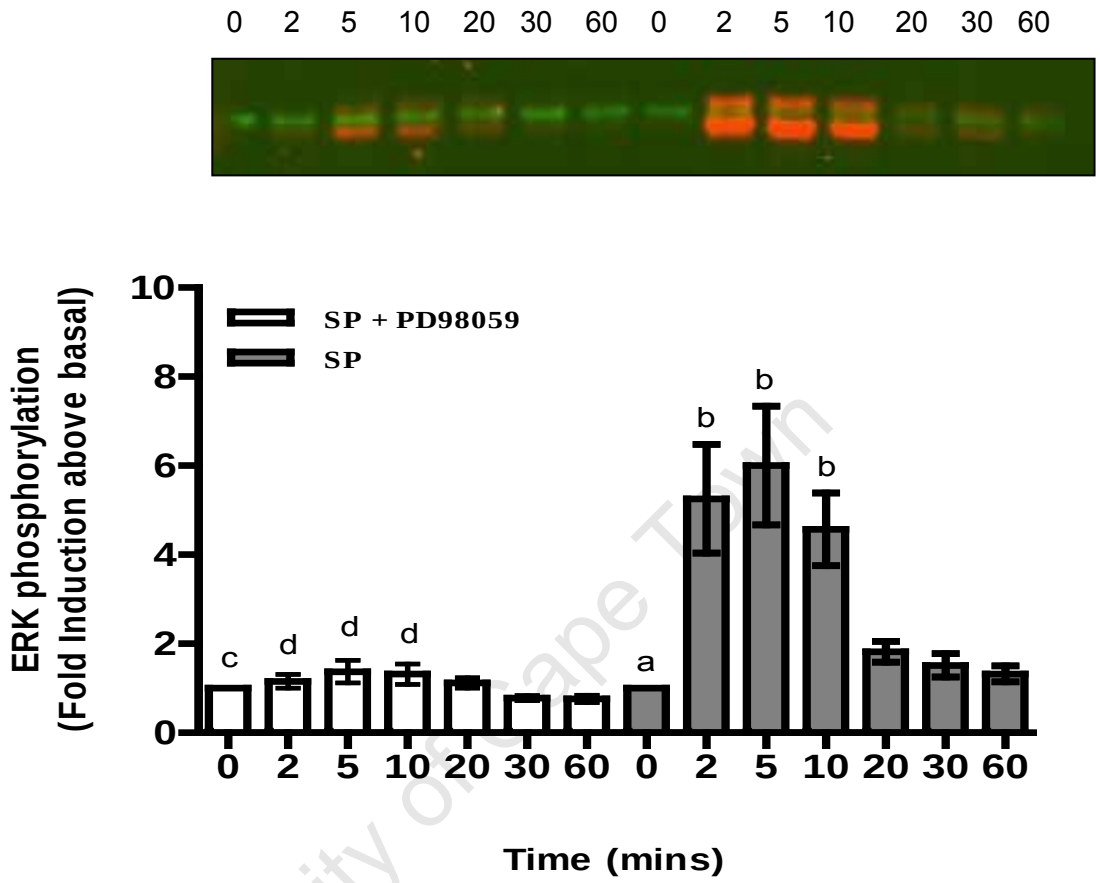

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Figure 4.2 Extracellular signal-regulated kinase (ERK) phosphorylation in WT HeLa cells in response to SP (1:100) treatment for 2, 5, 10, 20, 30 and 60min in absence/presence of inhibitor PD-98059 or left untreated (0min). Representative Western blot analysis revealed SP (1:100) significantly induced ERK phosphorylation following treatment for 2, 5 and 10min when compared to untreated (0min). b is significantly different from a ($P<0.05$*). The co-incubation of SP and PD-98059 abolished the phosphorylation of ERK for the same treatment periods 2, 5 and 10min when compared to untreated (0min). d is not significantly different from c. Data presented as representative blots together with semi-quantitative analysis of ERK phosphorylation shown as mean ± SEM from four independent experiments.

Next, it was decided to determine whether EGFR activation is required for SP-induced phosphorylation of ERK as it is well established that in some cases, the activation/phosphorylation of ERK is via transactivation of EGFR (Muller *et al.*, 2006; Milling-Smith *et al.*, 2006). Figure 4.3 shows like in the previous figure, that ERK phosphorylation in WT HeLa cells treated with SP (1:100) significantly increased after 2, 5 and 10 min when compared to the untreated (0 min) (5.54 ± 1.27-, 6.09 ± 1.95- and 6.91 ± 1.67-fold increase respectively; Figure 4.3) (all $P<0.05$). Despite SP-induced ERK phosphorylation being slightly elevated at 20, 30 and 60 min, neither treatment period was shown to be statistically significant. Treatment with AG-1478 (an inhibitor of EGFR transactivation) partially abolished the phosphorylation of ERK for 2, 5 and 10 min when compared to the untreated (0 min) (2.07 ± 0.40-, 3.49 ± 0.66- and 1.86 ± 0.80-fold increase respectively; Figure 4.3) (all $P<0.05$). This reduced activation of ERK represents a 62%, 43% and 63% inhibition of ERK induction respectively; Figure 4.3 (all $P<0.05$). These results demonstrate that EGF transactivation plays a major role in ERK activation but also suggests that other pathways are involved in ERK activation by SP.

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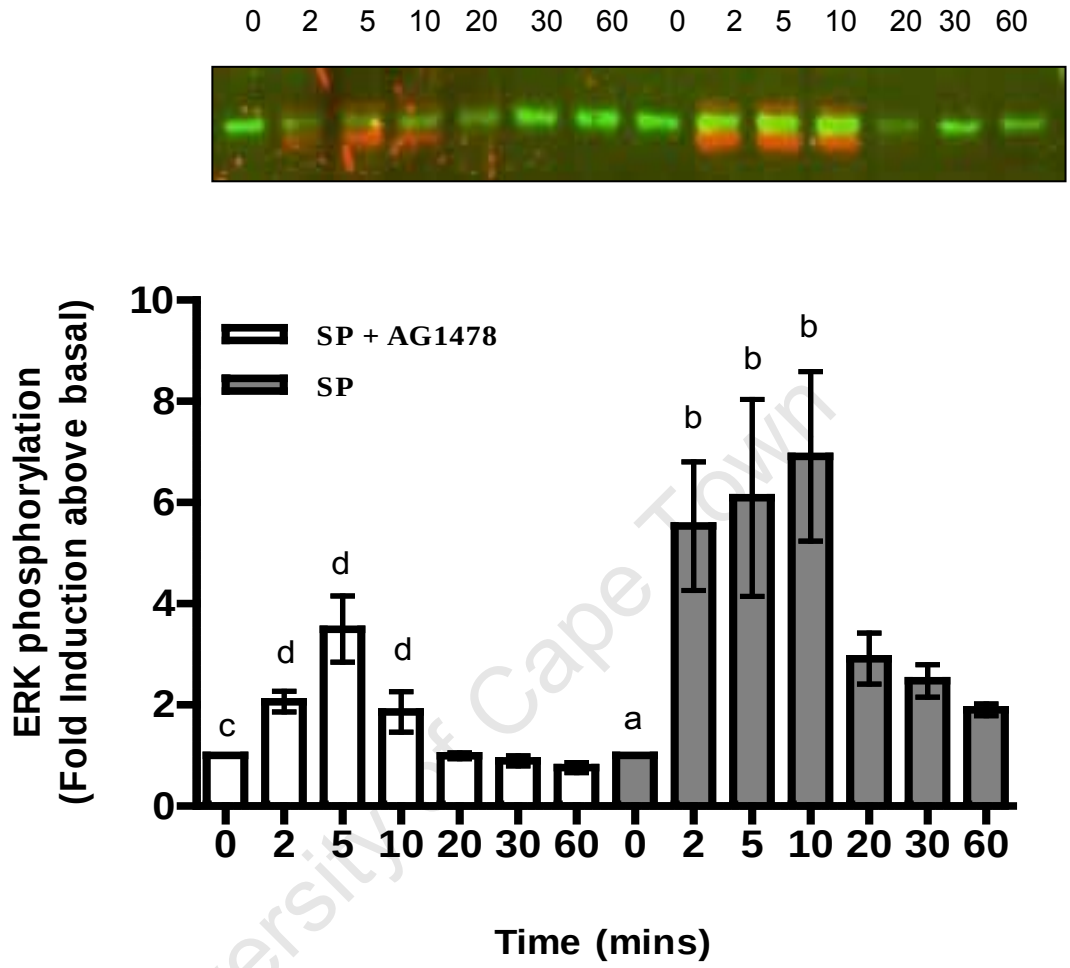

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Figure 4.3 Extracellular signal-regulated kinase (ERK) phosphorylation in WT HeLa cells in response to SP (1:100) treatment for 2, 5, 10, 20, 30 and 60min in absence/presence of inhibitor AG-1478 or left untreated (0min). Representative Western blot analysis revealed SP (1:100) significantly induced ERK phosphorylation following treatment for 2, 5 and 10min when compared to untreated (0min). b is significantly different from a ($P<0.05$*). Although incubation of SP and AG-1478 only partially abolished the induction of ERK for 2, 5 and 10min when compared to the untreated (0min), d is significantly different from c ($P<0.05$*), this did significantly inhibit the SP-induced phosphorylation of ERK for the same treatment periods when compared to SP (1:100) incubation alone. d is significantly different from b ($P<0.05$*). Data presented as representative blots together with semi-quantitative analysis of ERK phosphorylation shown as mean ± SEM from four independent experiments.

The inhibitor for PTGS-2 (NS-398) was tested in order to ascertain whether PTGS-2 activation is necessary for SP-induced phosphorylation of ERK. PTGS-2 expression is associated with apoptosis resistance, angiogenesis and metastasis in cancer of the colon and non-small-cell lung cancer (NSCLC) (Tsujii *et al.,* 1997; Tsujii *et al.,* 1998; Krysan *et al.,* 2008) and can be upregulated by SP in cervical adenocarcinoma cells (Sales *et al.,* 2002b). The inflammatory and tumourigenic signalling which results from PTGS-2 overexpression often converges on the ERK cascade (McKay *et al.,* 2007; Kim *et al.,* 2009). Measuring the induction of ERK by SP in the presence of NS-398 (PTGS-2 inhibitor) demonstrated that the ERK activation was hardly inhibited as shown by 9.5%, 6.5% and 1.9% inhibition of induction for 2, 5 and 10min treatment periods respectively and did not achieve statistical significance, while no induction was observed at treatment periods of 20, 30 and 60 min (Figure 4.4). Therefore, ERK induction is a direct result of SP stimulation and is not dependent of PTGS-2 as expected as ERK is a very early signalling event.

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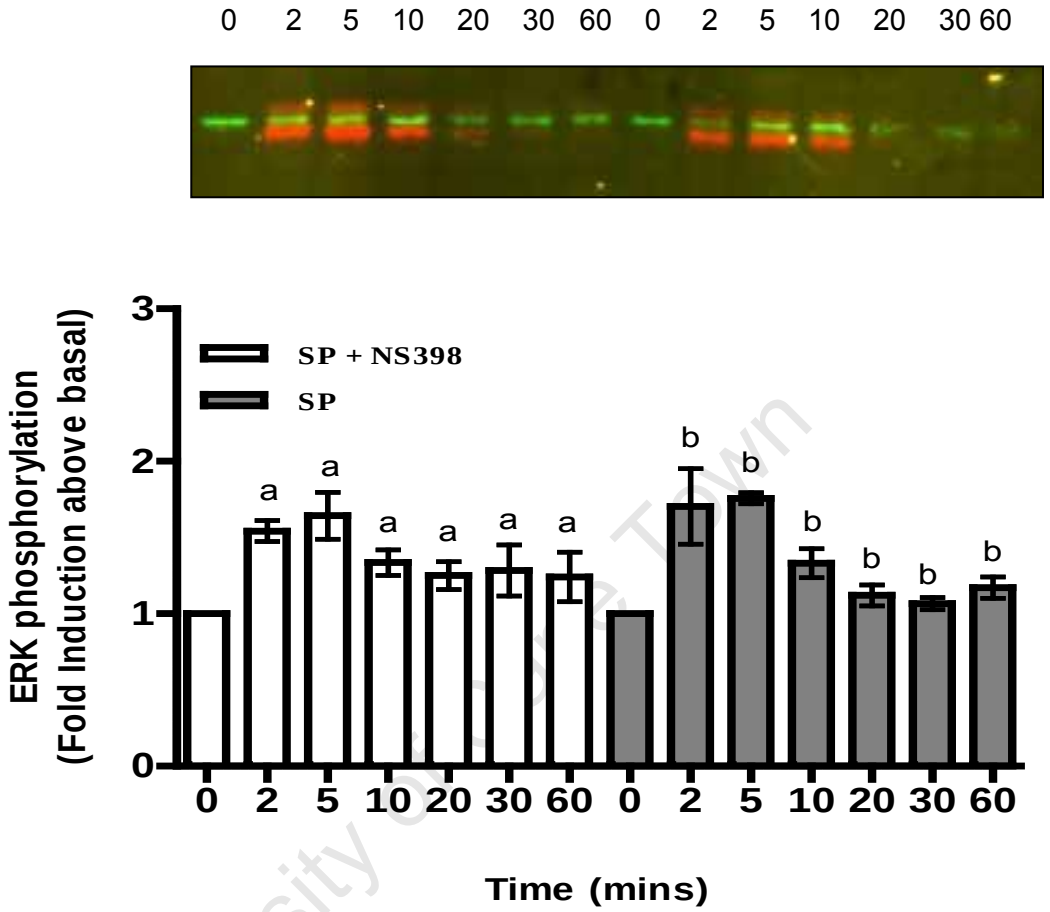

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Figure 4.4 Extracellular signal-regulated kinase (ERK) phosphorylation in WT HeLa cells in response to SP (1:100) treatment for 2, 5, 10, 20, 30 and 60min in absence/presence of inhibitor NS-398 or left untreated (0min). Representative Western blot analysis revealed that NS-398 did not significantly reduce the phosphorylation of ERK induced by treatment with SP (1:100). a is not significantly different from b. Data presented as representative blots together with semi-quantitative analysis of ERK phosphorylation shown as mean ± SEM from four independent experiments.

Since, PTGS-2 does not appear to have a role in SP induction of ERK, it was decided to determine whether PTGS-1 signalling was involved. PTGS-1, like PTGS-2, is upregulated in cancers of the cervix and endometrium (Jabbour and Sales 2004; Subbaramaiah *et al.,* 1997) and using WT HeLa cells as a model system, we therefore wanted to establish if it was central in SP-activated ERK. Results showed (Figure 4.5) that PTGS-1 inhibitor (SC-560) was not successful in reducing the phosphorylation of ERK induced by treatment with SP (1:100) at any of the time points investigated (1.77 ± 0.09 vs 1.63 ± 0.13), (1.77 ± 0.22 vs 1.69 ± 0.10), (1.38 ± 0.11 vs 1.45 ± 0.01), (1.31 ± 0.06 vs 1.18 ± 0.03), (1.00 ± 0.08 vs 1.11 ± 0.05) and (1.05 ± 0.05 vs 1.20 ± 0.05) for 2, 5, 10, 20, 30 and 60min respectively; Figure 4.5). In conclusion ERK induction is a direct result of SP stimulation and is not dependent on PTGS-1 or PTGS-2.

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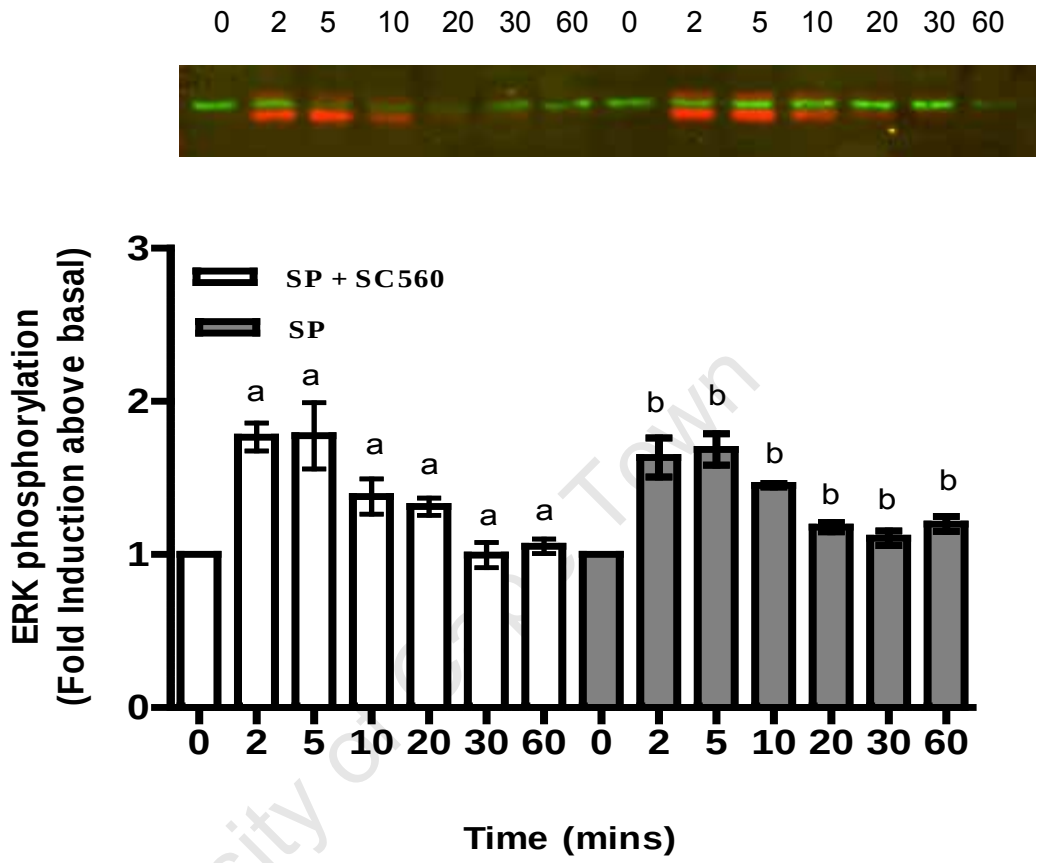

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Figure 4.5 Extracellular signal-regulated kinase (ERK) phosphorylation in WT HeLa cells in response to SP (1:100) treatment for 2, 5, 10, 20, 30 and 60min in absence/presence of inhibitor SC-560 or left untreated (0min). Representative Western blot analysis revealed that SC-560 did not significantly reduce the phosphorylation of ERK induced by treatment with SP (1:100) at any of the treatment periods. b is not significantly different from a. Data presented as representative blots together with semi-quantitative analysis of ERK phosphorylation shown as mean ± SEM from four independent experiments.

The immunosuppressive potential of SP is attributed in part to its high TGFβ content (Robertson *et al.,* 2002). Furthermore, the expression of TGF-β is closely associated with the onset and progression of cancer and is also known to induce ERK activation in pancreatic cancer cells (Ellenrieder *et al.,* 2000). We therefore wished to ascertain whether TGF-β signalling was necessary to mediate the activation of ERK by SP. Figure 4.6 shows that ERK phosphorylation in WT HeLa cells treated with SP (1:100) significantly increased after 2, 5 and 10 min when compared to the untreated (0 min) (2.63 ± 0.30-, 3.04 ± 0.56- and 2.55 ± 0.64-fold increase respectively; Figure 4.6) (all $P<0.05$) and thereafter although ERK phosphorylation remained slightly increased at 20, 30 or 60 min, this did not attain statistical significance. Results also revealed that the TGF-β1 inhibitor did not reduce the phosphorylation of ERK induced by treatment with SP (1:100) at 2, 5 and 10 min as shown by 3.1%, 3.0% and 9.4% inhibition of induction respectively (Figure 4.6). This finding indicates that SP-activation of ERK is not mediated by TGF-β.

Based on these results together, it can therefore be concluded that the induction of ERK is rapid and is partially mediated via the transactivation of EGF as well as other pathways that are activated by SP and this is not attributed to the TGF-β present in SP but to other constituents. Moreover, ERK activation does not occur via PTGS-1/-2 and therefore the upregulation of prostaglandin synthase enzymes appears to be downstream from ERK induction.

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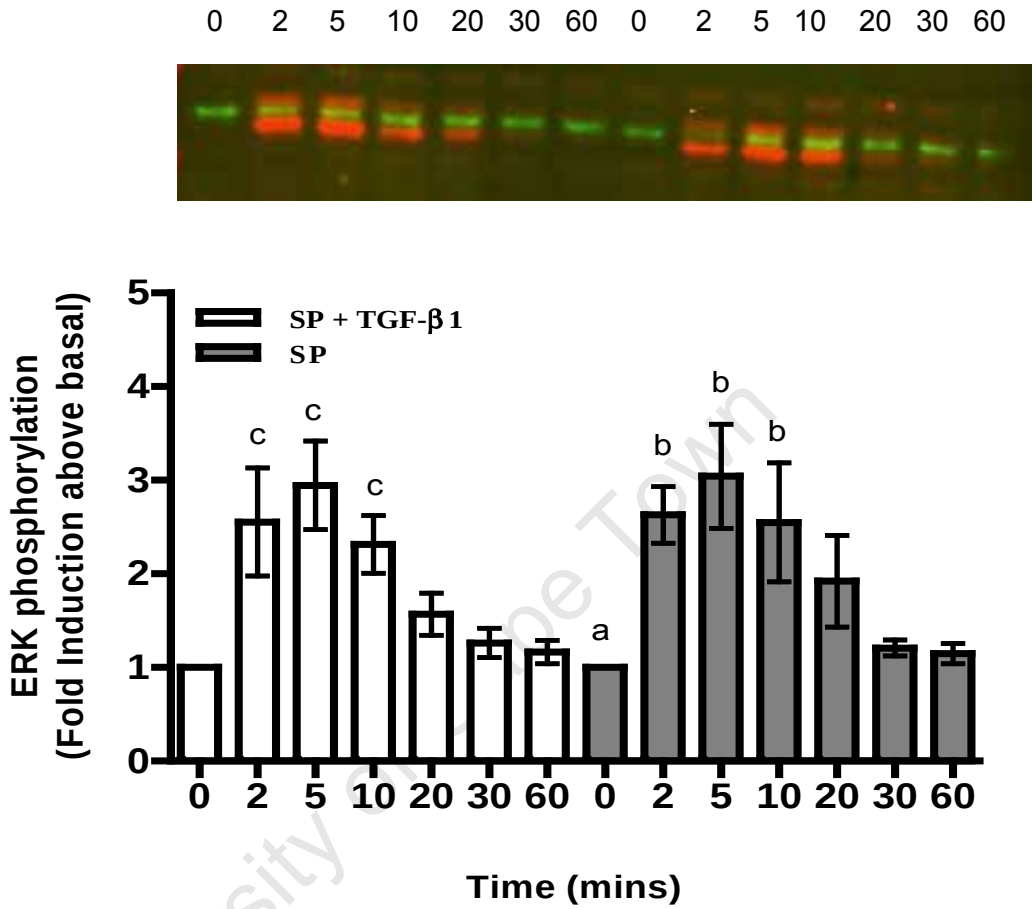

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Figure 4.6 Extracellular signal-regulated kinase (ERK) phosphorylation in WT HeLa cells in response to SP (1:100) treatment for 2, 5, 10, 20, 30 and 60min in absence/presence of inhibitor TGF-β1 or left untreated (0min). Representative Western blot analysis revealed SP (1:100) significantly induced ERK phosphorylation following treatment for 2, 5 and 10min when compared to untreated (0min). b is significantly different from a ($P<0.05$*). The co-incubation of SP andTGF-β1 did not significantly reduce the phosphorylation of ERK when compared to SP (1:100) incubation alone. b is not significantly different from c. Data presented as representative blots together with semi-quantitative analysis of ERK phosphorylation shown as mean ± SEM from four independent experiments.

4.4.3 Identification of Signalling Pathways Required for the Transcriptional Activation of PTGS-1 and PTGS-2 in WT HeLa Cells Treated with SP

PTGS-1 and PTGS-2 transcripts were both shown to be significantly upregulated in normal cervical tissue when treated with SP in comparison to the respective controls. SP treatment also promoted the induction of other pro-tumourigenic processes such as pro-inflammatory cytokines and pro-angiogenic factors and therefore suggests that SP may have a possible role in mediating cervical tumourigenesis. In addition, elevated levels of PTGS-1, PTGS-2 and PGE_2 synthesis have been observed in cervical carcinomas and this raises the question as to which signalling pathways are predominantly involved in the transcriptional activation of these genes (Sales *et al.,* 2001; Sales *et al.,* 2002a). As results from the previous chapter demonstrate that SP can induce the upregulation of genes that are also overly expressed in cervical cancer, here we are using a HeLa cell model system to determine whether SP stimulates the transcription of prostaglandin synthases (PTGS-1 and PTGS-2) and utilize specific chemical inhibitors to gain insight on the signalling pathways in effect.

Firstly, WT HeLa cells were pre-treated with inhibitors of PD-98059 (MAPK kinase inhibitor), AG-1478 (EGFR tyrosine kinase inhibitor), NS-398 (PTGS-2 inhibitor), SC-560 (PTGS-1 inhibitor), NF-κB (NF-κB inhibitor) and TGF-β1 (TGF-β1 inhibitor) for 1hr prior to treatment with SP (1:100) for 6hr. Thereafter, total RNA was extracted, followed by cDNA synthesis and then quantitative RT-PCR analysis was performed and fold induction calculated against the control. WT HeLa cells treated with SP for 6hr revealed that PTGS-1 expression was significantly elevated compared to the control (7.3 ± 2.7-fold increase; Figure 4.7A) ($P<0.005$) and confirms the use of WT HeLa cells as a suitable model system. In addition, the SP induction of PTGS-1 was reduced in the presence of inhibitors PD-98059 and AG-1478 (3.7 ± 0.8- and 2.9 ± 0.5-fold increase respectively; Figure 4.7A) (both $P<0.05$). Since reduction of PTGS-1 is only partial (50% and 63% respectively), and not completely inhibited, this implicates that signalling mechanisms other than ERK and EGFR are in use to mediate SP effects on PTGS-1 transcription. It can also be postulated that since AG-1478 inhibits SP-induced PTGS-1 slightly more than PD-98059, EGFR is required for ERK and the

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extra inhibition is due to an additional role EGFR has in the induction of PTGS-1. In contrast, the other inhibitors; NS-398, SC-560, NF-κB inhibitor and TGF-β1 inhibitor did not reduce the induction fold of PTGS-1. The reduction observed by PD-98059 and AG-1478 demonstrates that MAPK kinases (ERK1/2) and EGFR activation are required for the SP-induced transcription of PTGS-1 while inhibitors of PTGS-1, PTGS-2, NF-κB and TGF-β1 are not. This indicates that the transactivation of PTGS-1 in response to SP is partially activated via EGFR/ERK and additional pathways that do not involve PTGS-1, PTGS-2, NF-κB or TGF-β are also implicated. Furthermore, SP was found to significantly upregulate the expression of PTGS-2 in comparison to the control (3.4 ± 0.5-fold increase; Figure 4.7B) ($P<0.005$). Like PTGS-1, the SP induction of PTGS-2 was reduced in the presence of inhibitors PD-98059 and AG-1478 (2.1 ± 0.2- and 1.3 ± 0.2-fold increase respectively; Figure 4.7B) ($P<0.05$ and $P<0.005$). This equates to 38% and 62% inhibition of induction respectively and in a similar fashion to PTGS-1, because AG-1478 inhibited SP-induced PTGS-2 slightly more than PD-98059, EGFR is required for ERK and the extra inhibition is due to an additional role EGFR has in the induction of PTGS-2. In addition, NS-398 significantly inhibited the fold-induction of SP-activated PTGS-2 by 41%, (1.4- ± 0.2-fold increase respectively; Figure 4.7B) ($P<0.05$). Although this shows that PTGS-2 is required for its own upregulation, the induction was only partially reduced and therefore signalling apparatus other than ERK, EGFR and PTGS-2 are required for the SP-activated transcription of PTGS-2 and this does not involve PTGS-1, NF-κB or TGF-β1 as their respective inhibitors did not reduce the induction fold of SP-induced PTGS-2.

Transcriptional activation of PTGS-1 and PTGS-2 by SP is partially mediated by the ERK/EGFR pathway. However, PTGS-2 activity is also required for its own upregulation and signalling pathways involving molecules other than PTGS-1, PTGS-2, NF-κB and TGF-β are implicated in mediating SP-activated PTGS-1 whereas molecules other than PTGS-1, NF-κB and TGF-β are implicated in mediating SP-activated PTGS-2.

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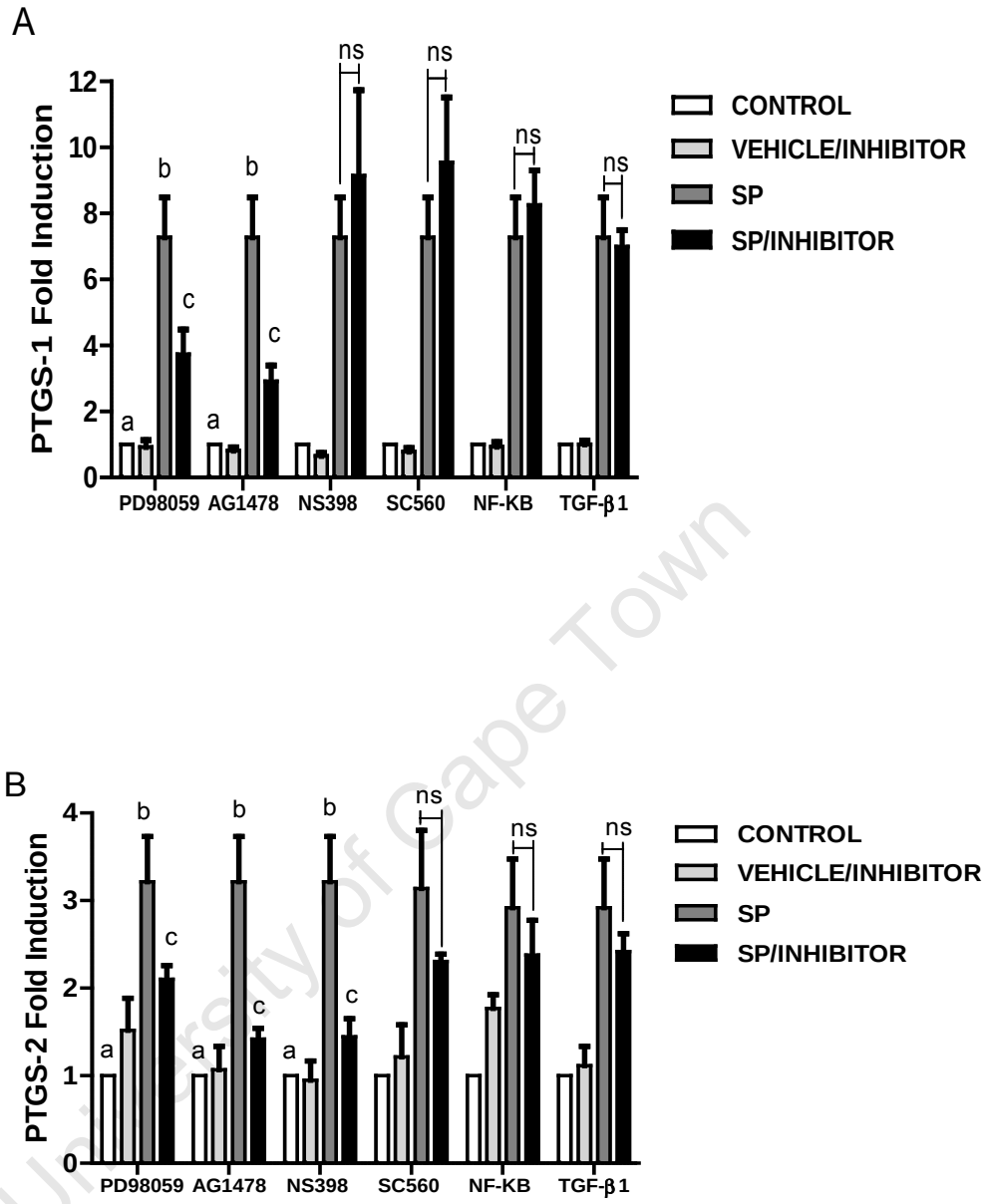

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Figure 4.7: Quantitative RT-PCR analysis determined that **(A)** PTGS-1 expression was significantly increased by SP (1:100) treatment (P<0.005**) in WT HeLa cells, b is significantly different from a, and this induction was significantly reduced in presence of the inhibitors PD-98059 and AG-1478 (both P<0.05*), c is significantly different from a. **(B)** Expression of PTGS-2 was significantly elevated by SP (1:100) treatment (P<0.005**) in WT HeLa cells, b is significantly different from a, and this induction was significantly down-regulated by PD-98059 AG-1478 and NS-398 (P<0.05*, P<0.005** and P<0.05* respectively), c is significantly different from b. All data represented as mean ± SEM from four independent experiments.

4.4.4 Identification of Signalling Pathways Required for the Transcriptional Activation of Pro-Inflammatory Cytokines IL-6, IL-8 and IL-11 in WT HeLa Cells Treated with SP

Having demonstrated in chapter 3 that SP (1:500) treatment induces increased expression of pro-inflammatory cytokines IL-6, IL-8 and IL-11 in normal human cervix and that they are also found to be upregulated in cancer (Robertson *et al.,* 1992; Ernst *et al.,* 2008; Fujimoto *et al.,* 2000), we wanted to determine the role of signalling pathways activated by SP in the transcription of these genes. WT HeLa cells were pre-treated with inhibitors of ERK, EGFR, PTGS-2, PTGS-1, NF-κB and TGF-β1 for 1hr prior to treatment with SP (1:100) for 6hr. Thereafter, total RNA was extracted, followed by cDNA synthesis and then quantitative RT-PCR analysis was performed and fold induction calculated against the control. QRT-PCR analysis of IL-6 revealed that its expression was significantly increased in comparison to the control (19.8 ± 2.3-fold increase; Figure 4.8A) ($P<0.0001$). Results generated from testing the inhibitors found that inhibitors PD-98059, AG-1478, NS-398 and NF-κB reduced the induction of IL-6 relative to induction in the presence of SP alone (6.8 ± 1.0-, 5.8 ± 0.2-, 1.9 ± 1.1- and 5.9 ± 1.9-fold increase respectively; Figure 4.8A) (all $P<0.005$). This equates to reductions of approximately 66%, 71%, 90% and 70% respectively. In contrast, the inhibitor SC-560 slightly reduced IL-6 expression whereas inhibitor TGF-β1 marginally increased IL-6 expression but neither result attained statistical significance. The reduction observed by inhibitors PD-98059, AG-1478, NS-398 and NF-κB demonstrates that ERK, EGFR, PTGS-2 and NF-κB are required for the SP-activated transcription of IL-6 while PTGS-1 and TGF-β1 are not. However, the observation that NS-398 reduced the inhibition by 90% demonstrates that PTGS-2 signalling is totally obligatory in the transactivation of SP-induced IL-6. Despite ERK, EGFR and NF-κB being strongly involved, their respective inhibitors only partially blocked IL-6 induction and therefore other pathways/signalling molecules are implicated in mediating IL-6 production. QRT-PCR analysis of IL-8 showed that it was significantly upregulated relative to the control as demonstrated by a 199 ± 30 fold increase (Figure 4.8B) ($P<0.0001$). Furthermore, all inhibitors tested; PD-98059, AG-1478, NS-398, SC560, NF-κB and TGF-β1 significantly inhibited the levels of SP-activated IL-8 (74-, 30-, 19-, 80-, 18- and 83-fold increase respectively; Figure 4.8B) ($P<0.05$, $P<0.005$,

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P<0.0001, P<0.05, P<0.0001 and P<0.005 respectively). The reduced levels of SP-activated IL-8 observed by these inhibitors (63%, 85%, 90%, 60%, 91% and 59% respectively) shows that many signalling molecules (EGFR, ERK, PTGS-1, PTGS-2, NF-κB and TGF-β1) are central in its transcriptional activation. The almost total inhibition by NS-398 and the NF-κB inhibitor indicate that both PTGS-2 and NF-κB are absolutely required for IL-8 transcription although their order of action needs to be required. The other molecules, ERK, EGFR, PTGS-1 and TGF-β1 are required but only play a partial role and it is quite likely that ERK and EGFR are upstream to them, while the order of PTGS-1 and TGF-β in the pathway is not known. Analysis of the transcription of IL-11 in response to SP reveals a significant increase of 28.8 ± 3.8-fold increase (Figure 4.8C) (P<0.0001) in comparison to the control. PD-98059, AG-1478, SC-560, NF-κB and TGF-β1 inhibitors were shown to significantly decrease the induction of SP-activated IL-11 (11.1-, 9.3-, 16.0-, 15.8, and 13.7-fold increase respectively; Figure 4.8C) (P<0.05, P<0.005, P<0.05, P<0.05 and P<0.05 respectively). This equates to an inhibition of IL-11 transcription by 61%, 68%, 44%, 45% and 52% respectively. In contrast, NS-398 did not reduce the induction fold. The marked but partial inhibition shows that ERK, EGFR, PTGS-1, NF-κB and TGF-β1 are involved, however, since their inhibition only partially blocked IL-11 transcription, they may function in concert and/or that additional signalling factors are required for activation of IL-11.

To summarize, it appears as though SP-activation of IL-6, IL-8 and IL-11 is mediated through several different signalling pathways. Inhibitors for PTGS-1 and TGF-β did not have any effect on the upregulation of SP-activated IL-6 and therefore it can be deduced that these signalling molecules are not involved whereas ERK, EGFR, PTGS-2 and NF-κB are and that PTGS-2 signalling is obligatory. Furthermore, all the inhibitors that were used to inhibit SP-activated IL-8 were successful and suggest that pathways involving PTGS-1, PTGS-2, ERK, EGFR, NF-κB and TGF-β are opertated upon by signalling molecules within SP in order to enhance the expression of IL-8 but PTGS-2 and NF-κB are absolutely required. As for IL-11, it appears as though PTGS-2 signalling does not participate in SP induction of this chemokine whereas PTGS-1, ERK, EGFR, NF-κB and TGF-β signalling are strongly implicated in its transcriptional

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activation, along with others, although the correct sequential order of these signalling events is unclear.

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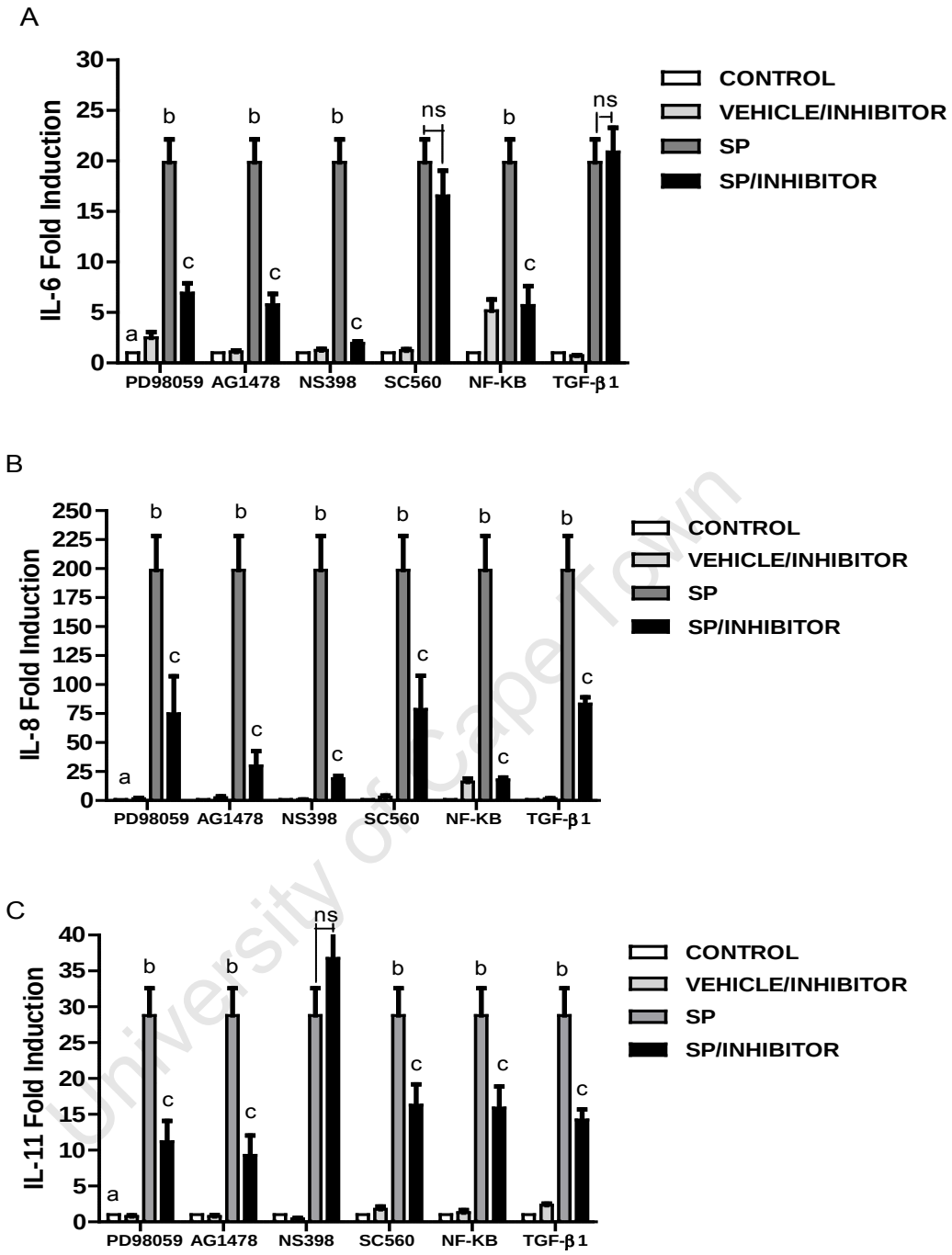

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Figure 4.8: Quantitative RT-PCR analysis revealed **(A)** IL-6 expression to be significantly upregulated by SP(1:100) treatment in WT HeLa cells when compared to the control (P<0.0001***) while PD-98059, AG-1478, NS-398 and NF-κB significantly inhibited this induction (all P<0.005**). **(B)** IL-8 expression was significantly increased by SP (1:100) treatment in WT HeLa cells (P<0.0001***) and this fold induction was significantly reduced by PD-98059, AG-1478, NS-398, SC560, NF-κB and TGF-β1 (P<0.05*, P<0.005**, P<0.0001***, P<0.05*, P<0.0001*** and P<0.005** respectively). **(C)** IL-11 expression was significantly increased by treatment with SP (1:100) in WT HeLa cells (P<0.0001) and this induction was significantly inhibited by PD-98059, AG-1478, SC-560, NF-κB and TGF-β1 (P<0.05*, P<0.005**, P<0.05*, P<0.05*, and P<0.05* respectively) Data are presented as mean ± SEM from four independent experiments.

