THE ROLE OF SEMINAL PLASMA IN THE REGULATION OF INFLAMMATION AND INFLAMMATORY PATHWAYS IN THE CERVIX: POTENTIAL FOR CERVICAL CANCER PROGRESSION AND HIV TRANSMISSION IN SOUTH AFRICAN WOMEN

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DECLARATION

I herewith declare that 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.

Anthonio O. Adefuye

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ANOVA	analysis of variance
AP1	activator protein-1
BCP	1-bromo-3-chloropropane
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
CAM	cell-surface adhesion molecule
cAMP	adenosine 3',5'-cyclic monophospate
cDNA	complementary DNA
CIN	cervical intraepithelial neoplasia
COX	cyclooxygenase
DAB	3'3-diaminobenzidine
DC-SIGN	dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
DEPC	diethylpyrocarbonate
dH_20	distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
ECM	extracellular matrix
Ect-1	ectocervical cell line
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
FIGO	international federation of obstetricians and gynaecologists

GPCR	G protein-coupled receptor
H&E	haemotoxylin and eosin
HSIL	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)-triphosphate
Kb	base pairs
kDa	kilodalton
LPS	lipopolysaccharides
LSIL	low-grade squamous intraepithelial lesion
Mg	milligram
µg	microgram
Ml	millilitre
µl	microlitre
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase
Mins	minutes
MMP	matrixmetalloproteinases
mPGES-1	membrane-bound prostaglandin E synthase-1
mPGES-2	membrane-bound prostaglandin E synthase-2
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-kappa B

Nm	nanometers
NSAID	non-steroidal anti-inflammatory drug
Os	opening of the cervix
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
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
PGH2	hydroxy cyclic endoperoxide
PGI_2	prostacyclin
PGIS	prostacyclin I synthase
PGT	prostaglandin transporter
PI3K	phosphatidylinositol 3-kinase
PLA_2	phospholipase A_2
PPAR	peroxisome proliferators-activated
$PTGER_1$	prostanoid receptor EP_1
$PTGER_2$	prostanoid receptor EP_2
$PTGER_3$	prostanoid receptor EP_3
$PTGER_4$	prostanoid receptor EP_4
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
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SIL	squamous intra-epithelial lesions
SP	seminal plasma
STAT3	signal transducers and activators of transcription
Taq	thermus aquaticus
TBS	tris-buffered saline
TGF	transforming growth factor
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor-α
Tris	tris (hydroxymethyl) amino methane
TSP-1	thrombospondin
TXA_2	thromboxane A_2
TXS	thromboxane synthase
UTR	untranslated region
VEGF	vascular endothelial growth factor
WHO	world health organisation
WT	wild type

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SYNOPSIS

Cervical cancer is a chronic inflammatory disease of multifactorial etiology accounting for an annual estimated 266,000 deaths worldwide and usually present in sexually active women. In sub-Saharan Africa, cervical cancer is the most common cancer among women and the leading cause of cancer related deaths in this region. The obvious association of HIV infection and cervical cancer has long been established. High incidence and prevalence rate of HIV infection has been recorded in many areas with high incidence of cervical cancer suggesting that cervical cancer and premalignant cervical lesions may increase transmission and acquisition of HIV infection. Seminal plasma (SP) has been shown to initiate inflammatory response within the female genital tract. Exposure of neoplastic cervical epithelial cells to SP has been shown to promote the growth of cancer cells in vitro and tumors in vivo by activating several pro-inflammatory pathways. In addition to the regulation of tumor growth, SP-mediated inflammatory responses within the female genital tract have been suggested to contribute to the transmission of HIV and other sexually transmitted infections (STIs).

The initial aim of this study was to determine the role of SP in the regulation of pro-inflammatory pathways in neoplastic cervical epithelial cells. TaqMan 96-well array revealed that SP regulates the activation of eicosanoid, toll-like receptor-NFκB, kallikrien-bradykinin-bradykinin receptor, cytokine, and chemokine signaling pathways to mediate the expression of inflammatory mediators in cervical cancer cells. These data highlight the potential of SP to exacerbate inflammatory processes within the local cervical cancer microenvironment creating conditions favorable for cervical tumor progression.

IL-1α is a pleotropic pro-inflammatory cytokine found to be robustly regulated by SP in the array. IL-1α expression is up-regulated in several human cancers and has been associated with virulent tumor phenotype and poorer prognosis. Hence, I investigated the role of SP in the regulation of IL-1α in neoplastic cervical epithelial cells HeLa, neoplastic and normal cervical tissue and the molecular mechanism underlying this regulation.

Quantitative real-time PCR and ELISA confirmed the role of SP in the induction of IL-α expression in neoplastic cervical epithelial cells. Using selected receptor antagonists and agonists and small molecule chemical inhibitors known to inhibit specific biological pathways, I established that SP regulated IL-1α mRNA and protein expression via the activation of EP_2/EGFR/PI3 kinase-Akt signaling in a HeLa (adenocarcinoma) cell line model system. PGE_2 and EGF were identified as possible ligands responsible for SP-mediated induction of IL-1α in these neoplastic cells.

Furthermore, qRT-PCR confirmed overt expression of IL-1α mRNA in cervical squamous cell carcinoma and adenocarcinoma tissue explants, compared with normal cervix. Using immunohistochemistry, IL-1α was localized to the neoplastically transformed squamous, columnar and glandular epithelium in all cases of squamous cell carcinoma and adenocarcinomas explants studied. In addition, SP was found to induce IL-1α expression in normal and cancerous cervical tissue explants via similar pathways as obtained in HeLa cells. The SP-mediated induction of a pleotropic pro-inflammatory cytokine IL-1α in normal and neoplastic cervical epithelial cells suggests that SP may promote cervical inflammation as well as progression of cervical cancer in sexually active women.

To elucidate the relationship between cervical cancer and HIV, I initially investigated the expression of HIV receptor and chemokine co-receptor CD4 and CCR5 in in both normal and neoplastic cervical tissues explants by real-time RT-PCR analysis and immunohistochemistry. CD4 and CCR5 expression was elevated in cervical cancer tissue compared with normal cervix. Ex vivo studies conducted on cervical (normal and neoplastic) tissues and HeLa cells, showed that SP significantly increases expression of CD4 and CCR5. Using a panel of chemical inhibitors of intracellular signaling and a cyclooxygenase (COX)-1 doxycycline-inducible expression system, I found that regulation of CCR5 expression by SP occurred via the epidermal growth factor receptor and COX-1-prostaglandin E_2 pathway. These data provide a link between activation of inflammatory pathways and regulation of HIV receptor expression in cervical cancer cells and suggests that repeated exposure of cervical epithelium and neoplastic cervical epithelial cells to seminal plasma can impact on HIV susceptibility in women.

In conclusion, this study reports that repeated exposure of neoplastic cervical epithelium to seminal plasma in sexually active women with cervical cancer could exacerbate cervical cancer development and result in poorer patient outlook as well as increase susceptibility to HIV infection in the cervical cancer milieu.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Cervical cancer; cancer of the cervix uteri is the fourth most common cancer in women and the seventh overall, with an estimated 528,000 new cases and 266,000 deaths worldwide in 2012 [1]. As with liver cancer, a large majority (around 80-85%) of the global burden occurs in the less developed regions (Figure 1), where it accounts for almost 12% of all female cancers [1,2].

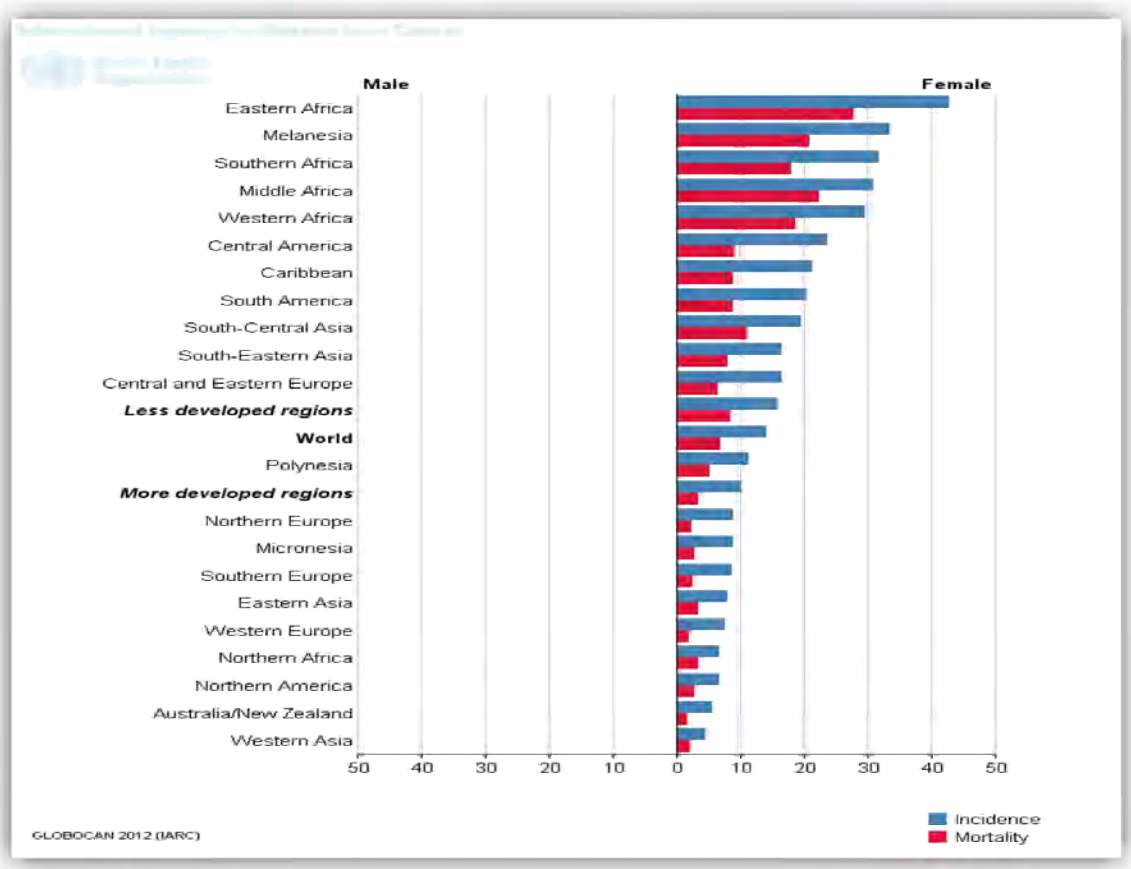


Figure 1.1: Cervical cancer estimated incidence and mortality Worldwide in 2012 [2].

In sub-Saharan Africa, cervical cancer is the most common cancer among women, accounting for 22.2% of all cancer cases and also the leading cause of cancer related deaths in this region [3]. The occurrence of the disease is gradually having a decline trend in the developed countries as a result of increased awareness, routine screening and early detection/intervention, while in many countries in sub-Saharan Africa incidence rate is on the rise as 60-75% of women affected reside in the rural areas [4] where there is poor access to health facilities; malnutrition and co-morbid conditions e.g. HIV infection [5,6]; poor awareness; late presentation with disease [7]; limited facilities for treatment; high rate of loss to follow-up [8]; and failure to complete treatment.

1.2 Etiology and risk factors

The etiology of the disease is multifactorial. However, the single most common risk factor attributed to cervical cancer is infection with high risk *human papillomavirus* (HPV) (type 16 and 18). HPV DNA is found in 90-99.7% of cervical cancer cases [1] and many of the factors that could increase HPV acquisition and promote its oncogenic effect are wide spread in sub-Saharan Africa. Such factors include; high parity, early marriage, and polygamous marriages [9,10]. It has been estimated that the life-time risk of contracting HPV is 80% [11] and averagely, it takes about 12-15 years before a persistent HPV infection in the phase of premalignant lesion (CIN) leads to cervical cancer [12,13]. Although infection with HPV is highly prevalent among sexually active women, only a few of these infected women will develop cervical cancer thus suggesting that other co-factors are necessary for the development of neoplasia [14].

Other risk factors include low socio-economic status; immunosuppression from co-morbidities such as TB, HIV, and *herpes simplex virus* (type 2) [15]; non-HPV sexually transmitted infection (STI) such as; *Neisseria gonorrhea* [16,17], *Chlamydia trachomatis* [18,19], and bacterial vaginosis [20]. The presence of these STIs in the cervix elicit chronic inflammatory responses leading to the release of free radical and subsequent activation of various inflammatory pathways which have been found to play a vital role in disease progression. Similarly, cigarette smoking has also been identified as an independent risk factor for the development of cervical cancer [21-23] as tobacco-specific carcinogens and polycyclic aromatic hydrocarbons have been detected in the cervical epithelium or mucus of smokers [24,25]. These compounds can cause malignant transformation by binding and damaging cellular DNA, hence enhancing the effect of HPV [1].

1.2.1 *HIV; a co-factor in cervical cancer*

In January, 1993, the surveillance case definition of AIDS was expanded to include cervical cancer in women infected with human immunodeficiency virus, thus classifying it, Kaposi Sarcoma, and Non-Hodgkin Lymphoma as an AIDS-defining disease [26]. This makes HIV an important risk factor/co-factor in the development of cervical neoplasia. The role of HIV in the development of cervical cancer has been largely attributed to immunosuppression, but recent findings suggests a molecular interaction between HIV and HPV playing a vital role in disease occurrence and progression [3,27].

1.3 Classifications

Cervical cancer can be classified into three major histological categories as recognized by WHO (World health organization) [28] these include; squamous cell carcinoma, adenocarcinoma, and other undifferentiated carcinomas.

1.3.1 *Squamous cell carcinoma of the cervix*

This is the most common histological type of cervical cancer accounting for approximately 70 - 80% of all cases [1]. Cervical squamous cell carcinomas have been further classified into; large cell keratinizing, large cell non-keratinizing, and small cell carcinomas according to the predominant cell type. Large cell non-keratinizing squamous cell carcinomas account for the majority of tumors.

1.3.2 *Adenocarcinoma of the cervix*

Adenocarcinoma of the cervix represents the second most common histological type accounting for approximately 20-25% of tumors [1]. It is derived from the glandular cells of the cervix. Over the past decade, there has been an increase in the incidence of adenocarcinoma especially in women younger than 35 years of age, attributed to an increase in prevalence of HPV infection [29,30].

1.3.3 *Other uncommon subtypes:*

1.3.3.1 *Verrucous carcinoma of the cervix*

Verrucous carcinoma is a rare subtype of well differentiated squamous cell carcinoma and it has been associated with HPV 6. Histological features comprises of well differentiated squamous cells with fond-like papillae and minimal stromal invasion. It is a slow growing locally invasive tumor [31].

1.3.3.2 *Adenosquamous carcinoma of the cervix*

Adenosquamous carcinoma of the cervix contains an admixture of glandular and malignant squamous cells. It accounts for approximately 1-2% of cases [1].

1.3.3.3 *Neuroendocrine (small-cell) carcinoma of the cervix*

Neuroendocrine carcinoma of the cervix is a rare malignancy, representing less than 5% of all cases of cervical cancer. It is characterized by frequent and early nodal and distant metastases, resulting in a relatively poor prognosis [32].

1.3.3.4 *Adenoma mallignum of the cervix*

Adenoma mallignum of the cervix is a well differentiated adenocarcinoma that is difficult to recognize as malignant condition. It is associated with peutz-jeghers syndrome and account for 1% of adenocarcinomas [33].

1.3.3.5 *Adenoid cystic carcinoma of the cervix*

Adenoid cystic carcinoma of the cervix is an uncommon variant of adenocarcinoma and occurs more commonly in postmenopausal women and multiparous black women in sixth and seventh decades of life. However, it is known to develop before age 40 in certain women [34].

1.3.3.6 *Metastatic tumors*

Metastatic tumors within the cervix are uncommon if one excludes endometrial carcinoma, which involves the cervix by direct spread. A variety of other neoplasms rarely metastasize to the cervix [35]. These include tumor occurring as direct extension from the bladder, and rectum and lymphatic or vascular metastasis from distant organs such as ovaries, breast, colon, and kidneys [35].

1.4 Clinical presentation

Abnormal vaginal bleeding is the most common symptom of invasive cancer of the cervix and it could present as a blood stained discharge, scant spotting, or frank bleeding [36]. Abnormal vaginal discharge is usually purulent, odorous and non-puritic. A positive history of post-coital bleeding may occur. Patients can also complain of pelvic pain which is often unilateral and radiating to the hip or thigh in advanced cases. Symptoms of systemic illness such as generalized weakness, weight loss and anemia can also be present [37].

Signs and symptoms of local metastasis may include loss of urine and faeces per-vagina indicating fistula formation with the bladder and rectum respectively [1].

1.5 Diagnosis

The natural history of cervical cancer as a continuous single disease progressing steadily from mild cervical intraepithelial neoplasia (CIN1) to more severe degrees of neoplasia and microinvasive lesions (CIN 2 or CIN 3) and finally to invasive carcinoma has been the basis for diagnosis [38]. The median age of cervical cancer diagnosis is 47 years [1]. However, disease occurrence can begin as early as third decade of life [1] and during pregnancy in predisposed women [39]. The primary method for the detection of high-risk HPV is the Papanicolaou-stained (Pap) smear [40], which is a screening tool that detects changes in cells of the transformation zone of the cervix often caused by HPV. Invasive carcinoma of the cervix may be suspected on the analysis of Pap smear or on visualizing a lesion on the cervix. Pap smear reporting classification has evolved and been refined over time. The current reporting system is the Bethesda System (Table 1) [40,41].

Pap smear may in some cases be falsely negative or non-diagnostic, hence any suspected lesion on the cervix must be biopsied and examined histologically for neoplastic/dysplastic epithelial cells [1]. If biopsy reveals cells suggestive of carcinoma in situ/or micro-invasion, or in the setting of a negative colposcopy with significantly abnormal cervical cytology smear cone biopsy (conization) should be done to diagnose the presence or absence of invasion. However, greater than 90% of early onset diagnosis can be cured.

Table 1.1: The Bethesda and CIN Classification System for cervical squamous cell dysplasia[40]

Bethesda System	CIN System	Interpretation
Negative for intraepithelial lesions or malignancy	Normal	No abnormal cells
ASC ASC-US (atypical squamous cells of undetermined significance) ASC-H (atypical squamous cells, cannot exclude HSIL)		Squamous cells with abnormalities greater than those attributed to reactive changes but that do not meet the criteria for a squamous intraepithelial lesion.
LSIL (low-grade squamous intraepithelial lesion)	CIN 1	Mildly abnormal cells; changes are almost always due to HPV
HSIL (high-grade squamous intraepithelial lesion) with features suspicious for invasion (if invasion is suspected)	CIN2/3	Moderately to severely abnormal squamous cells.
Carcinoma	Invasive squamous cell carcinoma Invasive glandular cell carcinoma (adenocarcinoma)	The possibility of cancer is high enough to warrant immediate evaluation but does not mean that the patient definitely has cancer.

1.6 Clinical staging

Once tissue diagnosis of invasive carcinoma has been made, it is important to estimate the extent of the disease for both prognostic purposes and treatment planning. The most widely used staging system is that of FIGO (International Federation of Gynecology and Obstetrics) staging of cervical cancer (Table 2). Staging is done at the time of primary diagnosis and is determined clinically using the size of the cervical tumor and/or its extent of spread to the pelvis [1].

Table 1.2: Cervical cancer staging according to FIGO staging system [1].

Stage	Description
Stage 0	Carcinoma-in-situ, intraepithelial carcinoma
Stage I	Invasive carcinoma strictly confined to the cervix
Stage IA	Invasive carcinoma identified microscopically
Stage IA1	Measured invasion of stroma 3 mm or less in depth and no wider than 7 mm
Stage IA2	Measured invasion of stroma more than 3 mm but no greater than 5 mm in depth and no wider than 7 mm
Stage IB	Preclinical lesions greater than stage IA or clinical lesion confined to the cervix.
Stage IB1	Clinical lesions 4 cm or less in size
Stage IB2	Clinical lesions more than 4 cm in size
Stage II	Carcinoma extending beyond the cervix but not to the pelvic sidewall; carcinoma involves vagina but not its lower third
Stage IIA	Involvement of upper two-third of the vagina, no parametrial involvement
Stage IIB	Obvious parametrial involvement

Stage III	
	Carcinoma extending onto the pelvic wall; no cancer free space between the tumor and pelvic sidewall (on rectal examination). Involvement of the lower third of the vagina.
Stage IIIA	Involvement of lower third of the vagina; no extension to pelvic sidewall
Stage IIIB	Extension to pelvic sidewall and/or hydronephrosis or nonfunctioning kidney
Stage IV	
	Carcinoma extends beyond true pelvis or clinically involves mucosa of bladder or rectum.
Stage IVA	Spread of tumor to adjacent organs
Stage IVB	Spread of tumor to distant organs

1.7 Molecular Pathogenesis

1.7.1 Papillomaviruses

The papillomaviruses belong to the family Papovaviridae. A mature virion is about 52-55 nm in diameter with a supercoiled circular double stranded DNA (8kb in length) enclosed by an icosahedral structure composed of 72 capsomers [42,43]. All the reading frames are on one strand of the DNA. The virus infects squamous epithelia (or cells with potential for squamous maturation) [43], inducing chronic infections that have no obvious systemic sequellae and rarely cause mortality in the infected host, but periodically shed large amounts of infectious virus that can be transmitted to naïve individuals [44]. Infection can also give rise to the induction of proliferative lesions e.g. skin warts and occasionally neoplasia [43]. They are exclusively species specific hence they could be grouped/classified using the host specie i.e. human papilloma virus (HPV) infecting only human. Papillomaviruses are also tissue-tropic having tendency to infect cutaneous and /or internal squamous mucosal surfaces in their host [43].

Papillomaviruses are classified by the host species infected and the degree of DNA sequence homology (genotype) within specific viral genes. To date, about 200 HPV types have been identified and cloned based on sequencing gene encoding the capsid protein L1 [40,45], of these types 16, 18, 31, 35, 39, 45, 51, 52, 56, 58, 66, and 70 have been implicated in most cases of cervical cancer [1,40] (Table 3). Furthermore, based on their oncogenic potential and frequency of occurrence in neoplastic lesions, they have been classified into; low risk types such as HPV 6b, 11, 40, 42, 43, and 44 associated with low grade lesions such as cervical intraepithelial neoplasia 1 (CIN 1) and cervical condylomas; intermediate (or high in some literatures) risk types such as HPV 33, 31, 35, 45, 51, 52, 56, 58, and 59 found infrequently in all grades of CIN [46-48]; high risk types HPV 16 and 18 found in 90% of cervical cancer and about 50-80% of

CIN 2 and CIN 3 [49-51] (Table 3). HPV 16 is the most prevalent type in squamous cell carcinoma while type 18 the most prevalent in adenocarcinoma. The mere presence of HPVs alone doesn't confer neoplasia as the virus can be found in about 3-30% of asymptomatic patients [50], thus indicating that other events such as viral persistence and/or altered viral gene expression are required to initiate neoplastic process.

HPV types have different effects on the transition from CIN to cancer and the approximate measure of the risk of transition from intraepithelial neoplasia to invasive cancer can be calculated using the cancer:CIN prevalence ratio [42]. Studies have shown that HPV 18 is associated with a higher cancer:CIN ratio than HPV 16, suggesting that it confers a more aggressive neoplastic phenotype than type 16 [52,53].

Table 1.3: HPV types and disease association [54,55]

Disease	HPV type
Plantar warts	1, 2, 4, 63
Common warts	2, 1, 7, 4, 26, 27, 29, 41, 57, 65, 77, 1, 3, 4, 10, 28
Flat warts	3, 10, 26, 27, 28, 38, 41, 49, 75, 76
Other cutaneous lesions (e.g., epidermoid cysts, laryngeal carcinoma)	6, 11, 16, 30, 33, 36, 37, 38, 41, 48, 60, 72, 73
Epidermodysplasia verruciformis	2, 3, 10, 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 50
Recurrent respiratory papillomatosis	6, 11
Focal epithelial hyperplasia of Heck	13, 32
Conjunctival papillomas/carcinomas	6, 11, 16
Condyloma acuminata (genital warts)	6, 11, 30, 42, 43, 45, 51, 54, 55, 70
Cervical intraepithelial neoplasia	
Unspecified	30, 34, 39, 40, 53, 57, 59, 61, 62, 64, 66, 67, 68, 69
Low risk	6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52, 74
High risk	16, 18, 6, 11, 31, 34, 33, 35, 39, 42, 44, 45, 51, 52, 56, 58, 66
Cervical carcinoma	16, 18, 31, 45, 33, 35, 39, 51, 52, 56, 58, 66, 68, 70

1.7.2 Transmission and immune evasion

HPV infectious cycle begins when the cervical epithelium is exposed to virus present in the saliva, seminal fluid or skin of infected partner during coitus. The virus enters the body through mild abrasion or micro-trauma to the cervico-vaginal epithelium by first binding to the heparin sulfate proteoglycans (HSPGs) on the basal membrane exposed after tissue disruption/abrasion [56] and proceed to infect primitive basal keratinocytes (Figure 2) [57], with high level expression of viral proteins and viral assembling confined to the upper layers of the stratum spinosum and granulosum of the epithelia [58] (Figure 2).

Post transmission the virus remains confined to the epithelia mucosa where it does not come in contact with the bloodstream hence evading the innate immune system. The ability of the virus to evade immune detection and immune-invasion has been considered to play a vital role in viral persistence [43]. For the most part of its infectious cycle, there is no local release of pro-inflammatory cytokines that are important for dendritic cell (DC) activation and migration, and the essential signals required to activate immune response is also absent in the infected epithelia. Even though there is no viraemia, viral-induced cytolysis, or cell death, infected keratinocytes are expected to activate the antiviral defense system, the type 1 interferons (IFNs). The type 1 INFs (IFNα and IFNβ) possess anti-proliferative, anti-angiogenic, antiviral and immune-stimulatory properties acting as a bridge between innate and adaptive immunity activating immature DCs [59]. The virus is capable of actively inducing mechanism that de-regulate the interferon pathway via down regulation of pattern recognition receptors such as Toll-like receptor 9 (TLR-9), hence enabling infection to proceed undetected [43]. Since these receptors augment innate and adaptive responses to pathogens by activating the antigen presenting cells and phagocytes [60,61].

Similarly, the high risk HPV (16 and 18) viral oncoprotein E6 and E7 also directly interact with components of the IFN signaling pathway [62]. Type 16 E7 inhibit IFNα-mediated signal transduction by binding to IFN regulatory factor (P48/IRF-9), this prevents translocation to the nucleus, thus inhibiting the formation of the IFN-stimulated gene factor 3 (ISGF-3) transcription complex that binds IFN-specific response element (ISRE) in the nucleus [63]. It also impedes intermediate IFN-mediated signaling by forming a complex with IRF-1, inhibiting IRF-1 mediated activation of IFNβ promoter and recruiting histone deacetylase to the promoter, in so doing preventing transcription [64]. Type 18 E7 oncoprotein on the other hand reduces the expression of IRF-1 target gene e.g. monocyte chemotactic protein 1 (MCP-1), transporter associated with antigen processing 1 (TAP1) and IFNβ by inhibiting the transactivation of IRF-1 [65]. E6 can bind to IRF-3, inhibiting its transactivating function, thereby preventing IFNα mRNA transcription [66], it can also inhibit phosphorylation of Tyk2, STAT (signal transducer and activator of transcription) 1 and 2, impairing JAK (Janus kinase)/STAT activation, and therefore inhibiting specific IFNα-mediated signal transduction [67].