4.4.5 Identification of Signalling Pathways Required for the Transcriptional Activation of Pro-angiogenic Factors GRO-α and VEGF in WT HeLa Cells Treated with SP

Having ascertained that SP triggers the induction of many signalling molecules in WT HeLa cells and that the majority of this activation occurs downstream from EGFR and ERK, we next wished to determine if upregulation of these pathways are required for increases in the transcripts of pro-angiogenic factors Gro-α and VEGF. WT HeLa cells were pre-treated with PD-98059, AG-1478, NS-398, SC-560, NF-κB and TGF-β1 inhibitors for 1hr prior to treatment with SP (1:100) for 6hr. Thereafter, total RNA was extracted, followed by cDNA synthesis and then quantitative RT-PCR analysis was performed and fold induction calculated against the control. QRT-PCR analysis revealed levels of Gro-α were significantly elevated by SP treatment and exhibited a 17.0 ± 3.9-fold increase in comparison to the control (Figure 4.9A) ($P<0.005$), Furthermore, inhibitors of PD-98059, AG-1478, NS-398, SC-560, NF-κB and TGF-β1 were statistically significant in hampering production of SP-mediated Gro-α as displayed by the lower fold-inductions when compared to SP treatment alone (6.5 ± 1.6-, 3.5 ± 0.9-, 3.6 ± 1.2-, 5.3 ± 0.3-, 4.6 ± 1.2- and 6.9 ± 1.7-fold increase respectively; Figure 4.9A) ($P<0.05$, $P<0.005$, $P<0.05$, $P<0.05$, $P<0.05$ and $P<0.05$ respectively), equating to approximate reductions of 62%, 80%, 79%, 69%, 73% and 52% respectively. The reduced expression of SP-mediated Gro-α by PD-98059, AG-1478, NS-398, SC-560, NF-κB and TGF-β1 inhibitors reveals that EGFR, ERK, PTGS-2, PTGS-1, NF-κB and TGF-β1 are involved in the SP-induced transcriptional activation of Gro-α. Since there was no total inhibition for either of the inhibitors used, this indicates that each one only plays a partial role but it is possible that they work together and also implies the involvement of other signalling molecules. VEGF expression was significantly raised through SP-stimulation as determined by a 2.7 ± 0.3-fold induction in comparison to the control (Figure 4.9B) ($P<0.05$). Moreover, inhibitors of PD-98059, AG-1478, NS-398 and NF-κB significantly reduced the SP-activated induction of VEGF as revealed by fold inductions that are 47%, 56%, 47% and 30% less than that of SP treatment alone (1..4 ± 0.2-, 1.2 ± 0.3-, 1.4 ± 0.4- and 1.9 ± 0.2-fold increase respectively; Figure 4.9B) (all $P<0.05$). In contrast, SC-560 and TGF-β1 inhibitor hardly reduced the fold induction of SP-induced VEGF. These

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results show that ERK, EGFR, PTGS-2 and NF-κB are required for the transcriptional activation of VEGF while PTGS-1 and TGF-β1 are not. In a similar manner to Gro-α, since no inhibitor completely blocked SP-activated induction of VEGF, this indicates that each one has a partial role and again it is possible they work in synergy but other signalling apparatus is being operated upon in order to mediate the effects of SP.

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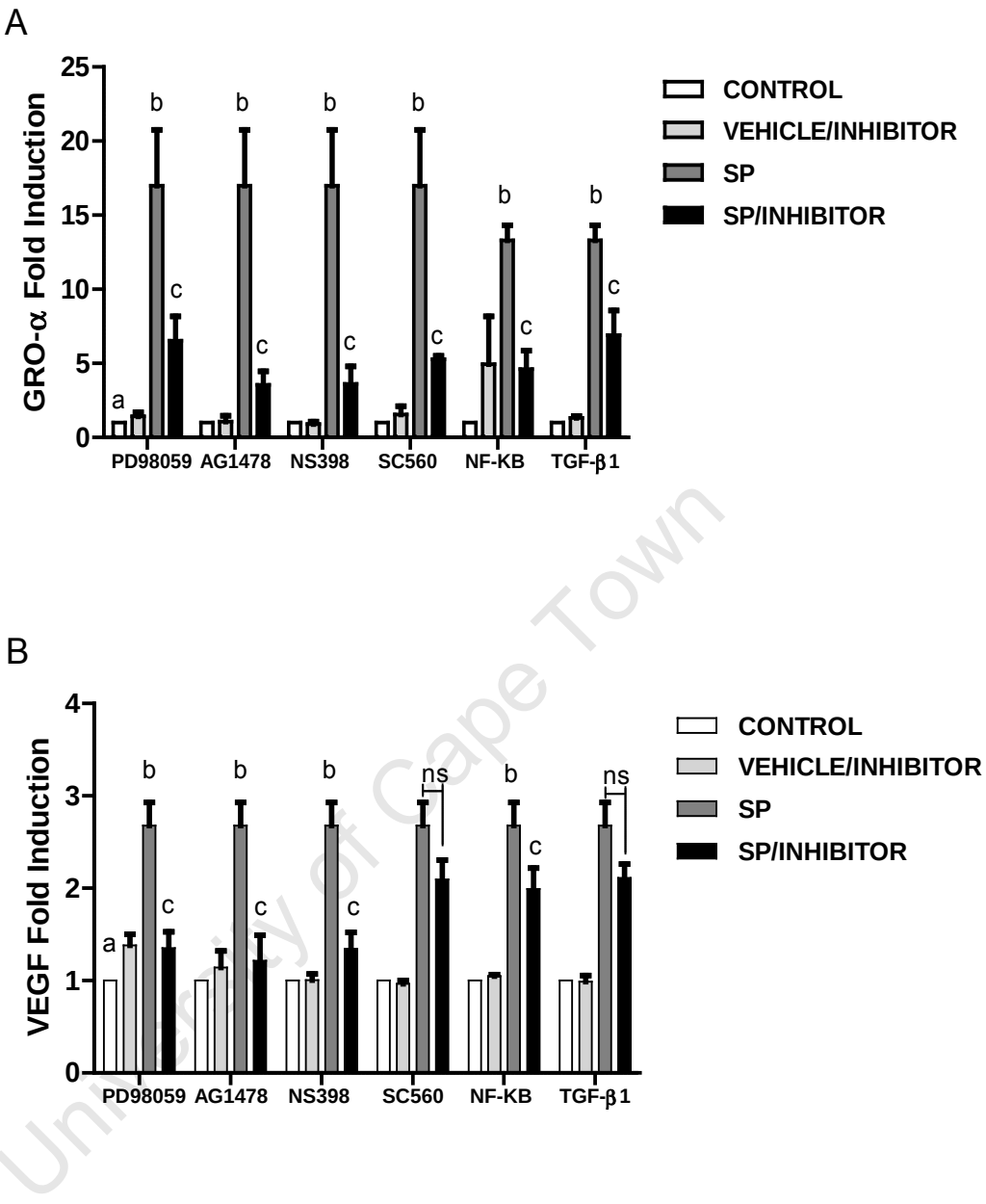

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Figure 4.9: Quantitative RT-PCR analysis was used to determine the levels of SP-induced Gro-α and VEGF in response to specific inhibitors and revealed **(A)** Gro-α expression mediated by SP (1:100) was significantly elevated in WT HeLa cells in comparison to the control ($P<0.005$**), b is significantly different from a, and this induction fold was significantly reduced by PD-98059, AG-1478, NS-398, SC-560, NF-κB and TGF-β1 ($P<0.05$*, $P<0.005$**, $P<0.05$*, $P<0.05$*, $P<0.05$* and $P<0.05$* respectively), c is significantly different from b. **(B)** VEGF expression induced by SP (1:100) was significantly upregulated in WT HeLa cells in comparison to the control ($P<0.05$*), b is significantly different from a, and this induction fold was significantly reduced by PD-98059, AG-1478, NS-398 and NF-κB (all $P<0.05$*), c is significantly different from b. Data represented as mean ± SEM from four independent experiments.

4.4.6 Expression of the Proliferative Marker Ki-67 in WT Hela Cells Treated with SP

The expression of Ki-67 was shown to be upregulated in cervical carcinomas when compared to normal cervix (Looi *et al.*, 2008), thus confirming that cervical tumour tissue has a higher proliferative capacity. In order to investigate whether SP could induce cellular proliferation, WT HeLa cells were employed as a model system to measure Ki-67. QRT-PCR analysis was used to determine the expression levels of Ki-67 in WT Hela cells treated with SP (1:100) for 6hr and fold induction calculated against the control. Results showed that SP treatment significantly upregulated the expression of Ki-67 as demonstrated by a 3.6 ± 0.9-fold increase in proliferation rate in comparison to the control (Figure 4.10; $P<0.05$). This suggests that SP exposure may pose as a potential risk to the cervical microenvironment by enhancing the rate of cellular proliferation.

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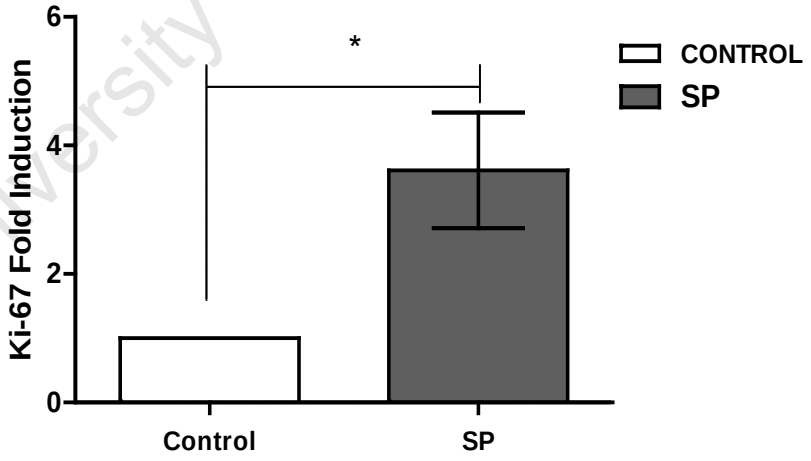

Figure 4.10: Quantitative RT-PCR analysis revealed expression levels of Ki-67 in WT HeLa cells treated with SP (1:100) for 6hr to be significantly upregulated in comparison to the control ($P<0.05$*). Data represented as mean ± SEM from four independent experiments.

4.4.7 Stereological Quantification of the Proliferative Marker Ki-67 in WT Hela Cells Treated with SP

The increase in Ki-67 in SP-treated WT HeLa cells was further confirmed by immunohistochemistry which was used to determine the expression of Ki-67 in response to SP (1:100) stimulation in WT HeLa cells *in vitro* after 24, 48 and 72 hrs of treatment. Quantitative stereology revealed treatment with SP was increased in comparison to the control after 24 hrs (75.0 ± 2.5 vs. 51.1 ± 3.3; P<0.005) 48 hrs (65.8 ± 2.6 vs 53.8 ± 3.1; P<0.05) and 72 hrs (84.5 ± 1.8 vs 55.9 ± 4.2; P<0.005) respectively (Figure 4.11).

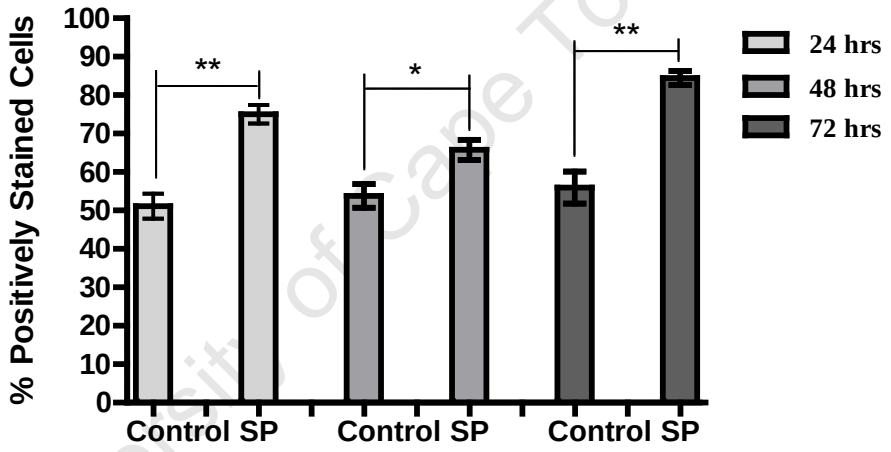

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Figure 4.11: Number of positively stained cells for Ki67 was quantified and is significantly increased when treated with SP (1:100) in comparison to the control treatment group for 24, 48 and 72 hrs, (P<0.005**, P<0.05* and P<0.005**) respectively. Data are represented as mean ± SEM from three independent experiments.

4.4.8 Effect of SP on WT HeLa Cell Proliferation

Having previously shown that SP has the capacity to induce the proliferation index of WT HeLa cells, as determined by the upregulation of Ki-67 by means of QRT-PCR analysis and stereological quantification, it was decided to ascertain whether SP exposure leads to increases in cellular proliferation. WT HeLa cells were seeded and allowed to propogate overnight before serum-starvation for 24hr. Thereafter, cells were treated with SP (1:100) for periods of 24, 48 and 72hr. Following cessation of the respective treatment periods and by means of spectrophotometry detection using the Proliferation Reagent (Promega) as per the manufacturers guidelines, absorbances were recorded, calculated and fold difference determined. Experiments revealed that SP treatment increases cellular proliferation in WT HeLa cells in comparison to the control following treatment periods of 24 hrs (1.2 ± 0.03-fold increase; P<0.005), 48 hrs (1.4 ± 0.09-fold increase; P<0.005) and 72 hrs (4.9 ± 0.4- fold-increase; P<0.0001) respectively (Figure 4.12). Although SP treatment induced increases in cellular proliferation after 24 and 48 hrs, this enhanced growth was very prominent after 72hr treatment. This data corresponds with the earlier finding that SP enhances the proliferative index as determined by the upregulation of Ki-67.

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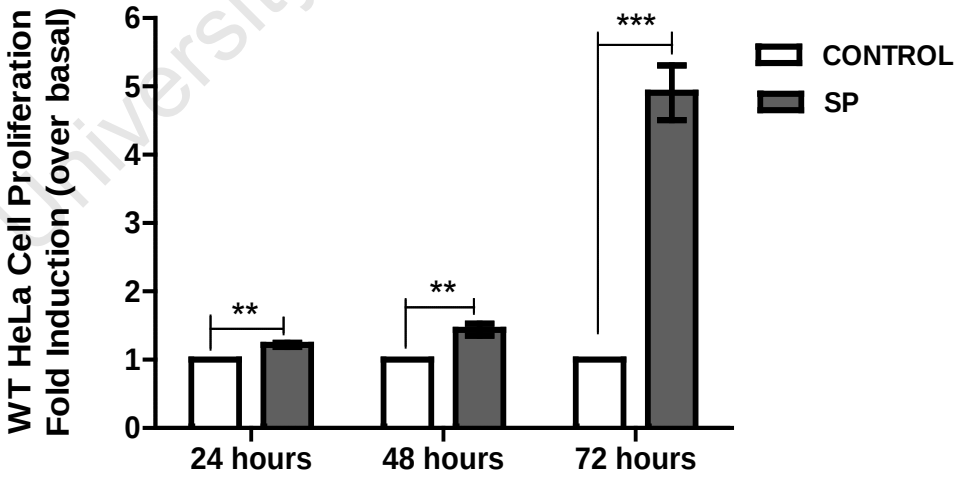

Figure 4.12: Proliferation assay results revealed SP (1:100) significantly increased the cellular proliferation of WT HeLa cells in comparison to the control treatment group for 24, 48, and 72 hrs, (P<0.005**, P<0.005** and P<0.0001*** respectively). Data are represented as mean ± SEM from fifteen independent experiments.

4.4.9 Expression of the Proliferative Marker Ki-67 in Normal Cervix, Cervical Adenocarcinoma and Cervical Squamous Carcinoma Samples

Since we have found that SP enhances WT HeLa cell proliferation and upregulate expression of Ki-67, we decided to confirm that Ki-67 is upregulated in cervical carcinoma. This was done by immunohistochemical staining for the proliferation marker Ki-67 on fixed tissue blocks of normal cervical tissue, cervical adenocarcinoma and cervical squamous carcinoma (n=5 per group). The increase in Ki-67 expression in cervical squamous carcinoma tissue in comparison to normal cervical tissue was confirmed as indicated by the differences in brown staining (arrowed; Figure 4.13A and 4.13B). Quantitative stereology for the proliferation marker Ki-67 found cervical squamous carcinoma to contain 23% ± 4% of positively-stained Ki-67 cells in comparison to normal cervix (0.95% ± 0.33%; Figure 4.13C; $P<0.05$) and is approximately a 24-fold increase. The number of Ki-67 positively stained cells in cervical adenocarcinoma displayed a higher mean percent in comparison to the normal cervix (8.4 ± 6.8 vs 0.95 ± 0.33 respectively; Figure 4.13C) and although this data amounted to an 8.8-fold increase, due to extreme variability, results were not deemed statistically significant. Nevertheless, these results clearly show that in general there is increased Ki-67 staining in cervical cancer tumours which is indicative of increased cell proliferation and is consistent with the effects this thesis has shown SP exhibits on WT HeLa cells.

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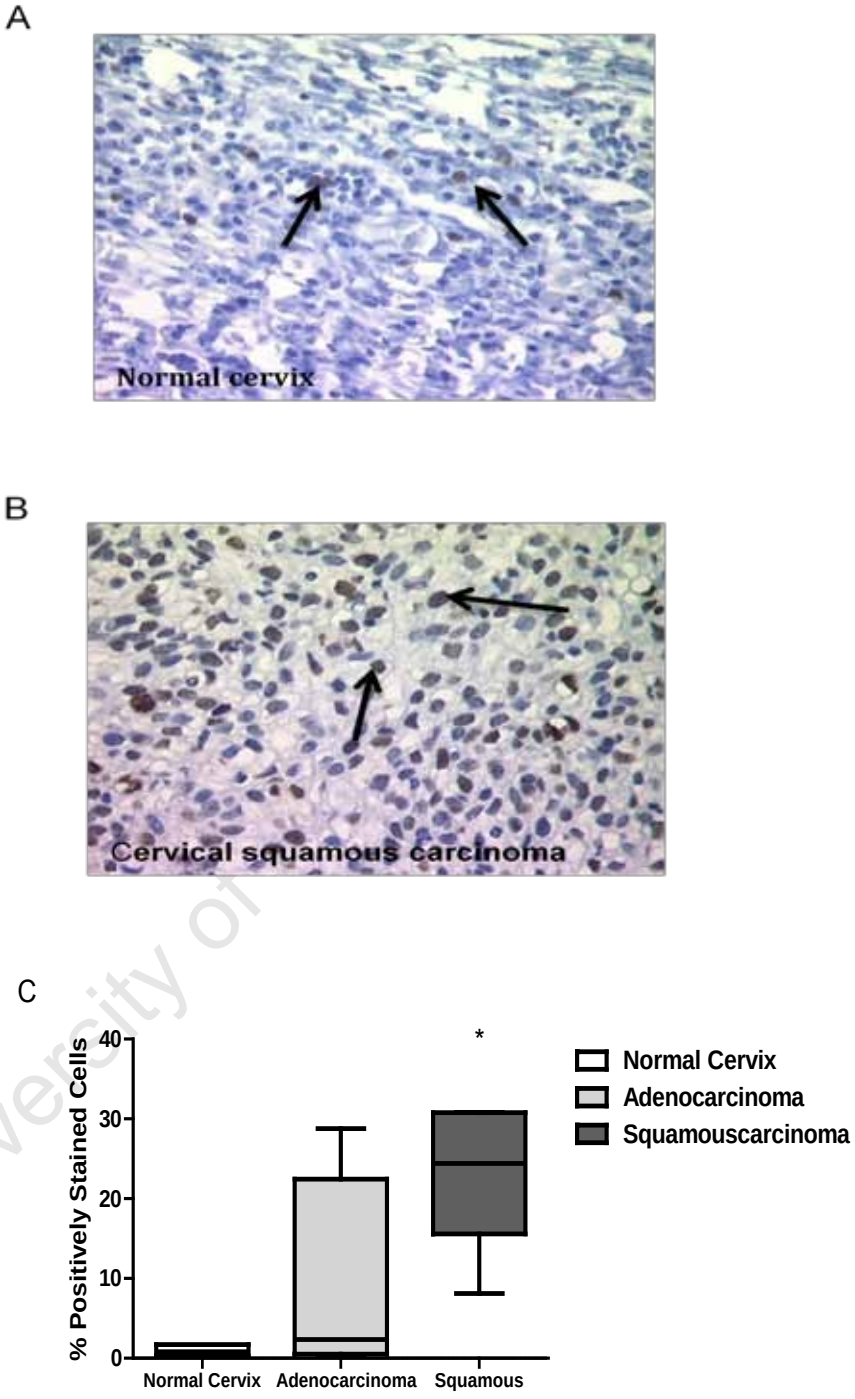

Figure 4.13 Immunohistochemical staining of Ki67 in **(A)** normal cervical tissue and **(B)** cervical squamous carcinoma (n=5 per group). **(C)** Number of positively stained cells for Ki67 was quantified for normal cervical tissue and cervical squamous carcinoma which was found to be significantly increased in comparison to the normal tissue ($P<0.05$*). Stereology quantification for cervical adenocarcinoma was not siginificant in comparison to the normal cervix and therefore is not shown. Data are represented as mean ± SEM from five independent experiments.

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4.5 Discussion

Prior to carrying out any signalling experiments in WT HeLa cells, it was deemed necessary to perform a titration assay with varying concentrations of SP over different treatment periods so that the optimum conditions for upregulating genes of interest could be determined. The preceding chapter demonstrated that normal cervical tissue treated with SP at a concentration of 1:500 for 24hr induced a significant increase in PTGS enzymes, prostanoid signalling, IL-6, IL-8, IL-11, Gro-α, VEGF and CD31. Work undertaken by Muller and colleagues (2006) have previously shown SP to enhance VEGF and PTGS-2 in WT HeLa cells expressing the PTGER4 receptor at a 1:500 dilution, whereas other data published by our research group revealed SP used at a concentration of 1:250 significantly induced the activation of ERK and promoted cAMP accumulation via the PTGER2 receptor in endometrial adenocarcinoma cells (Battersby *et al.,* 2007). However, results herewithin show that an SP concentration of 1:100 was required for marked induction and a treatment period of 6hr were the optimum conditions for which to investigate SP-activated cell signalling pathways as determined by the upregulation of IL-6, IL-8 and Gro-α. It must be noted that an SP concentration of 1:50 elicited an enhanced response in comparison to 1:100 but the concentration at 1:50 was considered too high to inhibit with chemical inhibitors.

These resulting experimental conditions are slightly different to that of others in our laboratory as Muller and colleagues (2006) attained highest fold-induction of VEGF and PTGS-2 in HeLa cells expressing the PTGER4 receptor using SP at a dilution of 1:500 and a treatment period of 8hr whereas Sales and colleagues (2002b) generated significant induction of PTGS-2 and PG receptor signalling in cervical adenocarcinoma cells by treating with SP (1:50) for 24 hrs. Another study performed in our laboratory by Battersby and colleagues (2007) demonstrated the upregulation of fibroblast growth factor-2 expression in endometrial adenocarcinoma cells following 6hr treatment using SP at a concentration of 1:250 and previously in this thesis, SP (1:500) treated normal cervical tissue for 24 hrs was seen to induce the upregulation of pro-inflammatory mediators and pro-angiogenic cytokines. To reason these differences, it is possible that the effects of SP are cell specific and that is why its signalling properties are being mediated differently between the varying types reported

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as being tested (cervical adenocarcinoma cells, endometrial adenocarcinoma cells and normal cervical tissue). Additionally, the studies carried out by Muller and colleagues (2006) and Battersby and colleagues (2007) use cell lines that overexpress the PTGER4 and PTGER2 receptors respectively, whereas the cervical adenocarcinoma cells used by Sales and colleagues (2002b) and the normal cervical tissue samples used within do not. As a result, the variation in model systems between tissue and different cell lines appears to play a significant role in determining the response time to SP stimulation. Although the WT HeLa cells used here do not overly express any of the PTGER1-4 receptors seen in the studies of Muller *et al.,* (2006) and Battersby *et al.,* (2007), these cells are being stimulated by SP at different dilution, 1:100 vs 1:500 and 1:250 respectively and the cells may respond quicker to higher concentrations of SP.

ERK is a key signalling molecule involved in gene transcription and regulates many cellular processes such as cellular transformation and growth (Battersby *et al.,* 2007). Furthermore, ERK activation has been shown to occur downstream from PTGER4 receptor signalling in response to SP and has the potential to promote cancer development by means of activating signalling complexes with EGFR, TGF-β and NF-κB (Muller *et al.,* 2006; Dumont and Arteaga, 2003; Zhan and Desiderio, 2010). In order to determine the mechanism of activation of ERK in SP-activated signalling in WT HeLa cells, several inhibitors were applied in the presence/absence of SP and demonstrated that PD-98059 as control and AG-1478 inhibited the rapid SP-induced phosphorylation of ERK which occurred between 2-10 min. However, since AG-1478 only partially blocked the SP-mediated induction, ERK activation must also operate on alternative pathways to those involving EGFR. Specific chemical inhibitors for PTGS-1 and PTGS-2 (SC-560 and NS-398 respectively) did not block ERK activation. This proposes that ERK activation is triggered by SP directly and not secondary downstream effects of these signalling molecules. This result was expected as ERK activation is very rapid after the addition of SP and is observed already after 2 min exposure. Although TGF-β1 is implicated in the initation and progression of cancer (Ranganathan *et al.,* 2007), and has been shown to mimic some aspects of the seminal response (Denison *et al.,* 1999a; Robertson *et al.,* 2002; Gutsche *et al.,* 2003), the TGF-β inhibitor did not inhibit SP-activated ERK signalling and implies that SP

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induction of ERK is not dependent on this cytokine. Muller and colleagues (2006) demonstrated similar effects by displaying that SP induced ERK signalling in WT Hela cells expressing the PTGER4 receptor, while here we show this induction of ERK can also occur in untransfected HeLa adenocarcinoma cells. Results generated in the study by Muller and colleagues (2006) revealed that SP (1:500) induced a 9-fold increase in ERK activation following treatment for 3 min whereas results in this thesis show that using a SP concentration of 1:100 induced a 6-fold increase of ERK after 5 min. It therefore appears that the differing SP concentrations (1:100 vs 1:500) and the involvement of the PTGER4 receptor are significant factors in the phosphorylation of ERK signalling in HeLa adenocarcinoma cells. Further data from the work of Muller and colleagues (2006) demonstrated that this SP-induced activation of ERK was in a partially dependent manner of EGFR (as also confirmed here) and Raf and although these signalling molecules are strongly involved, other pathways are implicated in mediating the effects of SP.

Interestingly, Battersby and colleagues (2007) performed a study in which Ishikawa cells expressing the PTGER2 receptor (comparable with PTGER2 levels in endometrial adenocarcinoma biopsy tissue) were treated with SP and the results showed that the EGFR inhibitor (AG-1478), protein kinase A inhibitor (4C3MQ) and PTGER2 receptor antagonist (AH6809) were all able to inhibit the rapid SP activation of ERK. This demonstrates that SP can activate EP2 receptor signalling in a similar manner to that proposed for endogenously secreted PGE_2 (Sales *et al.*, 2004b), and suggests that SP-induced EGFR signalling involed in endometrial carcinoma may also be applicable in the development of cancer of the cervix. Therefore, several receptors and signalling mechanisms appear to be involved in mediating the SP response and therefore the type of receptor expressed by the cells may be of major significance.

EGFR signalling is important for the regulation of essential normal cellular processes such as proliferation, cell differentiation and the modulation of apoptosis but aberrant receptor signalling is closely associated in the pathophysiology of cancer (Fischer *et al.*, 2003). Furthermore, the molecular mechanisms of EGFR signal transactivation are known to involve the processing of transmembrane growth factor precursors by

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metalloproteases and carcinomas of the cervix have previously shown to over-express matrix metalloproteinases MMP-2 and MMP-9 when compared with normal cervix (Fischer *et al.,* 2003; Moser *et al.,* 1999). Therefore, increases in EGFR signalling that are induced by SP stimulation may explain the resultant elevation of matrix metalloproteases known to exist in cervical carcinoma which in turn may lead to further EGFR signalling.

The upregulation of PTGS-1 and PTGS-2 have previously been reported in cervical cancer and this is known to influence pro-tumourigenic events such as tumour invasion, apoptosis and angiogenesis (Sales *et al.,* 2002a,b; Kulkarni *et al.,* 2001; Tsujii *et al.,* 1995). More recent studies have examined the usefulness of prostaglandin synthase enzymes as prognostic markers in attempts to determine the extent of disease progression and the likely rates of survival (Athavale *et al.,* 2006; Joo *et al.,* 2005). Results herewithin display that seminal plasma can upregulate the expression of PTGS-1 and PTGS-2 in normal cervical tissue and that PTGS-1/-2 are both involved in enhancing the levels of the pro-inflammatory mediator IL-8 and pro-inflammatory cytokine Gro-α in WT HeLa cells, although using this cell line as a model system also suggest PTGS-2 to have a greater role as demonstrated by its further participitation in promoting SP-activated expression of pro-tumourigenic factors IL-6 and VEGF.

In addition, PD-98059, an inhibitor of the MAPK kinase/ERK1/2 signalling pathway, and AG-1478, an inhibitor of EGFR, both only partially blocked the SP-induced transcription of PTGS-1 and PTGS-2. The close association of these signalling molecules and PTGS-1/-2 in cancer development is verified in studies undertaken by Matsuoka and colleagues (2010) who showed that increased gastric carcinoma metastasis in mice was linked with PTGS-1 and ERK phosphorylation while Muller and colleagues (2006) demonstrated that PD-98059 reduced the protein expression of SP-provoked PTGS-2 and a study carried out by Kulkarni and colleagues (2001) displayed that PD-98059 inhibited EGF-mediated induction of PTGS-2 in cervical cancer cell lines. Therefore, SP-induced ERK signalling certainly appears to be associated with cell growth in cancer of the cervix by playing a role in mediating the

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expression of SP-induced PTGS-1 and PTGS-2 and this process probably involves the transactivation of EGFR.

NS-398, an inhibitor for PTGS-2, effectively blocked SP-induced expression of PTGS-2, although the expression of PTGS-1 remained unaffected. This finding coincides with data published by Smith and colleagues (2000) which revealed that NSAIDs, such as NS-398, decreases products biosynthesized from PTGS-2 but not PTGS-1. In addition, a study undertaken by Nakata and colleagues (2003) demonstrated that NS-398 inhibits PTGS-2 induction of granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) in lung cancer cell lines mediated by the MAPK pathway or NF-κB activity whereas other work released by Banu and colleagues (2007) showed NS-398 to significantly reduce EGFR phosphorylation in colorectal cancer cells. Our results show that ERK and EGFR are required for both PTGS-1 and PTGS-2 and that EGFR appears to be required for the transactivation of ERK. Since inhibition of these signalling molecules only partially blocks PTGS-1 and PTGS-2, this suggests that other pathways could be involved. Undertaking experiments that used two inhibitors together would provide more insight as to the mechanisms involved and determine if the respective pathways combine to give an additive effect. Furthermore, PTGS-2 activity was also seen to be required to upregulate itself and taken together, these observations confirm that PTGS-2 is strongly involved in the progression of several cancers and that the use of NS-398 as a chemopreventative measure is a good possibility for future clinical use.

This study demonstrates that SP significantly induced transcription of inflammatory mediators IL-6, IL-8 and IL-11 in WT HeLa cells. One noteable finding is that every inhibitor that was used (PD-98059, AG-1478, NS-398, SC-560, NF-κB and TGF-β1) could successfully block production of IL-8 generated in response to SP exposure. The biological activity of IL-8 is invovled in many interactions of the microenvironment that contributes to tumour progression and these include angiogenesis, cell growth, tumour cell motion and modification of immune responses (Yuan *et al.*, 2005). Therefore, due to the multi-tasking nature of IL-8, it is possible that all the respective pathways which were inhibited (ERK, EGFR, PTGS-1, PTGS-2, NF-κB and TGF-β1),

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are involved in its transcriptional activation, whether it be a primary or a secondary mechanism. Inhibitors that blocked 85%+ are obligatory in the transactivation of IL-8 and it is therefore likely that they work in parallel and are upstream to another signalling molecule/transcription factor that is required for the SP-induced activation of IL-8. A study undertaken by Sharkey and colleagues (2007) displayed that SP could regulate IL-8 and IL-6 production in cell lines derived from the cervix whereas work published by Scott and colleagues (2009) revealed that SP increased IL-8 secretion in endometrial epithelial cells. Although the results here show that transcriptional activation of ERK, EGFR, PTGS-2 and NF-κB are strongly implicated in mediating the upregulation of SP-induced IL-8, using a combination of inhibitors would illustrate whether these respective signalling molecules operate within the same pathway or act upon different mechanisms. Since the signalling molecules in question only partially blocked SP-activated IL-8, it may be that these pathways function together or that other signalling molecules are involved.

IL-6 expression levels were shown to be increased in response to SP with PD-98059, AG-1478, NS-398 and NF-κB inhibitor shown to significantly obstruct the stimulatory effects of SP. This finding shows that like IL-8, SP-induced IL-6 is mediated by several different pathways. Research carried out by O'Leary and colleagues (2004) displayed that SP can impact on embryo development in the pig by inducing the over-production of PTGS-2 as well as IL-6 and this is likely to be through direct effects on local epithelial cell cytokine synthesis. Since NS-398 inhibited 90% of SP-induced IL-6, PTGS-2 therefore appears to play a major role in the activation of IL-6 in our WT HeLa cell model system but other pathways must be responsible for the remaining 10%, or it could be that inhibiton of PTGS-2 is not complete. The results also show the involvement of ERK, EGFR and NF-κB signalling in SP-induced IL-6 activation but they are not exclusively involved as inhibiton of these pathways only blocked IL-6 by approximately 70%. It is possible that ERK and EGFR are required for the upregulation of PTGS-2 and its subsequent activity, however, PTGS-2 appears to be necessary to mediate SP-induced IL-6 as demonstrated by the basal levels of PTGS-2 and that its inhibitor completely blocks its activity resulting in 90% inhibition. NF-κB signalling also has a role but this is not exclusive and may operate on at least two

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pathways which are via PTGS-2 that is downstream from ERK and EGFR and the other is NF-κB. In order to gain further insight, an experiment with both inhibitors of PTGS-2 and NF-κB may show 100% inhibiton and therefore illustrate that SP-induced transactivation of IL-6 arises exclusively via these mechanisms, whether they act in series or converging pathways.

IL-11 is another pro-inflammatory mediator that was activated in response to SP stimulation and that PD-98059, AG-1478, SC-560, NF-κB inhibitor and TGF-β1 inhibitor significantly reduced its upregulation. The data displaying links between IL-11 and cervical cancer is limited but this chemokine appears to perform a central role in gastric tumourigenesis (Merchant, 2008), is being considered as a prognostic marker in patients with prostate cancer (Furuya *et al.*, 2005) and can be induced by increased levels of PTGS-2 in breast cancer (Singh *et al.*, 2006). One striking difference is that unlike IL-6 and IL-8, the activation of IL-11 by SP in WT HeLa cells does not involve PTGS-2 and although pathways involving EGFR and ERK are again strongly implicated, other upstream signalling molecules must therefore be responsible for activating SP-induced IL-11. Furthermore, NF-kB signalling appears to have a lesser role in the transcriptional activation of IL-11 when compared to IL-6 and IL-8 and this may be a subsequent effect of PTGS-2 not being involved and suggests that NF-κB signalling, to a certain extent, is dependent on upstream PTGS-2.

Angiogenesis is an integral feature of most cancers and cervical cancer is no different (Kulkarni *et al.*, 2001). Gro-α and VEGF are known angiogenic factors with both being shown to be upregulated by SP in WT HeLa cells in this study. Employing WT HeLa cells as a model system has shown that multiple signalling pathways are engaged in the SP-induced transcriptional activation of Gro-α and VEGF. EGFR, ERK, PTGS-1, PTGS-2, NF-κB and TGF-β1 appear to mediate the upregulation of Gro-α whereas EGFR, ERK, PTGS-2 and NF-κB mediate the upregulation of VEGF but PTGS-1 and TGF-β does not. The results also suggest that ERK and EGFR activation are required for the upregulation of SP-activated Gro-α and VEGF by possibly mediating signalling via many receptors. The observation that inhibitors for ERK and EGFR partially blocked SP induction of Gro-α and VEGF suggest that induction is via receptors that

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are capable of coupling to both, possibly ERK activation via EGFR transactivation and this is direct and immediate. The activation of ERK/EGFR may also be required for the upregulation of PTGS-1/-2 which in turn is required for the transactivation of Gro-α and VEGF. The ERK/EGFR signalling may be upstream from PTGS-1/-2 but any receptor activated by SP to induce PTGS-1/-2 can be carried out via ERK activation and this is likely to be downstream from PG receptor signalling. The observation that the NF-κB inhibitor partially blocks both Gro-α and VEGF implicates that pathways involving NF-κB signalling are strongly implicated in mediating the angiogenic effects of SP, however, the transcription of this signalling molecule may, in part, be dependent on the activation of others, such as ERK, EGFR and PTGS-2. TGF-β1 partially mediated the SP-mediated induction of Gro-α and may be activated in the same direct manner as NF-κB, however, since TGF-β1 is not involved in the activation of VEGF, it is suggested that other transcription factors are in play.

Data published by Lee and colleagues (2006) demonstrated Gro-α to be secreted from ovarian cancer cell lines in culture but these cells ceased producing this cytokine in serum-free medium. This finding implies that Gro-α is not constitutively expressed but rather in response to exogenous growth factors, thus suggesting that the increases being observed in normal cervix and WT HeLa cells are a direct result of SP exposure. Furthermore, the expression of Gro-α is known to be regulated in part at the transcription level with binding sites for several transcription factors such as NF-κB and Sp1 having been found in its promoter region (Ye, 2001). It is therefore possible that SP may be a primary regulator of Gro-α mediated angiogenesis in cervical carcinoma by promoting upregulation of NF-κB. The induction of VEGF by seminal plasma exposure in WT HeLa adenocarcinoma cells has previously been shown to be mediated by ERK and EGFR signalling (Muller *et al.*, 2006). However, Wei and colleagues (2003) utilized cervical cancer C33A cells to demonstrate that VEGF induced angiogenesis was dependent on the upregulation of IL-6. Although SP-activated IL-6 may aid in the promotion of VEGF in normal cervical tissue and WT HeLa cells, these results indicate that PTGS-2 is involved as determined by NS-398 significantly inhibiting the SP-induced fold induction of VEGF and shows that SP may activate VEGF expression by upregulating levels of PTGS-2 and IL-6.

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Results from this thesis have demonstrated that SP triggers the over-expression of pro-inflammatory mediators and pro-angiogenic factors in normal cervical tissue and WT HeLa cells through the activation of signalling pathways such as EGFR and MAPK/ERK1/2 and has also highlighted the potential proliferative ability of SP by means of showing it to upregulate Ki-67 and induce growth in WT HeLa cells. The expression levels of Ki-67 can be used to determine the proliferative capacity of cancer cells with previous studies having employed Ki-67 as a prognostic marker in an attempt to gauge a patient's survival chances and likelihood of reoccurrence following surgery (Silva-Filho *et al.*, 2004). In addition, the upregulation of Ki-67 has been displayed in cervical carcinoma and immunoquantification of this protein can distinguish CIN from normal or benign cervical squamoepithelial lesions (Kruse *et al.*, 2001).

WT HeLa cells were treated with SP and QRT-PCR analysis demonstrated a significant elevation in the proliferation index as determined by the fold-induction of Ki-67. Literature released by Baselga and colleagues (2003) demonstrated distinct differences in Ki-67 expression in skin and in breast tumours when treating with the anti-EGFR reagent, gefitinib. The skin was choosen as a comparable target tissue for its established role of EGFR in renewal of the dermis (Jost *et al.*, 2000) and the study showed that following treatment with gefitinib in the skin, there was an inhibition of all EGFR-dependent pathways, including inhibition of phosphorylation of mitogen-activated protein kinase (MAPK) and a decrease in the proliferation of Ki-67. The SP induced expression of Ki-67 in our model cell line system was further confirmed by immunohistochemical staining in which WT HeLa cells were stimulated with SP and the number of positive stained cells for Ki-67 proved that this proliferative marker was significantly upregulated following treatment for 24, 48 and 72 hr periods.

Proliferation and the subsequent cellular growth that ensues is an important process required for angiogenesis (Keightley *et al.*, 2010) and since angiogenesis is a major contributor to cancer progression, it was decided to ascertain whether SP has the potential to promote cellular growth by using WT HeLa cells as a model system. WT

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HeLa cells were exposed to SP for treatment periods of 24, 48 and 72hr and assessed for cellular proliferation rates. SP was shown to significantly enhance cellular proliferation after 24, 48 and 72 hr periods which corresponds with the enhanced levels of Ki-67 for the same treatment periods and demonstrates that SP has the potential to promote cellular proliferation in the reproductive tract. Although the SP-activated increases in cellular proliferation were statistically significant at 24, and 48 hr, the augmented proliferative rate assessed at 72 hr was very prominent and suggest that despite SP showing pro-angiogenic properties, the effects elicited is on WT HeLa adenocarcinoma cells is not an immediate one.

Since unabated cellular proliferation is central in the progression of cancer (Papantoniou *et al.,* 2004), one of the objectives in this study was to establish whether SP exerted any proliferative effects on WT HeLa cells and this was determined by investigating expression of the proliferative marker Ki-67 and cellular proliferation rates. Researchers are now examining the use of Ki-67 as a prognostic marker in cervical cancer (Silva-Filho *et al.,* 2004) and we needed to ascertain whether Ki-67 induction was increased in cervical carcinomas in comparison to normal cervical tissue. Immunohistochemical staining was performed on cervical samples that included; normal, adenocarcinoma and squamous carcinoma and revealed that the expression of Ki-67 was significantly upregulated in squamous carcinoma samples in comparison to normal cervix. Although the expression levels of Ki-67 were also found to be increased in cervical adenocarcinoma samples, this result was not statistically significant due to extreme variability. Various studies have displayed that the expression of Ki-67 was significantly elevated in cervical adenocarcinoma tissue and squamous carcinoma tissue (Silva-Filho *et al.,* 2004; Hongyu *et al.,* 2006) and a larger assemblage of samples is required to validate a statistically significant finding in cervical adenocarcinomas in this study.

Interpretation of these results suggest that whatever proteins, prostaglandins, chemokines or other activator molecules are being activated by SP, it takes between 48hr and 72hr for the exerted proliferative effects to be observed in WT HeLa cells. This period is therefore a worthy timeframe in which to study the signalling molecules

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that are upregulated. The findings implicate that the activation of MAPK/ERK1/2, EGFR and PTGS-2 are strongly involved in cellular proliferation and supports earlier data that associates these signalling pathways with pro-tumourigenic events such as increased production of PTGS enzymes, prostanoid receptor activation and over-expression of pro-inflammatory mediators and pro-inflammatory cytokines. Furthermore, ERK1/2 is known to be a potent regulator of cell growth, differentiation and development (Lewis *et al.,* 1998) and SP has been shown to activate ERK1/2 signalling in cervical adenocarcinoma cells via the PTGER4 receptor (Muller *et al.,* 2006). As seen here, WT HeLa cells treated with PD-98059 restricted cellular proliferation induced by SP and the resultant decrease further cements the findings of Muller and colleagues (2006) as it provides supplementary evidence that pro-angiogenic factors and/or other activator compounds within SP are promoting cellular growth via ERK1/2 signalling. Furthermore, AG-1478 also inhibited the proliferative effects of SP in WT HeLa cells and this was shown to be closely associated with the activation of ERK1/2 which is in accordance with the findings of Battersby and colleagues (2007) who found that SP promoted growth in endometrium adenocarcinoma cells was due to ERK signalling via transactivation of EGFR and this pathway could be of significance in cervical tumourigenesis. NF-κB signalling is also strongly implicated in cancer development and was seen to participate in mediating the effects of SP because presence of the NF-κB inhibitor blocked the upregulation of SP-activated IL-6, IL-8, IL-11, Gro-α and VEGF in WT HeLa cells. The remaining inhibitor which made a significant impact was NS-398 decreasing PTGS-2 expression. PTGS-2 is heavily involved in many biological processes and can be rapidly induced by numerous growth factors, tumour promoters and oncogenes (DuBois *et al.,* 2004; Smith *et al.,* 2000). The vast consortium of cytokines and prostaglandins contained within seminal plasma have been shown to increase PTGS-2 (Sales *et al.,* 2002b), henceforth, SP can promote the proliferative effects regulated by PTGS-2 as revealed by the inhibitory ability of NS-398 and although the exact signalling apparatus is still to be defined, ERK1/2, EGFR and NF-κB appear to be at the fore.

Cervical cancer is a multistage process with many different signalling pathways involved in the development and progression of the disease. Women infected with

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HPV are at a predisposed risk of cervical cell neoplasticity and several of the cancer promoting pathways involved in the neoplastic transformation can be activated by SP and this not only promotes cell growth via mechansisms such as ERK and EGFR as seen here, but also makes unravelling the complexities of cervical cancer more difficult. The majority of research that is undertaken to investigate the links between cervical cancer and seminal plasma tends to focus on PTGS-1 and PTGS-2 signalling that is induced by exposure to SP (Sales *et al.,* 2002b; Muller *et al.,* 2006; Sharkey 2007) and whilst evidence from this study confirms these findings, results also suggest that other cellular signalling that is involved is derived from growth activators such as NF-κB and TGF-β, albeit in conjunction with PTGS enzyme activation, inflammatory mediators IL-6, IL-8 and IL-11 and pro-angiogenic factors Gro-α and VEGF.

With regard to the use of WT HeLa cells as a model system, it can be concluded that the activation of MAPK/ERK1/2 and EGFR is central in mediating the effects of SP and that the downstream involment of PTGS-1/-2 and is also strongly implicated. The signalling pathways that ERK and EGFR are involved in leads to the upregulation of pro-inflammatory mediators such as IL-6, IL-8 and IL-11 and pro-angiogenic factors Gro-α and VEGF. The SP-activated induction of NF-κB signalling and to a lesser extent TGF-β signalling, are also associated with the transcriptional activation of these target genes, thus highlighting the multiple pathways that SP can operate upon. In addition, SP promotes WT HeLa cell proliferation as determined by the upregulation of Ki-67 and elevated cellular proliferation rates and confirms the use of WT HeLa cells as a suitable model system to study the effects of SP. However, caution is needed to interpret chemical inhibitor data due to the possibility that inhibitors can work in tandem or parallel and exert non-specific effects.

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Chapter 5

SEMINAL PLASMA MEDIATES AN INFLAMMATORY RESPONSE AND PROMOTES ANGIOGENESIS TO ENHANCE GROWTH OF HUMAN CERVICAL CELLS IN MF-1 NUDE MICE

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5.1 Introduction

In previous chapters, employing human normal cervical tissue and HeLa cell lines has established that SP upregulates genes involved in tumour initiation and progression and directly enhances cell proliferation. As a result, it was decided to determine whether the use of an animal model system would produce similar findings.

Cervical tumours are normally invasive, causing many deaths each year with high incidence rates being observed not only in parts of Africa but also in Southeast Asia and Latin America (Potter 1997). Over 95% of all cervical carcinomas contain DNA of some human papillomavirus (Motoyama *et al.,* 2004; Mora *et al.,* 2007), however, with the transition of HPV to cervical cancer being shown to be a prolonged process, coupled with the knowledge that not everyone who develops HPV develops cancer, additional factors appear to be involved. Subbaramaiah and Dannenberg (2007) demonstrated that HPV oncoproteins E6 and E7 mediates the transcription of PTGS-2 which in turn increases cellular proliferation. SP has been shown to increase PTGS-2 in results herewithin which is in agreement with other studies from our laboratory (Sales *et al.,* 2002b; Muller *et al.,* 2006) and in a similar fashion to E6 and E7 by means of activating EGFR signalling, SP induction of PTGS-2 may enhance cell proliferation to promote tumour growth.

Other pro-tumourigenic events implicated in the development and progression of cancer are inflammation and angiogenesis. Many activator molecules involved in the inflammatory response and angiogenesis are contained within SP, such as TGF-β, pro-inflammatory mediators IL-6 and IL-8, TNF-α, PGE_2 and VEGF (Robertson *et al.,* 1992; Denison *et al.,* 1999a; Sharkey *et al.,* 2007). The exact mechanisms responsible for the development of cervical carcinoma are not yet fully understood but the vast variety of cytokines contained within SP and the tumour-promoting processes these can trigger such as PTGS-PTGER signalling, cellular proliferation, altering existing vasculature and enhanced inflammatory response suggest its properties may be of certain importance in sexually active women infected with HPV.

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Results generated so far in this thesis have shown that SP increased the expression of PTGS-1/-2, PGE_2 receptor signalling, pro-inflammatory cytokines IL-6, IL-8 and IL-11, pro-angiogenic factors Gro-α and VEGF and the endothelial cell marker CD31 in normal cervical tissue. The use of the WT HeLa cell line as a model system further demonstrated the tumour-promoting potential of SP by inducing the transcription of PTGS-1/-2, pro-inflammatory cytokines IL-6, IL-8 and IL-11 and pro-angiogenic factors Gro-α and VEGF as mirrored in normal cervical tissue as well as increasing cellular proliferation, upregulating the expression of Ki-67 and the involvement of signalling pathways such as ERK, EGFR, PTGS-1, PTGS-2, TGF-β, and NF-κB.

Subsequent to determining the upregulation of PTGS enzymes, prostaglandin receptors and a host of pro-inflammatory mediators and angiogenic factors in normal cervical tissue and WT HeLa adenocarcinoma cells in response to SP stimulation, it was decided to perform an *in vivo* study in an attempt to see whether the increased expression of these cancer favouring characteristics could also be induced in transgenic nude mice through treatment with seminal plasma.

Seminal plasma has previously been shown to trigger the upregualtion of prostanoid receptor signalling in cervical adenocarcinoma cells in addition to inducing over-expression of PTGS-2 and VEGF in HeLa cells expressing the PTGER4 receptor (Muller *et al.,* 2006). Although HPV infection is deemed as the initiating step in the development of cervical neoplasias, increases in prostanoid biosynthesis, PTGS-2 and VEGF are considered pro-tumourigenic events and as a result, SP stimulation may enhance progression or the onset of cervical cancer (Kulkarni *et al.,* 2007; Muller *et al.,* 2006). Furthemore, Jeremias and Witkin (1999) revealed that repeated exposure of neoplastic cervical epithelial cells to SP promoted the release of matrix metalloproteinases, the upshot of which, is the degradation of the extracellular matrix and cervical tumourigenesis.

Battersby and colleagues (2007) demonstrated that SP and PGE_2 similarly activated increased PTGER2 signalling in endometrial carcinomas and work undertaken by Sales and colleagues (2002b) showed evidence of SP and PGE_2 to correspondingly

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elevate the expression of PTGS-2 in cervical adenocarcinoma cells via increases in cAMP. Taken together, this therefore suggests that cervical tumourigenesis could be regulated in an autocrine/paracrine manner by PGE_2 in SP and PTGER receptor interactions.

Although these arachidonic cascade factors are mainly concerned with the maintenance of homeostatic state, PTGS enzyme expression, prostaglandin synthesis, prostaglandin cognate receptor expression and the resulting signalling pathways they activate are found to be dysregulated in numerous reproductive pathologies, including cervical adenocarcinoma (Narumiya *et al.,* 1999; Sales *et al.,* 2002a; Jabbour *et al.,* 2006). Unfortunatley, data detailing the effects of SP *in vivo* is relatively scarce and it is anticipated that the PGE_2 content within SP, or another potent suppressor factor contained within the ejaculate pool, may induce similar effects in nude mice that allow progression of cervical cancer.

Over the years, multiple studies in animal models have greatly assisted our understanding of the physiological and pathophysiological mechanisms that are regulated by PTGS enzymes, the major metabolites that PTGS enzymes biosynthesize called prostaglandins, the cognate receptors that prostaglandins adhere to and the production of chemical activators that result (Coleman *et al.,* 1994; Langenbach *et al.,* 1999). Whether the approach applied was generating knockout models that lack the prostaglandin receptors PTGER1-4, ablation of genes in mice encoding for PTGS-1 and PTGS-2, or the use of agonists and antagonists to examine signalling pathways, it would be very hard to perceive how else the valuable information generated from these mouse studies would have come to light and as a consequence the significant clinical benefit of NSAIDs seen in today's society would not have been possible.

Normal cervical tissue and WT HeLa adenocarcinoma cells have thus far in this thesis shown a propensity towards encouraging tumourigenesis by over-expressing PTGS enzymes, increasing PTGER receptor synthesis and upregulating pro-inflammatory mediators and pro-angiogenic factors in response to SP stimulation. As a result, this work culminates in an *in vivo* study to illustrate whether SP enhances cervical growth

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in a xenograft model by switching on the inflammatory pathways that include PTGS-1/-2 enzymes and chemokines such as IL-6 and IL-8, whether SP promotes cellular proliferation as determined by the proliferative marker Ki-67 and whether SP induces angiogenesis as determined by the expression of VEGF and endothelial cell marker CD31.

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5.2 Aim of the Study

The aim of this study was to establish whether SP treatment mediated an inflammatory response to enhance the growth *in vivo* of WT HeLa adenocarcinoma cells xenografts inoculated into nude mice. Previous studies have successfully displayed that the expression of PTGS enzymes, prostaglandin receptors (PTGER1-PTGER4), pro-inflammatory cytokines and pro-angiogenic factor VEGF are increased in response to seminal plasma stimulation *in vitro*. Whether SP treatment could replicate the induction of these known tumourigenic events *in vivo* was undertaken by examining the following;

(i) *Establish whether nude mice xenografts display an increase in proliferation, growth rate and size in response to seminal plasma treatment.*

(ii) *Does SP affect the expression of PTGS-1, PTGS-2 and prostaglandin subtype receptors PTGER1, PTGER2, PTGER3 and PTGER4.*

(iii) *Does SP affect the expression of pro-inflammatory cytokines in response to seminal plasma stimulation.*

(iv) *Does SP affect the expression of the pro-angiogenic factor VEGF, and as a result, promote the induction of CD31.*

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5.3 Materials and Methods

5.3.1 Animals

MF-1 nude mice were obtained from the University of the Witwatersrand, Johannesburg, South Africa. The mice were used between 6-18 wks of age and housed as per the conditions stated in section 2.4.1 as directed by the ethics committee protocol. On day 1 of the experimental procedure, mice were inoculated with WT HeLa cells and assigned into treatment groups as described in section 2.4.2.

5.3.2 Tissue Collection and RNA Extraction

Following the ten week treatment period, animals were sacrificed and tumours excised and weighed as previously described in section 2.4.2. A proportion of the tumour was fixed in 4% paraformaldehyde for wax-embedding and immunohistochemistry and the remainder used for RNA extraction. RNA was extracted as described in section 2.6.2 and the concentration and purity of the RNA was determined by measuring the absorbance at 260nm and 280nm using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

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5.3.3 cDNA Synthesis and Quantitative Real-Time PCR

Quantitative real-time PCR (QRT-PCR) was performed to investigate the relative expression of PTGS-1, PTGS-2, prostaglandin receptor subtypes (PTGER1-PTGER4), IL-6, IL-11, VEGF and Ki-67 in MF-1 nude mice xenografts arising from inoculation with HeLa adenoocarcinoma cells. Approximately 1µg of total RNA was reverse transcribed in the presence of PCR reaction buffer (1x), $MgCl_2$ (5.5mM), dNTPs (2mM), random hexamers (2.5µM), RNAse inhibitor (0.4U/µl) and Multiscribe™ reverse transcriptase (1.25U/µl) (PE Applied Biosystems, Warrington, UK)) as described in section 2.6.5. Thereafter, QRT-PCR was carried out as outlined in section 2.6.6 using specific primers and probes listed in table 2.5 (250nM each). The reaction mix for QRT-PCR contained Taqman buffer (5.5mM $MgCl_2$, 200µM dATP, 200µM dCTP, 200µM dGTP and 400µM dUTP), AmpliTaq Gold DNA polymerase (0.025U/µl), ribosomal 18S forward and reverse primers and probe (all at 50nM), forward and reverse primers for genes of interest (all at 250nM) and 2µl of cDNA.

5.3.4 Immunohistochemistry (IHC)

Nude mice xenograft tumours derived from inoculating with WT HeLa cells and treated with either SP (1:500) or PBS were taken for immunohistochemical evaluation of the proliferative marker Ki-67 and also to determine blood vessel size by the expression of endothelial cell marker CD31. Briefly, tumours were excised and fixed as described in 2.4.2 and thereafter sections were made as described in 2.8.1. IHC standard protocol procedures for Ki-67 and CD31 differed slightly in that sections being stained for Ki-67 were blocked in normal swine serum and the concentration of the primary antibody specific for Ki-67 was 1:200 whereas sections being stained for CD31 were blocked in normal goat serum and the concentration of the primary antibody specific for CD31 was 1:250. Furthermore, the secondary antibody used for Ki-67 was goat anti-mouse (1:200) whereas the secondary antibody used for CD31 was swine anti-rabbit (1:250). The number of positively stained cells for Ki-67 and vessels positively stained for CD31 were quantified and measured respectively using standard stereological techniques.

5.3.5 Statistical Analysis

All data are presented as mean ± SEM. Statistical significant differences were determined by unpaired student's *t*-test or ANOVA using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) (*, $p<0.05$; **, $p<0.005$; ***, $p<0.0001$).

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5.4 Results

5.4.1 Pilot Study to Determine the Number of HeLa Cells to Inject into MF-1 Nude Mice

Initially, a pilot study was undertaken to determine how many WT HeLa cells to inject into the MF-1 nude mice in order to obtain visible tumours. The mice (n=12) were equally divided into three groups and subcutaneously injected in the right dorsal flank with $5x10^5$, $1x10^6$ or $2x10^6$ cells in 0.2 ml PBS. After 4 weeks, all mice were examined for tumour development and each group was found to have 0/4, 1/4 and 3/4 xenografts respectively (Figure 5.1). As a result, it was decided to inject a total of $2x10^6$ cells per mouse in the subsequent study.

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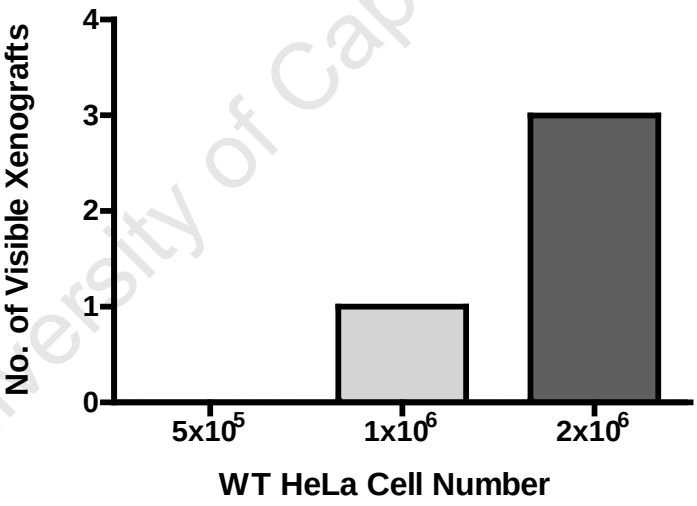

Figure 5.1 The effects of cell volume in obtaining visible xenografts. The MF-1 nude mice were engrafted with $5x10^5$, $1x10^6$ or $2x10^6$ WT HeLa cells and xenografts grown for 4 weeks.

5.4.2 SP Administration Does Not Affect Bodyweights of MF-1 Nude Mice

The effect of seminal plasma on the bodyweights of MF-1 nude mice injected with WT HeLa cell xenograft tumours was investigated over a ten week period. The nude mice were subcutaneously injected with $2x10^6$ WT HeLa cells into the right dorsal flank prior to commencement of treatment. Mice were then subjected to 3x weekly injections of 100µl containing control PBS or SP (1:500) and bodyweights recorded weekly. Mice treated with SP displayed no significant differences in mean bodyweight during, or at the end of the treatment period when compared to the control (Figure 5.2).

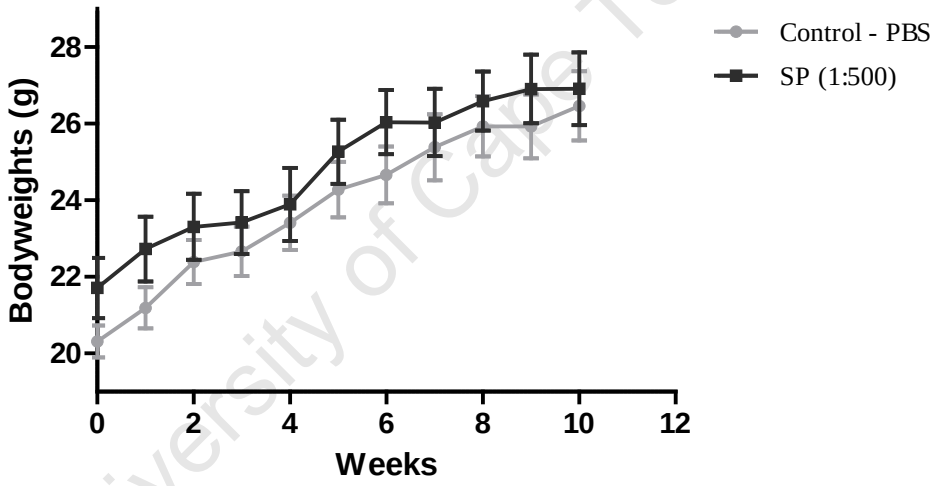

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Figure 5.2 The effects of SP on bodyweight. The MF-1 nude mice were engrafted with WT Hela cells and treated with SP (n=6) or PBS controls (n=8). Bodyweights of mice treated with SP (1:500) were not significantly different at any point during the 10 week experimental period in comparison to the controls treated with PBS.

5.4.3 SP Enhances Tumour Growth and Tumour Weight in WT HeLa Cell Xenografts *in vivo*

Next to be investigated was the effect of seminal plasma on the growth and weight of WT HeLa cell xenograft tumours *in vivo*. Tumours treated with SP displayed a marked increase in growth rate in comparison to the control group which was significantly different at week 10 of the treatment period (Figure 5.3A; $P<0.05$). In addition, we found that upon excising the tumours at the end of the 10 week treatment period, the tumours removed from the SP group weighed significantly more than the control group (Figure 5.3B; $P<0.05$) thereby confirming that SP treatment on WT HeLa cell xenografts produces a significant increase in tumour size and weight.

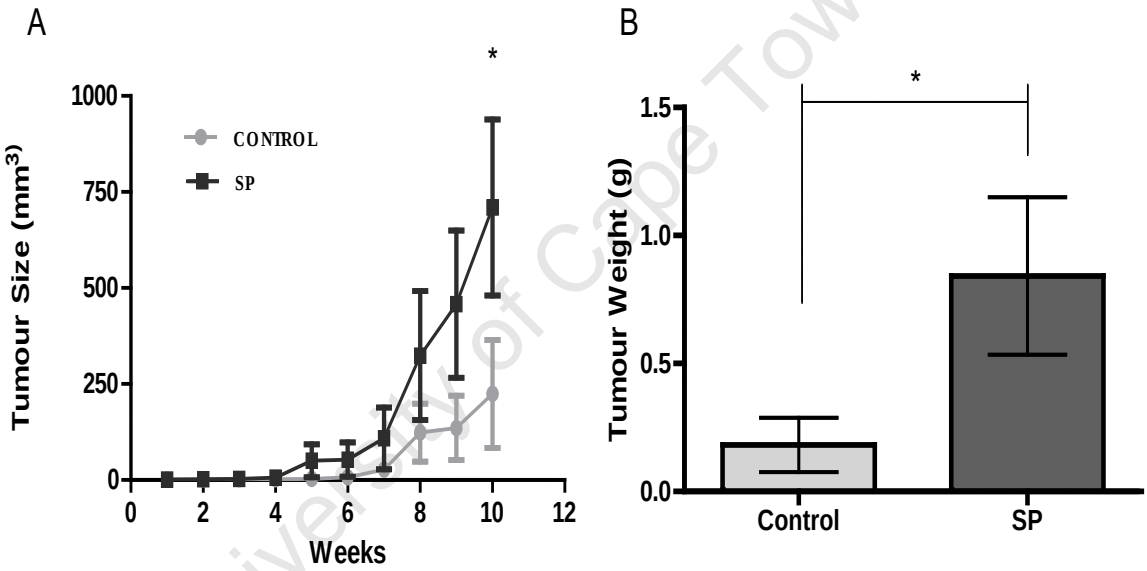

Figure 5.3 The effects of SP (1:500) on tumour growth and tumour weight. The MF-1 nude mice were engrafted with WT Hela cells and treated with SP (n=6) or PBS controls (n=8). **(A)** Tumour size in the nude mice xenografts is significantly different after 10 weeks of SP injections in comparison to PBS treated mice ($P<0.05$*). **(B)** The weight of the tumours was determined at the end of the 10 week experimental period. Mice injected with SP displayed a significantly increased tumour weight in comparison to the control group ($P<0.05$*). Data are represented as mean ± SEM.

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5.4.4 SP Enhances Expression of Proliferative Marker Ki-67 in WT HeLa Cell Xenografts *in vivo*

Having ascertained a significant increase in tumour size and weight of SP-treated WT HeLa cell xenografts, next to be confirmed was whether the enhanced tumour size and weight was due to induction of cellular proliferation by SP. Quantitative RT-PCR analysis for the proliferation marker Ki-67 revealed a 7.5 ± 2.2-fold elevation in proliferation rate in SP-treated WT HeLa cell xenograft tumours compared with control xenografts (Figure 5.4A; $P<0.05$). This increase in proliferation in SP-treated xenografts was further confirmed by immunohistochemistry as indicated by the differences in brown staining (arrowed; Figure 5.4B and 5.4C). Quantitative stereology displayed a 66 ± 4 percent increase in Ki-67 positive cells in comparison to control 35 ± 9 (Figure 5.4D; $P<0.05$).

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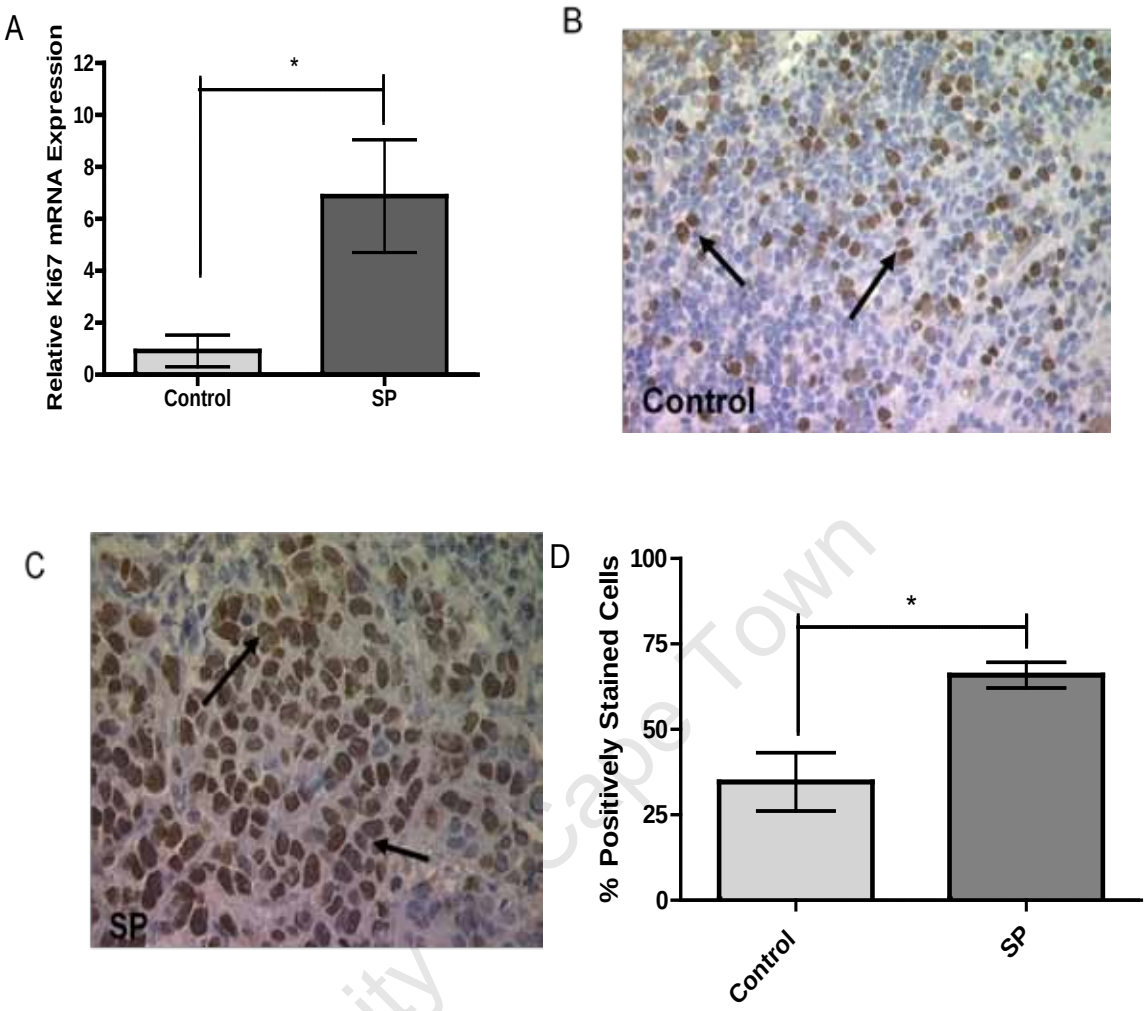

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Figure 5.4 (A) Ki-67 mRNA expression was measured by quantitative RT-PCR and is significantly increased by SP (1:500) as compared to the controls, ($P<0.05$*). Immunohistochemical staining of Ki67 in **(B)** control tumours and **(C)** SP-treated tumours from WT Hela cell xenografts (n=5 per group). **(D)** Number of positively stained cells for Ki67 was quantified and is significantly increased when treated with SP (1:500) in comparison to the control group ($P<0.05$*). Data are represented as mean ± SEM.

5.4.5 SP Enhances the Upregulation of PTGS-1 and PTGS-2 Expression in WT HeLa Cell Xenografts *in vivo*

The majority of solid tumours, if not all, are infiltrated with immune and inflammatory cells, highlighting the increasingly recognised close relationship between inflammation and tumourigenesis with the inflammatory component greatly contributing to increases in cellular growth and proliferation (Grivennikov and Karin, 2009; Wu and Zhou, 2009). The role of SP in WT HeLa cell xenografts on the expression of the PTGS-PTGER pro-inflammatory pathway known to be involved in cervical cancer (Subbaramaiah and Dannenburg, 2007) was investigated by quantitative RT-PCR analysis. Results revealed that SP treatment of WT HeLa cell xenografts significantly elevated the expression of PTGS-1 and PTGS-2 mRNA compared to control xenografts (2.4 ± 0.3 and 5.0 ± 0.3-fold increase respectively; Figure 5.5A; P<0.005 and Figure 5.5B; P<0.0001). Previous in vitro studies from our laboratory have shown that induction of PTGS enzymes coincidently upregulate prostaglandin receptor expression and signalling (Jabbour and Sales, 2004; Sales and Jabbour, 2003).

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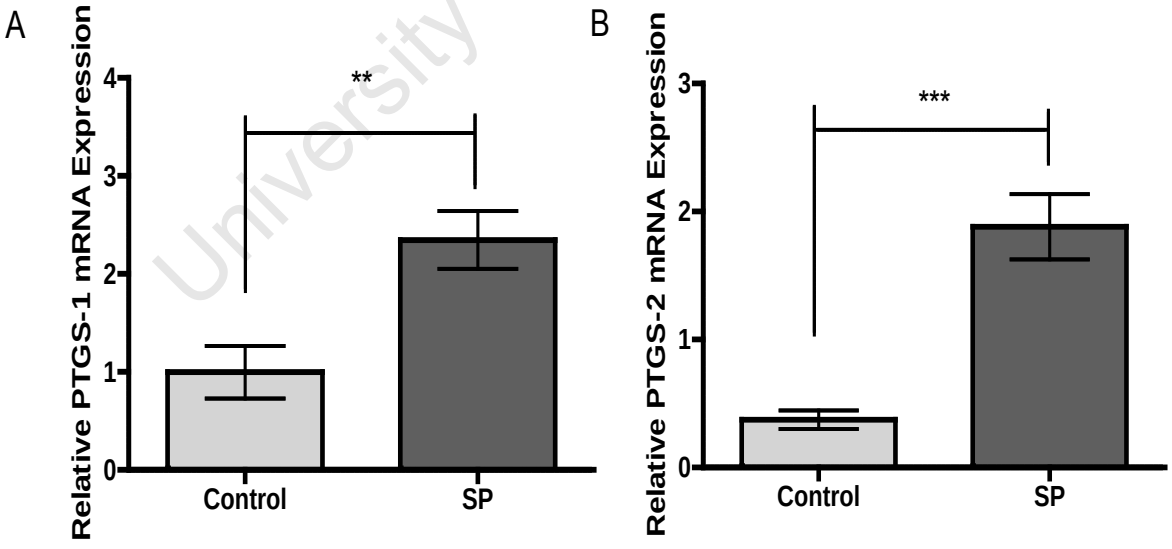

Figure 5.5 (A) PTGS-1 and **(B)** PTGS-2 mRNA expression was measured by quantitative RT-PCR and is significantly increased by SP (1:500) as compared to the control, (P<0.005** and P<0.0001***) respectively. Data are represented as mean ± SEM from three independent experiments.

5.4.6 SP Enhances the Upregulation of PTGER Expression in WT HeLa Cell Xenografts *in vivo*

We investigated the effect of SP treatment of WT HeLa cell xenografts on expression of prostaglandin receptors *in vivo*. Quantitative RT-PCR was used and the treatment of nude mice xenografts with SP induced the mRNA expression of PTGER1, PTGER2 and PTGER4 (2.8 ± 0.4-, 2.4 ± 0.4-, 2.1 ± 0.3-fold induction respectively; Figure 5.6A; $P<0.005$, Figure 5.6B and Figure 5.6D; $P<0.05$), but had no significant effect on expression of PTGER3 mRNA (Figure 5.6C) suggesting a role for prostaglandin receptor signalling in regulating tumour growth.

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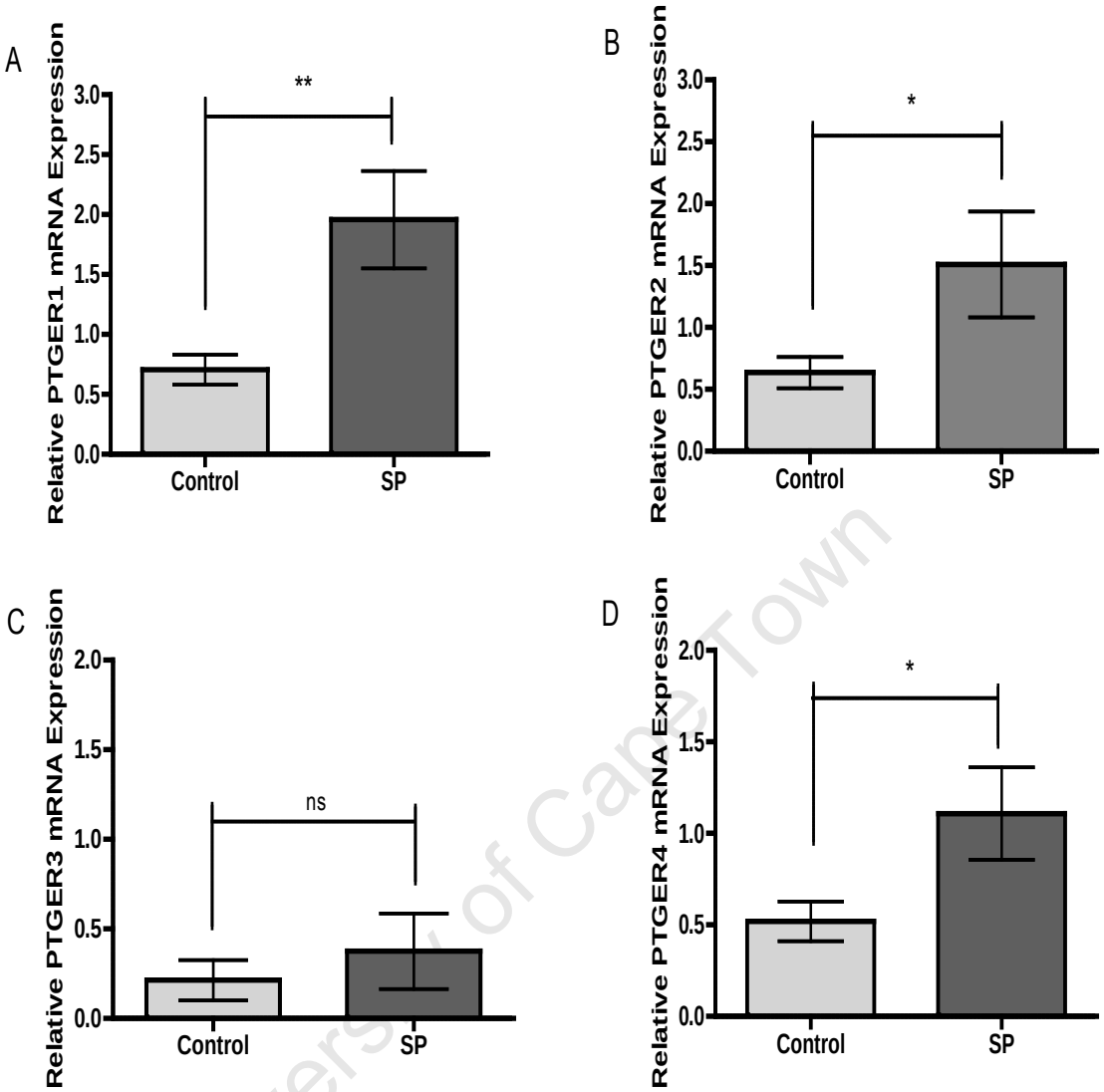

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Figure 5.6 The activity of the 4 different subtypes of the PGE_2 receptors were investigated and namely PTGER1, PTGER2 and PTGER4 were increased in response to SP as measured by mRNA production **(A)** PTGER1 (P<0.005**) **(B)** PTGER2 (P<0.05*) and **(D)** PTGER4 (P<0.05*). **(C)** SP had no significant effect on the expression of the PTGER3 receptor (P<0.5). Data are represented as mean ± SEM from three independent experiments.

5.4.7 SP Enhances Expression of Pro-inflammatory Cytokines IL-6 and IL-11 in WT HeLa Cell Xenografts *in vivo*

Inflammatory effectors, including growth factors and prostaglandins are known to regulate the tumour microenvironment by inducing pro-inflammatory cytokines and increasing vascular permeability (Tsujii *et al.,* 1998; Takehara *et al.,* 2006; Toomey *et al.,* 2009). Next to be investigated was the effect of SP on the expression of potent pro-inflammatory cytokines and angiogenic factors with known roles in tumour development. Quantitative RT-PCR analysis showed a significant increase in expression of IL-6 and IL-11 mRNA expression in WT HeLa cell xenografts treated with SP when compared to the control treatment group (2.5 ± 0.14-, 1.9 ± 0.16-fold increase respectively; Figure 5.7A; P<0.005 and Figure 5.7B; P<0.05).

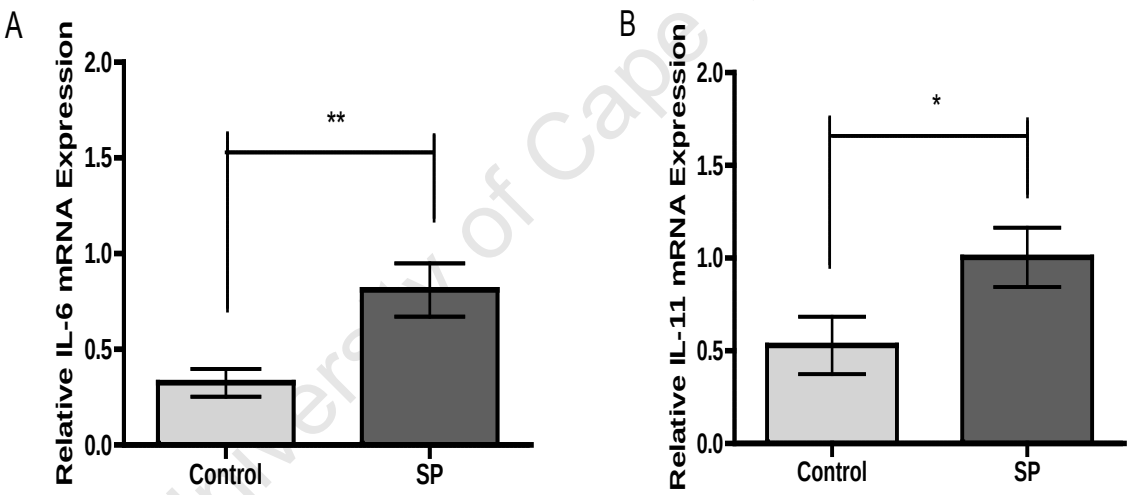

Figure 5.7 Quantitative RT-PCR analysis revealed **(A)** IL-6 mRNA expression is significantly increased in nude mice xenografts when injected by SP (1:500) in comparison to the control treatment group (P<0.005**). Increased IL-11 **(B)** mRNA production is seen in SP-treated (1:500) xenografts in comparison to the control (P<0.005*). Data represented as mean ± SEM from three independent experiments.

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5.4.8 SP Enhances Expression of Pro-angiogenic factor VEGF in WT HeLa Cells Xenografts *in vivo*

Furthermore, in addition to showing that IL-6 and IL-11 production was enhanced in response to treatment with SP, it was found that VEGF mRNA expression, which has the potential to enhance cervical tumourigenesis (Cheng *et al.*, 1999) was highly significantly elevated in WT HeLa cell xenografts treated with SP in comparison to the control treatment group (3.4 ± 0.2-fold increase; Figure 5.8; P<0.0001).

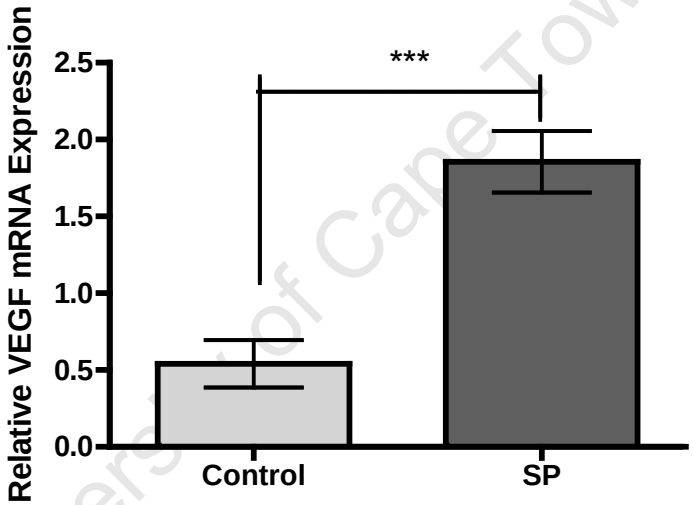

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Figure 5.8 Relative mRNA expression of VEGF seen in SP-treated (1:500) xenografts in comparison to the control (P<0.0001***) as determined by quantitative RT-PCR. Data are represented as mean ± SEM from three independent experiments.

5.4.9 SP Enhances Expression of CD31 in WT HeLa Cell Xenografts *in vivo*

Having identified that the WT HeLa cell xenograft tumours treated with SP had elevated VEGF expression, we next investigated whether these tumours also displayed alteration in their vasculature. Immunohistochemistry (Figure 5.9A and 5.9B) and quantitative stereology (Figure 5.9C) performed on WT HeLa cell xenograft tumours stained with the endothelial cell marker CD31 revealed that SP-treated xenograft tumours exhibited an increase in blood vessel size (388 ± 22 vs. 247 ± 19 um^2 respectively; Figure 5.9C; $P<0.005$) as depicted by the brown staining (arrowed) on the perimeter of the vessels (Figure 5.9A and 5.9B).