In addition, studies have shown that HPV type 16 generally alters the expression of three groups of gene which are; cell-cycle signaling genes, IFN-response genes and NF-κB (nuclear factor kappa B) - stimulated genes [68-70]. It is therefore evident that the viral oncoprotein E6 and E7 play a fundamental role in host resistance to infection and immune function by direct alteration of various genes and pathway complexes required for immune detection and invasion.

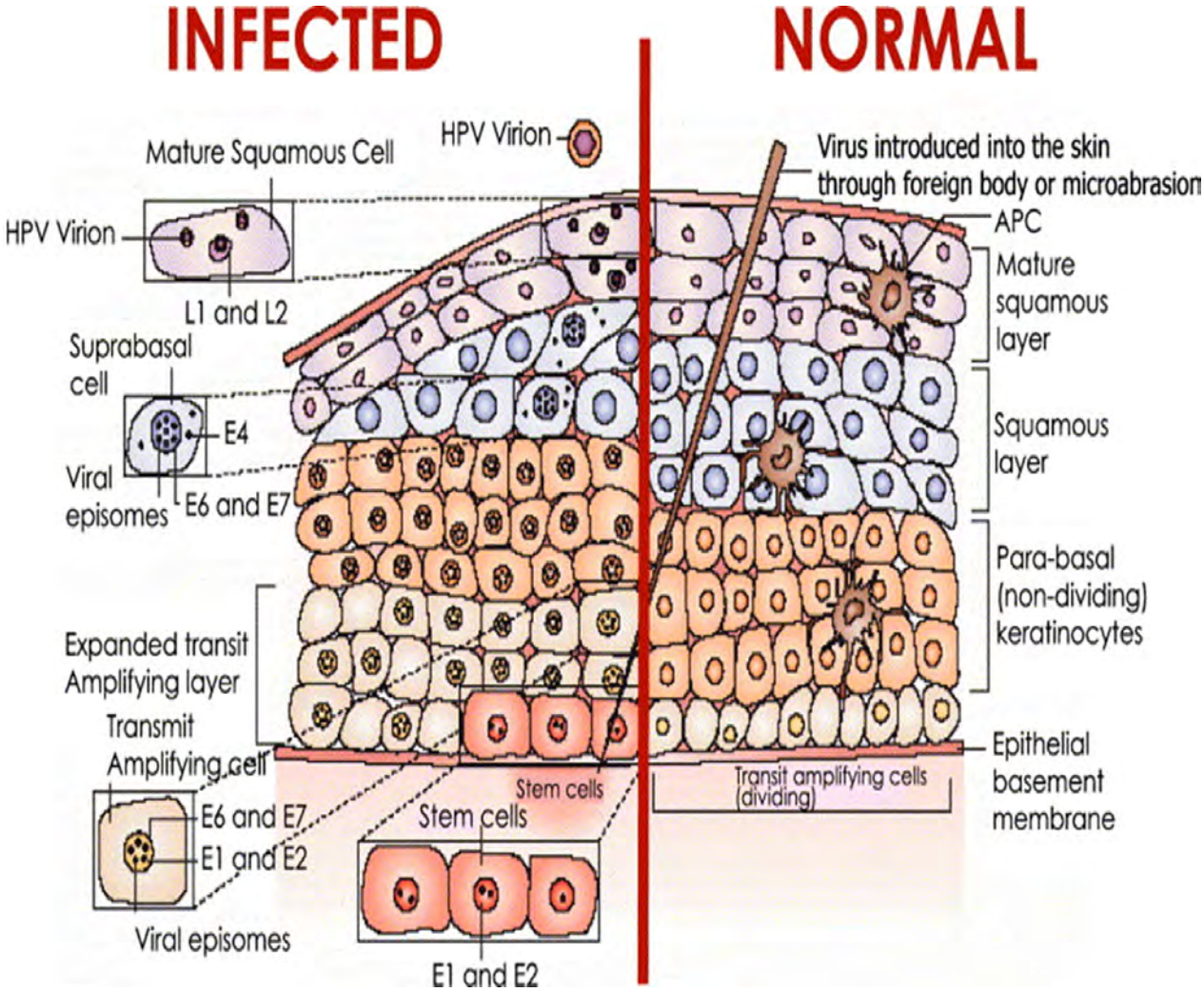


Figure 1.2: Schematic presentation of the infectious cycle of HPV showing strong E6 and E7 expression in differentiated epithelial cells. Infectious cycle begins when the virus present in the saliva, seminal fluid or skin of infected partner during coitus. The virus enters the body through mild abrasion or microtauma to the cervico-vaginal epithelium and proceeds to infect primitive basal keratinocytes, targeting stem cells. Daughter cells of epithelial stem cells divide along the basement membrane and then mature vertically through the epithelium without further division (right side). After the introduction of HPV into the stem cells in the basal layer of the epithelium, expression of viral non-structural proteins occurs. Under the regulation of these proteins, the dividing-cell population expands vertically and epithelial cell differentiation is delayed and is less complete. Viral proteins are expressed sequentially with differentiation, as shown and mature virions are produced only in the most superficial layers of the epithelium. Intraepithelial antigen-presenting cells (APCs) are depleted in the HPV-infected epithelium [57].

1.7.3 Genome Integration and Oncogene expression

Within the primitive basal cells, the viral oncogenes are incorporated into the host's DNA, where they utilize the host's DNA replication machinery to bring about viral DNA replication. This round of DNA replication process is independent of the cell cycle and help to amplify the viral copy to about 50-100copies/cell [43]. This is then followed by a phase of plasmid maintenance during which there is minimal viral gene expression. At this stage the, expression of the viral oncogene (especially E6 and E7) is also under strict control with their transcript barely detectable [43].

However, as the infected cells mature and enter the differentiation stage, there is massive rapid viral DNA replication with associated up-regulation of viral gene expression. This brings the number of viral copy to about 1000 copies/cell with abundant expression of the early genes (E6 and E7) and expression of the late genes from the late promoter [71]. As these cells mature they migrate vertically towards the epidermal layer. On getting to the superficial epidermal layers, they then undergo programmed cell death (apoptosis) which results in the release of viral particles. The whole replication cycle is said to take about 3weeks which coincide with the time taken by keratinocyte to mature, differentiate and desquamate [43]. There exist no blood-borne phase of infection as infectivity is completely intra-epithelial, however, some of the replicating virus may be exposed to the immune system [43]. On detection, the immune (innate and adaptive) systems initiate inflammatory responses against the virus, thus eradicating it in majority of infected cases [20,72]. However, in about 15-20% of cases this inflammatory response fails to clear the virus [73,74], hence leading to persistent infection, which together with inadequate resolution or exacerbation of activation of inflammatory pathways can promote malignant progression [43,75].

1.7.4 HPV oncogenes and cervical tumorigenesis

The viral genome is made up of 3 domains; a non-coding upstream regulatory region (URR) of about 1kb, an early region containing open reading frames (ORFs) E6, E7, E1, E2, E4, and E5 and a late region encoding the major and minor caspid proteins (L1 and L2) (Figure 3) [43]. Once infection ensue, the E6 and E7 early genes are actively transcribed to express the E6 and E7 oncoproteins, both of which are highly implicated in neoplastic transformation [71]. Although E6 and E7 oncogenes appears to be the major HPV oncogenes involved in neoplastic change, recent studies have demonstrated a role for the E5 oncogene in tumorigenesis and immune cell modulation [76] and regulation of late viral functions together with E4 oncogene. E1 and E2 oncogenes encode replication factors and are thought to play a role in HPV resistance by allowing episomal copies of the virus to be maintained in the nucleus and partitioned into daughter cells during mitosis [77]. Many of the observations of the roles of HPV oncogenes have been derived using in vitro model systems of HPV-containing cell lines where specific oncogenes have been ablated by RNA interference, or HPV negative cells that have been transfected with cDNA constructs containing specific oncogenes. These studies have shown that both oncoproteins target different molecular pathways in the cell to promote neoplastic state. E6 oncoprotein binds to P53 protein and targets it for ubiquitin-dependent degradation, while the E7 binds to the family members of the retinoblastoma protein (Rb) and disrupts the complex between Rb and the E2F transcription factor family (Table 4) [78].

These effects on P53 and Rb tend to cause an alteration in the cell cycle as both proteins are negative growth regulators that control transit from G_0/G_1 to S phase [42].

Similarly, P53 is also involved in DNA response to damage, by inducing either growth arrest or cell death by apoptosis. Hence, HPV mediated loss of Rb and P53 function facilitates immortalization of the cells and triggers the early steps in malignant transformation by disrupting cell cycle control [43,78]. Resulting into uncontrolled replication and a build-up of damaged DNA - ideal conditions for cancer development.

Animal studies using transgenic mouse models of cervical cancer have highlighted more specifically the contribution of individual oncogenes to tumorigenesis and have shown that the E7 proteins induces high-grade cervical dysplasia and invasive tumors, whereas the E6 proteins induces low-grade cervical dysplasia. Furthermore, they have highlighted that both oncogenes work in synergy to promote cervical cancer progression, since co-expression of E6 and E7 proteins produce larger and more extensive tumor compared to E7 alone [79].

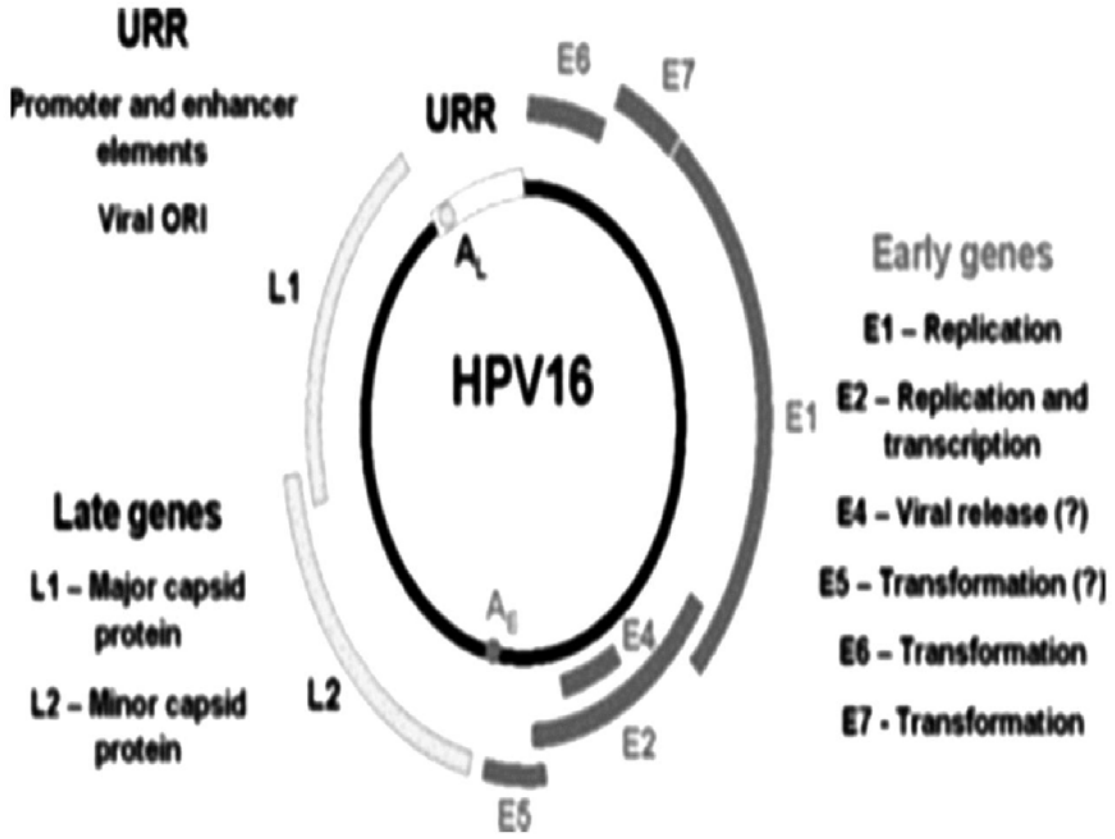


Figure 1.3: Genome organization of high risk HPV 16 [43].

Table 1.4: High risk HPV ORFs and their role in tumorigenesis [43].

ORF	Role in tumorigenesis
E6	Binds to P53 protein and targets it for ubiquitin-dependent degradation
E7	Binds to the family members of the retinoblastoma protein (Rb) and disrupts the complex between Rb and the E2F transcription factor family
E1	ATP binding protein essential for viral DNA replication
E2	Viral transcription factor; binds to E1 to facilitate initiation of vital DNA replication important in genome encapsidation.
E5	Possess weak transforming activity, but contributes to up-regulation of growth factor receptor
E4	Interacts with cytoskeletal proteins, allows viral assembly.

1.8 The role of inflammation and inflammatory pathways in tumorigenesis

Inflammation is a complex process involving a coordinate effort by the body to maintain homeostasis in the face of insult by disease pathogens (e.g. bacterial, viral or fungi) or by injury. It involves a host of resident and recruited immune cell types working together to promote the removal of the insult or injury and initiate tissue repair [80]. When successful, these results in the restoration of tissue homeostasis, a process termed resolution [81-83].