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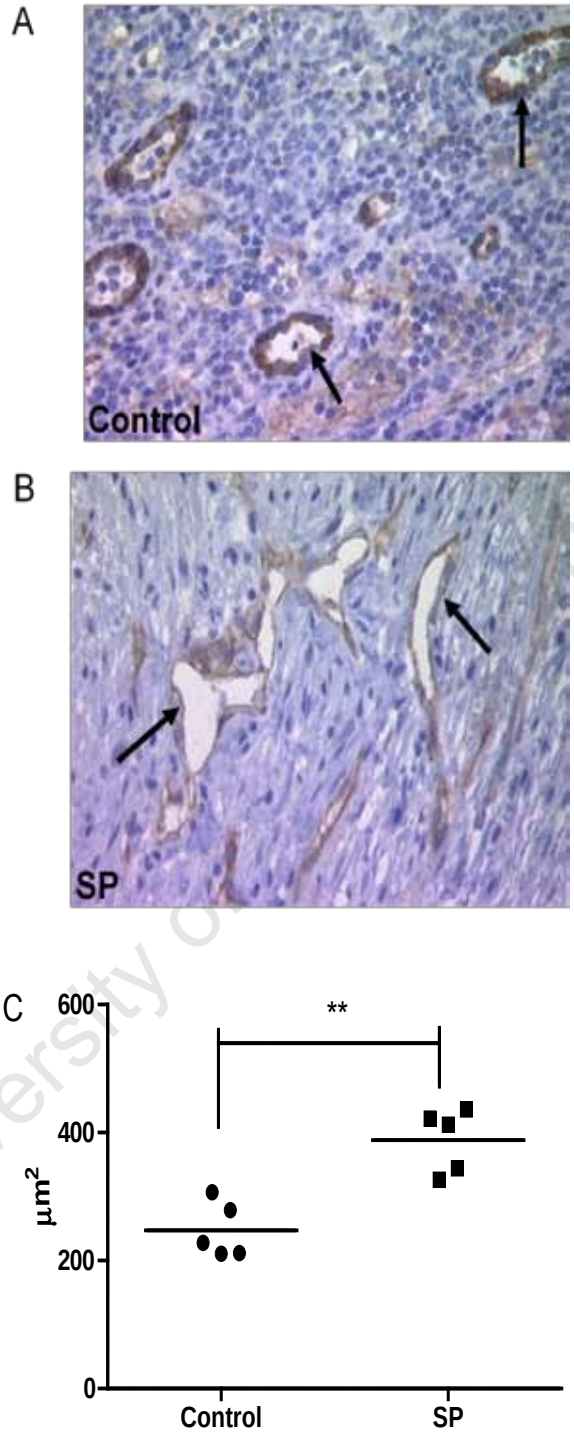

Figure 5.9 Imunohistochemical staining of CD31 in **(A)** SP-treated and **(B)** control tumours from WT Hela cell xenografts (n=5 per group). **(C)** The size of positively stained blood vessels for CD31 was quantified and is significantly increased when treated with SP (1:500) in comparison to the control ($P<0.05$*). Data are represented as mean.

5.5 Discussion

The results show that xenograft tumours in MF-1 nude mice originating from inoculation with neoplastic cervical epithelial (HeLa) cells grew significantly larger in both size and weight when seminal plasma was administered to the mice via an intraperitoneal injection compared to control xenografts following a ten week treatment period. The data also demonstrated that the increase in growth rate and tumour size was a consequence of increased cellular proliferation induced by SP as determined by the elevation in expression of the proliferation marker Ki-67 in SP-treated WT HeLa cell xenografts.

Seminal plasma is known to contain many inflammatory agents such as prostaglandins, transforming growth factor-ß (TGF-ß), and glycoprotein signalling molecules including cytokines and growth factors (Robertson, 2005). These molecules are independently thought to induce cellular and molecular changes in the cervical epithelium by binding to specific target cells in the female reproductive tract, thus activating modifications in gene expression, cellular composition and structure and function of local tissue via cytokine synthesis (Aumuller and Riva, 1992). Tumour cells have been widely reported as expressing raised levels of TGF-ß and a supplementary response to exogenous TGF-ß (contained within SP in high concentrations), may provide some epithelial cells with a growth advantage that ultimately leads to malignant expression (Jakowlew, 2006).

A recently performed study investigating pro-inflammatory mediators in hepatocellular injury revealed an increase in TGF-ß in response to elevated PTGS-2 production (Claria *et al.,* 2008). Hence, it is feasible to suggest that exposure of the neoplastic epithelium to SP could mount an inflammatory response leading to cervical tumourigeneis as a result of increasing the production of PTGS-2 coupled with high concentrations of TGF-ß being delivered into the female reproductive tract.

In addition, prostaglandins are present at a 10,000 fold higher concentration in SP than at the site of inflammation, with PGE_2 being one of the predominant types that is detected (Templeton, 1978). Prostaglandins perform several actions that are relevant to

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the control of cytokine release, cell growth, differentiation and changes in vasculature (Kelly *et al.,* 2001) and as a result, prostaglandins located within SP may therefore exacerbate cervical pathologies that are linked with an increase in PTGER signalling (Sales *et al.,* 2002b, Jabbour and Sales, 2004). In support of this, the current data generated from the nude mice study shows that in SP-treated WT HeLa cell xenografts, PTGS-1, PTGS-2 expression and expression of the E-series prostaglandin receptors PTGER1, PTGER2 and PTGER4 were elevated compared with control xenografts. Previous data from our laboratory have shown that in human cervix cancers, seminal plasma promotes expression of tumourigenic and angiogenic genes which possess the capability to modify cellular function via their actions on their respective receptors (Sales *et al.,* 2002b; Muller *et al.,* 2006; Battersby *et al.,* 2007). Therefore, inflammatory mediators contained in SP (such as PGE_2) operate on the female reproductive apparatus to enhance expression of PTGS-1, PTGS-2, thereby, adjusting molecular mechanisms to favour conditions of uncontrolled growth in cervical cancer (Sales *et al.,* 2002b; Muller *et al.,* 2006; Battersby *et al.,* 2007).

Furthermore, the present nude mice study also demonstrates that SP can dramatically elevate the expression of pro-inflammatory cytokines IL-6 and IL-11. Although the molecular mechanism whereby SP regulates these cytokines is unclear, additional evidence has came to fruition which confirms that SP can enhance IL-6 as well as other pro-inflammatory cytokines including IL-8, IL-10 and MCP-1 in vitro (Sharkey *et al.,* 2007; Denison *et al.,* 1999a). Epithelial cells of the female reproductive tract are a major responding cell lineage (Robertson *et al.,* 1996) and seminal plasma is believed to regulate the enhanced expression of these pro-inflammatory mediators in the cervix by means of contributing together with ovarian steroid hormones and inflammatory cytokine stimulation which in turn governs epithelial cell cytokine output (Sharkey *et al.,* 2007; Wira *et al.,* 2002; Wira *et al.,* 2005). Moreover, the increased production of IL-6 has been reported in carcinoma of the cervix, acting as an autocrine growth factor, and the induction of elevated PTGS2 and PGE_2 synthesis in cervical tissue following insemination would be expected to further augment the immune regulatory response (Takano *et al.,* 1996; Eustace *et al.,* 1993). This is further supported by studies in mice and pig that shows that seminal plasma possesses the

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ability to interact with cervical and uterine epithelial cells and trigger the release of pro-inflammatory cytokines such as IL-6 which resultantly induces local cellular and molecular changes that resemble an inflammatory response (Robertson, 1992; Robertson *et al.,* 1996; Robertson *et al.,* 2005). Therefore, SP can be considered as a regulator of pro-inflammatory cytokines that in turn contributes to controlling the immune response and promotes tumour progression. This is bolstered by the observation that the upregulation of pro-tumourigenic events seen in cervical cancer development and in findings generated here in normal human cervical tissue and WT HeLa cells is also replicated in the animal model in response to SP stimulation.

In order for a tumour to grow, an enhanced blood supply is crucial for the provision of necessary nutrients and oxygen (Zumsteg *et al.,* 2009). The majority of tumours are infiltrated with immune and inflammatory cells and a key observation to emerge in recent years is that tumour growth and metastasis are accompanied by the notable formation of new blood vessels, a process called angiogenesis (Nguyen, 1997). Angiogenesis is an essential process involved in normal cell growth and differentiation and if angiogenesis occurs excessively or deficiently, it can lead to the onset of many human pathological conditions, including tumour growth (Racia *et al.,* 2009).

There are least 15 known angiogenic peptides that have been identified to date which are either released or recruited by the tumour and one of the major proangiogenic factors regulating vascular function in tumour biology is vascular endothelial growth factor (VEGF) (Nguyen, 1997; Chang *et al.,* 2009). The primary function of VEGF is to create new blood vessels after injury or to bypass ones that are blocked and cancers which over express this angiogenic promoter enable the respective tumour to grow unabated (Xiong *et al.,* 2001). Enhanced expression of VEGF has been shown in several cancers such as ovarian, breast, renal and cervical (Garzetti *et al.,* 1999; Yoshiji *et al.,* 1996; Slaton *et al.,* 2001; Guidi *et al.,* 1995). Data generated from the present study shows that seminal plasma significantly induced expression of VEGF in nude mice xenografted with WT HeLa cells and suggests that SP not only induces a pro-inflammatory environment but also potentially enhances the angiogenic capacity. Although the exact molecular mechanism mediating the role of SP on VEGF

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expression is unknown, a previous study from our laboratory have shown SP to increase the expression of VEGF via the prostaglandin receptor PTGER4 and may potentially enhance cervical tumourigenesis (Muller *et al.,* 2006). Furthermore, TGF-β, several cytokines such as MMPs and interleukins IL-6, IL-8 and IL-10 have been shown to participate in the regulation of angiogenesis as a result of exposure to SP and it is therefore likely that the combination of all these signal transduction events can synergise to enhance the overall effect of SP on tumourigenesis (Lloyd *et al.,* 2003; Robertson, 1996; Sharkey *et al.,* 2007; Dension *et al.,* 1999). A study carried out by Tentes and colleagues (2007) demonstrated MMP-2 and MMP-9 to be contained within seminal plasma and since MMPs are capable of activating growth factors and cytokines and play a possible role in angiogenesis (Fowlkes and Winkler, 2002), this further highlights the potential of SP to promote angiogenesis in the female reproductive tract.

Moreover, in the present nude mice study results demonstrated that SP treatment of WT HeLa cell xenografts enhances blood vessel size. Although this may not exclusively be a direct result of increased synthesis of VEGF, several studies have previously illustrated that VEGF influences vessel size and therefore have an impact on the angiogenic process in carcinoma of the cervix (Racia *et al.,* 2009; Cheng *et al.,* 1999b; Guidi *et al.,* 1995). Having established an enhanced production of the proangiogenic factor VEGF in nude mice xenografts by means of treatment with seminal plasma, we wanted to confirm that SP was accountable for recruitment of larger blood vessels, hence, a resultant increase in angiogenesis and therefore larger tumours in comparison to the control group. Using CD31 as a marker, several cancers have shown a correlation between angiogenesis as determined by the intratumoural microvessel density and metastases, including carcinomas of breast, lung, prostate, rectum and bladder (Darai *et al.,* 1998; Horak *et al.,* 1992; Yamakzi *et al.,* 1994; Fregene *et al.,* 1993; Saclarides, 1994). A recent study performed by Mazibrada and colleagues (2008) revealed invasive cervical cancer to have a significant increase in CD31 in comparison to normal epithelium. This finding was given extra weight by results from the current nude mouse study whereby SP treatment instigated the development of larger blood vessels when compared to the controls and displays that

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seminal plasma can augment angiogenesis in cervical epithelial cells, thus giving rise to the strong likelihood of cervical tumourigenesis occurring.

In conclusion, it is known that cervical cancer development is multistage and that HPV infection alone is not sufficient to arise in progression of the disease. The fact that treatment with seminal plasma enhances the proliferation index in nude mice xenografts suggests that a particular mechanism may be involved whereby SP exposure in sexually active women triggers an increase in inflammatory mediators IL-6 and IL-11. This may then subsequently cause the pre-malignant lesion to become fully transformed, encouraging enhanced growth rate and growth size of tumours exposed to seminal plasma. In addition, highly expressed levels of PTGS2 and VEGF, coupled with an increase in tumour vascular density as revealed by immunohistochemical staining with CD31 implies that seminal plasma may extenuate development of cervical cancer in sexually active women.

This data highlights the potential for seminal plasma exposure to regulate neoplastic cell function, tumourigenesis and angiogenesis in cervical adenocarcinoma *in vivo.*

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Chapter 6

DISCUSSION AND CONCLUSIONS

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6.1 General Discussion

It is estimated that approximately 80% of cervical cancers occur in developing countries (Kent, 2010). The main reason for the high prevalence within these populations is the lack of awareness in women for its early detection and management (Srivastava *et al.,* 2009). The fact there are no adequate screening programmes in the countries that these women habitat and that symptoms often do not present themselves makes cervical cancer a much more deadly and widespread disease.

Cervical cancer development is multistage and although HPV infection is a prerequisite in the development of 99.7% cases (Walboomers *et al.,* 1999), onset of the disease does not occur immediately and therefore other factors may be responsible. This has prompted the suggestion that SP can trigger particular machinery within the cervical microenvironment of sexually active women that results in the upregulation of an array of growth activator molecules which act to aggravate the pre-malignant lesion to become fully transformed, encouraging growth via events such as angiogenesis.

HPV is an etiological agent attributed to the development of cervical cancer because infection with a high-risk strain of human papillomavirus is present in virtually all cases of the disease (Lowy *et al.,* 2008). The HPV operates by triggering alterations in cells of the cervix which can lead to the development of cervical intraepithelial neoplasia and then subsequently to cancer (Sales *et al.,* 2002b; Lowy *et al.,* 2008). However, not all women who have HPV infections develop cervical carcinoma and because it typically takes upto 15 years before invasive cervical cancer develops, this poses the likelihood that other factors are involved. There is mounting evidence to suggest that on the background of HPV infection, exposure of cervical epithelial cells to seminal plasma can promote the expression of tumourigenic and angiogenic factors such as PTGS enzymes, prostaglandins and VEGF (Castellsague *et al.,* 2002; Sales *et al.,* 2002b; Muller *et al.,* 2006) and this may result in development of carcinoma of the cervix.

SP can promote favourable conditions for tumour growth by exacerbating local pathologies by means of altering HPV-derived precursor lesions following vaginal,

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oral or anal intercourse. This is demonstrated by the resultant progression of cervical, endometrial, throat and anal cancer following exposure to SP (Wang *et al.*, 2010; Battersby *et al.*, 2007; D'Souza *et al.*, 2007; Melbye *et al.*, 1994). In fact, the components contained with seminal plasma are so versatile that they have been shown to gain access to the circulation if they are deposited in the gastrointestinal tract, especially if it has been traumatized (James and Hargreave, 1984).

Seminal plasma has been highlighted as containing a diverse array of components that includes, prostaglandins, cytokines, lipids, proteins, angiogenic factors, signal transduction molecules such as IL-1β, TNF-α and soluble necrosis factor p55, protein kinases, peptides, growth factors, immune response proteins and pro-inflammatory mediators such as IL-6 (Ness and Grainger, 2008; Robertson *et al.*, 2005; Fung *et al.*, 2003; Robertson *et al.*, 2002; Sabatte *et al.*, 2007; Fraser *et al.*, 2006), but the exact effect of SP repotoire of molecules on cells in the female gential tract is not yet fully understood. Seminal plasma concentrations of prostaglandins are 10,000-fold greater than detected at site of inflammation (Templeton, 1978) and that has raised the hypothesis that SP prostaglandins may exacerbate cervical pathologies by activating intracellular signalling via elevated prostanoid receptors (Jabbour *et al.*, 2006). PGE_2 is mostly present in SP as the 19-hydroxy form and experiments with cultured human cervical explants show that exposure to this PG promotes expression of chemotactic IL-8 and inhibits expression of the anti-inflammatory molecule secretory leukocyte protease inhibitor (SLPI) (Denison *et al.*, 1999b). Moreover, there is much published data to support a central role for PTGS enzymes, prostaglandins and their cognate receptors in cervical cancer development as well as other reproductive pathologies such as endometrial adenocarcinoma, endometriosis, dysmenorrhoea and ovarian cancer (Sales *et al.*, 2002b; Kino *et al.*, 2005; Jabbour *et al.*, 2006).

Another moiety contained within SP that can be attributed with having cytokine-inducing effects in the female tract is TGF-β (Robertson *et al.*, 2002). Seminal TGF-β has been demonstrated to promote granulocyte macrophage colony-stimulating factor (GM-CSF) production in mice within hours of insemination (Robertson *et al.*, 1992) and the consequence of its release is a recruitment and activation of leukocytes which

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resemble a classical inflammatory response (McMaster *et al.*, 1992; Robertson *et al.*, 1996). Furthermore, IL-4, IL-6 and IL-10 are known to be secreted from endocervical and endometrial cells in women and their interaction with exogenous TGF-β, PGE_2 and other seminal factors could further amplify the immunosuppressive properties of SP (Fichorova and Anderson, 1999).

Previous studies from our laboratory have shown SP to activate PTGS-2 and prostaglandin E_2 receptor expression and signalling in cervical adenocarcinoma cells (Sales *et al.*, 2002b) and to promote expression of tumourigenic and angiogenic genes in adenocarcinoma cells via the PTGER4 receptor (Muller *et al.*, 2006) and to upregulate fibroblast growth factor 2 expression in endometrial adenocarcinoma cells via the PTGER2 receptor (Battersby *et al.*, 2007). Further supporting data from Sharkey and colleagues (2007) revealed that SP can regulate cytokine gene expression in human cervical and vaginal epithelial cells by inducing production of pro-inflammatiory mediators such as IL-6, IL-8, GM-CSF and monocyte chemotactic protein-1 (MCP-1). Therefore, sexually active women are exposed to exogenous prostaglandins and other tumour-promoting cofactors contained within seminal plasma, which in turn can provide cervical epithelial cells with a growth advantage that eventually leads to malignant expression (Jakowlew, 2006).

In this study, expression of PTGS-1, PTGS-2, prostaglandin receptor expression, pro-inflammatory mediators and pro-angiogenic factors were assessed in normal human cervical tissue in reponse to SP. The upregulation of these tumour promoting molecules has already been established in several cancers (Lee *et al.*, 1995; DuBois *et al.*, 1998; Jabbour *et al.*, 2006) but the purpose here was to determine whether SP could induce their upregulation in normal cervical tissue samples, thereby showing evidence that components within SP can provoke changes in cervical epithelial cells that would favour tumourigenic events such as an enhanced inflammatory response and angiogenesis.

The findings concluded that following 24 hr treatment of normal cervical tissue, SP induced the upregulated expression of prostaglandin synthase enzymes 1 and 2,

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increased prostaglandin receptors PTGER1, PTGER3 and PTGER4 and this can lead to increased autocrine and paracrine signalling. SP also stimulated the production of a host of pro-inflammatory and pro-angiogenic factors that includes IL-6, IL-8, IL-11, Gro-α and VEGF. In addition, SP provoked alterations in tissue vasculature as determined by augmenting expression levels of the angiogenic marker CD31 following treatment for 24 hours and it appears that the effects of SP are being mediated directly as well as indirectly. The increases in PTGS synthesis that lead to further PG ligand production and enhanced PTGER receptor expression is a direct result of SP whereas the indirect effect is resultant from the increase in PG ligands which in turn has the potential to create an autocrine/paracrine feedback loop and as a consequence, additional increased signalling. These observations concur with results from other authors who employed cervical adenocarcinoma cell lines and human carcinoma samples and demonstrated that PTGS-1 and PTGS-2 expression are upregulated in carcinomas of the cervix and leads to a possible autocrine/paracrine regulation of neoplastic cell function by prostaglandins and their cognate receptors. SP can induce PTGS-2, VEGF, IL-6 and IL-8 in cervical adenocarcinoma cell lines and the enhanced levels of CD31 observed in uterine cervical specimens correlate to different stages of cervical carcinogenesis (Sales *et al.,* 2001; Sales *et al.,* 2002a; Sales *et al.,* 2002b; Muller *et al.,* 2006; Sharkey *et al.,* 2007; Mazibrada *et al.,* 2008). These results show for the first time that pro-tumourigenic events normally observed in cervical adenocarcinoma cell lines and carcinomas of the cervix can be induced in normal human cervical tissue via exposure to SP.

In summary, SP appears to promote cervical tumourigenesis by modulating neoplastic cell function in normal epithelial cells through its ability to induce tumour promoting factors such as PTGS-1, PTGS-2, PTGER receptor expression, IL-6, IL-8, IL-11, Gro-α and VEGF. Cervical epithelium is constantly shed and regenerated as a result of the menstruation cycle and this process must be tightly regulated so that the rates of cell death and cell renewal are finely balanced. However, when the cytokines that control this process are over produced, increased cellular proliferation can result (Macdonald, 2010). This can occur by SP augmenting the upregulation of prostaglandin synthase enzymes and prostaglandin receptor signalling which subsequently stimulates

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production of growth factors such as Gro-α and VEGF, the production of which induces changes in vasculature to encourage angiogenesis that will resultantly bring about the onset of cervical cancer.

WT HeLa cells were utilized as a model system to determine the role of certain signalling pathways suspected/known to be activated by SP in the activation of transcription of the genes IL-6, IL-8, IL-11, Gro-α and VEGF shown to be upregulated in normal cervical tissue in response to exposure to SP as described in chapter 3. Initially, the cell line was tested to ascertain whether it was a suitable system to examine if these genes were upregulated and following this, we proceeded to investigate the signalling pathways involved and the effects of SP on cellular proliferation. The findings generated in this part of this study were in agreement with those found earlier as the data confirmed that SP treatment induced enhanced expression of cofactors PTGS-1, PTGS-2, IL-6, IL-8, IL-11, Gro-α and VEGF. Results also demonstrated that the proliferative marker Ki-67 is significantly overexpressed in squamous carcinomas when compared to normal cervix as well as being upregulated in SP-treated WT HeLa cells. The SP induction of cell growth is further supported by the finding that SP treatment increased the rate of cellular proliferation in WT HeLa cells, a process central in angiogenesis.

Although SP induced similar pro-tumourigenic effects in WT HeLa cells to those observed in normal cervical tissue, the experimental conditions under which these inductions occurred were slightly different. Results generated in normal cervical tissue were brought about by using an SP concentration of 1:500 and treating for 24 hours whereas the upregulation of prostaglandin synthase enzymes, pro-inflammatory mediators and angiogenic factors in WT HeLa cells used an SP concentration of 1:100 and a treatment period of 6 hours. The reasoning behind this is that normal cervical tissue and WT HeLa cells are different experimental model systems and that induction in cell lines tends to be faster in comparison to tissue (6hr vs. 24hr). This observation is consistent with previous findings of our laboratory demonstrating that treating HeLa adenoncarcinoma cells expressing PTGER4 and endometrial adenocarcinoma cells expressing PTGER2 with SP showed significant upregulation at 8hr and 6hr

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respectively (Muller *et al.*, 2006; Battersby *et al.*, 2007), whereas the SP-treated cervical adenocarcinomas and $PGF_{2\alpha}$-treated endometrial carcinoma biopsies showed significant induction at 24hr (Sales *et al.*, 2002b; Sales *et al.*, 2007). Furthermore, the animal model system used in this thesis was treated with an SP concentration of 1:500 which is significantly more dilute than that used to treat WT HeLa cells. However, the treatment period in nude mice was over 10 weeks and seeing as there was upregulation of PTGER receptors in xenograft tumours, this would imply the tumours to be more responsive to lower concentrations of SP.

Subsequent to confirming the upregulation of target genes in our cell line model system that were also induced by SP in normal cervical tissue, we wished to try and identify which pathways were required for their transcriptional activation. Data to arise from this study revealed MAPK kinase/ERK1/2 and EGFR signalling to be activated in reponse to SP exposure and these results were in agreement with findings in a study carried out by Muller and colleagues (2006) which demonstrated SP to upregulate the ERK1/2 pathway in adenocarcinoma cells via the PTGER4 receptor in an EGFR and Raf-dependent manner while Battersby and colleagues (2007) showed that SP stimulated the PTGER2 receptor in endometrial adenocarcinoma cells and this led to the activation of ERK via EGF receptor transactivation.

PTGS-1 and PTGS-2 have previously been shown by our laboratory to be upregulated in cervical cancer (Sales *et al.*, 2002a; Sales *et al.*, 2002b) and in this study we show that they are both induced by SP in our cell line model system. The induction of PTGS-1 is mediated by EGFR/ERK in direct response to SP and a parcrine effect on PG synthesis does not appear to play a role in its activation. As the upregulation of PTGS-1 was only partially blocked by inhibitors for EGFR and ERK regulated pathways, this suggests that other signalling is involved, although our results show that PTGS-2, NF-κB and TGF-β are not implicated. The SP-induced PTGS-2 is also dependent on activation of EGFR/ERK signalling, but as for PTGS-1, they only have a partial role. The inhibitor for PTGS-2 inhibited its own induction by SP, indicating that an autocrine/paracrine pathway regulates the transcription of PTGS-2. In addition, PTGS-2 has been shown to increase the inflammatory response in cancers of the

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prostate, breast and cervix (Danforth *et al.,* 2008; Dossus *et al.,* 2009; Dannenberg *et al.,* 2001) and here we show that this signalling molecule is involved in mediating the SP-induced transcriptional activation of pro-tumourigenic factors IL-6, IL-8, Gro-α and VEGF in WT HeLa cells and stresses the importance of this gene in cervical cancer development. SP can induce the over-expression of IL-6, IL-8, IL-11, Gro-α and VEGF by means of activating the EGFR, ERK1/2 and NF-κB pathways as well as upregulating IL-8, IL-11 and Gro-α through activating TGF-β signalling. SP can mediate its effects directly and indirectly by operating upon multiple pathways and its ability to upregulate PG synthesis to create a positive autocrine/paracrine feedback loop. Despite the observation that the majority of SP-induced signalling appears to involve the activation of EGFR and ERK1/2 regulated pathways, the induction of PTGS-2 and NF-κB mechanisms are also strongly involved, as well as TGF-β to a lesser extent and other signalling pathways not investigated in this thesis. The triggering of NF-κB signalling is a direct response of SP, is closely associated with cancer development and along with PTGS-2 has been shown to upregulate the expression of the pro-angiogenic factor VEGF in A549 cells (Jung *et al.,* 2003) whereas TGF-β is a potent transduction signalling molecule contained in SP, is implicated in cancer progression and metastases and can promote pro-inflammatory cytokines IL-1β and IL-6 in endometrial epithelial cells by synergising with IL-8 (Robertson *et al.,* 2002; Gutsche *et al.,* 2003).

The use of WT HeLa cells as a model system also demonstrated that SP has the potential to induce increases in cellular growth following treatment periods of 24, 48 and 72 hrs as determined by upregulation of the proliferative marker Ki-67 and enhanced cellular proliferation. Research performed by Robertson (2007) revealed that following insemination, SP can activate an inflammatory response in the female reproductive tract and downstream changes that occur can include the induction of pro-inflammatory cytokines and PTGS-2. This in turn causes recruitment of macrophages and dendrtitic cells that release cytokines capable of promoting epithelial cell proliferation and may therefore result in amplified angiogenesis which encourages tumour growth. SP can therefore be considered as a growth factor influencing cervical

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epithelial cell proliferation by means of its ability to induce cytokine production and augment angiogenesis as seen in our model cell system.

Next, an animal model system was utilized in order to determine whether the tumour-promoting cofactors that were enhanced by SP in normal cervical tissue and WT HeLa cells could also be induced *in vivo*. This was studied employing WT HeLa cell derived xenografts in nude mice and SP treatment was shown to significantly increase tumour size, tumour weight and induce increases in the proliferation index as determined by the proliferative marker Ki-67 when compared to the control. Furthermore, xenograft tumours treated with SP displayed elevated expression of PTGS-1, PTGS-2, PTGER receptor signalling, IL-6, IL-11 and VEGF and also enlarged blood vessel size that reflected augmented angiogenesis. Because the idea of using SP to treat cervical adenocarcinoma cell line–derived xenografts *in vivo* is a relatively new concept, the data in which to compare these findings is rather limited. Nevertheless, these results further highlight the potential for seminal plasma exposure to regulate neoplastic cell function, tumourigenesis and angiogenesis in cervical adenocarcinoma *in vivo.*

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6.2 Role of SP-activated Genes in the Development and Progression of Cervical Cancer and the Signalling Pathways Involved

6.2.1 Upregulation of PTGS and PTGER Expression in Response to SP Treatment

Prostaglandin synthase enzymes and prostaglandin signalling via the prostanoid receptors are known as important regulators of reproductive function but dysregulation of these molecules can promote tumourigenesis in a number of reproductive tract cancers that includes endometrial, ovarian and cervical (Jabbour *et al.,* 2006; Kino *et al.,* 2005; Sales *et al.,* 2002b). Recent work has implicated a role for seminal plasma in initiating PTGS and PTGER signalling in the development of cervical carcinoma (Muller *et al.,* 2006; Sales *et al.,* 2002b) and although the method by which SP components trigger activation are not yet fully defined, the data is supported by the findings of this study.

Although over-expression of PTGS-2 is more commonly associated with tumour-promoting events, elevated levels of PTGS-1 have also gained increased recognition as shown by Narko and colleagues (1997) who revealed that cells transfected with PTGS-1 became tumourigenic when injected into nude mice and by Sales and colleagues (2002a) who found PTGS-1 to be over-expressed in cervical carcinomas. Colorectal tumourigenesis is associtated with dysregulation of PTGS-1 and PTGS-2 and their enzymatic products (Pai *et al.,* 2003), thus it appears that the upregulation of both PTGS-1 and PTGS-2 play a role in tumour development. The transcriptional activation of PTGS-1 and PTGS-2 by SP in normal cervical tissue and cell and animal model systems suggest, as is the case in colorectal cancer, that both PTGS enzymes are involved in cancer development of the cervix and SP has the potential to induce their over-expression. Given that SP has the capability to support tumourigenic events such as promoting tumour maintenance and encouraging cell growth via induced production of PTGS and PTGER (Greenhough *et al.,* 2009), and that colorectal carcinoma development correlates with dysregulation of both PTGS enzymes, it is possible that SP could trigger the same effects in the cervix as seen in colon tumourigenesis. Furthermore, data published by Ablin and Stein-Werblowsky (1997) revealed that men and women who partake in unprotected anal intercourse are more susceptible to

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developing anal cancer and although it is not known whether the same PTGS and PTGER signalling mechanisms are involved, this finding hypothesizes that SP may provide anal epithelial cells with a growth advantage in a similar fashion to epithelial cells residing in the cervix (Melbye *et al.,* 1994).

6.2.2 Upregulation of Pro-inflammatory Cytokines in Response to SP Treatment

The expression of IL-6, IL-8 and IL-11 were shown to be significantly upregulated in response to SP treatment. These cytokines are involved in many regulatory roles but their increased expression has also been associated with several cancers such as cervical carcinoma (Eustace *et al.,* 1993; Fujimoto *et al.,* 2000).

IL-6 is a pleiotropic cytokine that can activate target genes involved in differentiation, survival, apoptosis and proliferation (Takano *et al.,* 1996; Heinrich *et al.,* 2003). This chemokine has been reported to act in an autocrine and paracrine fashion in proliferation of normal squamous epithelial cells (Takano *et al.,* 1996), whereas a study undertaken by Eustace and colleagues (1993) demonstrated IL-6 to operate as an autocrine growth factor for *in vitro* tumour cell growth. Here, we displayed that IL-6 was upregulated in normal human cervical tissue, in WT HeLa cells *in vitro* and in WT HeLa cell xengorafts *in vivo* in response to SP stimulation and supports the findings of Sharkey and colleagues (2007) who demonstrated that SP could trigger an inflammatory response in human cervical epithelial cells by promoting the upregulation of IL-6 as well as IL-8 and MCP-1. Research performed by Baker and colleagues (2009) highlighted that IL-6 mediated angiogenic signalling in pancreatic cancer patients by promoting VEGF and fibroblast growth factor b (FGFb) and therefore suggest a role for this cytokine in regulating the inflammatory response and encouraging angiogenesis in cervical cancer development. In addition, seminal plasma is known to contain IL-6 (Eggert-Kruse *et al.,* 2001) and as a result, not only is the cervix being stimulated by IL-6 that is being generated as a result of SP exposure, but also by the concentrations present in SP itself, thus providing the cells in the exposed regions with a growth advantage.

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Using a specific inhibitor for PTGS-2 significantly reduced the SP activated fold-induction of IL-6 and this finding is confirmed in a study carried out by Hinston and colleagues (1995) who revealed that elevated IL-6 is induced by increased PGE_2 synthesis in a murine model of inflammation and this is mediated by inducible PTGS-2 expression. It can therefore be suggested that SP-induced expression of IL-6 in the cervix is dependent on upregulation of PTGS-2. Additional signalling pathways seen to be involved with the SP activation of IL-6 include ERK, EGFR and NF-κB. These findings correspond with other authors as Castrilli and colleagues (1997) demonstrated that IL-1α and IL-6 promote the *in vitro* growth of both normal and neoplastic human cervical epithelial cells and this stimulatory effect is mediated by epidermal growth factor whereas other data published by Nishikori (2005) illustrated that the transcriptional activation of NF-κB is involved in myeloma cell growth and this signalling can be triggered by cytokines and growth factors, including IL-1α, TNF-α and VEGF, stimulatory molecules that are either contained within SP or induced by SP in the cervical microenvironment and as a result may activate NF-κB signalling in order to induce IL-6. NF-κB activation has also been shown to be dependent on ERK signalling in hepatocellular carcinoma (Cheng *et al.*, 2005), a signalling pathway shown here to be initiated by SP stimulation and highlights that IL-6 activation can be induced by a multitude of signalling events with SP having the potential to modulate their regulation in the cervix. However, despite the strong roles of ERK, EGFR, PTGS-2 and NF-κB in SP-mediated IL-6 production, these signalling mechanisms only partially inhibited activation and therefore implicates the involvement of other upstream pathways.

IL-8 is a chemoattractant chemokine and plays a role in angiogenesis, mitogenesis and inflammation (Vlahopoulos *et al.*, 1999). Data published by Fujimoto and colleagues (2000) revealed expression levels of IL-8 to increase significantly during the advancement of cervical cancer and may act as trigger for angiogenesis in the later stages of uterine cervical cancers by increasing the angiogenic potential of tumor necrosis factor-α (TNF-α). Results generated in this thesis reveal that IL-8 is over-expressed in normal cervical tissue and WT HeLa cells in response to SP stimulation

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and the pro-angiogenic and pro-inflammatory characteristics of this chemokine may contribute to the onset of cervical carcinoma in sexually active women.

The activation of IL-8 by SP was shown to be mediated via many signalling pathways, including; ERK, EGFR, PTGS-1, PTGS-2, TGF-β and NF-κB. These findings can be correlated in part by other researchers who have shown that exogenous PGE_2 up-regulates IL-8 in human colonic epithelial cells through coupling to PTGER4 receptors to elevate cAMP-dependent PKA signal transduction (Yu and Chadee, 1998) while a study carried out by Chelouche-Lev and colleagues (2004) in breast cancer cell lines found that VEGF expression correlated with high levels of IL-8 and that inhibitors of MAPK, P13K and ERK significantly reduced the expression of both these cytokines. Furthermore, a study undertaken by Arun and colleagues (2009) illustrated that IL-8 production in head and neck squamous carcinoma was mediated by NF-κB dependent PKA signalling whereas the upregulation of IL-8 in breast cancer metastases was shown by Singh and colleagues (2006) to be regulated by PTGS-2. It is evident that several signalling pathways are activated by the various molecules contained within SP and the generation of SP-activated IL-8 appears to be dependent on ERK, EGFR, PTGS-1, PTGS-2, TGF-β and NF-κB transactivation, however, inhibitors of these pathways only partially blocked the induction of IL-8 and implies additional activator molecules are operated upon to mediate SP-induced IL-8. Work carried out by Denison and colleagues (1999a) demonstrated that SP and its component prostaglandins PGE_2 and 19-hydroxy PGE, are capable of stimulating IL-8 and IL-10 release in cervical explants and suggest that induction of IL-8 in female tract mucosa may act together with PGE_2 and 19-hydroxy PGE contained within SP to induce leukocytosis, further increasing the inflammatory response. As well as its production being increased in response to SP, IL-8 has been shown to be contained within SP (Eggert-Kruse *et al.*, 2001) and in addition to SP-induced prostanoid synthesis and PTGS signalling causing the upregulation of IL-8 and other pro-tumourigenic cytokines, the IL-8 component within SP may also contribute to promoting angiogenesis by self-regulating itself and further increasing its expression. This suggests that both autocrine and paracrine regulation are involved in IL-8 expression as reported by Yao and colleagues (2005) in lung cancer cells via the NF-κB pathway.

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IL-11 is a multi-functional cytokine that can affect cell growth, differentiation and proliferation (Du and Williams, 1994). Here, it was shown to be upregulated by SP *in vivo* and *in vitro.* IL-11 is known to be produced by the human endometrium and endometrial cancer tissue where it performs roles in epithelial cell adhesion and trophoblast cell invasion (Yap *et al.,* 2010), two processes known to be important in cancer development and may explain why it is upregulated in SP-treated normal cervix.

Although PTGS-2 can induce IL-11 in breast cancer cells (Singh *et al.,* 2006), PTGS-2 was not involved with the SP induction of IL-11 in WT HeLa cells, thus suggesting its regulation is by other means in the cervix. Along with IL-6, IL-11 was shown to be greatly upregulated in the development of gastric tumours in mice and this was found to correlate with activation of STAT3 (Howlett *et al.,* 2005) while Jackson and colleagues showed in human gastric cancer tissue that IL-6 expression coincided with the activation of STAT3 and ERK1/2. Results here show that SP induced IL-11 by several signalling mechanisms, including TGF-β and this result was confirmed by Kang and colleagues (2005) by using a mouse model of high bone-metastatic activity and revealed that IL-11 induction was closely involved with TGF-β by means of Smad mechanisms which are strongly implicated in tumour progression. These findings implicate the SP-induced upregulation of IL-11 in promoting the development of cervical cancer in sexually active women by being able to encourage cell growth and tissue invasion.

It is therefore suggested that the mechanisms already in operation to sustain tumour-promoting events such as inflammation and angiogenesis are dependent on increased expression of pro-inflammatory mediators such as IL-6, IL-8 and IL-11. The expression of the chemokines contained within SP, in addition to the levels induced by the prostaglandins and other activator molecules contained within SP, act upon the cervical microenvironment in an autocrine and paracrine manner to promote cervical tumourigenesis and these pathways are central with PTGS enzyme activation.

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6.2.3 Upregulation of Pro-angiogenic Factors in Response to SP Treatment

Angiogenesis supports tumour growth and progression in all cancers since it stimulates vasculature development to provide the tumour with essential nutrients and oxygen (Racia *et al.,* 2009). Soslow and colleagues (2000) previously demonstrated that upregulation of PGTS-2 can activate angiogenesis and data from this study displays that SP treatment can induce the upregulation of PTGS-2. Enhanced expression of PTGS-2 has also been shown to induce pro-angiogenic factors such as VEGF, IL-6 and IL-8 and produces prostaglandins that have both autocrine and paracrine effects on proliferation and migration of endothelial cells (Tsujii *et al.,* 1998; Mazhar *et al.,* 2005). Evidence therefore suggests that SP can induce angiogenesis by stimulating the production of PTGS-2 and also by increasing the regulatory growth factor VEGF.

Although the machinery whereby SP enhances angiogenesis is unclear, several studies have previously shown that VEGF influences blood vessel size and therefore have an impact on the angiogenic process in cervical cancer (Racia *et al.,* 2009; Guidi *et al.,* 1995; Cheng *et al.,* 1999). Research performed by Wei and colleagues (2003) demonstrated that VEGF-dependent angiogenesis promoted cervical tumour growth through IL-6 induction whereas a previous *in vitro* study from our laboratory has shown that SP increases the expression of VEGF in cervical carcinoma cells via the PTGER4-mediated activation of extracellular signal-regulated kinase 1/2 and highlights that SP can activate VEGF to potentially enhance or sustain cervical tumourigenesis (Muller *et al.,* 2006).

Gro-α is another growth factor that was seen to be upregulated by SP treatment in this study. Gro-α is known to be contained within SP and is also capable of stimulating cytokine recruitment in endothelail cell cultures as well as regulating inflammation (Rajasekaran *et al.,* 1996; Ochsenkuhn *et al.,* 2008). Work undertaken by Wang and colleagues (2006) found that increased expression of Gro-α induced microvascular endothelial cell migration and tube formation of colorectal adenocarcinoma cells following its activation by PGE_2 and therefore upregulation of this pro-angiogenic chemokine can promote angiogenesis in colorectal cancer.

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The data produced in this study suggests that properties contained within SP can trigger unabated angiogenesis in cervical cancer by enhancing the production of PTGS-2, VEGF and Gro-α. The inhibitor for PTGS-2 (NS-398) significantly reduced the SP-activated induction of VEGF and Gro-α and thereby confirms the central involvement of PTGS-2 in mediating the transcriptional activation of these pro-angiogenic factors. The upregulation of VEGF and Gro-α was also found to be mediated by NF-κB signalling and constitutive activation of this pathway is involved various forms of cancer such as colon and ovarian (Rayet and Gelinas, 1999). NF-κB signalling is implicated as a mediator of growth regulatory signals in breast and prostate cancer cells and regulated by EGFR activation in colorectal tumours (Hoshiya *et al.,* 2003; Brandi *et al.,* 2008). Moreover, Karl and colleagues (2005) demonstrated that NF-κB activation was involved in pro-angiogenic signalling in head and neck tumours through its association with the anti-apoptotic protein Bcl-2 and upregulation of pro-angiogenic factors Gro-α and IL-8 and the same mechanisms may be playing a part here to promote SP-induced angiogenesis in the cervix.

In addition, TGF-β signalling was shown to play a partial role in mediating the transcriptional activation of SP-induced Gro-α. Work carried out by Bierie and colleagues (2009) recently showed that aberrant TGF-β signalling is linked to poor prognosis in human breast cancer as a consequence of enhanced chemokine production and is attributed, at least in part, to TGF-β regulated activation of Gro-α as well as CXCL5 and macrophage inflammatory protein-3A (MIP3A). Therefore, TGF-β signalling that promotes Gro-α expression in breast cancer may be applicable to SP-mediated cancer growth in the cervix. Although TGF-β signalling pathways have been shown to be modified in human cancers during tumour progression and metastasis, processes in which VEGF is centrally involved (Bierie and Moses, 2006), VEGF induction seen here was not found to be dependent on TGF-β and this mechanism of activation may only be engaged in the later stages of the disease and not the initial onset that is implicated with SP exposure.

Taken together, these findings suggest that SP may promote cervical tumourigenesis by inducing the transcripitional activation of EGFR, MAPK/ERK, NF-κB and TGF-β

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signalling as well as upregulating postanoid signalling and the overexpression of PTGS-2 which creates a positive feedback loop to further increase prostaglandin synthesis. As a result of this SP-induced signalling, pro-inflammatory cytokines and pro-inflammatory factors such as IL-6, IL-8, IL-11, Gro-α and VEGF can act in an autocrine and paracrine manner to promote cell growth, angiogenesis and cellular proliferation in adjacent cervical tissue as depicted in Figure 6.1.