Rudolf Virchow in 1863 demonstrated the presence of leukocytes in neoplastic tissues and made a link between inflammation and cancer (Table 5). Virchow proposed that the origin of cancer at sites of chronic inflammation is reflected by the lymphoreticular infiltrate at such site [72]. Over the past decades, our knowledge of the inflammatory microenvironment of malignant tissues has evolved to support Virchow's hypothesis. Hence, the alliance between cancer and inflammation is beginning to have an impact on mode of treatment and cancer prevention. Approximately 15-20% of the global cancer burden is attributable to infectious agents [84] and inflammation is an essential element of these chronic infections.

Similarly, chronic inflammatory responses mediated by certain chemicals and autoimmune diseases have also been associated with increased risk of neoplastic changes and ultimately invasive malignancy [85,86].

Table 1.5: Association between specific cancers and infectious agent/inflammatory stimulus [72]

Malignancy	**Infectious agent/inflammatory stimulus**
Cancer of the bladder	*Schistosomia hematobium* (Schistosomiasis)
Bronchial cancer	Silica (silicosis), asbestos (asbestosis), cigarette smoke.
Cervical cancer	Papillomavirus (Human papilloma virus)
Colorectal cancer	Inflammatory bowel disease
Esophageal cancer	Barrett's metaplasia
Gastric cancer	*Helicobacter pylori* (gastritis)
Hepatocellular cancer	Hepatitis virus (B and C)
Kaposi's sarcoma	Human herpes virus type 8
MALT lymphoma	*Helicobacter pylori*
Mesothelioma	Asbestos
Ovarian cancer	Pelvic inflammatory disease, talc, tissue remodeling.

1.8.1 Inflammatory cell component of tumors

The inflammatory microenvironment of tumors (including cancer of the cervix) is characterized by the presence of vast population of different host immune cells in the stroma and tumor areas at any given time – each capable of synthesizing cytokines or other factors which can alter the fate of the immune populations within the tumor. Leukocytes are recruited into the tissue in response to cytokines and chemokines released by tumor cells and are resident both in the stroma and in the tumor itself [87].

1.8.1.1 Polymorphonuclear leukocytes (PMN's or neutrophils)

Polymorphonuclear leukocytes (PMN's or neutrophils) are the first immune cell types to be recruited to sites of inflammation, followed by monocytes which are derived from haemopoietic progenitor cells [88]. When in the tissue, monocytes then differentiate into either macrophages or dendritic cells (called Langerhans cells in the epidermis). Langerhans cells generally constitute the first defense against pathogens. Recently tumor associated neutrophils have been described that are capable of polarizing the phenotype of other immune cells and altering the cellular composition of the tumor microenvironment [89]. Here PMN's are thought to exist in either an N1state, capable of killing tumor cells by producing and releasing cytotoxic compounds, or an N2 state capable of promoting tumor growth by modulating the cytokine/chemokine environment in the tumor [89].

1.8.1.2 Macrophages

A significant proportion of the immune infiltrate in tumors is comprised of tumor-associated macrophages (TAMs). These are derived from circulating monocyte precursors via the release of

monocyte chemotactic protein (MCP) chemokines [75]. Although TAMs are a heterogeneous cell population, early stage tumors generally have type 1 macrophages (M1). These produce pro-inflammatory cytokines and chemokines, such as CXCL10 and CXCL19 to recruit Th1, Th17 and natural killer (NK) cells [90]. In more advanced tumors, TAMs polarize towards a type 2 (M2) state to encourage Th2 differentiation and the production of potent angiogenic factors such as VEGF to facilitate tissue remodeling which is thought to promote tumorigenesis [91].

1.8.1.3 Dendritic cells (DC)

DCs play a crucial role in the activation of antigenic-specific immunity and the maintenance of tolerance, thus helping to provide a link between innate and adaptive immune systems. However, tumor-associated dendritic cells (TADCs) typically have an immature phenotype with defective ability to stimulate T cells [92], making them poor inducers of effective response to tumor antigens.

1.8.1.4 Lymphocytes

Natural killer cells (NK) are quite rare to find in the tumor microenvironment [87]. The predominant T-cell populations possess a "memory" phenotype. In cervical cancer these cells have been discovered to produce interleukins (IL) 4 and 5 [93] which are associated with the T-helper type 2 (Th2) cells. Polarized Th2 responses are generally ineffective against tumors and viruses. Similarly, T-cell receptor signaling has also been found to be defective in tumor-infiltrating T cells (TIL) [94].

1.9 Pro-inflammatory cytokines

The cytokine and chemoattractive cytokine (chemokine) networks vary in many tumor tissues, compared with normal tissue [95,96]. It has been discovered that the cytokine complex of many tumors is loaded with pro-inflammatory cytokines, chemokines and growth factors but commonly lacks specific cytokines required for immune sustaining responses [95]. These inflammatory cytokines and chemokines that can be produced by the neoplastic cells themselves and/or tumor-associated leukocytes, and platelets have been found to contribute immensely to tumorigenesis and malignant progression. The high level of hypoxia found in the tumor cells have been discovered to be responsible for the induction of many of these cytokines and chemokines [97]. A good example of some of the most important cytokine found in tumor microenvironment are; tumor necrosis factor (TNF), interleukin 1 (IL-1), interleukin 6 (IL-6) and chemokines.

1.9.1 *Tumor necrosis factor (TNF)*

Tumor necrosis factor (TNF) is a major cytokine involved in inflammation and tissue remodeling. It stimulates fibroblast growth and angiogenesis as well as the production of MCP-1 (monocyte chemotactic protein 1), which regulates the infiltration of macrophages and lymphocytes [75]. It has been found to play an essential role in several cancer models, and a critical mediator of inflammation in autoimmune disease in both humans and mice, acting primarily via the induction of NF-κB [98,99]. Similarly, the direct evidence to demonstrate the role of TNF in malignancy comes from the observation that mice lacking the TNF gene do not develop skin carcinogenesis [100].

1.9.2 *Interleukin 1 (IL-1)*

IL-1 represent a term used for two polypeptides (IL-1α and IL-1β) that possess a wide range of inflammatory properties [101]. It has been found to stimulate tumor growth, development and metastases. IL-1 also plays a role in leukocyte adherence to the vascular endothelium to promote extravasation of the vasculature and tumor infiltration [75]. In addition, IL-1 can activate NF-κB in a manner similar to TNF [102].

1.9.3 *Interleukin 6 (IL-6)*

IL-6 is a key cytokine that promotes cancer cell proliferation while also inhibiting their apoptosis via the activation of signal transducer and activator of transcription 3 (STAT 3) [103,104]. IL-6 acts as an angiogenic factor and has also been implicated in the same processes as TNF [102]. Additionally, IL-6 promotes the activation of the coagulation cascade, differentiation of B cells and T cell activation [105].

1.9.4 *Chemokines*

Inflammatory cytokines are also major inducers of chemokines in many resident and recruited cell types. Chemokines represent a group of small chemotactic protein (approximately 8-11 kDa in size) [106], which are grouped on the basis of the arrangement of the two N-terminal cysteine residues and are designated as CXC, CC, C, and CX_3C depending on whether the first two cysteine residues have one or more amino acid between them (CX..C) or are adjacent to one another (CC) [106]. The CXC and CC families are the most numerous of all known chemokines.

They interact with cell-surface G protein coupled receptors [106] and to date, of the 50 chemokines discovered only about 18 chemokine receptors have been described [107].

Chemokines are renowned for their ability to stimulate directional movement of virtually all classes of leukocytes and in addition stimulate the affinity of integrins on leukocytes for endothelial receptors such as intracellular adhesion molecule 1 and 2 and vascular adhesion molecule -1 (VCAM-1) to facilitate diapedesis into the inflamed/cancerous tissue [108]. CXC chemokines are generally active on neutrophils and lymphocytes, whereas CC chemokines act on monocytes, dendritic cells, lymphocytes and natural killer (NK) cells, but not neutrophils [72]. Similarly, the receptors for these chemokines can be found mainly on the infiltrating leukocytes and the tumor cells [72].

2.1 Molecular role of inflammatory cytokines/chemokines in tumorigenesis

Inflammatory cytokines and chemokines augment tumorigenesis by influencing growth and development, proliferation, mutation, and movement of both the neoplastic and stroma cells [72] via a variety of complex molecular mechanism such as: induction of angiogenesis, DNA damage, acting as growth factors, and enhancing cell metastasis.

2.1.1 *Induction of angiogenesis*

Prolonged and excessive pathological angiogenesis is an important feature of cancer and inflammatory diseases that may predispose to cancer [72,109]. Once established tumor cells may acquire further characteristic which stimulate new vessel formation and it has been established that various signals such as; metabolic stress (e.g. low pH, hypoxia, and hypoglycaemia), mechanical stress (e.g. pressure from proliferating cells), genetic mutations (e.g. deletion of

tumor-suppressor gene controlling production of angiogenic regulators), and immune/inflammatory response [110,111] can trigger neovascularization. Tumor vessels development involves various mechanism including; sprouting or intussusceptions from pre-existing vessels, mobilization of endothelial precursors from the bone marrow [112,113], and by growth of tumor cells around existing vessels to form perivascular cuff [114]. It is known that there are various molecular players involved in these different mechanisms, and among these are vascular endothelial growth factor (VEGF) and angiopoietin (Ang) [114].

Infiltrating immune cells (such as monocytes, macrophages, mast cells and platelets) secrete an array of angiogenic factors such as VEGF, TGF-β1, bFGF, Ang 1, insulin like growth factor 1 (IGF-1) etc. some of which contribute to tumor angiogenesis [115,116]. Cytokines (e.g. TNF, IL-1, and IL-6) can stimulate the production of angiogenic factors such as VEGF. Chemokines also augment angiogenesis in tumor microenvironment. The N terminus of some CXC chemokines contains three amino acid residues [Glu-Leu-Arg (ELR motif)] preceding the first cysteine amino acid residue of the primary structure of these cytokines [117,118]. Chemokines containing the ELR motif (ELR^+) are angiogenic factors and members of this angiogenic CXC chemokines include, CXCL1, CXCL 2, CXCL 3, CXCL 5, CXCL 6, CXCL 7 and CXCL 8 [117]. These chemokines act directly as chemotactic factor to endothelial cells and can also stimulate angiogenesis in vivo [117,119]. Furthermore, CXCL 12 an ELR^- CXC chemokine has also been discovered to promote angiogenesis by increasing the expression of VEGF.

2.1.2 *Acting as growth factors*

Cytokines and chemokines found in tumor microenvironment do not only attract infiltrating cells into tumor site but also have the potential to stimulate tumor cell growth, proliferation and

survival by acting as autocrine growth factors for neoplastic cells. The best described chemokines in this group are the growth-related (GRO) family chemokines (e.g. CXCL 1, CXCL 2, and CXCL 3) and CXCL 8 [120-122]. Similarly, cytokines (IL-1 and IL-6) have also been found to act as growth factors for various tumors such as hematological and gastric malignancies [123,124].

2.1.3 *DNA damage*

DNA damage is a hallmark of cancers. Inflammatory cytokines (e.g. TNF) induces nitric oxide synthase (NO synthase) to produce reactive oxygen in the form of nitric oxide (NO) [125]. This NO can then directly oxidise DNA to cause mutagenic changes and damage to some DNA repair proteins [125]. Cytokines may also disrupt genome integrity by inhibiting cytochrome p450 or glutathione S-transferase isoenzymes [72].

Furthermore, inflammatory cytokines can induce DNA damage by causing functional inactivation of the tumor-suppressor protein p53.

2.1.4 *Enhancing cell metastasis and invasion*

Inflammatory cytokines and chemokines influence various stages in the process of metastasis. Cytokines and CC chemokines promote tumor cell invasion and metastasis by inducing the production of proteases which are required for cell metastasis [72]. IL-1 and TNF have been shown to augment the expression of adhesion molecules on endothelial cell [86,126]. Recent findings have suggested that the chemokine receptor CCR7 and ligand CCL21 involved in the emigration of activated DC to lymph nodes are likely involved in nodal metastasis [106]. Similarly, activation of the chemokine receptor CXCR4 by its ligands CXCL12 enhances

metastatic properties by rapidly increasing the affinity of the β1 integrin on B16 cells for VCAM-1 [127]. Furthermore, the chemokine complex CXCR4/CXCL12 activates several migratory, proliferative, and survival signaling pathways, including the mitogen-activated protein kinase pathway, phosphatidylinositol-3 kinase (PI-3K)-Akt pathway and possibly certain Janus tyrosine kinases 2 and 3 [128].

3.1 Inflammatory pathways

3.1.1 Cyclooxygenase-Prostaglandin (COX-PG) pathway

The inflammatory cyclooxygenase (COX)-prostaglandin (PG) axis is a central pathway regulating inflammation and cancer [129,130]. There are two COX enzyme isoforms in humans, namely COX-1 and COX-2 [131]. COX-1 is found to be constitutively expressed in health in a wide range of cells and tissues where it mediate various physiological functions [132], while COX-2 is normally absent in healthy tissues but is rapidly induced in response to inflammatory cytokines, oncogenes, hormones and tumor promoters [133]. However, studies have shown that both COX enzymes and/or their products may function in promoting and maintaining neoplastic conditions [28]. Initially thought to be induced by HPV E6 and E7 oncogenes, following infection by high risk HPV [134], the mechanisms by which COX enzymes promote cervical tumorigenesis are complex. The enzymes act via a cell-autonomous pattern to promote conversion of premalignant cells to malignancy. COX enzymes can augment malignant cell progression by inducing genome instability via reactive oxygen (ROS) and lipid peroxidase induced DNA mutations [129]. Furthermore, COX enzymes have also been shown to initiate oncogenic signaling networks and transcription factors leading to deregulation of oncoproteins or tumor suppressor genes [135,136].