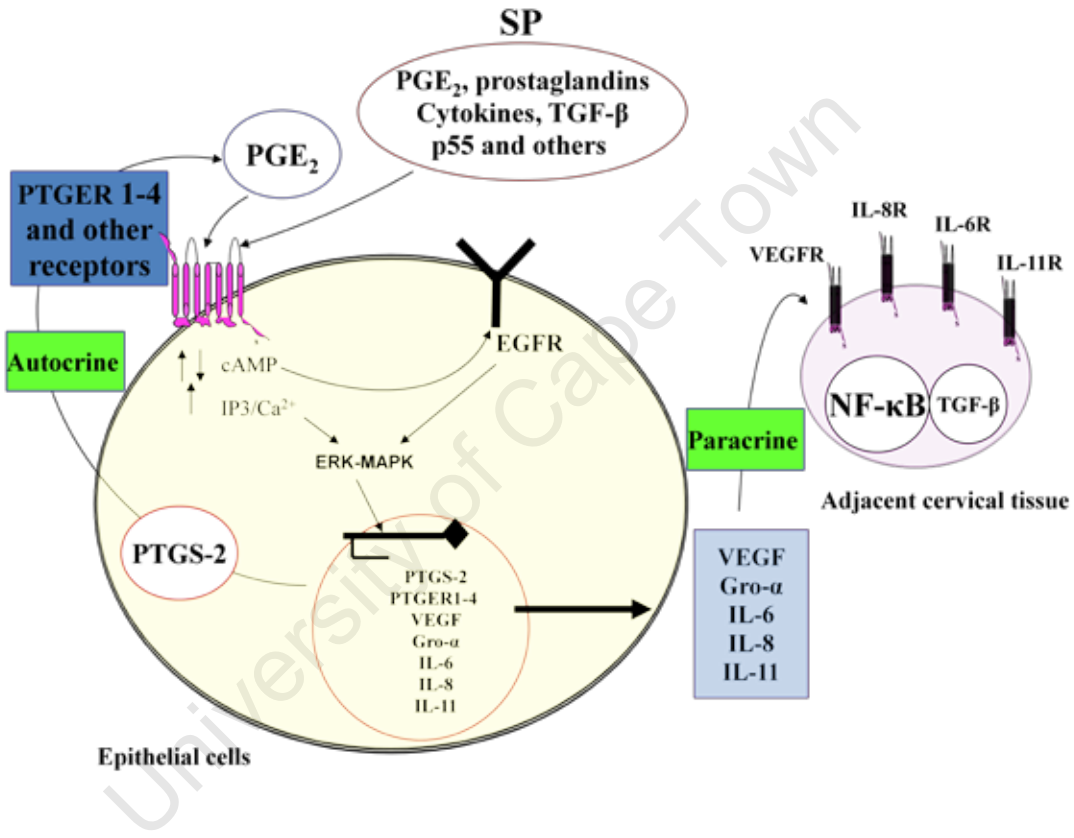

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Figure 6.1 Autocrine and paracrine regulation of SP on PTGS-2 and prostaglandin receptors and possible downstream effects on cervical carcinoma development. SP is synthesized by the PTGS-2 enzyme pathway and activates a few of the cognate receptors PTGER1-4 in epithelial cells from the cervix to initiate target gene expression such as PTGS-2, VEGF, Gro-α, IL-6, IL-8 and IL-11 genes. Thereafter, PTGS-2 can auto-regulate a positive feedback loop to activate further prostaglandin signalling and further enhance PGE_2 levels while VEGF, Gro-α, IL-6, IL-8 and IL-11 can bind to their specific receptors on adjacent cervical tissue to promote angiogenesis and tumour growth in a paracrine manner.

6.2.4 Upregulation of Proliferative Marker Ki-67 and Blood Vessel Marker CD31 in Response to SP Treatment

In this study, Ki-67 and CD31 were shown to be upregulated in response to SP. These markers were employed to determine cellular proliferation and alterations in vasculature respectively with both being found to be increased in nude mice xenografts and WT HeLa cells exposed to SP. In addition, CD31 was shown to be significantly enhanced in normal cervical tissue following treatment with SP for 24 hr. Several studies have attempted to incoroporate the use of markers such as Ki-67 and CD31 for prognostic use in patients with carcinoma of the cervix and this may pose as a useful adjunct for predicting clinical outcome in the future (Mazibrada *et al.,* 2008; Leung *et al.,* 2004). In addition to Ki-67 and CD31, SP also induced the upregulated expression of IL-6 and VEGF in normal cervix, nude mice xenografts and WT HeLa cells and this is supported by data published by Takano and colleagues (1996) whose group ascertained that high levels of IL-6 exist in cervical cancer samples whilst Cheng and colleagues (1999) focused on the expression levels of VEGF in patients with cancer of the cervix and determined that the intratumoral protein level of VEGF correlated with local tumour progression and tumour metastasis.

The upregulation of Ki-67, CD31, IL-6 and VEGF in carcinoma of the cervix is in agreement with the findings of Mazibrada *et al.,* (2008); Leung *et al.,* (2004); Takano *et al.,* (1996) and Cheng *et al.,* (1999) who found that these pathological characteristics of cervical cancer were significantly increased in their respective studies. Other work by Handra-Luca and colleagues (2003) revealed Ki-67 expression in human salivary gland mucoepidermoid carcinoma to be activated via ERK1/2. In this study, the elevated expression is a direct result of SP exposure. Although the levels of Ki-67, CD31, IL-6 and VEGF can be correlated with disease progression and likelihood of survival, the information cannot help patients who have already developed cancer of the cervix. Seminal plasma is capable of inducing several of the traits associated with cervical cancer as shown here, but could become a useful tool if employed in studies aimed at identifying factors associated with the onset of the disease to enable early detection.

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Furthermore, recently published data by Srivastava *et al.,* (2009) provides a novel approach for investigating different stages of cervical cancer as the findings suggest that increased lipid peroxidation (LPO) and reduced antioxidant levels may be taken as predicitive markers of carcinoma of the cervix and in addition to chemo-radiotherapy, the administration of antioxidants may delay recurrence. Other work carried out by Ahmed and colleagues (1999) showed that oxygen free radical levels were raised in cervical dysplasia and cervical cancer as compared to controls. SP is known to exhibit varying levels of reactive oxygen species (ROS) (Lissak *et al.,* 2004) and although they have a potential role in normal fertilization (Agarwal *et al.,* 2006), high levels can damage the spermatozoa and ultimately leads to male infertility (Aitken, 1994). However, supplementary evidence has indicated that ROS are involved with initiation and progression of carcinogenesis and that continued oxidative stress may lead to various neoplastic transformations (Halliell, 1996; Srivastava *et al.*, 2009).

Taken together, seminal plasma, and in particular seminal plasma derived from infertile men possesses the capability to mediate cervical cancer cell growth by increasing the production of ROS and oxidative stress (Agarwal *et al.,* 2003; Agarwal *et al.,* 2006). Unfortunately, markers of these parameters are only examined once the onset of disease has occurred and although they may illustrate valuable prognostic significance, the benefit to patients with ongoing development is minimal. Again this underlines the need to establish markers that can be correlated to the initial changes in the cervical microenvironment and halt the cancer from ever developing in the first place.

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6.2.5 Upregulation of Cellular Proliferation in Response to SP Treatment

SP was shown to significantly increase the proliferative index in nude mice xenografts and in WT HeLa cells as determined by the proliferative marker Ki-67. Employing WT HeLa cells as our model system also demonstrated that SP has the potential to increase cellular growth which is a key process required for angiogenesis and subsequent cancer development. Battersby and colleagues (2007) showed that SP can increase endothelial cell proliferation and migration by inducing expression of the pro-angiogenic factor FGF2 and this was mediated by c-Src, EGFR and ERK

signalling as well as PTGER2. Results herewithin display that EGFR, ERK1/2 and PTGER receptor expression are activated in response to SP stimulation and the mechanisms operating in response to SP to promote cellular growth in the endometrium could be applied in a similar fashion in order to mediate an increased proliferative rate in WT HeLa cells. NF-κB signalling is a known mediator of growth signals in breast and prostate cancer cells (Hoshiya *et al.,* 2003) and its activation observed in our model cell line system may also be involved in regulating SP-induced cellular proliferation in the cervix.

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6.3 Animal Model and the Resulting Implications

The results obtained from the *in vivo* studies showed that SP activated the upregulation of prostaglandin synthase enzymes and prostanoid signalling, induced the expression of a vast host of pro-inflammatory mediators and pro-angiogenic cytokines and increased the proliferation index and angiogenesis as determined by Ki-67 and CD31 respectively. It was then decided to use MF-1 nude mice as an animal model system in an attempt to see whether these same target genes were induced by SP *in vivo.*

The findings confirmed the results generated *in vitro* by demonstrating that SP induced faster tumour growth and increased tumour size of WT HeLa cell derived xenografts and this was made further evident by enhanced expression of Ki-67. In addition SP activated the upregulation of PTGS-1, PTGS-2, PTGER receptor signalling, IL-6, IL-11, VEGF and augmented angiogenesis as determined by the over-expression of CD31. Other *in vivo* studies have shown SP to activate the expression of several pro-inflammatory cytokines and chemokines in uterine epithelial cells of mice and pigs and include IL-1A, IL-1B, IL-6 IL-8, PTGS-2 and Gro-β (Robertson *et al.,* 1992; Robertson *et al.,* 1996; O'Leary *et al.,* 2004). The upregulation of these factors amplify the actions of seminal plasma chemotactic agents which can result in changes in vasculature and enhance the inflammatory response by activating macrophage recruitment (Sharkey *et al.,* 2007). As seen here in our animal model system, the culmination of SP inducing the upregulation of these pro-inflammatory cytokines and pro-angiogenic factors is to promote vasculature alterations that resultantly increases tumour growth and tumour size. The implication of these findings suggest that SP has the potential to exacerbate local pathologies in cervical epithelium and throughout the female reproductive tract by regulating the production of inflammatory cytokines to promote cell growth and tumourigenesis. However, caution must be taken when analysing animal model data because it is not a true reflection of what happens to the cervix. Firstly, the mouse is being treated i.p. whereas the cervix is exposed and secondly, the mode of SP introduction is also likely to be limiting as one route is systematic whereas the other is local. Nevertheless, this model was used to give an indication and is a much better model system than using in vitro cell culture.

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6.4 Conclusion

From the findings generated in this study it can be concluded that SP induces the upregulated expression of PTGS-1, PTGS-2, PTGER1, PTGER3, PTGER4, pro-flammatory cytokines IL-6, IL-8 and IL-11, pro-angiogenic factors Gro-α and VEGF and induce alterations in vasculature as determined by CD31 in normal human cervical tissue.

In addition, this study demonstrates that SP enhances cellular proliferation as well as inducing the expression of PTGS-1, PTGS-2, IL-6, IL-8, IL-11, Gro-α, VEGF and Ki-67 in WT HeLa cells. In order for SP to mediate the transcriptional activation of these cancer-promoting genes, the use of inhibitors herewithin has found that SP components act upon several signalling molecules to mediate its effects. Pathways involving ERK and EGFR are strongly involved in the upregulation of these target genes and this response is immediate and direct. PTGS-2 is central in not only mediating the SP-induced activation of the target genes highlighted here, but also in upregulating itself to form a positive autocrine/paracrine feedback loop leading to further increases in the expression levels of PTGER signalling, IL-6, IL-8, IL-11, Gro-α and VEGF. The transactivation of NF-κB signalling can be a direct effect of SP and as a secondary result of SP-triggered EGFR, ERK and PTGS-2 activation. TGF-β signalling is also activated by direct and secondary means although its involvement is to a much lesser extent. Despite evidence showing the importance of ERK, EGFR, PTGS-2 and NF-κB activation in mediating the pro-inflammatory and pro-angiogenic effects of SP, other signalling apparatus appears to be required.

Moreover, this study confirmed that SP promotes increases in tumour growth and tumour size in nude mice xenografts *in vivo*. Further results show that SP-treated xenografts also displayed significantly enhanced expression of PTGS-1, PTGS-2, PTGER1, PTGER2, PTGER4, IL-6, IL-11, VEGF, the proliferative marker Ki-67 and angiogenic marker CD31 when compared to the controls. The consequential increase in proliferation index and changes in vasculature highlight the potential of SP to augment the cervical microenvironment to favour tumour growth.

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Taken together, these data suggest that SP may directly modulate cervical tumour growth by increasing the production of PTGS-1, PTGS-2 and prostanoid receptor-ligand binding, which in turn generates vast increases in complex cell signalling. The subsequent activation of ERK and EGFR are the primary mechanisms involved, although, SP also mediates its effects via PTGS-2, the NF-κB pathway and to a lesser extent TGF-β. The outcome is the recruitment of inflammatory mediators, pro-angiogenic factors, chemokines and prostaglandins, all of which can act upon epithelial cells to promote favourable conditions for cancer growth. This is conveyed by means of changes in tissue vasculature to increase the supply of oxygen and nutrients to the tumour, increasing cellular proliferation to aid quicker tumour growth and an irregulated inflammatory response that calls upon unrequired inflammatory cells which happen to be strong participants of the neoplastic process, all resultantly combining to enhance tumour mass and support cervical tumourigenesis.

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6.5 Future Studies

At present, there are many studies which have been undertaken in an attempt to determine the underlying causes responsible for the development and progression of cervical cancer. There is also a multitude of data available which has scrutinized the components of seminal plasma and shown the ejaculate pool to exhibit powerful immunosuppressive properties (Kelly *et al.*, 1995; Robertson *et al.*, 2005). Current findings suggest that multiple sexual partners and sexual habits are important factors in the development of cervical cancer (Herbert and Coffin 2008), therefore implying that exposure to SP could be central to events which results in disease development. However, research investigating cervical carcinoma and seminal plasma under the same barometer is somewhat limited.

This study clearly displays that SP exhibits tumour-favouring capabilities through being able to prime the cervical microenvironment for tumour growth, thus adding further scope to the growing body of evidence from others as well as within our laboratory that suggests SP is heavily involved in the promotion of gynaecoligical malignancies (Sharkey *et al.*, 2007; O'Leary *et al.*, 2004; Sales *et al.*, 2002b; Battersby *et al.*, 2007).

To our knowledge, this is one of the first studies undertaken that has employed the use of seminal plasma *in vivo* to investigate how SP might induce cervical cancer and further research of this nature would be most helpful in trying to ascertain the importance of SP in cervical tumourigenesis. In continuing in line with our work, future studies that could be carried out would employ the same inhibitors used to identify SP-activated signalling pathways in WT HeLa cells and see whether these pathways are also operated upon by SP in human cervical tissue. In addition, using inhibitors in combination would determine whether these signalling molecules reside in the same pathways, and if not, their effect is an additive one. Furthermore, antagonists for various receptors could be utilized to ascertain which receptors mediate SP responses in human tissue and WT HeLa cells as a model system. Other studies could attempt to try and determine whether pathologies arising from particular strains of HPV are more susceptible to augmentation by SP exposure and sub-Saharan Africa

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would be an ideal location for human tissue sample collection due to the high prevalence of cervical cancer cases and the multiplicity of strains that exist within these populations. Research intent on identifying cervical tumour markers such as Ki-67 and CD31 may provide welcomed diagnostic and prognostic value to the clinic but performing microarray studies to determine what other genes can be upregulated by SP and identifying whether any of these are involved in metastasis may also be of significant benefit.

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REFERENCES

Aareleid, T., Pukkala, E., Thomson, H., and Hakama, M. (1993) Cervical cancer incidence and mortality trends in Finland and Estonia: a screened vs. an unscreened population. *European Journal of Cancer* **29A(5)**, 745-749.

Ablin, R.J., and Stein-Werblowsky, R.S. (1997) Sexual behaviour and increased anal cancer. *Immunology and Cell Biology* **75**, 181-183.

Abramovitz, M., Boie, Y., Nyguyen, T., Rushmore, T.H., Bayne, M.A., Metters, K.M., and Grygorczyk, R. (1994) Cloning and expression of a cDNA for the human prostanoid FP receptor. *Journal of Biological Chemistry* **269**, 2632-2636.

Abramovitz, M., Adam, M., Boie, Y., Grygorczyk, R., Rushmore T.H., Nguyen T., Funk, C.D., Bastien, L.,, Sawyer, N., and Rochette C. (1995) Human prostanoid receptors: cloning and characterization. *Advances in Prostaglandin, Thromboxane, and Leukotriene Research* **23**, 499-504.

Agarwal, A., Saleh, R.A., and Bedaiwy, M.A. (2003) Role of oxygen species in the pathophysiology of human reproduction. *Fertility and Sterility* **79(4)**, 829-843.

Agarwal, A., Sharma, R.K., Nallella, K.P., Thomas, A.J., Alvarez, J.G., and Sikka, S.C. (2006) Reactive oxygen species as an independent marker of male factor fertility. *Fertility and Sterility* **86**, 878-885.

Ahmed, M.I., Fayed, S.T., Hossein, H., and Tash, F.M. (1999) Lipid peroxidation and antioxidant status in human cervical carcinoma. *Disease Markers* **15**, 283-291.

Ahuja, S.K., and Murphy, P.M. (1996) The CXC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. *Journal of Biological Chemistry* **34**, 20545-20550.

Aitken, R.J. (1994) A free radical theory of male infertility. *Rep. Fert. Dev* **6**, 19-23.

Akira, S., Taga, T., and Kishimoto, T. (1993) Interleukin-6 in biology and medicine. *Advanced Immunology* **54**, 1-78.

Alexander, N.J., and Anderson, D.J. (1987) Immunology of semen. *Fertility and Sterility* **47**, 192-205.

Ali, S.F., Ayub, S., Manzoor, N.F., Azim, S., Afif, M., Akhtar, N., Jafery, W.A., Tahir, I., Farid-ul-Hasnian, S., and Uddim, N. (2010) Knowledge and awareness about cervical cancer and its prevention amongst interns and nursing staff in tertiary care hospitals in Karachi, Pakistan. *PLoS ONE* **5(6)**, e11059.

University of Cape Town

Allen, R.D., and Roberts, T.K. (1986) The relationship between the immunosuppressive and cytoxic effects of human seminal plasma. *American Journal Reprod. Immunol. Microbiol.* **11**, 59-64.

Allan, B., Marais, D.J., Hoffman, M., Shapiro, S., and Williamson, A.L. (2007) Cervical human papillomavirus (HPV) infection in South African women: implications for HPV screening and vaccine strategies. *Journal of Clinical Microbiology* **46(2)**, 740-742.

Amano, H., Hayashi, I., Endo, H., Kitasato, H., Yamashina, S., Maruyama, T., Kobayashi, M., Satoh, K., Narita, M., Sugimoto, Y., Murata, T., Yoshimura, H., Narumiya, S., and Majima, M. (2003) Host prostaglandin E(2)-EP3 signaling regulates tumor-associated angiogenesis and tumor growth. *Journal of Experimental Medicine* **197(2)**, 221-232.

An, S., Yang, J., So, S.W., Zeng, L., and Goetzl, E.J. (1994) Isoforms of the EP3 subtype of human prostaglandin E2 receptor transduce both intracellular calcium and cAMP signals. *Biochemistry* **33(48)**, 14496-14502.

Ansari, K.M., Rundhaug, J.E., and Fischer, S.M. (2008) Multiple signaling pathways are responsible for prostaglandin E2-indcued keratinocyte proliferation. *Molecular Cancer Research* **6(6)**, 1003-1016.

Appleby, P., Ristimaki, A., Neilson, K., Narko, K., and Hla, T. (1994). Structure of the human cyclo-oxygenase-2 gene. *Biochemical Journal* **302**, 723-727.

Appleby, P., Beral, V. and Berrington de Gonzalez, A., (International Collaboration of Epidemiological Studies of Cervical Cancer) (2003) Carcinoma of the cervix and tobacco smoking: colloborative reanalysis of individual data on 13,541 women with carcinoma of the cervix and 23,017 women without carcinoma of the the cervix from 23 epidemiological studies. *International Journal of Cancer* **118**, 1481-1495.

Arenburg, D.A., Kunkel, S.L., Polverini, P.J., Glass, M., Burdick, M.D., and Strieter, R.M. (1996) Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. *Journal of Clinical Investigation* **97(12)**, 2792-2802.

Arteaga, C.L., Carty-Dugger, T., Moses, H.L., Hurd, S.D., and Pietenpol, J.A. (1993) Transforming growth factor β1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. *Cell Growth and Differentiation* **4**, 193-201.

Arun, P., Brown, M.S., Ehsanian, R., Chen, Z., and Van Waes, C. (2009) Nuclear NF-kappaB p65 phosphorylation at serine 276 by protein kinase A contributes to the malignant phenotype of head and neck cancer. *Clinical Cancer Research* **15(19)**, 5974-5984.

Athavale, R., Clooney, K., O'Hagan, J., Shawki, H., Clark, A.H., and Green, J.A. (2006) COX-1 and COX-2 expression in stage I and stage II invasive cervical

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carcinoma: relationship to disease replapse and long-term survival. *International Journal of Gynecological Cancer* **16(3)**, 1303-1308.

Athirakul, K., Kim, H.S., Audoly, L.P., Smithies, O., and Coffman, T.M. (2001) Deficiency of COX-1 causes natiuresis and enhanced sensitivity to ACE inhibition. *Kidney International* **60**, 2324-2329.

Audoly, L.P., Ruan, X., Wagner, V.A., Goullet, J.L., Tilley, S.L., Koller, B.H., Coffman, T.M., and Arendshorst, W.J. (2001) Role of EP_2 and EP_3 PGE_2 receptors in control of murine renal hemodynamics. *American Journal of Physiology Heart and Circulatory Physiology* **280**, H327-H333.

Aumuller, G., and Riva, A. (1992) Morphology and functions of the human seminal vesicle. *Andrologia* **24**, 183-196.

Ausprunk, D.H. and Folkman, J. (1977) Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvascular Research* **14(1)**, 53-65.

Bader, B.L., Rayburn, H., Crowley, D., and Hynes, R.O. (1998) Extensive vasculogenesis, angiogenesis and organogenesis precede lethality in mice lacking all alpha v integrins. *Cell* **95**, 507-519.

Bae, S.K., Bae, M.H., Ahn, M.Y., Son, M.J., Lee, Y.M., Bae, M.K., Lee, O.H., Park, B.C., and Kim, K.W. (1999) Egr-1 mediates transcriptional activation of IGF-II gene in response to hypoxia. *Cancer Research* **59**, 5989-5994.

Bai, X.M., Jiang, H., Ding, J.X., Peng, T., Ma, J., Wang, Y.H., Zhang, L., Zhang, H., and Leng, J. (2010) Prostaglandin E_2 upregulates survivin expression via the EP1 receptor in hepatocellular carcinoma cells. *Life Sciences* **86(5-6)**, 214-223.

Bailie, R.S., Selvey, C.E., Bourne, D., and Bradshaw, D. (1996) Trends in cervical cancer mortality in South Africa. *International Journal of Epidemiology* **25(3)**, 488-493.

Baker, A.F., Dragovich, T., Cui, A., Laheru, D., Campen, C., Von Hoff, D.D., and Hidalgo, M. (2009) Plasma IL-6 level and survival of pancreatic cancer patients treated with a VEGFR inhibitor, vatalanib (PTK/ZK). *Journal of Clinical Oncology* **27(15S)**, e15514.

Balentien, E., Mufson, B.E., Shattuck, R.L., Derynck, R., and Richmond, A. (1991) Effects of MGSA/GRO alpha on melanocyte transformation *Oncogene* **6**, 1115-1124.

Balkwill, F., and Mantovani, A. (2001) Inflammation and cancer: back to Virchow ? *Lancet* **357**, 539-545.

Bancroft, J., and Stevens, A. (1982) Theory and practice of histological techniques. Edinburgh: Churchill Livingston, 1st edition.

University of Cape Town

Banu, N., Buda, A., Chell, S., Elder, D., Moorghen, M., Paraskeva, C., and Qualtrough, D., Pignatelli, M. (2007) Inhibition of COX-2 with NS-398 decreases colon cancer cell motility through blocking epidermal growth factor receptor transactivation: possibilities for combination. *Cell Proliferation* **40(5)**, 768-779.

Barbieri, F., Bajetto, A., and Florio, T. (2010) Role of chemokine network in the development and progression of ovarian cancer: a potential novel pharmacological target. *Journal of Oncology* **426956**, 1-15.

Barnett, J., Chow, J., Ives, D., Chiou, M., Mackenzie, R., Osen, E., Nguyen, B., Tsinng, S., Bach C., and Freire, J. (1994) Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochim Biophys Acta* **1209(1)**, 130-139.

Barnhill, R.L., Fandrey, K., Levy, M.A., Mihm, M.C., and Hyman, B. (1992) Angiogenesis and tumor progression of melanoma: quantitation of vascularity in melanocyte nevi and cutaneous melanoma. *Laboratory Investigation* **67**, 331-337.

Barnholtz-Sloan, J.S., Sloan, A.E., and Davis, F.G. (2004) Incidence proportions of brain metastases in patients diagnosed (1973 to 2001) in the Metropolitan Deroit Cancer Surveillance System. *Journal of Clinical Oncology* **22**, 2865-2872.

Barton, B.E. (2005) Interleukin-6 and new strategies for the treatment of cancer, hyperproliferative diseases and paraneoplastic syndromes. *Expert Opinion on Therapeutic Targets* **9(4)**, 737-752.

Baselga, J., Albanell, A., Ruiz, A., Lluch, A., Gascon, P., Gonzalez, S., Guillen, V., Sauleda, S., Averburch, S., and Rojo, F. (2003) Phase II and tumor pharmacodynamic study of genfinitib (ZD1839) in patients with advanced breast cancer. *Proceedings of the American Society of Clincal Oncology* **22(7)** (Abstract 24).

Bastien, L., Sawyer, N., Grygorczyk, R., Metters, K.M., and Adam, M. (1994) Cloning, functional expression and characterization of the human prostaglandin E_2 receptor EP_2 subtype. *Journal of Biological Chemistry* **269(16)**, 11873-11877.

Battersby, S., Critchley, H.O., de Brum-Fernandes, A.J., and Jabbour, H.N. (2004) Temporal expression and signalling of prostacyclin receptor in the human endometrium across the menstrual cycle. *Reproduction* **127**, 79-86.

Battersby, S., Sales, K.J., Williams, A.R., Anderson R.A., Gardner, S., and Jabbour, H.N. (2007) Seminal plasma and prostaglandin E_2 up-regulate fibroblast growth factor 2 expression in endometrial adenocarcinoma cells via E-series prostanoid-2 receptor-mediated transactivation of the epidermal growth factor receptor and extracellular signal-regulated kinase pathway. *Human Reproduction* **22(1)**, 36-44.

University of Cape Town

Bauer, A.K., Dwyer-Nield, L.D., and Malkinson, A.M. (2000) High cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) contents in mouse lung tumors. *Carcinogenesis* **21(4)**, 543-550.

Becker, K.P., Garnovskaya, M., Gettys, T., and Halushka, P.V. (1999) Coupling of thromboxane A2 receptor isoforms to Galpha 13: effects on ligand binding and signalling. *Biochim. Biophys. Acta* **1450(3)**, 288-296.

Becker, S., Quay, J., Koren, H.S., and Haskill, J.S. (1994) Constitutive and stimulated MCP-1, GRO alpha, beta, and gamma expression in human airway epithelium and bronchoalveolar macrophages. *American Journal of Physiology* **266**, L278-L286.

Beral, V., Hermon, C., Munoz, N., and Devesa, S.S. (1994) Cervical cancer. *Cancer Surv.* **19-20**, 265-285.

Berridge, M.V., and Tan, A.S. (1993) Charaterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch. Biochem. Biophys.* **303**, 474-482.

Bhagavan, N.V. (2002) Medical Biochemistry (4th ed.) Academic Press. p 499.

Bierie, B., and Moses, H.L. (2006) TGF-beta and cancer. *Cytokine Growth Factor Review* **1-2**, 29-40.

Bierie, B., Chung, C.H., Parker, J.S., Stover, D.G., Cheng, N., Chytil, A., Aakre, M., Shyr, Y., and Moses, H.L. (2009) Abrogation of TGF-β signaling enhances chemokine production and correlates with prognosis in human breast cancer. *Journal of Clinical Investigation* **119(6)**, 1571-1582.

Biscotti, C.V., Dawson, A.E., Dziura, B., Galup, L., Darragh, T., Rahemtulla, A., and Willis-Frank, L. (2005) Assissted primary screening using the automated ThinPrep imaging system. *American Journal of Clinical Pathology* **123**, 281-287.

Blake, P.R. (1995) Tumours of the uterine cervix and corpus uteri., Horwich, A. (ed). 1st edition, Chapman and Hall Medical, p 305-326.

Bobrovnikova-Marjon, E.V., Marjon, P.L., Barbash, O., Vander Jagt, D.L., and Abcouwer, S.F. (2004) Expression of angiogenic factors vascular endothelial growth factor and interleukin-8/CXCL8 is highly responsive to ambient glutamine availability: role of nuclear factor-kappaB and it activating protein-1. *Cancer Research* **64**, 4858-4869.

Boie, Y., Rushmore, T.H., Darmon-Goodwin, A., Grygorczyk, R., Slipetz, D.M., Metters, K.M., and Abramovitz, M. (1994) Cloning and expression of a cDNA for the human prostanoid IP receptor. *Journal of Biological Chemistry* **269(16)**, 12173-12178.

University of Cape Town

Boie, Y., Sawyer, N., Slipetz, D., Metters, K., and Abramovitz, M. (1995) Molecular cloning and characterization of the human prostanoid DP receptor. *Journal of Biological Chemistry* **270**, 18910-18916.

Bolger, P.M., Einser, G.M., Ramwell, P.W., Slotkoff, L.M., and Corey, E.J. (1978) Renal action of prostacyclin. *Nature* **271**, 467-469.

Boniface, K., Bak-Jensen, K.S., Li, Y., Blumenschein, W.M., McGeachy, M.J., McClanahan, T.K., McKenzie, B.S., Kastelein, R.A., Cua, D.J., and de Waal Malefyt, R. (2009) Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. **206(3)**, 535-548.

Bonvalet, J.P., Pradelles, P., and Farman, N. (1987) Segmental synthesis and actions of prostaglandins along the nephron. *American Journal of Physiology Renal Physiology* **253**, F377-F87.

Bottazzi, M.E., and Assoian, R.K. (1997) The extracellular matrix and mitogenic growth factors control G1 phase cyclins and cyclin-dependent kinase inhibitors. *Trends in Cell Biology* **7(9)**, 348-352.

Brandi, G., Pantaleo, M.A., and Biasco, G. (2008) Activated NF-κB in colorectal cancer: predictive or prognostic factor ? *Journal of Clinical Oncology* **26(8)**, 1388-1389.

Brat, D.J., Bellail, A.C., and Van Meir, E.G. (2005) The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro-Oncology* **7**, 122-133.

Breder, C.D., DeWitt, D.L., and Kraig, R.P. (1995) Characterization of inducible cyclooxygenase in rat brain. *Journal of Comparative Neurology* **355(2)**, 296-415.

Brenneis, C., Maier, T.J., Scmidt, R., Hofacker, A., Zulauf, L., Jakobsson, K., and Geisslinger, G. (2006) Inhibition of prostaglandin E_2 synthesis by SC-560 is independent of cyclooxygenase 1 inhibition. *The FASEB Journal* **20**, 1352-1360.

Breyer. R.M., Bagdassarian, C.K., Myers, S.A., and Breyer, M.D. (2001) Prostanoid receptors: subtypes and signalling. *Annual Review of Pharmacology and Toxicology* **41**, 661-690.

Brightling, C.E., Bradding P., Pavord, I.D., and Wardlaw, A.J. (2003) New sights into the role of mast cells in asthma. *Clinical and Experimental Allergy* **33**, 550-556.

Brown, L.F., Dvorak, A.M., and Dvorak, H.F. (1989) Leaky vessels, fibrin deposition, and fibrosis: a sequence of events common to solid tumors and to many other types of disease. *American Review of Respiratory Disease* **140**, 1104-1107.

Bosch, F.X., Manos, M.M., Munoz, N., Sherman, M., Jansen A.M., Peto, J., Schiffman, M.H., Morenzo, V., Kurman, R., and Shak, K.V., (1995) Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International

University of Cape Town

biological study on cervical cancer (IBSCC) Study Group. *The Journal of the National Cancer Institute* **87**, 796-802.

Botting, R. (2003) COX-1 and COX-3 inhibitors. *Thrombosis Research* **110(5-6)**, 269-272.

Boutwell, R.K. (1974) The function and mechanism of promoters of carcinogenesis. *CRC Critical Review in Toxicology* **2**, 419-443.

Bugge, J.F., Stokke, E.S., Vikse, A., and Kiil, F. (1990) Stimulation of renin release by PGE2 and PGI2 infusion in the dog: enhancing effect of ethacrynic acid. *Acta Physiologica Scandinavica* **138**, 193-201.

Burri, P.H., Hluschuk, R., Djonov, V. (2004) Intussuceptive angiogenesis: its emergence, its characteristics and its significance. *Developmental Dynamics* **231(3)**, 474-488.

Cai, Z., Chen, Q., Chen, J., Lu., Xiao, G., Wu, Z., Zhou, Q., and Zhang, J. (2009) Monocyte chemotactic protein 1 promotes lung cancer-induced bone resorptive lesions in vivo. *Neoplasia* **11(3)**, 228-236.

Campbell, C.L., Guardiani, R., Ollari, C., Nelson, B.E., Quesenberry, P.J., and Savarese, T.M. (2001) Interleukin-11 receptor expression in primary ovarian carcinomas. *Gynecologic Oncology* **80(2)**, 121-127.

Cao, Y., Chen, C., Weatherbeem J.A., Tsang, M., and Folkman J. (1995) gro-β, a C-X-C- chemokine, is an angiogenesis inhibitor that suppresses the growth of Lewis lung carcinoma in mice. *Journal of Experimental Medicine* **182**, 2069-2077.

Cason, J. (1996) Perinatal acquisition of cervical cancer associated papillomaviruses. *British Journal of Obstetrics and Gynaecology* **103(9)**, 853-858.

Castellani, S., Paladini, B., Paniccia, R., Di Serio, C., Valloti, B., Ungar, A., Fumagalli, S., Cantini, C., Poggesi, L., and Neri Serneri, C.G. (1997) Increased renal formation of thromboxane A2 and prostaglandin F2 alpha in heart failure. *American Heart Journal* **133**, 94-100.

Castellone, M.D., Teramoto, H., Williams, B.O., Druey, K.M., and Gutkind, J.S. (2005) Prostaglandin E_2 promotes colon cancer cell growth through a G_s-Axin-β-catenin signaling axis. *Science* **310**, 1504-1510.

Castellsague, X., Bosch, F.X., Munoz, N., Meijer, C.J.L.M., Shah, K.V., Sivia de Sanjose, P.H., Eluf-Neto, J., Ngelangel, C.A., Chichareon, S., Smith, J.S., Herrero, R., Moreno, V., and Franceschi, S. (2002) Male circumcision, penile human papillomavirus infection, and cervical cancer in female partners. *New England Medical Journal* **346**, 1105-1112.

University of Cape Town

Castrilli, G., Tatone, D., Diodoro, M.G., Rosini, S., Piantelli, M., and Musiani, P. (1997) Interleukin 1α and interleukin 6 promote the in vitro growth of both normal and neoplastic human cervical epithelial cells. *British Journal of Cancer* **75(6)**, 855-859.

Caunt, M., Hu, L., Tang, T., Brooks, P.C., and Ibrahim, S. (2006) Growth-regulated oncogene is pivotal in thrombin-induced angiogenesis. *Cancer Research* **66(8)**, 4125-4132.

Cebe-Suarez, S., Zehnder-Fjallman, A.H., and Ballmer-Hofer, K. (2006) The role of VEGF receptors in angiogenesis; complex partnerships. *Cellular and Molecular Life Sciences* **63**, 601-615.

Chalabi, S., Easton, R.L., Patankar, M.S., Lattanzio, F.A., Morrison, J.C., Panico, M., Morris, H.R., Dell, A., and Clark, G.F. (2002) The expression of free oligosaccharides in human seminal plasma. *Journal of Biological Chemistry* **277**, 32562-32570.

Chaluda, P.C., Thompson, M.B., Mahler, J.F., Doyle, C.M., Gaul, B.W., Lee, C., Tiano, H.F., Morham, S.G., Smithies, O., and Langenbach, R. (2000) Genetic disruption of PTGS-1, as well as PTGS-2, reduces intestinal tumorigenesis in Min mice. *Cancer Research* **60**, 4705-4708.

Chan, B.S., Satriano, J.A., Pucci, M., and Schuster, V.L. (1998) Mechanism of prostaglandin E_2 transport across the plasma membrane of HeLa cells and xenopus oocytes expressing the prostaglandin transporter (PGT). *Journal of Biological Chemistry* **273**, 6689-6697.

Chan, J.K., Monk, B.J., Brewer, C., Keefe, K.A., Osann, K., McMeekin, S., Rose, G.S., Youssef, M., Wilczynski, S.P., Meyskens, F.L., and Berman, M.L. (2003) HPV infection and number of lifetime sexual partners are strong predictors for 'natural' regression of CIN 2 and 3. *British Journal of Cancer* **89**, 1062-1066.

Chandrasekharan, N.V., Dai, H., Roos, K.L., Evanson, N.K., Tomsik, J., Elton, T.S., and Simmons, D.L. (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure and expression. *Proceedings of the National Academy of Sciences USA* **99**, 13926-13931.

Chang, S.H., Liu, C.H., Conway, R., Han, D.K., Nithipatikom, K., Trifan, O.C., Lane, T.F., and Hla, T. (2004) Role of prostaglandin E_2-dependent angiogenic switch in cyclooxygenase-2 induced breast cancer progression. *Proceedings of the National Academy of Sciences USA* **101**, 591-596.

Chang, B.D., Cao, L.S., and Zhou, H.L. (2009) Expression of COX-2 and VEGF and their correlation with angiogenesis in human clear cell renal cell carcinoma. *Zhonghua Zhong Liu Za Zhi* **31**, 687-690.

Chariyalertsak, S., Sirikulchayanonta, V., Mayer, D., Kopp-Schneider, A., Furstenburger, G., Marks, F., and Muller-Decker, K. (2001) Aberrant cyclooxygenase isoenzyme expression in human intrahepatic cholangiocarcinoma. *Gut* **48(1)**, 80-86.

University of Cape Town

Cheifetz, S., Andres, J.L., and Massague J. (1988) The transforming growth factor-beta receptor type III is a membrane proteoglycan. *Journal of Biological Chemistry* **263(32)**, 16984-16991.

Chelouche-Lev, D., Miller, C.P., Tellez, C., Ruiz, M., Bar-Eli, M., and Price, J. (2004) Different signalling pathways regulate VEGF and IL-8 expression in breast cancer: implications for therapy. *European Journal of Cancer* **40(16)**, 2509-2518.

Cheng, J.C.H., Chou, C.H., Kuo, M.L., and Hsieh, C.Y. (2005) Radiation enhanced MMP-9 dependent migration/invasion in hepatocellular carcinoma cell through P13K/Akt/NF-κB or MAPK/ERK/NF-κB pathways. *Proceedings of the American Assocation of Cancer Research* **46**, Abstract 5690.

Cheng, H.F., Wang, J.L., Zhang, M.Z., Miyazaki, Y., Ichikawa, I., Mckanna, J.A., and Harris, R.C. (1999) Angiotensin II attentuates renal cyclooxygenase-2 expression. *Journal of Clinical Investigation* **103(7)**, 953-961.

Cheng, I., Liu, X., Plummer, S.J., Krumroy, L.M., Casey, G., and Witte, J.S. (2007) COX2 genetic variation, NSAIDs, and advanced prostate cancer risk. *British Journal of Cancer* **97**, 557-561.

Cheng, W.F., Chen, C.A., Lee, C.N., Chen, T.M., Hsieh, F.J., and Hsieh, C.Y. (1999) Vascular endothelial growth factor in cervical carcinoma. *Obstetrics and Gynecology* **93(5)**, 761-764.

Chopra, V. Dinh, T., and Hannigan, E.V. (1998) Circulating cytokines and angiogenic factors in patients with cervical cancer. *Cancer Investigation* **16**, 152-159.

Chou, C.H., wei, L.H., Kuo, M.L., Huang, Y.J., Lai, K.P., Chen, C.A. and Hsieh, C.Y. (2005) Up-regulation of interleukin-6 in human ovarian cancer cell via Gi/P13K-Akt/NF-κB pathway by lysophosphatidic acid, an ovarian cancer-activating factor. *Carcinoogenesis* **26(1)**, 45-52.

Christofori, G., and Semb, H. (1999) The role of cell adhesion molecule E-cadherin as a tumour-supressor gene. *Trends in Biochemical Sciences* **24**, 73-76.

Chu, J., Lloyd, F.L., Trifan, O.C., Knapp, B., and Rizzo, M.T. (2003) Potential involvement of the cyclooxygenase-2 pathway in the regulation of tumor-associated angiogenesis and growth in pancreatic cancer. *Molecular Cancer Therapy* **2**, 1-7.

Chulada, P.C., Doyle, C., Gaul, B., Tiano, H.F., Mahler, J.F., Lee, C.A., Morham, S.G., and Langenbach, R. (1998) Cyclooxygenase-1 and -2 deficiency decrease spontaneous intestinal adenomas in the *Min* mouse. *Proceedings of the American Association for Cancer Research* **39**, 195.

Claria, J., Horrillo, R., Martinez-Clemente, M., Moran-Salvador, E., Titos, E., Gonzalez-Periz, A., and Ferre, N. (2008) Basic mechanism of hepatocellular injury.

University of Cape Town

Role of inflammatory lipid mediators. *Journal of Gastroenterology and Hepatology* **31**, 682-692.

Coleman, R.A., and Kennedy, I. (1985) Characterisation of the prostanoid receptors mediating contraction of guinea-pig isolated trachea. *Prostaglandins* **29**, 363-375.

Coleman, R.A., Smith, W.L., and Narumiya, S. (1994a) International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *The American Society for Pharmacology and Experimental Therapeutics* **46(2)**, 205-229.

Coleman, R.A., Grix, S.P., Head, S.A., Louttit, J.B., Mallett, A., and Sheldrick, R.L.G. (1994b) A novel inhibitory prostanoid receptor in piglet saphenous vein. *Prostaglandins* **47**, 151-168.

Conesa-Zamora, P., Domenech-Peris, A., Orantes-Casado, F.J., Ortiz-Reina, S., Sahuquillo-Frias, L., Acosta-Ortega, J., Garcia-Solano, J., and Perez-Guillermo, M. (2009) Effect of human papillomavirus on cell cycle-related proteins p16, Ki-67, cyclin D1, p53, and ProEx C in precursor lesions of cervical carcinoma: a tissue mircoarray study. *American Journal of Clinical Pathology* **132(3)**, 378-390.

Cooper, K., Herrington, C.S., Graham, A.K., Evans, M.F., and McGee, J.O. (1991) In situ evidence for HPV 16, 18, 33 integration in cervical squamous cell cancer in Britain and South Africa. *Journal of Clinical Pathology* **44**, 406-409.

Coussens, L.M., Raymond, W.W., Bergers, G., Webster, M.L., Behrendtsen, O., Werb, Z., Caughey, G.H., and Hanahan, D. (1999) Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes and Development* **13**, 1382-1397.

Coussens, L.M., Tinkle, C.L., Hanahan, D., and Werb, Z. (2000) MMP-9 supplied by bone marrow derived cells contributes to skin carcinogenesis. *Cell* **103**, 481-490.

Coussens, L.M., and Werb, Z. (2002) Inflammation and cancer. *Nature* **420**, 860-867.

Cuenca, R.A., Azizkhan, R.G., and Haskill, S. (1992) Characterization of GRO α, β and γ expression in human colonic tumours: potential significance of cytokine involvement. *Surgical Oncology* **1(4)**, 323-329.