COX enzymes catalyze the enzymatic conversion of arachidonic acid (AA) after its release from the membrane glycerophospholipids by the enzymes phospholipase A_2 (PLA_2) to PGG_2, which is subsequently reduced to an unstable endoperoxide intermediate prostaglandin H_2 (PGH_2) (Figure 4). PGH_2 is then metabolized by specific PG synthases to produce five structurally related bioactive lipid molecules including PGE_2, PGI_2, PGD_2, $PGF_{2\alpha}$ and TxA_2 [137-139] (Figure 4). COX derived PGE_2 is the major prostaglandin produced in most human solid tumors including cancer of the cervix [28] and recent findings have indicated that COX derived PGE_2 and its signaling pathways is a key mediator of inflammatory responses, tumor growth and metastasis [28,140]. Similarly, $PGF_{2\alpha}$ have also been discovered to play a vital role in tumorigenesis. Sales et al. (2008) revealed that $PGF_{2\alpha}$ acting through its receptor (FP receptor) promotes adhesion and migration of endometrial cancer cells while TxA_2 has been reported to promote angiogenesis [141,142].

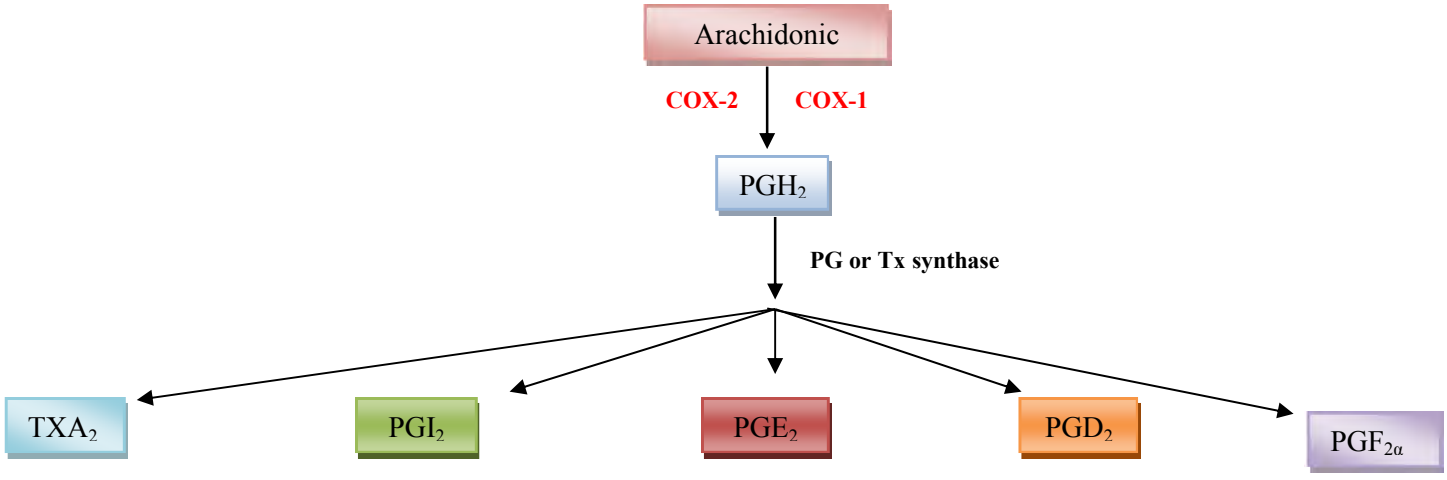


Figure 1.4: Diagram showing overview of prostaglandins (PG) synthesis. COX-1 and COX-2 both catalyzes the oxygenation and reduction of arachidonic acid (AA) after its release from glycerophospholipids to the intermediate form prostaglandin H_2 (PGH_2) which is then converted by prostaglandin or thromboxane synthase (PG or Tx synthase) to respective PG metabolites [140].

3.1.1.1 *PGE_2 regulation*

Cellular level of PGE_2 is relatively dependent on the COX/PGE synthase biosynthesis and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) degradation [140]. The two cytosolic (GSTM2-2 and GSTM3-3) and microsomal (mPGES1 and mPGES2) PGE_2 synthases can also catalyze the conversion of PGH_2 to PGE_2 [143-145]. The prostaglandin degrading enzyme 15-PGDH catalyzes the oxidation of the 15 (S)-hydroxyl group of PGE_2 converting it to a biologically inactive 15-keto PGE_2 form [146]. Hence the loss of 15-PGDH expression is suggested to contribute to tumor progression [140].

3.1.1.2 *PGE_2 receptors*

PGE_2 initiate its tumorigenic effects by binding to its cognate receptors. Four major subtypes of PGE_2 receptors have been described and identified (EP_1, EP_2, EP_3, EP_4) [147]. These receptors are G-protein-coupled seven-transmembrane-domain receptors which belong to the rhodopsin superfamily of serpentine receptors [148]. EP_1 receptor couples to Gq to stimulate phospholipase C activation, calcium mobilization, and subsequent protein kinase C activation [149]. EP_2 and EP_4 subtypes couples to Gs which in turn activates adenylcyclase, thus stimulating intracellular cAMP production [149], while EP_3 receptor couples to Gi which lowers intracellular cAMP via the inhibition of adenylcyclase [149]. However, there are splice variants of EP_3 that can couple to Gq, Gs or G_{12} [149].

The biological role of PGE_2 and its cognate receptor mediated signaling in tumorigenesis has been further substantiated by studies showing the impairment of tumor growth, angiogenesis, invasion, and metastasis in EP receptor knockout animals [150-152].

3.1.1.3 PGE_2-EP signaling and cancer progression

Profound evidence has shown that PGE_2 promotes tumor progression by stimulating EP receptor signaling. This subsequently enhances cellular growth and proliferation, angiogenesis, invasion and metastasis, and suppression of immune response [140].

3.1.1.3.1 *PGE_2-EP signaling in tumor growth/proliferation*

PGE_2 mediates tumor growth/proliferation by a complex circuit of signaling pathways that are found to be cell-type and cell content-dependent [129]. Activation of the EP_2 and EP_4 receptors subsequently leads to the downstream activation of Ras/Raf-1/ERK pathway which play an important regulatory role in the proliferative response of several tumor cells to PGE_2 [153-155]. Binding of PGE_2 to EP_2 transactivates epidermal growth factor receptor (EGFR) resulting in ERK activation and subsequent stimulation of cellular growth [156]. In addition, PGE_2 has been found to also activate ERK via EP_1-dependent activation of protein kinase C (PKC) [157].

3.1.1.3.2 *PGE_2-EP signaling in tumor angiogenesis*

Studies have shown that COX enzymes significantly influence tumor angiogenesis by inducing the release of VEGF and PGE_2 [158,159]. PGE_2 can stimulate angiogenesis directly by influencing endothelial cell response or indirectly by the induction of growth factor release [160,161]. Binding of PGE_2 to endothelial EP_2 and EP_4 receptors leads to the activation of NF-κB and AKT while its interaction with tumor cell surface EP_2/EP_4 receptors induces the

production of VEGF and plasminogen activator receptor (uPAR); both signaling events leading to tumor angiogenesis [162].

3.1.1.3.3 *PGE_2-EP signaling in tumor invasion/metastasis*

The AKT/PI3-kinase pathways have been discovered to play a central role in the transmission of PGE_2 pro-migratory signal to neoplastic cells [129]. PGE_2-AKT activation transactivates EGFR in a manner dependent on p60src [163]. In addition to its metastatic properties, PGE_2 signaling also augments tumor cell invasion by inducing extracellular matrix degradation, adhesion of tumor cells to extracellular matrix, and adhesion of cells to the vascular bed. These events are mediated by EP_4 receptor ligation leading to the up-regulation of CD44 expression and increased metalloprotease -2 (MMP-2) activity [157,164-166].

3.1.1.3.4 *PGE_2-EP signaling in tumor immune suppression*

PGE_2 mediates its immunosuppressive effects via various mechanisms such as suppression of T cell function, stimulation of T regulatory cell function, inhibition of dendritic cell activation, and polarization of activated macrophages [167-169]. PGE_2 has been shown to down-regulate Th1 cytokines such as TNFα, IFNγ, and IL-2 [170] and up-regulate Th2 cytokines such as IL-4, IL-6, and IL-10 (immunosuppressive cytokines) [171,172]. Furthermore, recent findings have also shown that PGE_2-EP_2 dependent signaling can stimulate accumulation of myeloid-derived suppressor cells in tumor microenvironment [173]. However, the downstream signaling events that mediate these PGE_2 immunosuppressive effects are poorly understood.

3.1.2 Epidermal growth factor receptor (EGFR) pathway

Several groups of growth factor receptors have been discovered to play a vital role in pathogenesis and progression of different carcinoma types and among these is the Epidermal growth factor receptor (EGFR) [174,175]. EGFR; a receptor tyrosine kinase (RTK) belongs to the ErbB family of receptors [176] that encompasses four related receptors namely; EGFR (ErbB1/HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) [177-179]. These receptors possess an extracellular ligand binding domain, a single hydrophobic transmembrane segment and an intracellular protein tyrosine kinase containing a regulatory carboxyl terminal segment. Activation of the receptor leads to recruitment and phosphorylation of several downstream intracellular substrates, hence leading to cellular signaling and other tumor promoting cellular activities [176].

3.1.2.1 Receptor activation and signaling

The ErbB receptor ligands, EGF-related growth factors are a family of small polypeptide ligands which are homologous to their specific ErbB receptor. They have been classified in to three major groups [179,180] which include; group 1[EGF, transforming growth factor α (TGF-α) and amphiregulin (AR)] which bind specifically to EGFR; group 2 [beta cellulin (BTC), heparin-binding growth factor (HB-EGF) and epiregulin (EPR)] which possess dual specificity by binding to both EGFR and ErbB4; group 3 [neuregulins (NRGs)] which is subdivided into NRG-1 and NRG-2 (binds to ErbB3 and ErbB4) and NRG-3 and NRG-4 (binds to ErbB4 only) [181-184].

However, none of the EGF family peptides have been found to bind to ErbB2. These peptides (EGF) can be found freely in the serum where they are being synthesized as inactive membrane

anchored precursors that must be cleaved and released by the metalloproteinases to give rise to the matured and active form of growth factor [185].

Upon binding of its cognate ligands to the extracellular domain, EGFR stimulate the formation of receptor homo or hetero dimers leading to the activation of the intrinsic receptor tyrosine kinase and autophosphorylation of specific tyrosine residues within its cytoplasmic domain [176,186]. These phosphotyrosine residues, then serves as a nucleation sites for proteins containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains, the recruitment of which leads to activation of various intracellular signaling pathways [187].

3.1.2.2 EGFR and cancer progression.

The roles of EGFR and EGF-family of peptide growth factor-driven signaling in human tumorigenesis have long been established [187]. This receptor and its associated ligand-binding peptides are often over-expressed in human carcinomas thus giving rise to a more clinically aggressive tumor variant [176,187] and several in vivo and in vitro studies have also demonstrated that these proteins can initiate cellular transformation [188-190]. Furthermore, the role of EGFR in autonomous proliferation of various carcinoma cells have also been formally demonstrated and blockade of EGFR significantly inhibits the *in vitro* and *in vivo* growth of several human derived cancer cell lines of various histological type [180].

3.1.2.2.1 *EGFR signaling in tumor proliferation*

One of the major downstream signaling routes for EGFR is via the Ras/Raf/MEK/MAP kinase pathway [191]. EGFR activate the Ras/Raf/MEK/MAP kinase via the growth factor receptor bound protein-2 (Grb2) or SH2-containing collagen-related proteins (Shc) [192,193]. Ras

activation leads to a multistep phosphorylation cascade that causes the activation of MAKs, ERK1, and ERK2 [194]. These (ERK1 and ERK2) then regulate the transcription of molecules that are linked to cell proliferation, survival and transformation [194].

3.1.2.2.2 *EGFR signaling in tumor invasion/motility*

Another important downstream signaling target for EGFR is the PI3K/AKT pathway [195,196]. AKT phosphorylation tranduces signals that activate a cascade of responses which include cell growth and proliferation, survival and motility [195]. Furthermore, EGFR can also signal via the stress-activated protein kinase pathway, involving protein kinase C (PKC) and JAK/STAT. Activation of these pathways translate in the nucleus to initiate cellular responses such as cell division, motility, invasion, adhesion, and cellular repair [179].

3.1.3 Phosphatidylinositol 3 kinase-AKT (PI3K-AKT) pathway

The phosphatidylinositol 3 kinases (PI3Ks) belongs to a family of lipid kinase that is distinguished by their ability to phoshorylate the inositol ring 3'-OH group of membrane inositol phospholipids to generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3) [197]. They have been grouped into three classical subfamilies according to their structure and substrate specificity [198] and these include:

3.1.3.1 Class I kinases

Members of class I are activated by cell surface receptors and have been divided into two subclasses (Ia and Ib) based on their mechanism of activation and associated adaptors [197,199]. The primary lipid generated in vivo by the members of class I kinases is phosphatidylinositol

(PtdIns)-3,4,5-trisphosphate (PI-3,4,5-P_3) and phosphatidylinositol-3,4-bisphosphate (PI-3,4-P_2), using phosphatidylinositol-4,5-bisphospahte (PI-4,5-P_2) as a substrate [200,201]. Class Ia PI3Ks consist of heterodimers of a p110 catalytic and a p85 regulatory/adaptor subunits [198,202] and they are activated by receptors with protein tyrosine kinase activity (RTK) while the class Ib are made up of heterodimers of a p110γ catalytic and p101 regulatory subunits and are activated by G-protein coupled receptors. The class I kinases (especially class Ia) are the most extensively investigated class of all the PI3Ks.

3.1.3.2 Class II Kinases

This class is made up of three distinct isoforms that are ubiquitously expressed. They are characterized into PIK3C2α, PIK3C2β, and PIK3C2γ (liver specific) on the basis of the presence of a C2 domain on the extreme C-terminus [203]. They are devoid of adaptor/regulatory subunits and use PtdIns(4)P and PtdIns as their principal substrates. In contrast to the class I which in the resting state are cytoplasmic, the class II PI3Ks are predominantly membrane-bound [199] where they play a suggestive role in membrane trafficking and receptor internalization [198]. Members of this class can also be activated by RTKs, integrins and cytokine receptors however, their effector response to these activators are poorly understood [204].

3.1.3.3 Class III kinases

Members of this class are heterodimeric enzymes with catalytic (Vps34) and regulatory (p150) subunits. They utilize PtdIns as their primary substrate and are implicated in the regulation of mammalian target of rapamycin (mTOR) activity, protein trafficking through the lysosomes, and

the regulation of autophagy response to cellular stress, thus indicating their importance in controlling cellular growth and survival [198].

3.1.3.4 Protein kinase B (AKT)

The downstream signaling of the PI3Ks is mediated most importantly via the serine/threonine kinase AKT (protein kinase B or PKB). AKT was initially characterized as the human homologue to *v-akt* (a viral oncogene known to cause a type of leukaemia in mice) from transforming retrovirus AKT8 [205,206]. Three isoforms of the human PKB/AKT family have been identified, they are named PKBα (AKT 1), PKBβ (AKT 2), and PKBγ (AKT 3) [207]. Although these isoforms are products of different genes, they are highly homologous to each other at the amino acid level [208,209]. Genes encoding these isoforms are differentially distributed in the tissues, with PKBα (AKT 1) and PKBβ (AKT 2) having a wider distribution than the PKBγ (AKT 3) isoform. Each isoform is composed of a pleckstrin homology (PH) domain (with approximately 100 amino acids at the N-terminal), a central kinase domain analogous to those of PKA and PKC [210,211], and a hydrophobic C-terminus containing a second regulatory site. The kinase domain and the C-terminus of the PKBα (AKT 1) contain threonine (Thr308) and serine (Ser473) residues respectively. The interaction of the PH domain with PI-3,4,5-P_3 initiate conformational changes resulting in the exposure of these sites (Thr308 and Ser473) for phosphorylation, an action essential for maximal AKT activation [212].

The PI3K/AKT pathway is known to play a fundamental role in the regulation of various cellular functions such as cell growth, survival and movement [208,213]. However, recent findings have shown that this pathway play a major role in tumorigenesis, as well as the tumor's potential response to cancer treatment [197].

3.1.3.5 PI3k-AKT Pathway activation and signaling

Activation of RTKs (e.g. EGFR, VEGFR, interleukin receptors, integrin receptors) by growth factors causes the activated receptor to interact with one or two SH2 domains in the phosphotyrosine adaptor unit, and localize PI3K to the plasma membrane. Similarly, intracellular proteins such as protein kinase C (PKC), SHP1, Rac, Rho, and Src can also activate PI3K in the cell [200]. Activated PI3K phosphorylates PI-4,5-P_2 to produce PI-3,4,5-P_3, and the lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) directly opposes the activity of PI3K by dephosphorylating PIP_3 into PIP_2, thus acting as the central negative regulator of PI3K [214]. PI-3,4,5-P_3 mediates its cellular effect by binding to a subset of proteins containing pleckstrin homology (PH), FYVE, Phox (PX), C1, and C2 domains, thus activating these downstream targets [203]. Proteins containing PH domains include Tec family tyrosine kinase; guanine nucleotide exchange factors (GEF) for Rac; adenosine diphosphate (ADP)-ribosylating factor 6 (ARF6); GTPase –activating proteins (GAPs); and AKT (PKB) which represent principal mediators for PI3K class Ia-induced signaling [200-202].

Association of the PH domain of AKT with PI-3,4,5-P_3 leads to its recruitment to the plasma membrane where it is been phosphorylated at the kinase domain (Thr 308 in Akt 1) by phosphoinositide-dependent kinase 1(PKD 1) [201,215]. Additional phosphorylation by PDK2 at the C-terminal domain (Ser 473 in Akt 1) is essential for full activation of AKT [200,216]. Post activation, AKT reverts to the cytoplasm and the nucleus where it phosphorylates a number of downstream targets involved in various cellular functions.

3.1.3.6 PI3K-AKT signaling and cancer progression

Carcinogenesis is a complex process arising as a result of imbalance between cell division/ growth and programmed cell death (apoptosis). In this context, various signaling pathways involved in the regulation of cellular processes such as cell growth, differentiation, and development undergo oncogenic changes more than any other molecule. Studies have shown that components of the PI3K-AKT signaling pathways are frequently altered in human cancers [197] with amplification and over expression of the gene encoding PI3K (*PIK3C*) being described in ovarian, cervical, gastric, and breast cancers [217,218]. Similarly, various studies have also demonstrated *akt* amplification in human cancers [219,220]. This deregulation/amplification of components of the PI3K-AKT signaling pathway exerts a key role in tumor growth, survival, metastasis, and angiongenesis. Inhibition of signaling molecules in this pathway has been shown to strongly reduce tumor growth and angiogenesis in animal model experiment [203].

3.1.3.6.1 *PI3K-AKT signaling in tumor angiogenesis*

Angiogenesis is an essential process in tumorigenesis that can be triggered by growth factors, genetic alterations, and mutation of tumor suppressor gene such as p53. The angiogenic process in tumors can occur via sprouting of new vessels from preexisting blood vessels or via protrusion of interstitial tissue columns into vascular lumen [109].

PI3K-AKT signaling has been found to augment tumor angiogenesis by regulating the expression of VEGF and hypoxia–inducible factor 1 (HIF-1) via the activation of p70S6K1 and HDM2. In addition, this pathway can also promote tumor angiogenesis through the activation of reactive oxygen species (e.g. eNOS) [221,222].

3.1.3.6.2 *PI3K-AKT signaling in tumor cell proliferation*

PI3K-AKT signaling augment tumor cell proliferation by modulating the functions of numerous cellular substrates involved in the regulation of cell proliferation. A good example of these substrates include glycogen synthase kinase-3 (GSK-3) [223,224], membrane translocation of glucose transporter-4 (GLUT4) [223], cyclin-dependent kinase inhibitors, p21/Waf1/Cip1and p27/Kip2 [216,225-228], mammalian target of rapamycin (mTOR) [229,230] and tuberous sclerosis complex 2(TSC2) [231].

PI3K-AKT inhibits GSK 3 activity by direct phosphorylation of the serine residue in the N-terminal regulatory domain of insulin-activated PI3K [232]. GSK 3 inhibition prevents the phosphorylation of β-catenin, thus impeding its degradation and facilitating its translocation into the nucleus. In the nucleus, β-catenin combines with various transcription factors such as TCF/LEF-1 to induce the expression of Cyclin D1 which induces cell cycle progression by regulating Rb hyperphosphorylation and inactivation. Furthermore, PI3K-AKT signaling also phosphorylate of p21/Waf1/Cip1 and p27/Kip2, inhibiting their anti-proliferative effects by reducing their ability to activate Cyclin D1/Cdk4 complex [233].

By increasing the phosphorylation of mTOR, PI3K-AKT signaling enhances protein synthesis an essential process required for cell proliferation [234]. In addition this signaling pathway also phosphorylate TSC2 complex to inhibit its growth suppressor function.

3.1.3.6.3 *PI3K-AKT signaling in tumor cell survival (inhibition of apoptosis)*

PI3K-AKT pathway regulates tumor cell survival by phosphorylating downstream substrates that directly or indirectly control the apoptotic machinery. The pathway inactivates the pro-apoptotic factors Bad and pro-caspase 9; activates the transcription of NF-κB regulated anti-apoptotic

genes via the activation of Ikappa B kinase (IKK); inactivates Forkhead family transcription factors (AFX, FKHR, and FKHRL1) thereby inhibiting the expression of pro-apoptotic gene such Fas lingand (*FasL*), *Bim*, and *IGFBP-1* [197,208,235].

3.1.3.6.4 *PI3K-AKT signaling in tumor cell metastasis*

The PI3k-AKT signaling contributes to tumor invasiveness and metastasis by inhibiting anoikis and stimulating MMPs secretion.

4.1 Regulation of cervical inflammation, inflammatory pathways, and tumorigenesis by HPV oncogenes

Inflammation involves extensive tissue remodeling events which are orchestrated by complex networks of cytokines, chemokines and bioactive lipids. These work across multiple cellular compartments to elicit their function. The classic hallmarks of inflammation are the recruitment of immune cells into the tissue and alteration of vascular function to allow for immune cell extravasation. This alteration in permeability causes edema and the redness generally associated with tissue inflammation. Several reports have correlated inflammatory cell infiltrate with HPV-induced high grade lesions. Infiltrating lymphocytes are thought to contribute to tumor growth and spread as well as immunosuppression, generally associated with malignant diseases [72]. Although the precise mechanism whereby HPV oncogenes regulate tissue remodeling events is unclear, HPV infections have been shown to promote the release of inflammatory mediators and cytokines from keratinocytes to alter the immune response and promote the infiltration of macrophages, lymphocytes and NK cells [236]. Changes in the vasculature to facilitate immune extravasation and angiogenesis require tissue remodeling of the extracellular matrix, a process

facilitated by MMP's [237]. Several studies have correlated HPV E6 and E7 transcription with MMP transcription [238,239] and genes in cervical epithelial cells involved in tissue differentiation and remodeling [240]. In addition, transfection studies have shown that E7 oncoprotein forms a complex with and down-regulates leukocyte elastase inhibitor [241]. This would facilitate the activation of neutrophils and promote neutrophil influx into the tissue. Furthermore, transgenic mouse models where the early region genes from HPV16 are expressed under the control of the human keratin 14 promoter have shown that macrophage recruitment to HPV-associated lesions occurs via the release of the chemokine CCL2 and interaction with its receptor CCR2 present on macrophages [242].

Once resident in the tissue, inflammatory cells are known to produce vast amounts of reactive oxygen species and nitric oxide which have been shown to induce DNA damage [243] and to contribute towards the progression of the disease in high grade cervical lesions [243]. Furthermore nitric oxide has been shown to induce transcription of E6 and E7 oncogenes in cervical epithelial cells [244], which can further enhance inflammation via an auto-amplifying positive feedback loop via activation of COX-2 and other parallel inflammatory pathways. Similarly, studies by Subbaramaiah and Dannenberg and Oh and colleagues have now emerged to show that in neoplastic cervical epithelial cells, HPV 16 E5, E6 and E7 oncogenes can induce the inflammatory COX-PG axis, by elevating expression of the immediate early oncogene COX-2 and the E-series prostaglandin receptors, EP_2 and EP_4 [76,245,246]. And several studies have also emerged from in vitro and in vivo model systems employing cell lines and rodents to demonstrate that prostaglandins, produced as a consequence of elevated COX enzyme expression, can promote extensive tissue remodeling within tumors by evoking all the classical hallmarks of cancer, namely: cellular proliferation, angiogenesis, inhibition of apoptosis and

alteration in vascular permeability to allow immune cell extravasation from the vasculature [80,247] as discussed above.

5.1 HIV, cervical cancer and inflammation

The human immunodeficiency virus (HIV); the only etiological factor attributed to the Acquired Immunodeficiency Syndrome (AIDS) belongs to the genus Lentivirus within the family *Retroviridae* [248]. About 33 million people harbor this virus worldwide [249] with high epidemic rates in sub-Saharan Africa. Mature HIV virion is spherical (approximate diameter of 100-120nm), with a genome of two copies of identical (9.2kb) single-stranded positive sense RNA molecule encoding the characteristic retrovirus proteins; *Gag*, *pol*, and *Env* [250]. HIV is divided into two main sub types HIV-1 and HIV-2. HIV-1 is further subtyped into phylo-genetically related clades; types A-K [251].

Transmission is via unprotected sexual intercourse, intravenous drug use, blood transfusion or infection with blood-derived products. To initiate infection, virus attaches to cellular surfaces via an interaction between the gp120 viral envelope protein and a receptor complex present on the host cell consisting of the CD4 receptor and G protein-coupled receptor (GPCR) co-receptor, usually CCR5 or CXCR4 [250]. Most primary HIV-1 variants are restricted to the use of CCR5 and CXCR4 [252,253], however they have been shown to use alternative receptors in vitro. HIV-2 variants are capable of infecting a wider range of cells expressing different co-receptors such as GPR15 and CXCR6 in addition to CCR5 and CXCR4 [254]. This leads to fusion between the viral and cellular membranes and ultimate release of the viral core into the cell cytoplasm [250]. Once inside the host cell the virus is reversed transcribed to full length double stranded DNA by

the reverse transcriptase enzyme and is integrated into the host genome [255]. The hallmark of infection is characterized by progressive depletion of $CD4^+$ and $CD8^+$ helper T-cells leading to an immunodeficiency state, paving the way for opportunistic infection and ultimately mortality [256].