Curry, M.C., Hussain, J.I., Smith, C.J., and Allen, J.C. (1980) Identification of a macromolecular inhibitor of in vitro lymphocyte proliferation in human seminal plasma as bound spermine. *European Journal of Cellular Biology* **21**, 180-182.

Daikoku, T., Wang, D., Tranguch, S., Morrow, J.D., Orsulic, S., DuBois, R.N., and Dey, S.K. (2005) Cyclooxygenase is a potential target for prevention and treatment of ovarian epithelial cancer. *Cancer Research* **65**, 3735-3744.

University of Cape Town

Danforth, K.M., Hayes, R.B., Rodriguez, C., Yu, K., Sakoda, L.C., Huang, W.Y., Chen, B.E., Chen, J., Andriole, G.L., Calle, E.E., Jacobs, E.J., Chu, L.W., Figueroa, J.D., Yeager, M., Platz, E.A., Michaud, D.S., Channock, S.J., Thun, M.J., and Hsing, A.W. (2008) Polymorphic variants in PTGS2 and prostate cancer reisk: results from two large nested case-control studies. *Carcinogenesis* **29(3)**, 568-572.

Daniel, T.O., Liu, H., Morrow, J.D., Crews, B.C., and Marnett L.J. (1999) Thromboxane A2 is a mediator of cyclooxygenase-2-dependent endothelial migration and angiogenesis. *Cancer Research* **59**, 4574-4577.

Dannenburg, A.J., Altorki, N.K., Boyle, J.O., Dang, C., Howe, L.R., and Weksler, B.B. (2001) Cyclooxygenase-2: a pharmalogical target for the prevention of cancer. *Lancet Oncology* **2**, 544-551.

Darai, E., Bringuier, A.F., Walker-Combrouze, F., Fauconnier, A., Couvelard, A., Feldmann, G., Madelenat, P., Scoazec, J.Y. (1998) CD31 expression in benign, borderline, and malignant epithelial ovarian tumors: an immunohistochemical and serological analysis. *Gynecologic Oncology* **71**, 122-127.

De Lorenzo, M.S., Cohen, E.G., Du, B., Chang, M., Lantowski, A., Boyle, J.O., Weksler, B.B., Subbaramaiah, K., and Dannenberg, A.J. (2005) Involvement of prostaglandin E receptor subtype EP_4 in head and neck squamous cell carcinoma (HNSCC). *Proceedings of the American Association for Cancer Research* **46**, abstract 5890.

DeMay, M. (2007) Practical principles of cytopathology., R.E (ed), Chicago I.L; AmericanSociety for Clinical Pathology Press

Demetrious, J., and Demetrious, G.J. (2008) Lung cancer metastasis to the scapula and spine: a case report. *Chiropractic and Osteopathy* **16**, 8.

Denison, F.C., Grant, V.E., Calder, A.A., and Kelly, R.W. (1999a) Seminal plasma components stimulate interleukin-8 and interleukin-10 release. *Molecular Human Reproduction* **5**, 220-226.

Denison, F.C., Calder, A.A., and Kelly, R.W. (1999b) The action of prostaglandin E2 on the human cervix: stimulation of interleukin-8 and inhibition of secretory leukocyte protease inhibitor. *American Journal of Obstetrics and Gynecology* **180**, 614-620.

Descamps-Latscha, B., Herbelin, A., Nguyen, A.T., Roux-Lombard, P., Zingraff, J., Moynot, A., Verger, C., Dahmane, D., de Groote, D., and Jungers, P. (1995) Balance between IL-1 beta, TNF-alpha, and their specific inhibitors in chronic renal failure and maintenance dialysis. Relationships with activation markers of T cells, B cells, and monocytes. *Journal of Immunology* **154(2)**, 882-892.

De Souza, C.P., and Osami, S.A. (2007) Mitosis, not just open or closed. *Eurkaryotic Cell* **6(9)**, 1521-1527.

University of Cape Town

Detmar, M. (2000) Tumor angiogenesis. *Journal of Investigative Dermatology Symposium Proceedings* **5**, 20-23.

Devalaraja, R.M., Naney, L.B., Du, J., Qian, Q., Yu, Y., Devalaraja, M.N., and Richmond, A. (2000) Delayed wound healing in CXCR2 knockout mice. *Journal of Investigative Dermatology Symposium Proceedings* **115(2)**, 234-244.

de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., and zur Hausen, H. (2004) Classification of papillomaviruses. *Virology* **324,** 17-27.

DeWitt, D.L., Day, J.S., Sonnenburg, W.K., and Smith W.L. (1983) Concentrations of prostaglandin endoperoxide synthase and prostaglandin I2 syhtnase in the endothelium and smooth muscle of bovine aorta. *Journal of Clinical Investigation* **72(6)**, 1882-1888.

DeWitt, D.L., El-Harith, E.A., Kraemer, S.A., Andrews, M.J., Yao, E.F., Armstrong R.L., and Smith W.L. (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *Journal of Biological Chemistry* **265**, 5192-5198.

DeWitt, D.L., (1991) Prostaglandin endoperoxide synthase: regulation of enzyme of enzyme expression. *Biochim. Biophys, Acta* **1083**, 121-134.

DiCarlo, E., Forni, G., Lollini, P., Colombo, M.P., Modesti, A., and Musiani, P. (2001) The intriguing role of polymorphonuclear neutrophils in antitumor reactions. *Blood* **97**, 339-345.

Dimitriadis, E., White, C.A., Jones, R.L., and Salamonsen, L.A. (2005) Cytokines, chemokines and growth factors in endometrium related to implantation. *Human Reproduction Update* **11(6)**, 613-630.

Dinchuk, J.E., Car, B.D., Focht, R.J., Johnston, J.J., Jaffee, B.D., Covington, M.B., Contel, N.R., Eng, V.M., Collins, R.J., Czernaik, P.M., Gorry, S.A., and Trzaskos, J.M. (1995) Renal abnomalities and an altered inflammatory response in mice lacking cyclo-oxygenase II. *Nature* **378**, 406-409.

Ding, G.F., Li, J.C., Xu, Y.F., Sun, Y., and Tao, L. (2005) Correlation between the expression of VEGF-C mRNA, VEGFR-3, CD31 and tumor metastases in Chinese with prostate cancer. *Shi Yan Sheng Wu Xue Bao* **38(3)**, 257-264.

Dixon, D.A., Kaplan, C.D., McIntyre, T.M., Zimmerman, G.A., and Prescott, S.M. (2000) Post-transcriptional control of cyclooxygenase-2 gene expression. *Journal of Biological Chemistry* **275**, 11750-11757.

Dixon, D.A. (2004) Dysregulated post-transcriptional control of COX-2 gene expression in cancer. *Current Pharmaceutical Design* **10(6)**, 635-646.

University of Cape Town

Doherty, G.A., Byrne, S.M., Molloy, E.S., Malhorta, V., Austin, S.C., Kay, E.W., Murray, F.E., and Fitzgerald, D.J. (2009) Proneoplastic effects of PGE2 mediated by EP4 receptor in colorectal cancer. *BMC Cancer* **9**, 207.

Dolcet, X., Llobet, D., Pallares, J., and Matias-Guiu, X. (2005) NF-κB in development and progression of human cancer. *Virchows Archives* **446**, 475-482.

Dong, Y.J., Jones, R.L., and Wilson, N.H. (1986) Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. *British Journal of Pharmacology* **87**, 97-107.

Dong, Y.L., Gangula, P.R.R., Fang, L., and Yallampalli, C. (1996) Differential expression of cyclo-oxygenase-1 and -2 proteins in rat uterus and cervix during the estrous cycle, pregnancy, labor and in myometrial cells. *Prostaglandins* **52(1)**, 13-34.

Dossus, L., Kaaks, R., Canzian, F., Albanes, D., Berndt, S.I., Boeing, H., Buring, J., Chanock, S.J., Clavel-Chapleton, F., Feigelson, H.S., Gaziano, J.M., Giovannucci, E., Gonzalez, C., Haiman, C.A., Hallmans, G., Hankinson, S.E., Hayes, R.B., Henderson, B.E., Hoover, R.N., Hunter, D.J., Khaw, K.T., Kolonel, L.N., Kraft, P., Ma, J., Le Marchand, L., Lund, E., Peeters, P.H., Stampfer, M., Stram, D.O., Thomas, G., Thun, M.J., Tjonneland, A., Trichopoulos, D., Tumino, R., Riboli, E., Virtamo, J., Weinstein, S.J., Yeager, M., Ziegler, R.G., and Cox, D.G. (2009) PTGS2 and IL6 genetic variation and risk of breast and prostate cancer: results from the breast and prostate cancer cohort consortium (BPC3). *Carcinogenesis* **31(3)**, 455-461.

Drach, J., Gattringer, C., Glassl, H., Drach, D., and Huber, H. (1992) The biological and clinical significance of the Ki-67 growth fraction in multiple myeloma. *Hematology Oncology* **10**, 125-134.

D'Souza, G., Kreimer, A.R., Viscidi, R., Pawlita, M., Fakhry, C., Koch, W.M., Westra, W.H., and Gillison, M.L. (2007) Case-control study of human papillomavirus and oorpharyngeal cancer. *New England Medical Journal* **356(19)**, 1944-1956.

Du, X.X., and Williams, D.A. (1994) Interleukin-11: a multifactorial growth factor derived from the hematopoietic microenvironment. *Blood* **83**, 2023-2030.

DuBois, R.N., Awad, J., Morrow, J., Roberts, L.J., and Bishop, P.R. (1994) Regulation of eicoosanoid production and mitogenesis in rat intestinal epithelial cells by transforming growth factor-α and phorbol ester. *Journal of Clinical Investigations* **93**, 493-498.

DuBois, R.N., Abramson, S.B., Crofford, L., Gupta, R.A., Simon, L.S., van de Putte, L.B., and Lipsky, P.E. (1998) Cyclooxygenase in biology and disease. *Journal of FASEB* **12**, 1063-1073.

Duchrow, M., Schluter, C., Wohlenberg, C., Flad, H.D., and Gerdes, J. (1996) Molecular characterization of the gene locus of the human cell proliferation-associated nuclear protein defined by monoclonal antibody Ki-67. *Cell Proliferation* **29**, 1-12.

University of Cape Town

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., and Saltiel, B. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proceedings of the National Academy of Sciences* **92**, 7686.

Dumont, N., and Arteaga, C.L. (2003) Targeting the TGF beta signaling network in human neoplasia. *Cancer Cell* **3(6)**, 531-536.

Dvorak, H.F. (1986) Tumours: wounds that do not heal. Similarities between tumour stroma generation and wound healing. *New England Journal of Medicine* **315**, 1650-1659.

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S., and DuBois R.N. (1994) Up-regulation of cyclooxygenase 2 gene expression in human and colorectal adenomas and adenocarcinomas. *Gastroenterology* **107**, 1183-1188.

Eggert-Kruse, W., Boit, R., Rohr, G., Aufenanger, J., Hund, M., and Strowitzki, T. (2001) Relationship of seminal plasma interleukin (IL) -8 and IL-6 with semen quality. *Human Reproduction* **16(3)**, 517-528.

Eibl, G., Bruemmer, D., Okada, Y., Duffy, J.P., Law, R.E., Reber, H.A., and Hines, O.J. (2003) PGE_2 is generated by specific COX-2 activity and increases VEGF production in COX-2-expressing human pancreatic cancer cells. *Biochem. Biophys. Res. Commun.* **306**, 887-897.

Ellenrieder, V., Fernandez Zapico, M.E., and Urrutia, R. (2001) TGF[beta]-mediated signaling and transcriptional regulation in pancreatic development and cancer. *Current Opinion in Gastroenterology* **17(5)**, 434-440.

Erdogan, M., Pozzi, A., Bhowmick, N., Moses, H.L., Zent, R. (2008) Transforming growth factor-beta (TGF-beta) and TGF-beta associated kinase 1 are required for R-Ras-mediated transformation of mammary epithelial cells. *Cancer Research* **68(15)**, 6224-6231.

Erickson, B.A., Longo, W.E., Panesar, N., Mazuski, J.E., and Kaminski, D.L. (1999) The effect of selective cyclooxygenase inhibitors on intestinal epithelial cell mitogenesis. *Journal of Surgical Research* **81(1)**, 101-107.

Ernst, M., Najdovska, M., Grail, D., Lundgren-May, T., Buchert, M., Tye, H., Mattews, V.B., Armes, J., Bhathal, P.S., Hughes, N.R., Marcusson, E.G., Karras, J.G., Na, S., Sedgwick, J.D., Hertzog, P.J., and Jenkins, B.J. (2008) STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice. *Journal of Clinical Invastigation* **118(5)**, 1727-1738.

Eustace, D., Han, X., Gooding, R., Rowbottom, A., Riches, L., and Heyderman, E. (1993) Interleukin-6 (IL-6) functions as an autocrine growth factor in cervical carcinomas in vitro. *Gynecologic Oncology* **50**, 15-19.

University of Cape Town

Ferrara, N., Heinsohn, H., Waldner, C.E., Bunting, S., and Thomas, G.R. (1995) The regulation of blood vessel growth by vascular endothelial growth factor. *Annals of the New York Academy of Sciences* **752**, 246-256.

Fichorova, R.N., and Anderson, D.J. (1999) Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. *Biology of Reproduction* **60(2)**, 508-514.

FIGO T.N.M. (1992) Atlas, 3rd edition, 2nd edition, Heidelberg; Springer-Verlag, p 196.

Fimmel, S., Devermann, L., Herrmann, A., and Zouboulis, C. (2007) Gro-alpha: a potential marker for cancer and aging slienced by RNA interference. *Annals of the New York Academy of Sciences* **1119**, 176-189.

Fischer, O.M., Hart, S., Gschwind, A., and Ullrich, A. (2003) EGFR signal transactivation in cancer cells. *Biochemical Society Transactions* **(31)6**, 1203-1208.

Fleming, E.F, Athirakul, K., Oliverio, M.I., Key, M., Goulet, J., Koller, B.H., and Coffman, T.M. (1998) Urinary concentrating function in mice lacking the EP3 receptors for prostaglandin E2. *American Journal of Physiology Renal Physiology* **275**, F955-F61.

Fletcher, B.S., Kujubu, D.A., Perrin, D.M., and Herschman, H.R. (1992) Structure of the mitogen-inducible TIS10 gene and demonstration that TIS-10-encoded protein is a function of prostaglandin G/H synthase. *Journal of Biological Chemsitry* **267**, 4338-4344.

Folkman, J. (1990) What is the evidence that tumors are angiogenesis dependent ? *National. Cancer Institute.* **82** 4-6.

Forman, B.M., Chen, J., and Evans, R.M. (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proceedings of the National Academy of Sciences USA* **94**, 4312-4317.

Fosslien E. (2000) Molecular pathology of cyclooxygenase-2 in neoplasia. *Annals of Clinical and Laboratory Science* **30**, 3-21.

Fossilen E. (2001) Review: Molecular pathology of cyclooxygenase-2 in caner-induced angiogenesis. *Annals of Clinical and Laboratory Science* **31(4)**, 325-348.

Fowlkes, J.L., and Winkler, M.K. (2002) Exploring the interface between metallo-proteinase activity and growth factor and cytokine bioavailability. *Cytokine Growth Factor Review* **13**, 277-287.

University of Cape Town

Fraser, L.R., Adeoya-Osiguwa, S.A., Baxendale, R.W., and Gibbons, R. (2006) Regulation of mammalian sperm capacitation by endogenous molecules. *Frontiers in Bioscience* **11**, 1636-1645.

Frazer, I.H., Cox, J.T., Mayeaux, E.J., Franco, E.L., Moscicki, A.B., Palefsky, J.M., Ferris, D.G., Ferenczy, A.S., and Villa, L.L. (2006) Advances in prevention of cervical cancer and other human papillomavirus-related diseases. *Pediatric Infectious Disease Journal* **25(2)**, S65-S81.

Fregenem T.A., Khanuja, P.S., Noto, A.C., Gehani, S.K., Van Egmont, E.M., Luz, D.A., and Pienta, K.J. (1993) Tumor-associated angiogenesis in prostate cancer. *Anticancer Research* **13**, 2377-2381.

Fujimoto, J., Sakaguchi, H., Aoki, I., and Tamaya, T. (2000) Clinical implications of expression of interleukin 8 related to angiogenesis in uterine cervical cancers. *Cancer Research* **60**, 2632-2635.

Fujino, H., Xu, W., and Regan, J.W. (2003) Prostaglandin E_2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-reduced kinases. *Journal of Biological Chemistry* **278**, 12151-12156.

Fulton, A.M., Ma, X., and Kundu, N. (2006) Targeting prostaglandin E EP receptors to inhibit metastasis. *Cancer Research* **66(20)**, 9794-9797.

Fung, K.Y., Glode, L.M., Green, S., and Duncan, M.W. (2004) A comprehensive characterization of the peptide and protein constituents of human seminal fluid. *Prostate* **61**, 171-181.

Funk, C.D., Furci, L., FitzGerald, G.A., Grygorczyk, R., Rochette, C., Bayne, M.A., Abramovitz, M., Adam, M., and Metters, K.M. (1993) Cloning and expression of a cDNA for the human prostaglandin E receptor EP_1 subtype. *Journal of Biological Chemistry* **268(35)**, 26767-26772.

Furuya, Y., Nishio, R., Junicho, A., Nagakawa, O., and Fuse, H. (2005) Serum interleukin-11 in patients with benign prostatic hyperplasia and prostate cancer. *International Urology and Nephrology* **37(1)**, 69-71.

Gaffney, D.K., Holden, J., Davis, M., Zemmpolich K, Murphy, K.J., and Dodson, M. (2001) Elevated cyclooxygenase-2 expression correlates with diminished survival in carcinoma of the cervix treated with radiotherapy. *Int J. Radiat Oncol Biol Phys.* **49**, 1213-1217.

Gall, O., Schiavone, N., Papucci, L., Sardi, I., Magnelli, L., Franchi, A., Masini, E., and Capacciolim, S. (2003) Down-regulation of nitric oxide synthase-2 and cyclooxygenase-2 pathways by p53 squamous cell carcinoma. *American Journal of Pathology* **163**, 723-732.

University of Cape Town

Gan, H.K., Walker, F., Burgess, A.W., Rigopoulos, A., Scott, A.M., and Johns, T.G. (2007) The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG1478 increases the formation of inactive untethered EGFR dimers. *The Journal of Biological Chemistry* **282**, 2840-2850.

Gardiner, P.J. (1986) Characterization of prostanoid relaxant/inhibitory receptors (psi) using a highly selective agonist, TR4979. *British Jounral of Pharmacology* **87**, 45-56.

Garland, S.M., Hernandez-Avila, M., Wheeler, C.M., Perez, G., Harper, D.M., Leodolter, S., Tang, G.W.K., Ferris, D.G., Steben, M., Bryan, J., Taddeo, F.J., Railkar, R., Esser, M.T., Sings, H.L., Nelson, M., Boslego, J., Sattler, C., Barr, E., and Koutsky, L.A. (2007). Quarivalent vaccine against human papillomavirus to prevent anogenital diseases. *New England Medical Journal* **356**, 1928-1943.

Garzetti, G.G., Ciavattini, A., Lucarini, G., Pugnaloni, A., De Nictolis, M., Amati, S., Romanini, C., and Biagini, G. (1999) Expression of vascular endothelial growth factor related to 72-kilodalton metallproteinase immuno-staining in patients with serous ovarian tumors. *Cancer* **85**, 2219-2225.

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., and Baumann, H. (1987) Interferon β2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proceedings of the National Academy of Sciences USA* **84**, 7251-7255.

Gearing, D.P., Corneau, M.R., Friend, D.J., Gimpel, S.D., Thut, C.J., McGourty, J., Brasher, K.K., King, J.A., Gillis, S., Mosley, B., Ziegler, S.F., and Cosman, D. (1992) The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* **255**, 1434-1437.

Gerdes, J., Schwab, U., Lemke, H., and Stein, H. (1983) Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *International Journal of Cancer* **31**, 13-20.

Ghosh, S., May, M.J., and Kopp, E.B. (1998) NF-κB and rel proteins: evolutionary conserve mediators of immune responses. *Annual Review of Immunology*. **16**, 225-260.

Giles, H., Leff, P., Bolofo, M.L., Kelly., M.G., and Robertson, A.D. (1989) The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *British Journal of Pharmacology* **96**, 291-300.

Gilmore, T.D. (2006) Introduction to NF-κB: players, pathways, perspectives. *Oncogene* **25(51)**, 6680-6684.

Gorman, C.M., Moffat, L.F., and Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Molecular Cellular Biology* **2**, 1044-1051.

University of Cape Town

Graupera, M., March, S., Engel, P., Rodes, J., Bosch, J., and Garcia-Pagan, J.C. (2004) Sinusoidal endothelial COX-1 dervied prostanoids modulate the hepatic vascular tone of cirrhotic rat livers. *American Journal of Gastointestinal Liver Physiology* **288**, G763-G770.

Green, A.R., Green, V.L., White, M.C., and Spiers, V. (1997) Expression of cytokine messenger RNA in normal and neoplastic human breast tissue: identification of interleukin-8 as a potential regulatory factor in breast tumours. *International Journal of Cancer* **72**, 937-941.

Greenhough, A., Smartt, H.J.M., Moore, A.E., Roberts, H.R., Williams, A.C., Paraskeva, C., and Kaidi, A. (2009) The COX/PGE_2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* **30(3)**, 377-386.

Grivennikov, S.I., and Karin, M. (2008) Autocrine IL-6 signalling: a key event in tumorigenesis?. *Cancer Cell* **13(1)**, 7-9.

Grivennikov, S.I., and Karin, M. (2009) Inflammation and oncongenesis: a vicious connection. *Current Opinion in Genetics and Development* **20**, 1-7.

Grivennikov, S.I., and Karin, M. (2010) Dangerous liasons: STAT3 and NF-κB collaboration and crosstalk in cancer. *Cytokine and Growth Factor Reviews* **21(1)**, 11-19.

Guan, Y., Zhang, Y., Breyer, R.M., Fowler, B., Davis, L., Hebert, R.L., and Breyer, M.D. (1998) Prostaglandin E2 inhibits renal collecting duct Na+ absorption by activating the EP1 receptor. *Journal of Clinical Investigation* **102**, 194-201.

Guidi, A.J., Abu-Jawdeh, G., Berse, B., Jackman, R.W., Tognazzi, K., Dvorak, H.F., and Brown, L.F. (1995) Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. *Journal of the National Cancer Institute* **87(16)**, 1237-1245.

Gupta, R.A., and DuBois, R.N. (2001) Colorectal cancer prevention and treatment by inhibiton of cyclooxgenase-2. *Nature Reviews Cancer* **1**, 11-21.

Gupta, R.A., Tejada, L.V., Tong, B.J., Das, S.K., Morrow, J.D., Dey, S.K., and DuBois, R.N., (2003) Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer. *Cancer Research* **63**, 906-911.

Gupta, V., Yeo, G., Kawakubo, H., Rangnekar, V., Ramaswamy, P., Hayashida, T., MacLaughlin, D.T., Donahoe, P.K., and Maheswaran, S. (2007) Mullerian-inhibiting substance induces Gro-β expression in breast cancer cells through a nuclear factor-κB-dependent and Smad1-dependent mechanism. *Cancer Research* **67**, 2747.

Gutsche, S., von Wolff, M., Strowitzki, T., and Thaler, C.J. (2003) Seminal plasma inducs mRNA expression of IL-β, IL-6 and LIF in endometrial epithelial cells in vitro. *Molecular Human Reproduction* **9**, 785-791.

University of Cape Town

Haas, T.L., Milkiewicz, M., Davis, S.J., Zhou, A.L., Eggington, S., Brown, M.D., Madri, J.A., and Hudlicka, O. (2000) Matrix metalloproteinase activity is required for activity-induced angiogenesis in rat skeletal muscle. *American Journal of Physiology. Heart and Circulation Physiology* **279(4)**, H1540-H1547.

Haghnegahdar, H., Du, J., Wang, D., Strieter, R.M., Burdick, M.D., Nanney, L.B., Cardwell, N., Luan, J., Shattuck-Brandt, R., and Richmond, A. (2000) The tumorigeniic and angiogenic effects of MGSA/GRO proteins in melanoma. *Journal of Leukocyte Biology* **67(1)**, 53-62.

Halliell, B. (1996) Antioxidant in human health and diseases. *Annual Review of Nutrition* **16**, 33-50.

Hammond, M.E., Shyamala, V., Siani, M.A., Gallegos, C.A., Feucht, P.H., Abbott, J., Lapointe, G.R., Moghadam, M., Khoja, H., Zakel, J., and Tekamp-Olson, P. (1996) Receptor recognition and specificity of interleukin-8 is determined by residues that cluster near a surface-accessible hydrophobic pocket. *Journal of Biological Chemistry* **271**, 8228-8235.

Han, C., and Wu, T. (2005) Cyclooxygenase-2-derived prostaglandin E_2 promotes human cholangiocarcinoma cell growth and invasion through EP_1 receptor-mediated activation of the epidermal growth factor receptor and Akt. *Journal of Biological Chemistry* **280**, 24053-24063.

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

Hanahan, D., and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86(3)**, 353-364.

Hanavadi, S., Martin, T.A., Watkins, G., Mansel, R.E., and Jiang, W.G. (2006) Expression of interleukin 11 and its receptor and their prognostic value in human breast cancer. *Annals of Surgical Oncology* **13(6)**, 802-808.

Handra-Luca, A., Bilal, H., Bertrand, J.C., and Fouret, P. (2003) Extra-cellular signal-regulated ERK-1/ERK-2 pathway activation in human salivary gland mucoepidermoid carcinoma. *American Journal of Pathology* **163**, 957-967.

Harlan, L.C., Bernstein, A.B., and Kessler, L.G. (1991) Cervical cancer screening: who is not screened and why ? *American Journal of Public Health* **81**, 885-890.

Harper, K.A., and Tyson-Capper, A.J. (2008) Complexity of COX-2 gene regulation. *Biochemical Society Transactions* **36**, 543-545.

Harris, C.C. (1996) p53 tumor suppressor gene: from the basic research laboratory to the clinic-an abridged historical perspective. *Carcinogenesis* **17**, 1187-1198.

Haskill, S., Peace, A., Morris, J., Sporn, S.A., Anisowicz, A., Lee, S.W., Smith, T., Martin, G., Ralph, P., and Sager, R. (1990) Identification of three related human GRO

University of Cape Town

genes encoding cytokine gene functions. *Proceedings of the National Academy of Sciences USA* **87(19)**, 7732-7736.

Hawk, E.T., Viner, J.L., Dannenberg, A., and DuBois, R.N. (2002) COX-2 in cancer – a player that's defining the rules. *Journal of the National Cancer Institute* **94(8)**, 545-546.

Hebert, R.L., O'Connor, T., Neville, C., Burns, K.D., Laneuville, O., and Peterson, L.N. (1998) Prostanoid signaling, localization and expression of IP receptors in rat thick ascending limb cells. *American Journal of Physiology and Renal Physiology* **275**, F904-F914.

Heinrich, P.C., Behrmann, I., Hann, S., Hermanns, H.M., Muller-Newen, G., and Schaper, F. (2003) Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochemical Journal* **374**, 1-20.

Herbert, J., and Coffin, J. (2008) Reducing patient risk for human papillomavirus infection and cervical cancer. *Jounral of the American Osteopathic Association* **108(2)**, 65-70.

Hernandez, G.L., Volpert, O.V., Iniguez, M.A., Lorenzo, E., Martinez-Martinez, E., Grau, R., Fresno, M., and Redondo, J.M. (2001) Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of the nuclear of the activated T cells and cyclooxygenase 2. *Journal of Experimental Medicine* **193**, 607-620.

Herschman, H.R. (1996) Prostaglandin synthase-2. *Biochimica et Biophysica Acta* **1299**, 125-140.

Heslin, , M.J., Cordon-Cardon, C., Lewis, J.J., Woodruff, J.M., and Brennan, M.F. (1988) Ki-67 detected by MIB-1 predicts distant metastasis and tumor mortality in primary, high grade extremity soft tissue sarcoma. *Cancer* **83**, 490-497.

Hinson, R.M., Williams, J.A., and Shacter, E. (1996) Elevated interleukin 6 is induced by prostaglandin E_2 in a murine model of inflammation. *Proceedings of the National Academy of Sciences USA* **93**, 4885-4890.

Hirano, T., Taga, T., Nakano, N., Yasukawa, K., Kashiwamura, S., Shimuzi, K., Nakajima, K., Pyun, K.H., and Kishimoto T. (1985) Purification to homogeneity and characterization of human B-cell differentiation factor (BCDF or BSF-2). *Proceedings of the National Academy of Sciences USA* **85**, 5490-5494..

Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S., and Narumiya, S. (1991) Cloning and expression of cDNA for a human thromboxane A_2 receptor. *Nature* **349**, 617-620.

Hirata, M., Kakazuka, A., Aizawa, M., Ushikubi, F., and Narumiya, S. (1994) Molecular characterization of a mouse prostaglandin D receptor and functional

University of Cape Town

expression of the cloned gene. *Proceedings of the National Academy of Sciences USA* **91**, 11192-11196.

Hirata, T., Kakizuka, A., Usfhikubi, F., Fuse, I., Okuma, M., and Narumiya, S. (1994). Arg^{60} to Leu mutation of the human thromboxane receptor in a dominantly inherited bleeding disorder. *Journal of Clinical Investigation* **94**, 1662-1667.

Hizaki, H., Segi, E., Sugimoto, Y., Hirose, M., Saji, T., Ushikubi, F., Matsuoka, T., Noda, Y., Tanaka, T., Yoshida, N., Narumiya, S., and Ichikawa, A. (1999) Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). *Proceedings of the National Academy of Sciences USA* **96**, 10501-10506.

Hladik, F., and McElrath, J.M. (2008) Setting the stage: a host invasion by HIV. *Nature Reviews of Immunology* **8**, 447-457.

Ho, G.Y., Kadish, A.S., Burk, R.D., Basu, J., Palan, P.R., Mikhail, M., and Romney, S.L. (1998) HPV-16 and cigarette smoking as risk factors for high-grade cervical intra-epithelial neoplasia. *International Journal of Cancer* **78(3)**, 281-285.

Hoffmann, A., Natoli, G., and Ghosh, G. (2006) Transcriptional regulation via the NFκB signalling module. *Oncogene* **25**, 6706-6716.

Holm, R., Kraus, I., Skomedal, H., Langerod, A., Kristensen, G.B., and Lyng, H. (2008) Human papillomavirus DNA and E6/E7 mRNA staus in relation to survival of patients treated fro cervical squamous cell carcinoma. *The Open Virology Journal* **2**, 74-81.

Holmes, K., Roberts, O.L., Thomas, A.M., and Cross, M.J. (2007). Vascular endothelial growth factor-2: structure, function, intracellular signalling and therapeutic inhibition. *Cellular Signalling* **19(10)**, 2003-2012.

Holmes, W.E., Lee, J., Kuang,W.J., Rice, G.C., and Wood, W.I. (1991) Structure and functional expression of a human interleukin-8 receptor. *Science* **253**, 1278-1280.

Honda, A., Sugimoto, Y., Namba, T., Watabe, A., Irie, A., Neigishi, M., Narumiya, S., and Ichikawa, A. (1993) Cloning and expression of a cDNA for mouse protaglandin E receptor EP_2 subtype. *Journal of Biological Chemistry* **268**, 7759-7762.

Hongyu, L., Hongling, S., Qian, X., Dongrui, D., Shixuan, W., Yunping, L., and Ding, M. (2006) Expression of pin1 nd Ki67 in cervical cancer and their significance. *Journal of Huazhong University of Science and Technology* **26(1)**, 120-122.

Horak, E.R., Leek, R., Klenk, N., LeJeune, S., Smith, K., Stuart, N., Greenall, M., Stepniewska, K., and Harris, A.L. (1992) Angiogenesis, assessed by platelet/endothelail cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet* **340**, 1120-1124.

University of Cape Town

Hoshiya, Y., Gupta, V., Segev, D.L., Hoshiya, M., Carey, J.L., Sasur, L.M., Tran, T.T., Ha, T.U., and Maheswaran, S. (2003) *Molecular and Cellular Endocrinology* **211(1-2)**, 605-617.

Howlett, M., Judd, L.M., Jenkins, B., La Gruta, N.L., Grail, D., Ernst, M., and Giraud, A.S. (2005) Differential regulation of gastric tumor growth by cytokines that signal exclusively through the corecptor gp130. *Gastroenterology* **129(3)**, 1005-1018.

Howley, P.M., and Lowy, D.R. (2007) Papillomaviruses. In Knipe, D.M., Howley, P.H., (ed) Fields Virology, 5th ed, vol 2. Philadelphia: Lippincott Williams and Wilkins, 2299-2354.

Huang, J.C., Wun, W.S.A., Goldsby, J.S., Egan, K., FitzGerald, G.A., and Wu, K.K. (2007) Prostacyclin receptor signalling and early embryo development in the mouse. *Human Reproduction* **22(11)**, 2851-2856.

Huang, X.Z.,Wu, J.F., Cass, D., Erie, D.J., Corry, D., Young, S.G., Farese (Jr.), R.V., and Sheppard, D. (1996) Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin. *Journal of Cellular Biology* **133**, 921-928.

Huber, A.R., Kunkel, S.L., Todd, R.F., and Weiss, S.J. (1991) Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science* **254**, 99-102.

Hwang, D., Scollard, D., Byrne, J., and Levine, E. (1998) Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. *Journal of the National Cancer Institute* **90**, 455-460.

Iida, N., and Grotendorst, G.R. (1990) Cloning and sequencing of a new gro transcript from activated human monocytes: expression in leukocytes and wound tissue. *Molecular Cellular Biology* **10(10)**, 5596-5599.

Iked, S., Yamada, T., Katsumata, N., Hida, K., Tanemura, K., Tsunematu, R., Ohmi, K., Sonoda, T., Ikeda, H., and Nomura, K. (1998) Cerebral metastasis in patients with uterine cervical cancer. *Japanese Journal of Clinical Oncology* **28(1)**, 27-29.

Imade, G.E., Baker, H.W.G., de Krester, D.M., and Hedger, M.P. (1997) Immunosuppressive activities in the seminal plasma of infertile men: relationship to sperm antibodies and autoimmunity. *Human Reproduction* **12(2)**, 256-262.

Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. (1995) Transcriptional regulation of human prostaglandin endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *Journal of Biological Chemistry* **270**, 24965-24971.

University of Cape Town

International collaboration of epidemiological studies of cervical cancer, (2007) Comparison of risk factors for invasive squamous cell carcinoma and adenocarcinoma of the cervix: collaborative reanalysis of individual data on 8097 women with adenocarcinoma from 12 epidemiological studies. *International Journal of Cancer* **120**, 885-891.

Jabbour, H.N., Milne, S.A., Williams, A.R.W., Anderson, R.A., and Boddy, S.C. (2001) Expression of COX-2 and PGE synthase and synthesis of PGE_2 in endometrial adenocarcinoma: a possible autocrine/paracrine regulation of neoplastic cell function via the EP2/EP4 receptors. *British Journal of Cancer* **85,** 1023-1031.

Jabbour, H.N., and Sales, K.J. (2004) Prostaglandin receptor signalling and function in endometrial pathology. *Trends in Endocrinology and Metabolism* **15(8)**, 398-404.

Jabbour, H.N. Sales, K.J., Smith, O.P., Battersby, S., and Boddy, S.C. (2006) Prostaglandin receptors are mediators of vascualr function in endometrial patholiges. *Molecular Cell Endocrinology* **252**, 191-200.

Jackson, C.B., Judd, L.M., Menheniott, T.R., Kronborg, I., Dow, C., Yeomans, N.D., Boussioutas, A., Robb, L., and Giraud, A.S. (2007) Augmented gp130-mediated cytokine signalling accompanies human gastric cancer progression. *Journal of Pathology* **213(2)**, 140-151.

Jackson, L.M., Wu, K.C., Jenkins, D., and Hawkey, C.J. (2000) Cyclooxygenase (COX) 1 and 2 in normal, inflamed and ucelrated human gastric mucosa. *Gut* **47**, 762-770.

Jakowlew, S.B. (2006) Transforming growth factor-beta in cancer and metastasis. *Cancer and Metastasis Reviews* **25** 435-457.

James, K., and Hargreave, T.B. (1984) Immunosuppression by seminal plasma and its possible clinical significance. *Immunology Today* **5**, 357-363.

Jang, M., Cai, L., Udeani, G.O., Slowing, K.V., Thomas, C.F., Beecher, C.W., Fong, H.H., Farnsworth, N.R., Kinghorn, A.D., Mehta, R.G., Moon, R.C., and Pezzuto, J.M. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**, 218-220.

Jenkins, B.J., Grail, D., Nheu, T., Najdovska, M., Wang, B., Waring, P., Inglese, M., McLoughlin, R.M., Jones, S.A., Topley, N., Baumann, H., Judd, L.M., Giraud, A.S., Boussioutas, A., Zhu., H.J., and Ernst, M. (2005) Hyperactivation of Stat3 in gp130 mutant mice promotes gastric hyperproliferation and densensitizes TGF-beta signalling. *Nature Medicine* **11(8)**, 845-852.

Jeremias, J., David, S.S., Toth, M., and Witkin, S.S. (1997) Induction of messenger RNA for the 70kDa heat shock protein in HeLa cells and the human endocervix following exposure to semen: implications for antisperm antibody production and susceptibility to sexually transmitted infections. *Human Reproduction* **12**, 1915-1919.

University of Cape Town

Jeremias, J., and Witkin, S.S. (1999) Effect of human seminal fluid on production of messenger ribonucleic acid for the metalloproteinase 2 and metalloproteinase 9 in cervical epithelial carcinoma cells. *American Journal of Obstetrics and Gynecology* **181**, 591-595.

Jimenez, P., Piazuelo, E., Cebrian C., Ortego, J., Strunk, M., Garcia-Gonzalez, A., Santander, S., Alcedo, J., and Lanas, A. (2010) Prostaglandin EP2 receptor expression is increased in Barrett's oesophagus and oesophageal adenocarcinoma. *Alimentary Pharmacology and Therapeutics* **31(3)**, 440-451.

Jones, R.L. and Wilson, N.H. (1990) An EP_3-receptor may mediate prostaglandin E-indcued potentiation of aggregation in human platelets. *British Journal of Pharmacology* **101**, 522.

Jones, M.K., Wang, H., Peskar, B.M., Levin, E., Itani, R.M., Sarfeh, I.J., and Tarnawski, A.S. (1999) Inhibition of angiogenesis by non-steroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Journal of the National Medical Association.* **5**, 1418-1423.

Joo, J.K., Lee, D.H., Kim, K.H., Na, Y.J., Lee, C.H., and Yoon, M.S. (2005) Relationship between the expression of COX-1, COX-2, p53 and VEGF and prognostic factors in squamous cell carcinoma of uterine cervix. *Korean Journal of Obstetrics and Gynecology* **48(11)**, 2661-2669.

Jost, M., Kari, C., and Rodeck, U. (2000) The EGF receptor – an essential regulator of multiple epidermal functions. *European Journal of Dermatology* **10**, 505-510.

Jung, Y.J., Isaacs, J.S., Lee, S., Trepel, J., and Neckers, L. (2003) IL-1β mediated up-regulation of HIF-1α via an NFκB/COX -2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *The FASEB Journal* 10.1096/fj.03-0329fje.

Kang, Y.J., He, W., Tulley, S., Gupta, G.P., Serganova, I., Chen, C.R., Manova-Todrova, K., Blasberg, R., Gerald, W.L., and Massague, J. (2005) Breast cancer bone metastasis mediated by the Smad tumour suppressor pathway. *Proceedings of the National Academy of Sciences USA* **102(39)**, 13909-13914.

Kang, Y.J., Mbonye, U.R., DeLong, C.J., Wada, M., and Smith, W.L. (2007) Regulation of intracellular cyclooxygenase levels by gene transcription and protein degradation. *Progress in Lipid Research* **46**, 108-125.

Karin, M. (2006) NF-κB and cancer: mechanisms and targets. *Molecular Carcinogenesis* **45**, 355-361.

Karl, E., Warner, K., Zeitlin, B., Kaneko, T., Wurtzel, L., and Jin, T. (2005) Bcl-2 acts in a pro-angiogenic signaling pathway through nuclear factor-κB and CXC chemokines. *Cancer Research* **65**, 5036-5039.

University of Cape Town

Karstrom-Encrantz, L., Runesson, E., Bostrom, E.K., and Brannstrom, M. (1998) Selective presence of the chemokine growth-regulated oncogene alpha (GROalpha) in the human follicle and secretion from cultured granulose-lutein cells at ovulation. *Molecular Human Reproduction* **4(11)**, 1077-1083.

Kawashima, I., Ohsumi, J., Mita-Honjo, K., Shimoda-Takano, K., Ishikawa, H., Sakakibara, S., Miyadai, K., and Takiguchi, Y., (1991) Molecular cloning of cDNA encoding adipogenesis inhibitory factor and identity with interleukin-11. *FEBS Letters* **283(2)**, 199-202.

Kawashima, I., Ohsumi, J., Miyadai, K., and Takiguchi, Y. (1992) Function, molecular structure and gene expression of interleukin-11 (IL-11/AGIF). *Nippon Risho* **50(8)** , 1833-1839.

Keatings, V.M., Collins, P.D., Scott, D.M., and Barnes, P.J. (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *American Journal of Respiratory and Critical Care Medicine* **153(2)**, 530-534.

Keightley, M.C., Brown, P., Jabbour, H.N., and Sales, K.J. (2010) F-prostaglandin receptor regulates endothelial cell function via fibroblast growth factor-2. *BMC Cell Biology* **11**, 8.

Kelly, R.W. (1995) Immunosuppressive mechanisms in semen: implications for contraception. *Human Reproduction* **10**, 1686-1693.

Kelly, R.W., King, A.E., and Crichtley, H.O.D. (2001) Cytokine control in human endometrium. *Reproduction* **121**, 3-19.

Kennedy, C.R., Zhang, Y., Brandon, S., Guan, Y., Coffee, K., Funk, C.D., Magnuson, M.A., Oates, J.A., Breyer, M.D., and Breyer, R.M. (1999) Salt-sensitive hypertension and reduced fertility in mice lacking the prostaglandin EP2 receptor. *Nature Medicine* **5**, 217-220.

Kent, A. (2010) HPV vaccination and testing. *Reviews in Obstetrics and Gynaecology* **3(1)**, 33-34.

Kerbel, R.S. (1993) Growth factors as mediators of malignant tumor progression. *Cancer and Metastasis Reviews* **12**, 215-217.

Khadra, A., Fletcher, P., Luzzi, G., Shattock, R., and Hay, P. (2006) Interleukin-8 levels in seminal plasma in chronic prostatitis/chronic pelvic pain syndrome and nonspecific urethritis. *BJU International* **97(5)**, 1043-1046.

Kim, J.J., and Goldie, S.J. (2008) Health and economic implications of HPV vaccination in the United States. *New England Journal of Medicine* **359**, 821-832.