5.1.1 *The interplay between HIV and HPV and their role in cervical cancer*

The interplay between HPV and HIV is complex; however their synergistic role in exacerbating pathology of the cervix has been well documented. For example, epidemiological studies have shown that women that are co-infected by HPV and HIV have an estimated 41 fold increase in the risk of developing neoplastic cervical lesions [257] and HIV infected immune-compromised women have been shown to have a higher prevalence of HPV-induced lesions [258]. Furthermore, studies in sub-Saharan women, where 67% of the population are living with HIV/AIDS have shown that women with HIV develop cervical cancer at an earlier age than women who are HIV negative [5,259,260].

Although the precise mechanisms predisposing women infected with HPV to HIV infection are unclear, there is compounding evidence that clearance of HPV infection from the female genital tract elicits an enormous cell-mediated immune response characterized by gross infiltration of lymphocytes and macrophages into the epithelium [261], which can enhance the risk of HIV infecting immune cells in the cervix in these women after unprotected sexual contact.

Central to the role of HIV in cervical cancer is its ability to ablate the systemic immune response to infection, including HPV infection. This can facilitate inadequate clearance of HPV in infected individuals, enhancing HPV persistence or re-infection, and increases the likelihood that

precancerous lesions will develop into cancer. To this end, HIV may modify HPV related carcinogenesis by altering the expression of inflammatory components (cytokines) in the cervix and diminution of local cervical cellular immunity, thus altering HPV regulation [249]. For example, HIV *tat* gene has been shown to increase the expression of HPV E1 and L1 genes, hence causing up-regulation of the HPV replication [262,263]. Furthermore, HIV-1 *tat* protein is capable of transactivating HPV16 transcription [264].

5.1.2 *The role of HIV in regulating inflammation and its potential contribution to cervical cancer.*

Systemic expression of several pro-inflammatory cytokines has been reported to be a major feature in HIV infection. The virus causes immune dysregulation leading to increase in the production of pro-inflammatory cytokines such as; TNFα, IL-1, and IL-6 which are detected in the plasma and lymph node of infected patient [265]. This link between HIV and production of pro-inflammatory cytokine was suggested by the observation that the virus and/or its surface glycoprotein gp120 can induce *in vitro* secretion of TNF, IL-1 and IL-6 by monocytes isolated from uninfected individuals [266-268]. Other studies also detected high levels of IL-1α, IL-1β, IL-6, and TNF in the serum and cerebrospinal fluid of sero-positive individuals [269,270]. Often associated with the production of these cytokines is the elevated secretion of CC-chemokines such as macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES [271,272]. These pro-inflammatory cytokines expressed as soluble factors or membrane binding molecules and are directly or indirectly involved in HIV entry and T cell apoptosis [256]. These cytokines have been found to be abundant in microenvironment of several tumors including cervical cancer (as

discussed above) where they are secreted by the tumor cells, endothelial cells and/or infiltrating activated immune cells where they act as endogenous tumor promoter by stimulating the production of transcription factors (e.g. NF-κB, AP-1), proliferative and angiogenic proteins (e.g. VEGF, MMPs) and adhesion molecules (e.g. E-selectin, VCAM), thus enhancing tumor growth and mediating tumor metastasis [273]. TNF, a major mediator of inflammation, can be detected in various human neoplasias where it is implicated in the induction of MCP-1 which can modulate the infiltration of immune cells in to the tumor microenvironment.

In addition to regulation of immune cell infiltrate, alteration in the expression profile of HIV receptors on cells within the cervico-vaginal region could impact on HIV acquisition and cervical cancer progression. The epithelial surface of the female reproductive tract expresses all the receptors necessary for HIV infection including CD4, CCR5 and CXCR4 [274]. Maher and colleagues have recently shown that HIV virions can bind the external surface of cervical epithelium and penetrate beneath the epithelial surface [275]. Thus, it is plausible that epithelial cells lining the cervico-vaginal interface could be the first cells to come into contact with HIV and might play a role in the replication of the virus and transmission to leukocytes present in the submucosa.

Expression of some of the HIV receptors on uterine epithelial cells display a temporal variation in expression during the menstrual cycle, indicating that they are hormonally regulated [274]. This could alter susceptibility to infection depending on the phase of the menstrual cycle. Chemokine receptors such as CXCR4 are elevated in cervical cancer and play a role in lymph node metastasis during advanced stage disease [276]. These receptors can also be hijacked by HIV for entry in such women. CXCR4 expression can be regulated by HPV oncogenes [277] and prostaglandins [278] in the female genital tract. These observations suggest that HPV infection

and inflammation can drive expression of HIV co-receptors on epithelial cells. It is unknown whether the inflammatory milieu of cervical cancer can also alter expression of HIV receptors on immune cells in the tumor periphery. It is plausible that in women with HPV infection or localized cervico-vaginal inflammation, alterations in HIV receptor expression could allow more virus to bind the epithelium and elevate the amount of virus present locally in the genital tract following intercourse. In women with cervical cancers or inflammatory cell infiltrate into the cervix, this could enhance susceptibility to infection.

6.1 Seminal plasma, cervical cancer, HIV and inflammation

Seminal plasma (SP) is the major constituent of the male ejaculate. It is a complex organic fluid comprising of secretions of the cowper's and littre glands (5%), prostate (15-30%), and the seminal vesicles (65-70%) [279]. Secretions from each of these reproductive organs are biochemically distinct, and on mixing as occurs at ejaculation, gives rise to the complex biochemical nature of the SP. Also called "Seminal Fluid", this endogenous fluid has been conventionally viewed as a nutritive, protective and transport medium for the mammalian spermatozoa [280]. However, recent studies both in rodents and humans have shown that SP contains signaling molecules which can bind to cognate epithelial receptors in the female reproductive tract to trigger inflammatory gene expression, thus causing modifications in cellular structure and composition in a sequence that resembles inflammatory response [280].

6.1.1 *Role of seminal plasma in regulating cervical inflammation and its potential contribution to cervical cancer.*

In mammals, the most evident physiological effects of insemination is a prompt and dramatic influx of inflammatory cells into the site of semen deposition [280]. These cellular changes, studied in detail in mice [281-283] was discovered to be mediated by SP as opposed to sperm [283] and is initiated when endogenous molecules present in SP interact with cervical and uterine epithelial cells to induce a surge in cytokines and chemokines synthesis [283]. These pro-inflammatory mediators stimulate the extravasation and infiltration of cervical sub-epithelial stroma by immune cells. Similarly, in humans coitus elicits immune cell (neutrophils, macrophages, dendritic cells, and lymphocytes) recruitment into the superficial epithelial and deep stroma tissues of the female reproductive tract [284]. In sexually active women, the degree at which SP normally activates the secretion of these pro-inflammatory components in any compartment of the female reproductive tract is poorly understood. However, exposure of the cervix to SP during coitus has been shown to elicit substantial changes in the leukocyte populations within the cervix, initiating a reaction reminiscent of inflammatory response with effects that penetrate through the stratified epithelial layer and deep into the stroma of the ectocervix [285]. The role of SP in mediating leukocyte influx in the cervix was supported when no inflammatory response was seen in the absence of coitus or with condom-protected coitus [285]. SP regulated the cervical leukocyte influx via the activation of pro-inflammatory cytokines and chemokines [286]. Similarly, in vitro studies have shown that SP induce the expression of inflammatory enzyme (COX-1 and COX-2), interleukin (IL) 6, 11 and chemokines CXCL1 and CXCL8 in cervical adenocarcinoma cells [287].

The influx of immune cells expands inducible regulatory T cell population, hence promoting immune tolerance there by preparing the reproductive tract for conception [285,288,289]. More broadly, the inflammatory response initiated by SP would be expected to impact on all physiological and pathophysiological events regulated by cervical leukocytes.

6.1.2 *Role of seminal plasma in regulating HIV infection and its potential contribution to cervical cancer*

In 1993, cervical cancer was classified as one of the AIDS-defining diseases in women infected with HIV [26]. This highlighted HIV as an important risk factor/co-factor in the development of invasive cervical neoplasia and highlighted cervical cancer as an infectious disease [27].

Since the beginning of the AIDS pandemic, greater than 35 million deaths have been reported in both adults and children worldwide [290]. It has been well documented that unprotected hetero or homo sexual intercourse is the major route of HIV infection with infected seminal plasma being the major transmission vector [291-293]. Post deposition of HIV on the recipient mucosa, infectious virus must cross the mucosal epithelium to interact with $CD4^+$ T lymphocytes, macrophages, and dendritic cells (DCs). These immune cells express the receptor CD4 and co-receptors CXCR4 or CCR5 which are required for HIV infection [292-295]. The exact molecular mechanisms through which HIV traverse the mucosal barriers and establish infection are poorly defined. The virions may transcytose through the genital epithelium [292,295] or pass through exposed genital lesions [296-298]. In addition, post coital epithelial microabrasions found in the vagina of most healthy women after consensual intercourse is suggested to constitute a frequent scenario for sexual transmission [299].

Beyond its role as a carrier for delivery of HIV during receptive vaginal or anal intercourse, SP has been suggested to play a more major role in HIV transmission. This is supported by studies which provided insight into the SP-mediated effects on both the virus and the mucosal HIV entry sites. A novel mechanism by which SP could enhance sexual transmission of HIV was demonstrated by Munch et al. (2007) [300]. To identify natural agents that might play a role in sexual transmission of HIV, Munch and colleagues screened a complex peptide/protein library derived from human SP for novel inhibitors and enhancers of HIV infection. They found that naturally occurring fragments of the prostatic acidic phosphatase (PAP) drastically enhance HIV infection [300].

Functional and structural analyses shows that PAP forms amyloid fibrils termed semen-derived enhancer of virus infection (SEVI) which markedly increased HIV infection. SEVI capture HIV and strongly heighten the number of productively infected cells by promoting the attachment and fusion of the virus to the cell surface. SEVI was shown to increase the infection by R5-, X4- and dual tropic HIV-1 clones in peripheral blood mononuclear cells (PBMCs), DCs and macrophages in vitro. In an in vivo experiment to demonstrate the enhancing effect mediated by SEVI, using hCD4/hCCR5-transgenic rats challenged with HIV-1 or SEVI-treated HIV-1 by tail vein injection. Munch and colleagues discovered that pre-treatment of HIV with SEVI resulted in a 5-fold increase in the number of HIV cDNA copies found in the splenocyte extracts from infected rats [300]. Taken together, the increase in HIV infectivity when treated with SEVI in vitro, suggests that it might favor sexual transmission of HIV. This however, has been shown to be highly dependent on the individual semen donor and correlates with the level of SEVI [301].

In addition, it has been suggested that SP-mediated inflammatory responses within the female genital tracts may contribute to the transmission of HIV and other STIs (sexually transmitted infections) through two major mechanisms; a) by disrupting the epithelial barrier, and b) by inducing the local recruitment of $CD4^+$ target cells favoring the dissemination of HIV infection [284,302,303].

Other proposed mechanisms of SP-mediated HIV transmission include; the role of spermatozoa in efficient transmission of HIV to dendritic cells [304], neutralization of the acidic pH of the vaginal fluid [305], and SP-mediated immunomodulation and suppression of both innate and adaptive immune response against HIV [306]. However, the detailed cellular and molecular mechanism by which SP can influence inflammatory pathways to regulate inflammation and HIV transmission in cervical cancer, which may play a major role in tumor progression, is yet to be studied.

6.1.3 Seminal plasma prostaglandins: potent pro-inflammatory factor

Seminal plasma is known to contain an enormous diversity of antigenically distinct molecules that include cytokines, angiogenic factors, prostaglandins, proteases, signal transduction molecules, protein kinases, transporter proteins, structural molecules and immune response proteins [307]. However, it is the highly expressed levels of prostaglandins found in human SP [308] that have attracted much interest of late.

Prostaglandins (PGs) are lipid autacoids that mediate both homeostatic functions and pathogenic mechanisms, including inflammatory response within the human body [309]. These potent pro-inflammatory lipids are generated from arachidonate by the action of cyclooxygenase enzymes,

and their biosynthesis is blocked by non-steroidal anti-inflammatory drugs. Of the PGs present in SP, PGE_2 has been identified as one of the predominant types detected [310,311].

Over the years, studies have shown that overt expression of PGE_2 and its signaling is found in numerous disorders including cervical cancer [28,312] and women infected with HIV and HPV [134,313]. It has been shown that PGE_2 potentiates the chronic inflammatory response seen in these diseases, leading to greater tumorigenesis (cervical cancer) [28] and enhanced viral replication (HIV infection) [314].

Similarly, recent study by Joseph et al. (2012) identified PGE_2 present abundantly in SP as the main constituent responsible for SP-mediated inflammatory effects in vaginal cells [315]. It is therefore very likely that SP-PGE_2 can influence inflammatory pathways to regulate inflammation and HIV transmission in cervical cancer. This is more evident as studies have shown that cervical cancer has up-regulated expression of PG receptors [28].

7.1 Statement of the problem

Presently there are more than 273,000 deaths occurring worldwide each year from cervical cancer thus accounting for 9% of total cancer deaths in women [316].

Mortality from cervical cancer is high in sub-Saharan Africa with a mortality rate of 35/100,000 women in East Africa, 23/100,000 in Southern Africa, and 24/100,000 in Western Africa as opposed to 2/100,000 in North America and 4/100,000 in Western Europe [317,318].

As previously mentioned infection with HPV (especially type 16) account for 50% of cases [10] and inflammation plays a major role in the pathogenesis of this disease. Seminal plasma has been shown to regulate the expression of inflammatory genes and initiate inflammatory processes and components in both normal and neoplastic human cervical epithelium in sexually active women [285,287].