University of Cape Town

Kim, S., Lewis, C., and Nadel, J.A. (2010) PGE2/EP3 receptor feedback exaggerates IL-8 production via EGFR reactivation. *American Journal of Respiratory and Critical Care Medicine* **181**, A2058.

Kim, Y.M., Lee, E.J., Park, S.Y., Cho, K.H., Kim, J.Y., and Pyo, H. (2009) Cyclo-oxygenase-2 up-regulates ataxia telangiectasia and Rad3 related through extracellular signal-regulated kinase activation. *Molecular Cancer Research* **7(7)**, 1158-1168.

Kino, Y., Kojima, F., Kiguchi, K., Igarashi, R., Ishizuka, B., and Kawai, S. (2005) Prostaglandin E_2 production in ovarian cancer cell lines is regulated by cyclooygenase-1, not cyclooxygenase-2. *Prostaglandins, Leukotrines and Essential Fatty Acids* **73(2)**, 103-111.

Kinsella, B.T., O'Mahony, D.J., and Fitzgerald, G.A. (1997) The human thromboxane A_2 receptor α isoform (TPα) functionally couples to the G proteins G_q and G_{11} in vivo and is activated by the isoprostane 8-epi prostaglandin $F_{2\alpha}$. *Journal of Pharmacology and Experimental Therapeutics* **281(2)**, 957-964.

Kinzler, K.W., and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell* **87**, 159-170.

Kirnbauer, R., Chandrachud, L., O'Neil, B.W., Wagner, E.R., Grindlay, G.J., Armstrong, A., McGarvie, G.M., Schiller, J.T., Lowy, D.R., and Campo, M.S. (1996) Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. *Virology* **219(1)**, 37-44.

Kirschbaum, N.E., Gumina, R.J., and Newman, P.J. (1994) Organization of the gene for the human platelet/endothelial cell adhesion molecule-1 shows alternatively spliced isoforms and a functionally complex cytoplasmic domain. *Blood* **84**, 4028-4037.

Kis, B., Snipes, J.A., and Busija, D.W. (2005) Acetaminophen and the cyclooxygenase-3 puzzle: sorting out facts, fiction and uncertainties. *Journal of Pharmacology* **315(1)**, 1-7.

Kiyici, A., Ibis, M., Akbulut, S., Koklu, S., Ucar, E., and Unlu, A. (2009) Serum and TNF-alpha levels in acute and chronic pancreatitis. *European Journal of Medicine* **6(2)**, 103-107.

Klenke, F.M., Gebhard, M.M., Ewerbeck, V., Abdollahi, A., Huber, P.E., and Sckell, A. (2006) The selective COX-2 inhibitor celecoxib suppresses angiogenesis and growth of secondary bone tumors: an intravital microscopy study in mice. *BMC Cancer* **6**, 9.

Kobayashi, T., and Narumiya, S. (2002) Function of prostanoid receptors: studies on knockout mice. *Prostaglandins Other Lipid Mediators* **68-69**, 557-573.

Kraemer, S.A., Meade, S.A., and DeWitt, D.L. (1992) Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5'-flanking regulatory sequences. *Arch. Biochem. Biophys* **293**, 391-400.

University of Cape Town

Kristensen, G.B., Holund, B., and Grinsted, P. (1989) Efficacy of the cytobrush versus the cotton swab in the collection of endocervical cells. *Acta Cytologica* **33(6)**, 849-851.

Krogan-Sakin, I., Cohen, M., Paland, N., Madar, S., Solomon, H., Molchadsky, A., Brosh, R., Buganim, Y., Goldfinger, N., Klocker, H., Schalken, J.A., and Rotter, V. (2009) Prostate stromal cells produce CXCL-1, CXCL-2, CXCL-3 and IL-8 in response to epithelia-secreted IL-1. *Carcinogenesis* **30(4)**, 698-705.

Kruse, A.J., Baak, J.P., de Bruin, P.C. van de Goot, F.R.W., and Kruten, N. (2001) Relationship between the presence of oncogenic HPV DNA assessed by polymerase chain reaction and Ki-67 immunoquantitative features in cervical intraepithelial neoplasia. *Journal of Pathology* **195**, 557-562.

Krysan, K., Lee, J.M., Dohadwala, M., Gardner, B.K., Reckamp, K.L., Garon, E., John, M.S., Sharma, S., and Dubinett, S.M. (2008) Inflammation, epithelial to mesenchymal transistion, and epidermal growth factor receptor tyrosine kinase inhibitor resistance. *Journal of Thoracic Oncology* **3(2)**, 107-110.

Kubo, H., Hosono, K., Suzuki, T., Ogawa, Y., Kato, H., Kamata, H., Ito, Y., Amano, H., Kato, T., Sakagami, H., Hayashi, I., Sugimoto, Y., Narumiya, S., Watanabe, M., and Majima, M. (2010) Host prostaglandin EP3 receptor signaling relevant to tumor-associated lymphangiogenesis. *Biomedicine and Pharmacotherapy* **64(2)**, 101-106.

Kulkarni, S., Rader, J.S., Zhang, F., Liapis, H., Koki, A.T., Masferrer, J.L., Subbaramaiah, K., and Dannenburg, A.J. (2001) Cyclooxygenase-2 is overexpressed in human cervical cancer. *Clinical Cancer Research* **7**, 429-434.

Kumar, L., Pokharel, Y.H., Dawar, R., and Thulkar, S. (1999) Cervical cancer metastatic to the breast: a case report and review of the literature. *Clinical Oncology* **11(6)**, 414-416.

Kundu N., and Fulton, A.M., (2002) Selective cyclooxygenase (COX)-1 or COX-2 inhibitors control metastatic disease in a murine model of breast cancer. *Cancer Research* **62**, 2343-2346.

Kuper, H., Adam, H.O., and Trichopolous, D. (2000) Infections as a major preventable cause of human cancer. *Journal of Internal Medicine* **248**, 171-183.

Lagunas-Martinez, A., Madrid-Marina, V., and Gariglio, P. (2010) Modulation of apoptosis by early human papillomavirus proteins in cervical cancer. *Biochimica et Biophysica Acta (BBA) – Reviews on Cancer* **1805(1)**, 6-16.

Lampiasi, N., Fodera, D., D'Alessandro, N., Cusimano, A., Azzolina, A., Tripodo, C., Florena, A.D., Minervini, M.I., Notarbartolo, M., Montalto, G., and Cervello, M. (2005) The selective cyclooxygenase-1 inhibitor SC-560 suppresses cell proliferation and induces apoptosis in human hepatocellular carcinoma cells. *International Journal of Molecular Medicine* **17**, 245-252.

University of Cape Town

Lands, W.E.M., and Hanel A.M. (1983) Inhibitors and activators of prostaglandin biosynthesis. In: C. Pace-Asciak and E. Granstrom (eds.), prostaglandins and related substances. Amsterdam: Elsievier/North-Holland Biomedical Press, p 203-223.

Lane, B.R., Liu, J., Bock, P.J., Schols, D., Coffey, M.J., Strieter, R.M., Polverini, P.J., and Markovitz, D.M. (2002) Interleukin-8 and growth-regulated oncogene alpha mediate angiogenesis in Kaposi's sarcoma. *Journal of Virology* **76**, 11570-11583.

Langenbach, R., Morham, S.G., Tiano, S.F., Loftin, C.D., Ghanayem, B.I., Chulada, P.C., Mahler, J.F., Lee, C.A., Goulding, E.H., Kluckman, K.D., Kim, H.S., and Smithies, O. (1995) Prostaglandin synthase I gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. *Cell* **83**, 438-492.

Langenbach, R., Loftin, C, Lee, C., and Tiano, H., (1999) Cyclooxygenase knockout mice. *Biochemical Pharmacology* **58**, 1237-1246.

Lash, G.E., Otun, H.A., Innes, B.A., Bulmer, J.N., Searle, R.F., and Robson, S.C. (2005) Inhibition of trophoblast cell invasion by TGFB1, 2, and 3 is associated with a decrease in active proteases. *Biology of Reproduction* **73**, 374-381.

Lawrence, D.A. (1996) Transforming growth factor-beta: a general review. *European Cytokine Network* **7**, 363-374.

Lawrence, R.A., Jones, R.L., and Wilson, N.H. (1992) Characterization of receptors involved in the direct and indirect actions of prostaglandins E and I on the guinea-pig-ileum. *British Journal of Pharmacology* **105**, 271-278.

Lee, G., and Ng, H.T. (1995) Clinical evaluations of a new ovarian cancer marker, COX-1. *International Journal of Gynecology and Obstetrics* **49** (supplement 1), S27-S32.

Lee, C.M., Fuhrman, C.B., Planelles, V., Peltier, M.R., Gaffney, D.K., Soisson, A.P., Dodson, M.K., Tolley, H.D., Green, C.L., and Zempolich, K.A. (2006) Phosphatidylinositol 3-kinase inhibition by LY294002 radiosensitizes human cervical cancer lines. *Clinical Cancer Research* **12**, 250.

Lee, Z., Swaby, R.F., Liang, Y., Yu, S., Liu, S., Lu, K.H., Bast, R.C., Mills, G.B., and Fang, X. (2006) Lysophosphatidic acid is a major regulator of growth-regulated oncogene α in ovarian cancer. *Cancer Research* **66**, 2740.

Leung, T.W., Xue, W.C., Cheung, A.N.Y., Khoo, U., and Ngan, H.Y.S. (2004) Proliferation to apoptosis ratio as a prognostic marker in adenocarcinoma of uterine cervix. *Gynecologic Oncology* **92**, 866-872.

Levy, D.E., and Darnell, J.E. (Jr) (2002) Stats: transcriptional control and biological impact. *Nature Reviews Molecular Cell Biology* **3**, 651-662.

University of Cape Town

Lewis, T.S., Shapiro, P.S., and Ahn, N.G. (1998) Signal transduction through MAP kinase cascades. *Advances in Cancer Research* **74**, 49-139.

Li, A., King, J.C., Eibl, G., Sugi, M., Strieter, R.M., Moro, A., Reber, H.A., and Hines, O.J. (2009) Dual inhibition of CXCR2 and VEGFR2 blocks pancreatic cancer growth. *Journal of Surgical Research* **151(2)**, 196.

Liabakk, N., Lien, E., Sundan, A., Sunde, A., Austgulen, R., and Espevik, T. (1993) Immunology: high concentrations of the soluble p55 tumour necrosis factor receptor in human seminal plasma. *European Society of Human Reproduction and Embryology* **8(11)**, 1837-1842.

Liang, Y., Brekken, R.A., and Hyder, S.M. (2006) Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones. *Endocrine-Related Cancer* **13(3)**, 905-919.

Lim, H., Paria, B.C., Das, S.K., Dinchuk, J.E., Langenbach, R., Trzaskos, J.M., and Dey, S.K. (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91**, 197-208.

Lin, D.L., Whitney, M.C., Yao, Z., and Keller, E.T. (2001) Interleukin-6 induces androgen responsiveness in prostate cancer cells through the up-regulation of androgen receptor expression. *Clinical Cancer Research* **7(6)**, 1773-1781.

Lin, Y.Z., Yao, S.Y., Veach, R.A., Torgerson, T.R., and Hawiger, J. (1995) Inhibition of nuclear translocation factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localisation sequence. *Journal of Biological Chemistry* **270**, 14255.

Lissak, A., Wiener-Megnazi, Z., Reznick, A.Z., Shnizerm, S., Ishai, D., Grach, B., Lahav-Baratz, S., Shiloh, H., Koifman, M., and Dirnfeld, M. (2004) Oxidative stress indices in seminal plasma, as measured by the thermochemiluminescence assay, correlate with sperm parameters. *Fertility and Sterility* **81**(supplement1), 792-797.

Lloyd, R.V., Vidal, S., Horvath, E., Kovacs, K., and Scheithauer, B. (2003) Angiogenesis in normal and neoplastic pituitary tissues. *Microscopy Research and Technique* **60(2)**, 244-250.

Looi, M.L., Dali, A.Z., Ali, S.A., Ngah, W.Z., and Yusof, Y.A. (2008) Expression of p53, bcl-2 and Ki-67 in cervical intraepithelial neoplasia and invasive squamous cell carcinoma of the uterine cervix. *Analytical and Quantitative Cytology and Histology* **30(2)**, 63-70.

Louttit, J.B., Head, S.A., and Coleman, R.A. (1992) Prostanoid EP-receptors in pig saphenous vein. *In Proceedings of 8th International Conference in Prostaglandins and Related Compounds* 257p.

University of Cape Town

Lowy, D.R., Kirnbauer, R., and Schiller, J. (1994) Genital human papillomavirus infection. *Proceedings of the National Academy of Sciences USA* **91,** 2436-2440.
Lowy, D.R., and Schiller J.T. (2006) Prophylactic human papillomavirus vaccines. *Journal of Clinical Investigation* **116**, 1167-1173.

Lowy, D.R., Solomon, D., Hildesheim, A., Schiller, J.T., and Schiffman, M. (2008) Human papillomavirus infection and the primary and secondary prevention of cervical cancer. *Cancer* (supplement) **113(7)**, 1980-1993.

Luan, J., Shattuck-Brandt, R., Haghnegahdar, H., Owen, J.D., Strieter, R., Burdick, M., Nirodi, C., Beauchamp, D., Johnson. K.N., and Richmond, A. (1997) Mechanism and biological significance of constitutive expression of MGSA/GRO chemokines in malignant melanoma tumour progression. *Journal of Leukocyte Biology* **62**, 588-597.

Luboshits, G., Shina, S., Kaplan, O., Engelberg, S., Nass, D., Lifshitz-Mercer, B., Chaitchik, S., Keydar, I., and Ben-Brauch, A. (1999) Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. *Cancer Research* **59**, 4681-4687.

Luff, R.D. (1992) The Bethesda System for reporting cervical/vaginal cytologic diagnoses: report of the 1991 worskshop. The Bethesda System Editorial Committee. *Human Pathology* **23**, 719-721.

Luppi, F., Longo, A.M., de Boer, W.I., Rabe, K.F., and Hiemstra, P.S. (2007) Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation. *Lung Cancer* **56**, 25-33.

Ma, X., Kundu, N., Rifat, S., Walser, T., and Fulton, A.M. (2006) Prostaglandin E receptor EP4 antagonism inhibits breast cancer metastasis. *Cancer Research* **66**, 2923-2927.

MacDonald, T.T. (2010) Cytokine regulation of intestinal epithelial cell proliferation. *Expert Review of Clinical Immunology* **6(4)**, 531-535.

Malik, A.I., (2005) The role of human papilloma virus (HPV) in the aetiology of cervical cancer. *Journal of the Pakistan Medical Association* **55(12)**, 553-558.

Marnett, L.J. (1992) Aspirin and the potential of prostaglandins in colon cancer. *Cancer Research* **52**, 5575-5589.

Martin-Hirsch, P.L., Paraskevaidis, E., and Kitchener, H. (2000) Surgery for cervical intraepithelial neoplasia. *Cochrane Database System Reviews* **2**, CD001318.

Massague, J., Blain, S.W., and Lo, R.S. (2000) TGFbeta signalling in growth control, cancer, and heritable disorders. *Cell* **103**, 295-309.

Matalliotakis, I., Goumenou, A., Koumantakis, E., Kyriakou, D., Fragouli, Y., and Loutradis, D. (1998) Detemination of interleukin-11 in seminal plasma and elevated

University of Cape Town

IL-11 in seminal plasma of infertile patients with urogenital infection. *System Biology in Reproductive Medicine* **41(3)**, 177-183.

Mathur, R.S., and Mathur, S.P. (2004) Vascular endothelial growth factor (VEGF) up-regulates epidermal growth factor (EGF-R) in cervical cancer in vitro: this action is mediated through HPV-E6 in HPV-positive cancers. *Gynecologic Oncology* **97(1)**, 206-213.

Matsuoka, T., Adair, J.E., Lih, F.B., Hsi, L.C., Rubino, M., Eling, T.E., Tomer, K.B., Yashiro, M., Hirakawa, K., Olden, K., and Roberts, J.D. (2010) Elevated dietary linoleic acid increases gastric carcinoma cell invasion and metastasis in mice. *British Journal of Cancer* **103**, 1182-1191.

Matsushima, K., Morishita, K., Yoshimura, T., Lavu, S., Kobayashi, Y., Lew, W., Appella, E., Kung, H.F., Leonard, E.J., and Oppenheim, J.J. (1988) Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDCNF) and the induction of MDCNF mRNA by interleukin-1 and tumor necrosis factor. *Journal of Experimental Medicine* **167**, 1883-1893.

May, M.J., and Ghosh, S. (1999) Signal transduction through NF-κB kinases: kinsmen with different crafts. *Science* **284**, 271-273.

Mayeaux, E.J., (2008) Reducing the economic burden of HPV-related diseases. *Journal of the American Osteopathic Association* **108(4)** (supplement 2), 2-7.

Mayer, B., Johnson, J.P., Letil, F., Jauch, K.W., Heiss, M.M., Schildberg, F.W., Birchmeier, W., and Funke, I. (1993) E-cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Res*earch **53**, 1690-1695.

Mazhar, D., Gillmore, P., and Waxman, J. (2005) COX and cancer. *QJM: An International Journal of Medicine* **98**, 711-718.

Mazibrada J., Ritta, M., Mondini, M., Andrea, M.D., Borgogna, C., Ciotti, M., Orlando, A., Surico, N., Chiusa, L., Landolfo, S., and Gariglio, M. (2008) Interaction between inflammation and angiogenesis during different stages of cervical carcinogenesis. *Gynecologic Oncology* **108**, 112-120.

McKay, M.M., and Morrison, D.K. (2007) Integrating signals from RTKs to ERK/MAPK. *Oncogene* **26**, 3113-3121.

McKinley, D., Wu, Q., Yang-Feng, T., and Yang, Y.C. (1992) Genomic sequence and chromosomal location of human interleukin-11 gene (IL-11). *Genomics* **13(3)**, 814-819.

McMaster, M.C., Newton, R.C., Dey, S.K., and Andrews, G.K. (1992) Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. *Journal of Immunology* **148**, 1699-1705.

University of Cape Town

McMichael, A.J., Beverley, P.C.L., Cobbold, S., Crumpton, M.J., Gills, W., Gotch, F.M., Hogg, N., Horton, M., Ling, N., MacLennan, I.C.M., Mason, D.Y., Milstein, C., Spiegelhalter, D., and Waldmann, S.H. (1987) Leukocyte typing III. White cell differentiation antigens. *Oxford University Press, Oxford* 589-590.

Melbye, M., Rabkin, C., Frisch, M., and Biggar, R.J. (1994) Changing patterns of anal cancer incidence in the United States. *American Journal of Epidemiology* **139**, 772-780.

Merchant, N.B., Rogers, C.M., Trivedi, B., Morrow, J., and Coffey, R.J. (2005) Ligand-dependent activation of the epidermal growth factor receptor by secondary bile acids in polarizing colon cancer cells. *Surgery* **138(3)**, 415-421.
Merchant, J.L. (2008) What lurks beneath: IL-11, via Stat3, promotes inflammation-associated gastric tumorigenesis. *Journal of Clinical Investigation* **118(5)**, 1628-1631.

Milling-Smith, O.P., Battersby, S., Sales, K.J., Critchley, H.O.D., and Jabbour, H.N. (2006) Prostacyclin receptor up-regulates the expression of angiogenic genes in human endometrium via cross talk with epidermal growth factor receptor and the extracellular signalling receptor kinase 1/2 pathway. *Endocrinology* **1474**, 1697-1705.

Misra, J.S., Gupta, H.P., and Das, V. (2004) Assessing the feasibility of single lifetime PAP smear evaluation between 41-50 years of age as strategy for cervical cancer control in developing countries from our 32 years experience of hospital-based routine cytological screening. *Diagnostic Cytopathology* **31(6)**, 376-379.

Mock, B.A., Nordan, R.P., Justice, M.J., Kozak, C., Jenkins, N.A., Copeland, N.G., Clark, S.C., Wong, G., and Rudikoff, S. (1989) The murine IL-6 gene maps to the proximal region of chromosome 5. *Journal of Immunology* **142**, 1372-1376.

Modi, W.S., Dean, M., Seuanez, H.N., Mukaida, N., Matsushima, K., and O'Brien, S.J. (1990) Monocyte-derived neutrophil chemotactic factor (MDNCF/IL-8) resides in a gene cluster along with several other members of the platelet factor 4 gene superfamily. *Human Genetics* **84(2)**, 185-187.

Mora, N., Rosales, R., and Rosales, C. (2007) R-Ras promotes metastasis of cervical cancer epithelial cells. *Cancer Immunology Immunotherapy* **56(4)**, 535-544.

Morham, S.G., Langenbach, R., Loftin, C.D., Tiano, H.F., Vouloumanos, N., Jennette, J.C., Mahler, J.F., Kluckman, K.D., Ledford, A., Lee, C.A., and Smithies, O. (1995) Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* **83**, 473-482.

Morita, I. (2002) Distinct functions of COX-1 and COX-2. *Prostaglandins and Other Lipid Mediators* **68-69**, 165-175.

Moser, B., Clark-Lewis, I., Zwahlen, R., and Baggiolini, M. (1990) Neutrophil-activating properties of the melanoma growth-stimulatory activity. *Journal of Experimental Medicine* **171(5)**, 1797-1802.

University of Cape Town

Moser, P.L., Kieback, D.G., Hefler, L., Temper, C., Neunteufel, W., and Gitsch, G. (1999) Immunohistochemical detection of matrix metalloproteinases (MMP) 1 and 2, and tissue inhibitor of metalloproteinase 2 (TIMP2) in stage 1B cervical cancer. *Anticancer Research* **19**, 4391-4393.

Motoyama S., Ladines-Llave, C.A., Luis-Villaneuva, S., and Maruo, T. (2004) The role of human papilloma virus in the molecular biology of cervical carcinogenesis. *Kobe Journal of Medical Science* **50**, 9-19.

Moustakas, A., Pardali, K., Gaal, A., and Heldin, C.H. (2002) Mechanisms of TGF-β signaling in regulation of cell growth and differentiation. *Immunology Letters* **82**, 85-91.

Mowa, C.N., Li, T., Jesmin, S., Folkesson, H.G., Usip, S.E., Papka, R.E., Hou, G. (2008) Delineation of VEGF-regulated genes and functions in the cervix of pregnant rodents by DNA microarray analysis. *Reproductive Biology and Endocrinology* **6**, 64.

Muller, M., Sales, K.J., Katz, A.A., and Jabbour, H.N. (2006) Seminal plasma promotes the expression of tumorigenic and angiogenic genes in cervical adenocarcinoma cells via the E-series prostanoid 4 receptor. *Endocrinology* **147(7)**, 3356-3365.

Munger, K., Phelps, W.C., Bubb, V., Howley, P.M., and Schlegel, R. (1989) The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *Journal of Virology* **63**, 4417-4421.

Munoz, N., Bosch, F.X., Castellsague, X., Diaz, M., de Sanjose, S., Hammouda, D., Shah, K.V., and Meijer, C.J.L.M. (2004) Against which human papillomavirus types shall we vaccinate and screen ? The International perspective. *International Journal of Cancer* **111**, 278-285.

Munoz, N., Castellsagueb, X., Berrington de Gonzalez, A., and Gissmann, L. (2006) Chapter 1 – HPV etiology of human cancer. *Vaccine* **24(3)**, S1-S10.

Murakami, M., Hibi, M., Nakagawa, T., Yasukawa K., Yamanishi, K., Taga, T., and Kishimoto, T. (1993) IL-6 induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science* **260(5115)**, 1808-1810.

Murakami, M., Naraba, H., Tanioka, T., Semmyo, N., Nakatani, Y., Kojima, F., Ikeda, T., Fueki, M., Ueno, A., Oh-isi, S., and Kudo, I. (2000) Regulation of prostaglandin E_2 biosynthesis by inducible membrane associated prostaglandin E_2 synthase that acts in concert with cyclooxygenase-2. *Journal of Biological Chemistry* **275**, 32783-32792.

Murakami, M., and Kudo, I., (2004) Recent advances in molecular biology and physiology of the prostaglandin E_2 biosynthetic pathway. *Progress in Lipid Research* **43**, 3-35.

University of Cape Town

Murata, T., Lin, M.I., Aritake, K., Matsumoto, S., Narumiya, S., Ozaki, H., Urade, Y., Hori, M., and Sessa, W. (2008) Role of prostaglandin D_2 receptor DP as a suppressor of tumor hyperpermeability and angiogenesis in vivo. *Proceedings of the National Academy of Scienes USA* **105(50)**, 20009-20014.

Murphy, P.M., and Tiffany, H.L. (1991) Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science* **253**, 1280-1283.

Musunaga, R., Kohno, H., Dhar, D.K., Ohno, S., Shibakita, M., Kinugasa, S., Yoshimura, H., Tachibana, M., Kubota, H., and Nagasue, N. (2000) Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. *Clinical Cancer Research* **6**, 4064-4068.

Nakagawa, O., Tanaka, I., Usui, T., Harada, M., Sasaki, Y., Itoh, H., Yoshimasa, T., Namba, T., Narumiya, S., and Nakao, K. (1994) Molecular cloning of human prostacyclin receptor cDNA and its gene expression in the cardiovascular system. *Circulation* **90(4)**, 1643-1647.

Nakata, H., Uemura, Y., Kobayashi, M., Harada, R., and Taguchi, H. (2003) Cyclooxygenase-2 inhibitor NS-398 suppresses cell growth and constitutive production of granulocyte-colony stimulating factor and granulocyte macrophage-colony stimulating factor in lung cancer cells. *Cancer Science* **94(2)**, 173-180.

Namba, T., Sugimoto, Y., Hirata, M., Hayashi, Y., Honda, A., Watabe, A., Negishi, M., Ichikawa, A., and Narumiya, S. (1992) Mouse thromboxane A_2 receptor: cDNA cloning, expression and Northern blot analysis. *Biochem. Biophys. Res. Commun.* **184**, 1187-1203.

Namba, T., Sugimoto, Y., Negishi, M., Irie, A., Ushikubi, F., Kakizuka, A., Ito, S., Ichikawa, A., and Narumiya, S. (1993) Alternative splicing of C-terminal tail of prostaglandin E receptor subtype EP3 determines G-protein specificity. *Nature* **365**, 166-170.

Narko, K., Ristimaki, A., Macphee, M., Smith, E., Haudenschild, C.C., and Hla, T. (1997) Tumorigenic transformation of immortalized ECV endothelial cells by cyclooxygenase-1 overexpression. *Journal of Biological Chemistry* **272**, 21455-21460.

Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999) Prostanoid receptors: structures, properties and functions. *Physiological Reviews* **79**, 1193-1226.

Nasrallah, R., Laneuville, O., Ferguson, S., and Hebert, R.L. (2001) Effect of COX-2 inhibitor NS-398 on expression of PGE_2 receptor subtypes in M-1 mouse CCD cells. *American Journal of Physiology Renal Physiology* **281(1)**, F123-F132.

Ness, R.B., and Grainger, D.A. (2008) Male reproductive proteins and reproductive outcomes. *American Journal of Obstetrics and Gynecology* **198**, 620 e621-624.

University of Cape Town

Nguyen, M. (1997) Angiogenic factors as tumor markers. *Investigational New Drugs* **15**, 29-37.

Nikolopoulos, S.N., Blaikie, P., Yoshioka, T., Guo, W., and Giancotti, F.G. (2004) Integrin β4 signaling promotes tumor angiogenesis. *Cancer Cell* **6(5)**, 471-483.

Nishigaki, N., Negishi, M., Honda, A., Sugimoto, Y., Namba, T., Narumiya, S., and Ichikawa, A. (1995) Identification of prostaglandin E receptor 'EP2' cloned from mastocytoma cells EP4 subtype. *FEBS Letters* **364**, 339-341.

Nishikori, M. (2005) Classical and alternative NF-κB activation pathways and their roles in lymphoid malignancies. *Journal of Clinical and Experimental Hematopathology* **45(1)**, 15-24.

Niwa, Y., Akamatsu, H., Niwa, H., Sumi, H., Ozaki, Y., and Abe, A. (2001) Correlation of tissue and plasma RANTES levels with disease course in patients with breast or cervical cancer. *Clinical Cancer Research* **7**, 285-289.

Oates, J.A., FitzGerald, G.A., Branch, R.A., Jackson, E.K., Knapp, H.R., and Roberts, L.J.D. (1988) Clinical implications of prostaglandin and thromboxane A2 formation, 1. *New England Journal of Medicine* **319**, 689-698.

Ochsenkuhn, R., Toth, B., Nieschlag, E., Artman, E., Friese, K., and Thaler, C.J. (2008) Seminal plasma stimulates cytokine production in endometrial epithelial cell cultures independently of the presence of leucocytes. *Andrologia* **40(6)**, 364-369.

Oida, H., Hirata, M., Sugimoto, Y., Ushikubi, F., Ohishi, H., Mizuno, N., Ichikawa, A., and Narumiya, S. (1997) Expression of messenger RNA for the prostaglandin D receptor in the leptomeninges of the mouse brain. *FEBS Letters* **417(1)**, 53-56.

Okamoto, S., Nomura, H., and Matsushima, K. (1992) Function, molecular structure and gene expression of IL-8. *Nippon Rinsho* **50(8)**, 16-20.

Okuda-Ashitaka, E., Sakamoto, K., Ezashi, T., Miwa, K., Ito, S., and Hayaishi, O. (1996) Suppression of the prostaglandin E receptor signalling by the variant form of EP_1 subtype. *Journal of Biological Chemistry* **271**, 31255-31261.

O'Leary, S., Jasper, M.J., Warnes, G.M., Armstrong, D.T., and Robertson, S.A. (2004) Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. *Reproduction* **128**, 237-247.

Omari, K.M., Lutz, S.E., Santambrogio, L., Lira, S.A., and Raine, C.S. (2009) Neuroprotection and remyelination after autoimmune demyelination in mice that inducibly overexpress CXCL1. *American Journal of Pathology* **174(1)**, 164-176.

Opalka, B., Dickopp, A., and Kirch, H.C. (2002) Apoptotic genes in cancer therapy. *Cells Tissues Organs* **172(2)** 126-132.

University of Cape Town

Oshima, M., Dinchuk, J.E., Kargman, S.L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J.M., Evans, J. F., and Taketo, M.M. (1995) Suppression of intestinal polyposis in *Apc*$^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2) *Cell* **87**, 803-809.

Owen, J.D., Strieter, R., Burdick, M., Haghnegahdar, H., Nanney, L., Shattuck-Brandt-R., and Richmond, A. (1997) Enhanced tumor-forming capacity for immortalized melanocytes expressing melanoma growth stimulatory activity/growth-regulated cytokine beta and gamma proteins. *International Journal of Cancer* **73(1)**, 94-103.

Paavonen, J., Jenkins D., Bosch, F.X., Naud, P., Salmeron, J., Wheeler, C.M., Chow, S.N., Apter, D.L., Kitchener, H.C., Castellsague, X., de Carvalho, N.S., Skinnner, S.R., Harper, D.M., Hedrick, J.A., Jaisamrarn, U., Limson, G.A., Dionne, M., Quint, W., Spiessens, B., Peeters, B., Struyf, F., Wieting, S.L., Lehtinen, M.O., and Dubin, G. (2007) Efficacy of a pro-phylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial. *Lancet* **369**, 2161-2170.

Paetkau, V. (1981) Lymphokines on the move. *Nature* **294**, 689-690.

Paganini-Hill, A., Hsu, G., Ross, R.K., and Henderson, B.E. (1991) Aspirin use and incidence of large-bowel cancer in a Californian retirement community. *Journal of the National Cancer Institute* 1182-1183.

Pai, R., Nakamura, T., Moon, W.S., and Tarnawski, A.S. (2003) Prostaglandins promote colon cancer cell invasion; signalling by cross-talk between two distinct growth factor receptors. *FASEB Journal* **17**, 1640-1647.

Palefsky, J.M., and Holly, E.A. (2003) Chapter 6: immunosuppression and coinfection with HIV. *National Cancer Institute Monograms* **31**, 41-46.

Papantoniou, V.J., Souvatzoglou, M.A., Valotassiou, V.J., Louvrou, A.N., Ambela, C., Koutsikos, J., Lazaris, D., Christodoulidou, J.K., Sotiropoulou, M.G., Melissinou, M.J., Perperoglou, A., Tsiouris, S., and Zerva, C.J. (2004) Relationship of cell proliferation (Ki-67) to ^{99m}Tc-(V) DMSA uptake in breast cancer. *Breast Cancer Research* **6(2)**, R56-R62.

Paradis, V., Lagha, N.B., Zeimoura, L., Blanchet, P., Eschwege, P., Ba, N., Benoit, G., Jardin, A., and Bedossa, P. (2000) Expression of vascular endothelial growth factor in renal cell carcinomas. *Virchows Archive* **436**, 351-356.

Paralkar, V.M., Borovecki, F., Ke, Z., Cameron, K.O., Lefker, B., Grasser, W.A., Owen, T.A., Li, M., DaSilva-Jardine, P., Zhou, M., Dunn, R.L., Dumont, F., Korsmeyer, R., Krasney, P., Brown, T.A., Plowchalk, D., Vukicevic, S., and Thompson, D.D. (2003) An EP2 receptor –selective prostaglandin E_2 agonist induces bone healing. *Proceedings of the National Academy of Sciences USA* **100(11)**, 6736-6740.

University of Cape Town

Parkin, D.M. (2006) The global health burden of infection-associated cancers in the year 2002. *International Journal of Cancer* **118**, 3030-3044.

Paul, S.R., Bennett, F., Calvetti, J.A., Kellehe, K., Wood, C.R., O'Hara, R.M., Leary, A.C., Sibley, B., Clark, S.C., and Williams, D.A. (1990) Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proceedings of the National Academy of Sciences USA* **87(19)**, 7512-7516.

Paul, W.E., and Seder, R.A. (1994) Lymphocyte responses and cytokines. *Cell* **76**, 241-251.

Paye, F., Nordlinger, B., Surgical resection in: Garden, O.J., Geraghaty, J.G., and Nagorney, D.M. (1998) Liver metastases: biology, diagnosis and treatment. Heidelberg, New York: Springer-Verlag Berlin, 65-79.

Pelus, L.M., and Fukuda, S. (2006) Peripheral blood stem cell mobilization: the CXCR2 ligand GRObeta rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. *Experimental Hematology* **34(8)**, 1010-1020.

Perez-Andino, J., Buck, C.B., and Ribbeck, K. (2009) Absorption of human papillomavirus 16 to live human sperm. *PLoS ONE* **4(6)**, e5847.

Petruzzelli, G.J., Snyderman, C.H., Johnson, J.T., and Myers, E.N. (1993) Angiogenesis induced by head and neck squamous cell carcinoma xenografts in the chick embryo chorioallantoic membrane model. *Annals of Otology, Rhinology and Laryngology* **102**, 215-221.

Pierce, K.L., Bailey, T.J., Hoyer, P.B., Gil, D.W., Woodward, D.F., and Regan, J.W. (1994) Cloning of a carboxyl-terminal isoform of the prostanoid FP receptor. *Journal of Biological Chemistry* **272(2)**, 883-887.

Pilbeam, C.C., Fall, P.M., Alander, C.B. and Raisz, L.G. (1997) Differential effects of nonsteroidal anti-inflammatory drugs on constitutive and inducible prostaglandin G/H synthase in cultured bone cells. *Journal of Bone and Mineral Research* **12**, 1198-1203.

Podsypanina, K., Du., Y.C.N., Jechlinger., M., Beverly, L.J., Hambardzumyan, D., and Varmus, H. (2008) Seeding and propagation of untransformed mouse mammary cells in the lung. *Science* **321(5897)**, 1841-1844.

Poli, V. (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *Journal of Biological Chemistry* **273**, 29279-29282.

Ponten J., Adami, H.O., Bergstrom, R., Dillner, J., Friberg, L.G., Gustafsson L., Miller A.B., Parkin, D.M., Sparen, P., and Trichopoulos D. (1995) Strategies for global control of cervical cancer. *International Journal of Cancer* **60,** 1-26.

University of Cape Town

Portus, B.J., Relias, T., and Katila, T. (2005) Effect of seminal plasma on uterine inflammation, contractility and pregnancy rates in mares. *Equine Veterinary Journal* **37(6)**, 515-519.

Potter, J.D. (1997) Food, nutrition and the prevention of cancer: a global perspective. *The American Institute for Cancer Research, Washington DC*, p670.

Pulai, J.I., Chen, H., Im, H.J., Kumar, S., Hanning, C., Hegde, P.S., and Loeser, R.F. (2005) NF-κB mediates the stimulation of cytokine and chemokine expression by human articular chondrocytes in response to fibronectin fragments. *Journal of Immunology* **174**, 5781-5788.

Putoczki, T., and Ernst, M. (2010) More than a sidekick: the IL-6 family cytokine IL-11 links inflammation to cancer. *Journal of Leukocyte Biology (ahead of print July 7 2010).*

Racia, M., Cimpean, A.M., and Ribatti, D. (2009) Angiogenesis and pre-malignant conditions. *European Journal of Cancer* **45**, 1924-1934.

Rajakariar, R., Yaqoob, M.M., and Gilroy, D.W. (2006) COX-2 in inflammation and resolution. *Molecular Interventions* **6(4)**, 199-207.

Rajasekaran, M., Hellstrom, W., and Sikka, S. (1996) Quantitative assessment of cytokines (GRO alpha and IL-10) in human seminal plasma during genitourinary inflammation. *American Journal of Reproductive Immunology* **36(2)**, 90-95.

Rajewsky, K., Gu, H., Kuhn, R.. Betz, U.A., Muller, W., Roes, J., and Schwenk, F. (1996) Conditional gene targeting. *Journal of Clinical Investigation* **98**, 600-603.

Rakoff-Nahoum, S. (2006) Why cancer and inflammation ? *Yale Journal of Biology and Medicine* **79**, 123-130.

Randall, L.M., Monk, B.J., Darcy, K.M., Tian, C., Burger, R.A., Liao, S.Y., Peters, W.A., Stock, R.J., and Fruehauf, J.P. (2009) Markers of angiogenesis in high-risk, early stage cervical cancer: a gynecologic group study. *Gynecologic Oncology* **112(3)**, 583-589.

Ranganathan, P., Agrawal, A., Bhushan, R., Chavalmane, A.K., Kalathur, R.K.R., Takahashi, T., and Kondaiah P. (2007) Expression profiling of genes regulated by TGF-beta: differential regulation in normal and tumour cells. *BMC Genomics* **8**, 98.

Rayet, B., and Gelinas, C. (1999) Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* **25**, 6685-6705.

Reddy, B.S., Tokumo, K., Kulkarni. N., Aligia, C., and Kelloff, G. (1992) Inhibition of colon carcinogenesis by prostaglandin synthesis inhibitors and related compounds. *Carcinogenesis* **13(6)**, 1019-1023.

University of Cape Town

Reese, J., Brown, N., Paria, B.C., Morrow, J., and Dey, S.K. (1999) COX-2 compensation in the uterus of COX-1 deficient mice during the pre-implantation period. *Molecular and Cellular Endocrinology* **150**, 23-31.

Reeves, J.J., Bunce, K.T., Sheldrick, R.L.G., and Stables, R. (1988). Evidence for the PGE-receptor subtype mediating inhibition of acid secretion in the rat. *British Journal of Pharmacology* **95**, 805.

Regan, J.W., Bailey, T.J., Pepperl, D.J., Pierce, K.L., Bogardus, A.M., Donello, J.E., Fairbairn, C.E., Kedzie, K.M., Woodward, D.F., and Gil, D.W. (1994) Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP2 subtype. *Molecular Pharmacology* **46,** 213-220.

Rent, C.S., Rent, G.S., and Northcutt, T.J. (Jr) (1972) Behavioural factors related to the onset of cervical cancer. *Journal of Health and Social Behaviour* **13**, 437-445.

Richart, R.M. (1990) A modified terminology for cervical intraepithelial neoplasia. *Obstetrics and Gynecology* **75**, 131-133.

Richmond, A., and Thomas, H. (1986) Purification of melanoma growth stimulatory activity. *Journal of Cellular Physiology* **129**, 375-384.

Richmond, A., Balentien, E., Thomas, H.G., Flaggs, G., Barton, D.E., Spiess, J., Bordoni, R., Francke, U., and Derynck, R. (1988) Molecular characterization and chromosomal mapping of melanoma growth stimulatory activity, a growth factor structurally related to beta-thromboglobulin. *EMBO Journal* **7**(7), 2025-2033.

Risau, W. (1997) Mechanism of angiogenesis. *Nature* **386**, 671-674.

Robertson, S.A., Mayrhofer, G., and Seamark, R.F. (1992) Uterine epithelial cells synthesize granulocyte-macrophage colony-stimulating factor and interleukin-6 in pregnant and nonpregnant mice. *Biology of Reproduction* **46**, 1069-1079.

Robertson, S.A., Mau, V.J., Tremellen, K.P., and Seamark, R.F. (1996) Role of high moleular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice. *Journal of Reproductive Fertility* **107**, 265-277.

Robertson, R.P. (1998) Dominance of cyclooxygenase-2 in the regulation of pancreatic islet prostaglandin synthesis. *Diabetes* **47(9)**, 1379-1383.

Robertson, S.A., Ingman, W.V., O'Leary, S., Sharkey, D.J., and Tremellen, K.P. (2002) Transforming growth factor β-a mediator of immune deviation in seminal plasma. *Journal of Reproductive Immunology* **57**, 109-128.

Robertson, S.A. (2005) Seminal plasma and male factor signalling in the female reproductive tract. *Cell and Tissue Research* **322**, 43-52.

University of Cape Town

Rocca, B., Spain, L.M., Pure, E., Langenbach, R., Patrono, C., and FitzGerald, G.A. (1999) Distinct and coordinated roles of prostaglandin H-synthesis 1 and 2 in T-cell development. *Journal of Clinical Investigation* **103**, 1469-1477.

Rome, L.H., and Lands, W.E.M. (1975) Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proceedings of the National Academy of Sciences USA* **72**, 4863-4865.

Rome, L.H., Lands, W.E.M., Roth, G.J., and Marejus, P.W. (1976). Aspirin as a quantitative acetylating reagent for the fatty acid oxygenase that forms prostaglandins. *Prostaglandins* **11**, 23-30.

Rose-John, S., Dufhues, G., Graeve, L., Kruttgen, A., Lutticken, C., Mullberg, J., Pietzko, D., Schooltink, H., Stoyan, T., Dam, T., Zohlnhoffer, D., and Heinrich, P.C. (1992) Studies on the structure and function of IL-6 and its receptor. *Fresenius Journal of Analytical Chemistry* **343(1)**, 16.

Rosenberg L., Palmer, J.R., Zauber, A. G., Warshauer, M.E., Stolley, P.D., and Shapiro, S. (1991) A hypothesis: nonsteroidal inflammatory drugs reduce the incidence of the large-bowel cancer. *Journal of the National Cancer Institute*. **83**, 355-358.

Roth, G.L., and Majerus, P.W. (1975) The mechanism of the effect of aspirin on human platelets I. Acetylation of a particulate fraction protein. *Journal of Clinical Investigation* **56**, 624-632.

Roth, G.J., Stanford, N., and Majerus, P.W. (1975) Acetylation of prostaglandin synthase by aspirin. *Proceedings of the National Academy of Sciences USA* **72**, 3073-3076.

Roux, P., Alfieri, C., Hrimech, M., Cohen, E.A., and Tanner, J.E. (2000) Activation of transcription factors NF-κB and NF-IL-6 by human immunodeficiency virus type 1 protein R (Vpr) induces interleukin-8 expression. *Journal of Virology* **74(10)**, 4658-4665.

Rozengurt, E. (1986) Early signs in the mitogenic response. *Science* **234(4773)**, 161-166.

Ruiz-Godoy, L., Rizo Rios, P., Sanchez Cervantes, F., Osornio-Vargas, A., Garcia-Cuellar, C., and Meneses Garcia, A. (2007) Mortality due to lung cancer in Mexico. *Lung Cancer* **58**, 184-190.

Ruutu, M., Frazer, I., and Liu, X. (2008) Therapeutic vaccination against cervical cancer – are we near ? *Cancer Forum* **32**, no2.

Sabatte, J., Ceballos, A., Raiden, S., Vermeulen, M., Nahmod, K., Maggini, J., Salamone, G., Salamon, H., Amigorena, S., and Geffner, J. (2007) Human seminal plasma abrogates the capture and transmission of human immunodeficiency virus type 1 to $CD4^+$ T cells mediated by DC-SIGN. *Journal of Virology* **81(24)**, 13723-13734.

University of Cape Town

Saclarides, T.J., Speziale, N.J., Drab, E., Szeluga, D.J., and Rubin, D.B. (1994) Tumor angiogenesis and rectal carcinoma. *Diseases of the Colon and Rectum* **37**, 921-926.

Sales, K.J., Katz, A.A., Davis, M., Hinz, S., Soeters, R.P., Hofmeyer, M.D., Millar, R.P., and Jabbour, H.N. (2001) Cyclooxygenase-2 expression and prostaglandin E_2 synthesis are up-regulated in carcinomas of the cervix: a possible autocrine/paracrine regulation of neoplastic cell function via EP2/EP4 receptors. *Journal of Clinical Endocrinology and Metabolism* **86(5)**, 2243-2249.

Sales, K.J., Katz, A.A., Howard, B., Soeters, R.P., Millar, R.P., and Jabbour, H.N. (2002a) Cyclooxygenase-1 is up-regulated in cervical carcinomas: autocrine/paracrine regulation of cyclooxygenase-2, prostaglandin E receptors and angiogenic factors by cyclooxygenase-1. *Cancer Research* **62**, 424-432.

Sales, K.J., Katz, A.A., Miller, R.P., and Jabbour H.N. (2002b) Seminal plasma activates cyclooxygenase-2 and prostaglandin E_2 receptor expression and signalling in cervical adenocarcinoma cells. *Molecular Human Reproduction* **8(12)**, 1065-1070.

Sales, K.J., and Jabbour, H.N. (2003) Cyclooxygenase enzymes and prostaglandins in reproductive tract physiology and pathology. *Prostaglandins Other Lipid Mediators* **71**, 97-117.

Sales, K.J., Milne, S.A., Williams, A.W.R., Anderson, R.A., and Jabbour H.N. (2004a) Expression, localisation, and signalling of prostaglandin F_2 receptor in human endometrial adenocarcinoma: regulation of proliferation by activation of the epidermal growth factor receptor and mitogen-activated protein kinase signalling pathways. *Journal of Clinical Endocrinology. Metab.* **89**, 986-993.

Sales, K.J., Maudsley, S., and Jabbour, H.N. (2004b) Elevated prostaglandin EP2 receptor in endometrial adencarcinoma cells promotes VEGF expression via cAMP-mediated transactivation of the EGF receptor and ERK1/2 signalling pathways. *Molecular Endocrinology* **18**, 1533-1545.

Sales, K.J., List, T., Boddy, S.C., Williams, A.R.W., Anderson, R.A., Naor, Z., and Jabbour H.N. (2005) A novel angiogenic role for prostaglandin $F_{2\alpha}$-FP receptor interaction in human endometrial adencocarcinomas. *Cancer Research* **65(17)**, 7707-7716.

Sales, K.J., Boddy, S.C., Williams, A.R., Anderson, R.A., and Jabbour H.N. (2007) F-prostanoid receptor regulation of fibroblast growth factor 2 signalling in endometrial adenocarcinoma cells. *Endocrinology* **148(8)**, 3635-3644.

Sales, K.J., Boddy, S.C., and Jabbour, H.N. (2008a) F-prostanoid receptor alters adhesion, morphology and migration of endometrial adenocarcinoma cells. *Oncogene* **27**, 2466-2477.

University of Cape Town

Sales, K.J., Grant, V., and Jabbour, H.N. (2008b) Prostaglandin E_2 and $F_{2\alpha}$ activate the FP receptor and up-regulae cyclooxygenase-2 expression via the cyclic AMP response element. *Molecular and Cellular Endocriology* **285(1-2)**, 51-61.

Salgado, R., Benoy, I., Weytjens, R., Van Bockstaele, D., Van Marck, E., Huget, P., Hoylaerts, M., Vermeulen, P., and Dirix, L.Y. (2002) Arterio-venous gradients of IL-6, plasma and serum VEGF and D-dimers in human cancer. *British Journal of Cancer* **87(12)**, 1437-1444.

Savage, M.A., Moummi, C., Karabatsos, P.J., and Lanthorn, T.H. (1993) SC-46275: a potent and highly seletive agonist at the EP3 receptor. *Prostaglandins Leukotrienes and Essential Fatty Acids* **49**, 939-943.

Schaefer, M., Hoffman, T., Schultz, G., and Gudermann, T. (1998) A new prostaglandin E receptor mediates calcium influx and acrosome reaction in human spermatozoa. *Proceedings of the National Academy of Sciences USA* **95**, 3008-3013.

Schendel, P.F., and Turner, K.J. (1998) Interleukin-11. *Cytokines* **11**, 169-182.

Schiffman, M.H., and Brinton, L.A. (1995) The epidemiology of cervical carcinogenesis. *Cancer* **76**, 1888-1901.

Schiffman, M., Herrero, R., Desalle, R., Hildesheim, A., Wacholder, S., Rodriguez, A.C., Bratti, M.C., Sherman, M.E., Morales, J., Guillen, D., Alfaro, M., Hutchinson, M., Wright, T.C., Solomon, D., Chen, Z., Schussler, J., Castle, P.E., and Burk, R.D. (2005) The carcinogenicity of human papillomavirus types reflects viral evolution. *Virology* **337**, 76-84.

Schlessinger, J. (2000) Cell signalling by receptor tyrosine kinases. *Cell* **103**, 211-225.

Scholzen, T., and Gerdes, J. (2000) The Ki-67 protein: from the known and unknown. *Journal of Cellular Physiology* **182**, 311-322.

Scott, J.L., Ketheesan, N., and Summers, P.M. (2009) Spermatozoa and seminal plasma induce a greater inflammatory response in the ovine uterus at oestrus that dioestrus. *Reproduction, Fertility and Development* **21(7)**, 817-826.

Scully, R.E., Bonfiglio, T.A., Kurman, R.J., Siverberg, S.G., and Wilkinson E.J. (1994) Histological typing of female genital tract tumours. R.E (ed), Berlin; Springer-Verlag, p 189.

Seed, M.P., Brown, J.R., Freemantle, C.N., Papworth, J.L., Coliville-Nash, P.R., Willis, D., Somerville, K.W., Asculai, S., and Willoughby, D.A. (1997) The inhibition of colon-26 adenocarcinoma development and angiogenesis by topical diclofenac in 2.5% hyaluronan. *Cancer Research* **57**, 1625-1629.

Sehgal, P.B., Zilberstein, A., Ruggieri, R., May, L.T., Ferguson-Smith, A., Slate, D.L., Revel, M., and Ruddel, F.H. (1986) Human chromosome 7 carries the beta 2 interferon gene. *Proceedings of the National Academy of Sciences USA* **83**, 5219-5222.

University of Cape Town

Sharkey, D.J., Macpherson, A.M., Tremellen, K.P., and Robertson, S.A. (2007) Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Molecular Human Reproduction* **13(7)**, 491-501.

Sheng, H., Shao, J., Kirkland, S.C., Isakson, P., Coffey, R., Morrow, J., Beauchamp, R.D., and DuBois, R.N., (1997) Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *Journal of Clinical Investigation* **99**, 2254-2259.

Shibuya, M. (2001) Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. *Cell Structure and Function* **26**, 25-35.

Shibuya, M., and Claesson-Welsh, L. (2005) Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Experimental Cell Research* **312**, 549-560.

Shoji, Y., Takahashi, M., Kitamura, T., Watanabe, K., Kawamori, T, Maruyama, T., Sugimoto, Y., Negishi, M., Narumiya, S., Sugimara, T., and Wakabayashi, K. (2005) Downregulation of prostaglandin E receptor subtype EP3 during colon cancer development. *Gut* **53**, 1151-1158.

Shoji, Y., Takahashi, M., Takasuka, N., Niho, N., Kitamura, T., Sato, H., Maruyama, T., Sugimoto, Y., Narumiya, S., Sugimura, T., and Wakabayashi, K. (2005) Prostaglandin E receptor EP_3 deficiency modifies tumor outcome in mouse two-stage skin carcinogenesis. *Carcinogenesis* **26(12)**, 2116-2122.

Shultz-Cherry, S., and Murphy-Ullrich, J.E. (1993) Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. *Journal of Cellular Biology* **122**, 923-930.

Shun, C.T., Wu, M.S., Huang, S.P., Wang, H.P., Chuang, S.M., and Lin, J.T. (2003) Cyclooxygenase-2 expression correlates with nuclear p53 accumulation in gastric carcinoma. *Hepatogastroenterology* **50**, 988-992.

Silva-Filho, A.L., Traiman, P., Triginelli, S.A., Reis, F.M., Pedroso, M.S., Miranda, D., Aberu, E.S., Macarenco, R.S.S., and Chuna-Melo, J.R. (2004) Expression of p53, K-67, and CD31 in the vaginal margins of radical hysterectomy in patients with stage 1B carcinoma of the cervix. *Gynecologic Oncology* **95**, 646-654.

Simpson, R.J., Hammacher, A., Smith, D.K., Matthews, J.M., and Ward, L.D. (1997) Interleukin-6: Structure-function relationships. *Protein Science* **6**, 929-955.

Singh, B., Berry, J.A., Shoher, A., and Lucci, A. (2006) COX-2 induces IL-11 production in human breast cancer cells. *The Journal of Surgical Research* **131(2)**, 267-275.

University of Cape Town

Singh, B., Berry, J.A., Shoher, A., Ayers, G.D., Wei, C., and Lucci, A. (2007) COX-2 involvement in breast cancer metastasis to bone. *Oncogene* **26**, 3789-3796.

Singh, R.K., and Lokeshwar, B.L. (2009) Deletion of intrinsic expression of interleukin-8 in prostate cancer cells causes cell cycle arrest, spontaneous apoptosis and increases in the efficacy of chemotherapeutic drugs. *Molecular Cancer* **8**, 57.

Sitas, F., Carrara, H., Terblanche, M., and Madhoo, J. (1997) Screening for cancer of the cervix in South Africa. *South Africa Medical Journal* **87**, 620-622.

Slaton, J.W., Inoue, K., Perrotte, P., El-Naggar, A.K., Swanson, D.A., Fidler, I.J., and Dinney, C.P. (2001) Expression levels of genes that regulate metastasis and angiogenesis correlate with advanced pathological state of renal cell carcinoma. *American Journal of Pathology* **158**, 735-743.

Smith, W.L., and Marnett, L.J. (1991) Review: prostaglandin endoperoxide synthase: structure and catalysis. *Biochim. Biophys. Acta* **1083**, 1-17.

Smith, C.J., Morrow, J.D., Roberts, L.J., and Martnett L.J. (1993) Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (COX-1). *Biochemical and Biophysical Research Communications* **192**, 787-793.

Smith, D.F., Galkina, E., Ley, K., and Huo, Y. (2005) GRO family chemokines are specialized for monocyte arrest from flow. *American Journal of Physiological Heart and Circulatory Physiology* **289(5)**, H1976-H1984.

Smith, W.L., and DeWitt, D.L. (1996) Prostaglandin endoperoxide H synthases-1 and -2. *Advances in Immunology* **62**, 167-215.

Smith, W.L., DeWitt, D.L., and Garavito, R.M. (2000) Cyclooxygenases: structural, cellular and molecular biology. *Annual Review of Biochemistry* **69**, 145-182.

Song, S., Liem, A., Miller, J.A., and Lambert, P.F. (2000) Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis. *Virology* **267**, 141-150.

Sonnenburg, W.K., and Smith W.L. (1988) Regulation of cyclic AMP metabolism in rabbit cortical collecting tubule cells by prostaglandins. *Journal of Biological Chemistry* **263**, 6155-6160.

Sonoshita, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., Oshima, M., and Taketo, M.M. (2001) Accerleration of intestinal polyposis through prostaglandin receptor EP2 in *Apc*$^{\Delta 716}$ knockout mice. *Nature Medicine* **7**, 1048-1051.

Soslow, R.A., Dannenburg, A.J., Rush, D., Woerner, B.M., Khan, K.N., Masferrer, J., and Koki, A.T. (2000) COX-2 is expressed in human pulmonary, colonic, and mammary tumors. *Cancer* **89**, 2637-2645.

University of Cape Town

Spurney, R.F., Ruiz, P., Pisetsky, D.S., and Coffman, T.M. (1992) Chronic thromboxane receptor blockade reduces renal injury in murine lupus nephritis. *Kidney International* **41**, 973-982.

Sri Kantha, S., Matsumura, H., Kubo, E., Kawase, K., Takahata, R., Serhan, C.N., and Hayaishi, O. (1994) Effects of prostaglandin D2, lipoxins and leukotrines on sleep and brain temperature of rats. *Prostaglandins, Leukotrines, and Essential Fatty Acids* **51**, 87-93.

Srivastava, S., Natu, S.M., Gupta, A., Pal, K.A., Singh, U., Agarwal, G.G., Singh, U., Goel, M.M., and Srivastava, A.N. (2009) Lipid peroxidation and antioxidants in different stages of cervical cancer: prognostic significance. *Indian Journal of Cancer* **46(4)**, 297-302.

Stanley, M., Pett, M., and Coleman, N. (20007) HPV: from infection to cancer. *Biochemical Society Transactions* **35**, 1456-1460.

Steckley, S.L., Pickworth, W.B., and Haverkos, H.W. (2003) Cigarette smoking and cervical cancer: Part II: a geographical variability study *Biomedicine and Pharmacotherapy* **57**, 78-83 (Abstract).

Steinwall, M., Akerlund, M., Bossmar, T., Nishii, M., and Wright, M. (2004) ONO-8815Ly, an EP2 agonist that markedly inhibits uterine contractions in women. *International Journal of Obstetrics and Gynaecology* **111(2)**, 120-124(5).

Steller, M.A., Delgado, C.H., and Zou, Z. (1995) Insulin-like growth factor II mediates epidermal growth factor-induced mitogenesis in cervical cancer cells. *Proceedings of the National Academy of Sciences* **92**, 11970-11974.

Strander, B. (2009) At what age should cervical screening stop? *British Medical Journal* **338**, 1022–1023.

Strong, P., Coleman, R.A., and Humphrey, P.P.A. (1992) Prostanoid-induced inhibition of lipolysis in rat isolated adipocytes: probable involvement of EP3 receptors. *Prostaglandins* **43**, 559-566.

Stuttfeld, E., and Ballmer-Hofer, K. (2009) Structure and function of VEGF receptors. *Life* **61(9)**, 915-922.

Su, B., and Karin, M. (1996) Mitogen-activated protein kinase cascades and regulation of gene expression. *Current Opinion in Immunology* **8,** 402-411.

Subbaramaiah, K., Telang, N., Ramonetti, J.T., Araki, R., DeVito, B., Weksler, B.B., Dannenberg, A.J. (1996) Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. *Cancer Research* **56**, 4424-4429.

University of Cape Town

Subbaramaiah, K., Zakim, D., Weksler, B.B., and Dannenberg, A.J. (1997) Inhibition of cyclooxygenase: a novel approach to cancer prevention. *Proceedings of the Society for Experimental Biology and Medicine* **216**, 201-210.

Subbaramaiah, K., Chung, W.J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J.M., and Dannenberg, A.J. (1998) Resveratol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells *Journal of Biological Chemistry* **273**, 21875-21882.

Subbaramaiah, K., and Dannenberg, A.J. (2007) Cyclooxygenase-2 transcription is regulated by human papillomavirus 16 E6 and E7 oncoproteins: evidence of a corepressor/coactivator exchange. *Cancer Research* **67(8)**, 3976-3985.

Sugimoto, Y., Negishi, M., Hayashi, Y., Namba, T., Honda, A., Watabe, A., Hirata, M., Narumiya, S., and Ichikawa, A. (1993) Two isoforms of the EP3 receptor with different carboxyl-terminal domains. Identical ligand binding properties and different coupling properties with Gi proteins. *Journal of Biological Chemistry* **268**, 2712-2718.

Sugimoto, Y., Yamasaki, A., Segi, E., Tsuboi, K., Aze, Y., Nishimura, T., Oida, H., Yoshida, N., Tanaka, T., Katsuyama, M., Hasumoto, K., Murata, T., Hirata, M., Ushikubi, F., Negishi, M., Ichikawa, A., and Narumiya, S. (1997) Failure of parturition in mice lacking the prostaglandin F receptor. *Science* **277**, 681-683.

Sugimoto, Y., Narumiya, S., and Ichikawa, A. (2000) Distribution and function of prostanoid receptors: studies from knockout mice. *Progress in Lipid Research* **39**, 289-314.

Sung, Y.M., Guobin, H., and Fischer, S.M. (2005) Lack of expression of the EP2 but not EP3 receptor for prostaglandin E2 results in suppression of skin tumor development. *Cancer Research* **65**, 9304-9311.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., and Kishimoto, T. (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. *Cell* **58**, 573-581.

Takai, Y., Wong, G.G., Clark, S.C., Burakoff, S., and Herrmann, S.J. (1988) B cell stimulatory factor-2 is involved in the differentiation of cytotoxic T lymphocytes. *Journal of Immunology* **140**, 508-512.

Takamori, H., Oades, Z.G., Hoch, R.C., Burger, M., and Schraufstatter, I.U. (2000) Autocrine growth effect of IL-8 and GRO(alpha) on a human pancreatic cell line, capan-1. *Pancreas* **21(1)**, 52-56.

Takano, H., Harigaya, K., Ishii, G., Sugaya, Y., Soeta, S., Nunoyama, T., Shirasawa, H., Shimizu, K., Tokita, H., Simizu, B., Makita, A., Seika, S. (1996) Interleukin-6 (IL-6) production in carcinoma of the cervix. *Archives of Gynecology and Obstetrics* **258**, 25-33.

University of Cape Town

Takehara, H., Iwamoto, J., Mizokami, Y., Takahashi, K., Ootubo, T., Miura, S., Narasaka, T., Takeyama, H., Omata, T., Shimokobe, K., Ito, M., and Matsuoka, T. (2006) Involvement of cyclooxygenase-2-prostaglandin E2 pathway in interleukin-8 production in gastric cancer cells. *Digestive Diseases and Sciences* **51**, 2188-2197.

Tanabe, T., and Tohnai, N. (2002) Cyclooxygenase isoenzymes and their gene structures and expression. *Prostaglandins and Other Lipid Mediators* **68-69**, 95-114.

Tang, D.G., Chen, Y.Q., Newman, P.J., Shi, L., Gao, X., Diglio, C.A., and Honn, K.V. (1993) Identification of PECAM-1 in solid tumor cells and its potential involvement in tumor cell adhesion to endothelium. *Journal of Biological Chemistry* **268(30)**, 22883-22894.

Templeton, A.A., Cooper, I., and Kelly, R.W. (1978) Prostaglandin concentrations in the semen of fertile men. *Journal of Reproduction and Fertility* **52**, 147-150.

Tentes, I., Asimakopoulos, B., Mourvati, E., Diedrich, K., Al-Hasani, S., and Nikolettos, N. (2007) Matrix metalloproteinase (MMP)-2 and MMP-9 in seminal plasma. *Journal of Assisted Reproduction and Genetics* **24(7)**, 278-281.

Thomas, D.W., Mannon, R.B., Mannon, P.J., Latour, A., Oliver, J.A., Hoffman, M., Smithies, O., and Koller, B.H. (1998). Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A_2. *Journal of Clinical Investigation* **102**, 1994-2001.

Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**, 5203.

Thompson, L.A., Barratt, C.L.R., Bolton, A.E., and Cooke, I.D. (1992) The leukocytic reaction of the human uterine cervix. *American Journal of Reproductive Immunology* **28**, 85-89.

Throat, M.A., Morimiya, A., Mehrotra, S., Konger, R., and Badve, S.S. (2007) Prostanoid receptor EP1 expression in breast cancer. *Modern Pathology* **21**, 15-21.

Thurston, G. (2003) Role of angiopoietins and Tie receptor tyrosine kinases in angiogenesis and lymphangiogenesis. *Cell and Tissue Research* **314(1)**, 61-68.

Tiano, H.F., Loftin, C.D., Akunda, J., Lee, C.A., Spalding, J., Sessoms, A., Dunson, D.B., Rogan, E.G., Morham, S.G., Smart, R.C, and Langenbach, R. (2002) Deficiency of either cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces moue skin tumorigenesis. *Cancer Research* **62(12)**, 3396-4010.

Tjalma, W., Weyler, J., Van Daele, A., Sonnemans, H., De Pooter, C., van Marck, E., Albertyn, G., and Van Dam, P. (1997) Computerized analysis of angiogenesis (CD31) in invasive cervical carcinoma: multivariate analysis. *European Journal of Cancer* **33 (**supplement 8), 50-51.

University of Cape Town

Tomayko, M.M., and Reynolds, P. (1988) Determination of subcutaneous size in athymic (nude) mice. *Cancer Chemotherapy and Pharmacology* **24(3)**, 148-154.

Toomey, D.P., Murphy, J.F., and Conlon, K.C. (2009) VEGF and tumour angiogenesis. *Surgeon* **7**, 174-180.

Toth, P., Li, X., Lei, Z.M., and Rao, C.V. (1996) Expression of human chorionic gonadotropin (hCG)/luteinizing hormone receptors and regulation of the cyclooxygenase -1 gene by exogenous hCG in human fetal membranes. *Journal of Clinical Endocrinology Metabolism* **81**, 1283-1288.

Tremellen, K.P., Seamark, R.F., and Robertson, S.A. (1998) Seminal transforming growth factor beta 1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. *Biological Reproduction* **58**, 1217-1225.

Tsai, H.H., Frost, E., Robinson, S., Ffrench-Constant, C., Geertman, R., Ransohoff, R.M., and Miller R.H. (2002) The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. *Cell* **110(3)**, 373-383.

Tsatsanis, C., Androulidaki, A., Venihaki, M., and Margioris, A.N. (2006) Signalling networks regulating cyclooxygenase-2. *International Journal of Biochemical Cell Biology* **38**, 1654-1661.

Tsujii, M., and DuBois, R.N. (1995) Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2. *Cell* **83**, 493-501.

Tsujii, M., Kawano, S., and DuBois, R.N., (1997) Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proceedings of the National Academy of Sciences USA* **94**, 3336-3340.

Tsujii, M., Kawano, S., Tsujii, S., Sawaoka, H., Hori, M., and DuBois R.N. (1998) Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* **93** 705-716.

Uchima, Y., Sawada, T., and Hirakawa, K. (2007) Action of antiproteases on pancreatic cancer cells. *Journal of Pancreas* **8(4**), 479-487.

Uhl, J.R., Bell, C.A., Sloan, L.M., Espy, M.J., Smith, T.F., Rosenblatt, J.E., and Cockerill, F.R. (2002) Application of rapid-cycle real-time polymerase chain reaction for the detection of microbial pathogens: the mayo-roche rapid anthrax test. *Mayo Clinic Proceedings* **77**, 673-680.

Urade, Y., Hayaishi, O. (1999) Prostaglandin D2 and sleep regulation. *Biochim. Biophys. Acta* **1436**, 606-615.

University of Cape Town

Use, P.P. (1981) Prolonged pill use, multiple sex partners may lead to cervical cancer or precursors, studies find. *International Family Planning Perspectives* **7(1)**, 26-28.

Ushikubi, F., Segi, E., Sugimoto, Y., Murata, T., Matsuoka, T., Kobayashi, T., Hizaki, H., Tuboi, K., Katsuyama, M., Ichikawa, A., Tanaka, T., Yoshida, N., and Narumiya, S. (1998) Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. *Nature* **395**, 281-284.

Van Der Ouderaa, F.J., Buytenhek, M., Nugteren, D.H., and Van Dorp, D. (1980) Acetylation of prostaglandin endoperoxide synthetase with acetylsalicylic acid. *European Journal of Biochemistry* **109**, 1-8.

Van der Poll, T., Keogh, C.V., Guirao, X., Buurman, W.A., Kopf, M., and Lowry, S.F. (1997) Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *Journal of Infectious Diseases* **176(2)**, 439-444.

Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature: New Biology* **231**, 232-235.

Versteeg, H.H., Van Bergen en Henegouwen, P.M., Van Deventer, S.J., Peppelenbosch, M.P. (1999) Cyclooxygenase-dependent signalling: molecular events and consequences. *FEBS Letters* **445**, 1-5.

Venkatakrishnan, G., Salgia, R., and Groopman, J.E. (2000) Chemokine receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells. *Journal of Biological Chemistry* **275**, 6868-6875.

Vicari, A.P., and Chaux, C. (2002) Chemokines in cancer. *Cytokine and Growth Factor Review* **13**, 143-154.

Vlahopoulos, S., Boldogh, I., Casola, A., and Brasier, A.R. (1999) Nuclear factor-kappa B-dependent induction of interleukin-8 gene expression by tumor necrosis factor alpha: evidence for an antioxidant sensitive activating pathway distinct from nuclear translocation. *Blood* **94(6)**, 1878-1889.

Vlahous, C., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *Journey of Biological Chemistry* **269**, 5241-5248.

Wadell, W.R., and Loughry, R.W. (1983) Sulindac for polyposis of the colon. *Journal of Surgical Oncology* **24**, 83-87.

Waggoner, S.E. (2003) Cervical cancer. *The Lancet* **361(9376)**, 2217-2225.

Wahl, L.M., and Kleinman, H.K. (1998) Tumor associated macrophages as targets for cancer therapy. *Cancer Institute* **90**, 1583-1584.

University of Cape Town

Wakui, S., Furusato, M., Itoh, H., Sasaki, H., Akiyama, A., Kinoshita, I., Asano, K., Tokuda, T., Aizawa, S., and Ushigome, S. (1992) Tumor angiogenesis in prostatic carcinoma with and without bone marrow metastasis: a morphometric study. *Journal of Pathology* **68**, 257-262.

Walboomers, J.M., Jacobs, M.V., Manos, M.M., Bosch, X., Kummer, J.A., Shah, K.V., Snijders, P.J.F., Petro, J., Meijer, C.J.L.M., and Munoz, N. (1999) Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology* **189(1)**, 12-19.

Wallin, K.L., Wiklund, F., Angstrom, T., Bergman, F., Stendahl, U., Wadell, G., Hallmans, G., and Dilner, J. (1999). Type-specific persistence of human papillomavirus DNA before the development of invasive cervical cancer. *New England Journal of Medicine* **341(22)**, 1633-1638.

Wallach, D., Varfolomeev, E.E., Malinin, N.L., Goltsev, Y.V., Kovalenko, A.V., and Boldin, M.P. (1999) Tumor necrosis factor and Fas signaling mechanisms. *Annual Review of Immunology* **17**, 331-367.

Walz, A., Peveri, P., Aschauer, H., and Baggiolini, M. (1987) Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. *Biochem. Biophys. Res. Comm.* **149**, 755-761.

Wang, B., Hendricks, D.T., Wamunyokoli, F., and Parker, M.I. (2006) A growth-related oncogene/CXC chemokine receptor 2 autocrine loop contributes to cellular proliferation in esophageal cancer. *Cancer Research* **66(6)**, 3071-3077.

Wang, D., and Richmond, A. (2001) Nuclear factor-κB activation by the CXC chemokine melanoma growth-stimulatory activity/grow-regulated protein involves the MEKK1/p38 mitogen-activated protein kinase pathway. *Journal of Biological Chemistry* **276**, 3650-3659.

Wang, D., Wang, H., Brown, J., Daikoku, T., Ning, W., Shi, Q., Richmond, A., Steiter, R., Dey, S.K., and DuBois, R. (2006) CXCL1 induced by prostaglandin E_2 promotes angiogenesis in colorectal cancer. *Journal of Experimental Medicine* **203(4)**, 941-951.

Wang, D., Dubois, R.N., and Richmond, A. (2009) The role of chemokines in intestinal inflammation and cancer. *Current Opinions in Pharmacology* **9(6)**, 688-696.

Wang, L.H., Cheng, G.Z., Park, S., Shu, S., He, L., Kong, W., Zhang, W., Yuan, Z., and Chen, J.Q. (2008) Advances of AKT pathway in human oncogenesis and as a target for anti-cancer drug discovery. *Current Cancer Drug Targets* **8**, 2-6.

Wang, M.T., Honn, K.V. and Nie, D. (2007) Cyclooxygenases, prostanoids, and tumour progression. *Cancer Metastasis Review* **26**, 525-534.

Wang, R., Luo, D., Ma, X., Yang, W., Chen, R., Liu, Y., Meng, L., Zhou, J., Xu, G., Lu, Y.P., Wang, S., and Ma, D. (2008) Antisense Ki-67 cDNA transfection reverses

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the tumorigenicity and induces apoptosis in human breast cancer cells. *Cancer Investigation* **26(8)**, 830-835.

Wang, X.S., and Lau, H.Y.A. (2006) Prostaglandin E_2 potentiates the immunologically stimulated histamine release from human peripheral blood-derived mast cells through EP1/EP3 receptors. *Allergy* **61**, 503-506.

Wang, X., Zhuang, J., Wu, K., Xu, R., Li, M., and Lu, Y. (2010) Human semen: the biological basis of sexual behaviour to promote human papillomavirus infection and cervical cancer. *Medical Hypotheses* **74(6)**, 1015-1016.

Watabe, A., Sugimoto, Y., Irie, A., Namba, T., Negishi, M., Ito, S., Narumiya, S., and Ichikawa (1993) Cloning and expression of cDNA for a mouse EP_1 subtype of prostaglandin E receptor. *Journal of Biological Chemistry* **268**, 20175-20178.

Watanabe, T., Nakao, A., Emerling, D., Hashimoto, Y., Tsukamoto, K., Horie, Y., Kinoshita, M., and Kurokawa, K. (1994) Prostaglandin F2 alpha enhances tyrosine phosphorylation and DNA synthesis through phospholipase C-coupled receptor via Ca(2+)-dependent intracellular pathway in NIH-3T3 cells. *Journal of Biological Chemistry* **269**, 17619-17625.

Watanabe, K., Kawamori, T., Nakatsugi, S., Ohta, T., Ohuchida, S., Yamamoto, H., Maruyama, T., Kondo, K., Ushikubi, F., Narumiya, S., Sugimara, T., and Wakabayashi, K. (1999) Role of the prostaglandin E receptor subtype EP_1 in colon carcinogenesis. *Cancer Research* **59**, 5093-5096.

Watson, J.M., Sensintaffar, J.L., Berek, J.S., and Martinex-Maza, O. (1990) Constitutive production of interleukin 6 by ovarian cancer cell lines and by primary ovarian tumor cultures. *Cancer Research* **50**, 6959-6965.

Wei, L.H., Kuo, M.L., Chen, C.A., Chou, C.H., Lai, K.B., Lee, C.N., and Hsieh, C.Y. (2003) Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. *Oncogene* **22**, 1517-1527.

Weissenbach, J., Chernajovsky, Y., Zeevi, M., Schulman, I., Soreqm H., Nir, U., Wallach, D., Perricaudet, M., Tiollais, P., and Revel, M. (1980) Two interferon mRNAs in human fibroblasts: in vitro translation and *Escherichia coli* cloning studies. *Proceedings of the National Academy of Sciences USA* **93**, 1-6.

Werness, B.A., Levine, A.J., and Howley, P.M. (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**, 76-79.

Wiesmann, C., Fuh, G., Christinger, H.W., Eigenbrot, C., Wells, J.A., and de Vos, A.M. (1997) Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor. *Cell* **91**, 695-704.

University of Cape Town

Williams, C.S., Smalley, W., and DuBois, R.N. (1997) Aspirin use and potential mechanisms for colorectal cancer prevention. *Journal of Clinical Investigation* **100**, 1-5.

Williams, C.S. Mann, M., and DuBois, R.N. (1999) The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* **18(55)**, 7908-7916.

Williams, C.S., Tsujii, M., Reese, J., Dey, S.K., and DuBois, R.N. (2000) Host cyclo-oxygenase-2 modulates carcinoma growth. *Journal of Clinical Investigation* **105**, 1589-1594.

Wilson, C., Seaton, A., Maxwell, P.J., Scullin, P., Johnston, P.G., and Waugh, D.J. (2006) CXCR2/NF-κB/BCL-2-survivin signalling pathway promotes resistance to oxaliplatin in metastatic prostate cancer cells. In Washington (DC): Proceedings of the 99th ACAR Annual Meeting.

Wira, C.R., Fahey, J.V., White, H.D., Yeaman, G.R., Givan, A.L., and Howell, A.L. (2002) The mucosal immune system in the female reproductive tract: influence of stage of the menstrual cycle and menopause o mucosal immunity in the uterus. In: Glasser, S.R., Alpin, J.D., Guidice, L., and Tabibzadeh, S. (eds.) *The Endometrium.* Taylor and Francis, London, 371-404.

Wira, C.R., Fahey, J.V., Sentan, C.L., Pioli, P.A., and Shen, L. (2005) Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunological Reviews* **206**, 306-335.

Wolpe, S.D., Sherry, B., Juers, D., Davatelis, G., Yurt, R.W., and Cerami, A. (1989) Identification and characterization of macrophage inflammatory protein 2. *Proceedings of the National Academy of Sciences* **86**, 612-616.

Woodworth, C.D., Bowden, P.E., Doniger, J., Pirisi, L., Barnes, W., Lancaster, W.D., and DiPaolo, J.A. (1988) Characterization of normal human exocervical epithelial cells immortalized *in vitro* by papillomavirus types 16 and 18 DNA. *Cancer Research* **48**, 4620-4628.

World Health Organization (2003). Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction, 4th edition Cambridge, UK: Cambridge University Press. p 60.

Wright, D.H., Nantel, F., Metters, K.M., and Ford-Hutchinson, A.W. (1999) A novel biological role for prostaglandin D2 is suggested by disruption studies of the rat DP prostanoid receptor. *European Journal of Pharmacology* **377**, 101-115.

Wright, T.C. (Jr) (2003) Chapter 10: Cervical cancer screening using visualization techniques. *Journal of the National Cancer Institute Monographs* **31**, 66-71.

Wu, Y., and Zhou, B.P. (2009) Inflammation: a driving force speeds cancer metastasis. *Cell Cycle* **8**, 3267-3273.

University of Cape Town

Xi, L.F., Toure, P., Critchlow, C.W., Hawes, S.E., Dembele, B., Sow, P.S., and Kiviat, N.B. (2003) Prevalence of specific types of human papillomavirus and cervical squamous intraepithelial lesions in consecutive, previously unscreened, West-African women over 35 years of age. *International Journal of Cancer* **103**, 803-809.

Xiong, S., Grijalva, R., Zhang, L., Nguyen, N.T., Pisters, P.W., Pollock, R.E., and Yu, D. (2001) Up-regulation of vascular endothelial growth factor in breast cancer cells by the heregulin-β1-activated p38 signaling pathway enhances endothelial cell migration. *Cancer Research* **61**, 1727-1732.

Xu, L., Xie, K., Mukaida, N., Matsushima, K., and Fidler, I.J. (1999) Hypoxia-induced elevation in interleukin-8 expression by human ovarian carcinoma cells. *Cancer Research* **59**, 5822-5829.

Xu, X.M., Tang, J.L., Chen, X.M., Wang, L.H., and Wu, K.K. (1997) Involvement of two Sp1 elements in basal endothelial prostaglandin H synthase-1 promoter activity. *Journal of Biological Chemistry* **272**, 6943-6950.

Yamakzaki, K., Abe, S., Takeawa, H., Sukoh, N., Watanabe, N., Ogura, S., Nakajima, I., Isobe, H., Inoue, K., and Kawakami, Y. (1994) Tumor angiogenesis in human lung adenocarcinoma. *Cancer* **74**, 2245-2250.

Yamamoto, K., Arakawa, T., Ueda, N., and Yamamoto, S. (1995) Transcriptional roles of nuclear factorκB and nuclear factor interleukin -6 in the tumor necrosis factor α-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *Journal of Biological Chemistry* **270**, 31315-31320.

Yan, S.F., Fujita, T., Lu, J., Okada, K., Zou, Y.S., Mackman, N., Pinsky, D.J., and Stern, D.M. (2000) Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nature Medicine* **6**, 1355-1361.

Yang, J., Xia, M., Goetzl, E.J., and An, S. (1994). Cloning and expression of the EP3-subtype of human receptors for prostaglandin E_2. *Biochem Biophys Res Commun.* **198,** 999-1006.

Yao, P.L., Lin, Y.C., Wang, C.H., Huang, Y.C., Liao, W.Y., Wang, S.S., Chen, J.J., and Yang, P.C. (2005) *American Journal of Respiratory Cell and Molecular Cell Biology* **32(6)**, 540-547.

Yap, J., Salamonsen, L.A., Jobling, T., Nicholls, P.K., and Dimitriadis, E. (2010) Interleukin 11 is upregulated in uterine lavage and endometrial cancer cells in women with endometrial carcinoma. *Reproductive Biology and Endocrinology* **8**, 63

Ye, R.D. (2001) Regulation of nuclear factor κB activation by G -protein-coupled receptors. *Journal of Leukocyte Biology* **70**, 839-848.

University of Cape Town

Yoshiji, H., Gomez, D.E., Shibuya, M., and Thorgeirsson, U.P. (1996) Expression of vascular endothelial growth factor, its receptor and other angiogenic factors in human breast cancer. *Cancer Res.* **56**, 2013-2016.

Yu, H., Kortylewski , M., and Pardoll, D. (2007) Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nature Reviews Immunology* **7**, 41-51.

Yu, Y., and Chadee, K. (1998) Prostaglandin E_2 stimulates IL-8 gene expression in human colonic epithelial cells by a posttranscriptional mechanism. *Journal of Immunology* **161**, 3746-3752.

Yuan, A., Chen, J.J., Yao, P.L., and Yang, P.C. (2005) The role of interleukin-8 in cancer cells and microenvironment interaction. *Frontiers in Bioscience* **10**, 853-865.

Zhan, X., and Desiderio, D.M. (2010) Signaling pathway networks mined from human pituitary adenoma proteomics data. *BMC Medical Genomics* **3**, 3-13.

Zhou, Y., Zhang, J., Liu, Q., Bell, R., Muruve, D.A., Forsyth, P., Arcellana-Panlilo, M., Robbins, S., and Yong, W.V. (2005) The chemokine GRO-α (CXCL1) confers to increase tumorigenicity to glioma cells. *Carcinogenesis* **26(12)**, 2058-2068.

Zumsteg, A., Baeriswyl, V., Imaizumi, N., Schwendener, R., Ruegg, C., and Christofori, G. (2009) Myeloid cells contribute to tumor lymphangiogenesis *PLOS One* **4(9)**, e7607.

zur Hausen, H. (2002) Papillomaviruses and cancer: from basic studies to clinical application. *Nature Reviews Cancer* **2**, 342-350.

http://www.emedicinehealth.com/pap_smear/article_em.htm

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APPENDIX A

Commonly Used Buffers and Solutions

Tissue Culture

Complete Medium for HeLa Cells: Dulbecco's Modified Eagles Medium (DMEM) Glutamax F-12 supplemented with (10%) (v/v) fetal bovine serum (FBS), (100 U/ml) penicillin and (100 µg/ml) streptomycin.

Serum Free Medium for HeLa Cells: Dulbecco's Modified Eagles Medium (DMEM) Glutamax F-12 supplemented with (10%) (v/v) (100 U/ml) penicillin and (100 µg/ml) streptomycin.

Complete Medium for HUVECs: Endothelial Basal Medium (EBM-2) supplemented with 2% fetal bovine serum (FBS) and Endothelial Growth Medium (EGM-2) containing growth supplements; VEGF, FGF, PDGF, IGF, EGF, ascorbic acid, heparin and gentamycin.

Serum Free Medium for HUVECs: Endothelial Basal Medium (EBM-2) supplemented with 1% fetal bovine serum (FBS) and the addition of ascorbic acid and gentamycin.

Medium for Cervical Tissue Treated with Seminal Plasma: Dulbecco's Modified Eagles Medium (DMEM) Glutamax F-12 without any supplements.

Immunohistochemistry

Citrate Buffer (0.1M):
42.02g Citric Acid Monohydrate
900ml Distiller Water
pH to 5.5 using concentrated NaOH
Make up to 2L and pH to 6.0

0.5M Tris-HCl stock x10:
121.14g Tris(Hydroxymethyl)methylamine
2L Distilled Water
pH to 7.4 using HCl

0.05 M TBS:
100ml 0.5M Tris-HCl
900ml Distilled Water
8.5g Sodium Chloride

Peroxidase Blocking Solution:
30ml of 30% (3% v/v) Hydrogen Peroxide
270ml Methanol

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Blocking Solution:
2ml non-immune serum
8ml PBS
0.5g BSA

Protein

Lysis Buffer:
150mM NaCl
10% Glycerol
0.6% (v/v) NP-40
50mM Tris/HCl pH 7.4
10mM EDTA
(add protease inhibitor cocktail tablet prior to use)

Running Buffer:
25mM Tris-HCl
0.2M Glycine
0.1% SDS

Loading Buffer:
125mM Tris-HCl pH 6.8
4% SDS
20% Glycerol
5% 2-mercaptoethanol
0.05% bromophenol blue

Transfer Buffer:
25mM Tris-HCl
0.192M Glycine
20% Methanol

Tris Buffered Saline (TBS):
50mM Tris-HCl
150mM NaCl pH 7.4

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Western Blotting

Citrate Buffer (0.1M):
42.02g Citric Acid Monohydrate
900ml Distiller Water
pH to 5.5 using concentrated NaOH
Make up to 2L and pH to 6.0

0.5M Tris-HCl stock x10:
121.14g Tris(Hydroxymethyl)methylamine
2L Distilled Water
pH to 7.4 using HCl

0.05 M TBS:
100ml 0.5M Tris-HCl
900ml Distilled Water
8.5g Sodium Chloride