Hence a complete and detailed understanding of the role and molecular mechanism by which seminal plasma regulates the expression of arrays of inflammatory genes to augment cervical tumorigenesis, alter susceptibility of women with cervical neoplasms to HIV infection and increase the development of AIDS related cervical cancer in sexually active women will serve as lead in developing new modalities for clinical intervention and treatment.

8.1 Hypothesis

We hypothesize that in sexually active women, repeated exposure of cervical epithelial cells to seminal plasma can regulate inflammatory pathways. In women with neoplastic cervical lesions, hyperactivation of inflammatory pathways could potentially enhance tumorigenesis and regulate pathways involved in HIV infection and AIDS-related cancer progression.

9.1 Aim

This study investigates the inflammatory and tumorigenic pathways regulated by SP in cervical epithelial cells and the molecular pathways underlying this regulation.

10.1 Specific objectives

1. To investigate the role of seminal plasma (SP) in regulating the expression of inflammatory genes in cervical adenocarcinoma cell in vitro.
2. To elucidate the various inflammatory pathways involved in SP induction of a pleotropic pro-inflammatory cytokine IL-1α and the molecular mechanisms underlying their action.
3. To investigate the inflammatory pathways and role of SP in HIV susceptibility in women and the mechanisms underlying this.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Ethics statement

Ethics approval for this study was obtained from the University of Cape Town Human Research Ethics Committee (HREC/REF: 067/2011). Written informed consent was obtained from all subjects before sample collection.

2.2 Chemicals and Reagents

All chemicals used for this study were of molecular biology grade, and were purchased from Merck Chemicals (PTY) Limited (Gauteng, South Africa) or Sigma Chemical Company (Cape Town, South Africa). Cell culture media, penicillin-streptomycin, and fetal-calf serum were purchased from Highveld Biological (PTY) Limited (Cape Town, South Africa). Bovine serum albumin (BSA), phosphate buffered saline (PBS), and Trizol® were all purchased from Sigma Chemical Company (Cape Town, South Africa). The chemical inhibitors: AG1478, epidermal growth factor receptor (EGFR) kinase inhibitor; LY294002, phosphoinositide-3-kinase (PI3K) inhibitor; SC560, cyclooxygenae-1 (COX-1) inhibitor; NS398, cyclooxygenase-2 (COX-2) inhibitor; SN50, nuclear factor kappa B (NFκB) inhibitor; and PD98059, extracellular signal-regulated kinases 1/2 kinase (ERK1/2) inhibitor were purchased from Calbiochem (Merck, Darmstadt, Germany). Prostaglandin E_2, Butaprost, PGE_2 receptor antagonist (AH6809), and human recombinant epidermal growth factor (EGF) were purchased from Sigma Chemical

Company (Cape Town, South Africa). Quantikine® Human IL-1α ELISA kit was purchased from R&D Systems, Minneapolis, USA. Polyclonal goat anti-IL-1α (sc-1253), β-actin (sc-1616), CCR5 (CKR5; sc-6128), CD4 (sc-19641), COX-1 (sc-1752), and biotin conjugated secondary donkey anti-goat IgG antibody (sc-2042) were purchased from Santa Cruz Biotechnology. Streptavidin-biotin peroxidase complex and 3.3'-diaminobenzidine were all purchased from Dako North America Incorporation, USA. Total PKB (AKT) (9272s) and phosphorylated PKB (S473) antibodies were purchased from Cell Signaling Technology. Pierce® BCA Protein Assay Kit and SuperSignal® West Pico Chemiluminescent Substrates were purchased from ThermoScientific, Rockford, USA and PVDF membrane was purchased from GE Healthcare, Amersham, United Kingdom.

2.3 Semen donors and preparation

Semen was collected from healthy male volunteers attending the Andrology Laboratory of the Reproductive Medicine unit at Groote Schuur Hospital, Cape Town, South Africa. All donors had at least 72 hours of total sexual abstinence (self-reported) prior to ejaculation. Ejaculates were collected in sterile specimen jars following a voluntary self-masturbation. Parameters such as sample volume, sperm number, sperm concentration, peroxidase-positive leukocytes, pH and viscosity were noted and compared with the 2010 WHO (World Health Organization) reference values for human semen characteristics [319]. Samples with below average parameters were excluded from the study. All samples were processed within 30 minutes of collection. The individual ejaculates were transported to the laboratory. Seminal plasma (SP; the cell free supernatant of the ejaculate) was isolated from ejaculate by centrifugation at 15000 g for 20

minutes. Ejaculates from 10 individual volunteers were pooled and then aliquoted (200µL) and stored at -80 ^{0}C until required. Individual ejaculates were analyzed for their ability to regulate expression of target genes before being pooled to minimize experimental variability. Prior to use seminal plasma was thawed on ice and diluted in sterile filtered serum free medium to use at a concentration of 1:50. SP has been shown to exert no toxic effect on HeLa cell viability up to and including concentration used in this study [287,320].

2.4 Cervical specimen

2.4.1 Cytobrush Sample

Cervical specimens were collected from women attending the Gynecology (Colposcopy) outpatient Clinic at Groote Schuur Hospital, Cape Town, South Africa. Specimens were obtained by cytobrush as described by Musey and colleagues [321]. Briefly, the cytobrush was inserted within the cervical os and gently rotated one 360 degree turn. The cytobrush was smeared onto a microscope slide for diagnosis and then placed into a 15ml collection tube containing serum free Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM) (Highveld Biological (PTY) Limited, Cape Town, RSA) with Glutamax-1and 1% penicillin-streptomycin. The tubes were immediately placed on ice and transported to the laboratory. The cells were dislodged from the cytobrushes by gentle agitation and each sample divided into two aliquots and was treated with vehicle (PBS) or 1:50 dilution of seminal plasma in serum free DMEM for 24 hours. After which, cells were pelleted by centrifugation at 1500 rpm and RNA extracted using Trizol (Sigma) following the manufacture's guidelines and reverse transcribed.

2.4.2 Cervical Tissue Explant

Cervical cancer tissue specimens were obtained at the time of surgery or biopsy from patients attending the Gynecologic Oncology Clinic at Groote Schuur Hospital (Cape Town) and who had previously been diagnosed with pre or invasive carcinoma of the cervix. Diagnostic cytology report was defined according to the Bethesda System for reporting cervical cytology [322] while pathological staging was defined according to the revised International Federation of Gynecology and Obstetrics (FIGO) staging for carcinoma of the cervix [323] upon physical examination. Punch biopsies were taken from the lesion by an experienced gynecologist with a special interest in gynecology oncology. Samples were immediately transported in PBS on ice to the laboratory where they were divided into experiment and control and then serum starved overnight prior to stimulation with seminal plasma. Histologically normal cervical tissues were collected from women undergoing Wertheim's hysterectomy for benign gynecological malignancies at Groote Schuur Hospital (Cape Town). Cervical (approximately 3 X 4mm) tissue samples collected at the time of surgery were immediately transported in PBS on ice to the laboratory where they were chopped into smaller pieces and divided into experiment and control before being serum starved overnight prior to SP stimulation.

2.5 Cell Line

2.5.1 HeLa S3

HeLa-S3 (HeLa) authenticated and verified as cervical adenocarcinoma cells positive for HPV-18 sequence with normal levels of pRB (retinoblastoma) and low levels of p53 tumor suppressor, were purchased from BioWhittaker (Berkshire, UK).

2.6 Cell Culture

Cells were routinely maintained in DMEM nutrient mixture F-12 with Glutamax-1 and pyroxidine supplemented with 10% fetal bovine serum (FBS) (Highveld Biological (PTY) Limited, Cape Town, RSA) and 1% penicillin-streptomycin (500 IU/ml penicillin and 500 µg/ml streptomycin) at 37 ^{0}C and 5% CO_2. The medium was stored at 4 ^{0}C, and was warmed to 37 ^{0}C before use. Monolayer cultures of HeLa cells were grown in 125 cm^2 tissue culture (TC) flasks and kept in a humidified incubator (37 ^{0}C , 95% relative humidity and 5% CO_2). Cellular-growth was monitored every 48 hours using an inverted fluorescent microscope and cells were passaged when confluent. To passage, cells were removed from the incubator and the media aspirated using sterilized Pasteur pipette attached to a pump. The cells were then washed twice in pre heated (37 ^{0}C) phosphate buffered saline (PBS) and dissociated from the TC flask with 5ml 1x trypsin-EDTA, and the flask placed back in the incubator for 5 min at 37°C. TC flask was then removed from the incubator and viewed under an inverted fluorescent microscope to confirm cell detachment after which excess trypsin-EDTA was aspirated and complete media added. The cells were then transferred into a new culture flask with complete medium at a ratio of 1 in 10 with a new passage number being recorded on each flask or seeded onto culture dishes

for experimentation or frozen down for later use. Early passage cells were frozen down for later use by re-suspending the trypsinized cells (~1×10^6 cells/ml) in FBS containing 5-10% DMSO and transferred into small sterile cryogenic vials. The vials containing cell aliquots (1ml each) were placed upright in a -80 ^{0}C freezer and later transferred to liquid nitrogen for long term storage.

2.7 Cellular Investigations

2.7.1 Cell treatment with seminal plasma

HeLa S3 cells were seeded in complete medium at density of $2x10^5$ cells in 3 cm diameter tissue culture dishes and allowed to attach and grow overnight. The following day, the complete medium was aspirated and cells were serum starved by incubating in serum free medium (Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM) with Glutamax-1 enriched with 1% penicillin-streptomycin (500 IU/ml penicillin and 500 μg/ml streptomycin) without FBS) for 24 hours. Cells were then treated with serum free media containing SP at a final dilution of 1:50 or serum free media with PBS as control for 4, 8, 16 and 24 hours. SP at this concentration has been shown to not affect cell viability [324]. The cells were then harvested after 4, 8, 16 and 24 hours using 1ml Trizol® (Sigma). In parallel, untreated wild type HeLa cell (positive control) was lysed in 1 ml of Trizol® (Sigma) and used as a reference control in each Real Time quantitative RT-PCR reaction. Samples were stored at -80°C until further use.

2.7.2 Treatment with chemical inhibitors

HeLa S3 cells at a density of $2x10^5$ were seeded in 6 well tissue culture plates and allowed to attach and grow overnight in a humidified incubator (at 37°C and 5% CO_2(v/v)). The next day, the complete medium was aspirated and the cells washed twice with PBS. The cells were then serum starved for 24 hours. After 24 hours, the serum free medium was then aspirated and cells pre-incubated with aforementioned chemical inhibitors (CalBiochem, Merck) at 37°C (Table 2.1) for 1 hour. The medium containing inhibitors was then aspirated and replaced with fresh serum free medium containing the inhibitors or vehicle (H_2O or DMSO) in the presence or absence of SP (1:50 dilution). The samples were incubated for 4, 8 or 16 hours. Post incubation the medium was aspirated and cells harvested with 1ml Trizol® (Sigma) and RNA extracted following the manufacture's guidelines and reverse transcribed as described previously [312]. The concentrations of the chemical inhibitors were determined empirically by titration as described [325-327].

Table 2.1: Inhibitors, the enzyme or molecule inhibited and concentration used [326,328,329]

Compound	Enzyme/molecule Inhibited	Concentration used
EGTA	Ca^{2+} chelator	1.5mM
NS-398	COX-2	8-10μM
SC-560	COX-1	10-15μM
PD-98089	ERK	50μM
AG-1478	EGFR	100-200nM
SN50	NFκB	100μg/mL
LY-294002	PI3K	25μM

2.8 RNA extraction

Throughout the RNA extraction, a ribonuclease (RNase) free environment was maintained by wiping all the surfaces with RNase-ZAP® (Sigma) and by using double distilled deionized water that was pretreated with 0.01% diethylpyrocarbonate (DEPC; Sigma). RNA was

extracted from cell samples using Trizol® (Sigma) following the manufacturer's protocol. Briefly, lysed cells in 1 ml of Trizol® (Sigma) were retrieved from -80 ^{0}C and thawed on ice after which 100µL BCP (1 bromo-2 chloropropane) (Merck) was added to each sample. The mixture was shaken vigorously for 15 seconds and incubated at room temperature for 10 minutes. Samples were then centrifuged for 15 minutes at 15,000 rpm at 4°C. The top colorless aqueous phase containing RNA was carefully transferred into a clean tube. The RNA was pelleted by the addition of 0.5mL of isopropanol (Merck) per 1ml of Trizol®, mixed thoroughly and allowed to stand for 10 minutes at room temperature. The samples were centrifuged at 15000 rpm for 10 minutes at 4°C. The isopropanol was then discarded and the resultant RNA pellet was washed with 75% (v/v) ethanol by centrifugation at 15000 rpm for 5 minutes at 4°C. The supernatant was then discarded and the pellet was air dried for 30 minutes. The pellet was re-suspended in nuclease free or 0.01% DEPC (Sigma Aldrich, UK) treated water and heated to 55°C for 2 minutes to remove any tertiary RNA structure. RNA quality and concentration was determined by Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The RNA was diluted using nuclease free or DEPC treated water to a final concentration of 200ng/µL and was stored at -80°C until future use.

2.9 Reverse transcription (cDNA synthesis)

The diluted RNA samples were thawed on ice and reverse transcribed to cDNA using reverse transcription reagent mix (Table 2.2) containing; $MgCl_2$ (5.5.mmol/L), (Applied Biosystems), 10x Reverse transcription buffer (1mmol/L), (Applied Biosystems), deoxynucleoside triphosphate (dNTP) mix (0.5mmol/L), (Applied Biosystems), RNase inhibitor (0.4 IU/µL) (Applied Biosystems), Random Primers (1.5µmol/L), (Invitrogen), oligo-dT (1.25 µmol/L),

(Applied Biosystems), and MultiScribe™ Reverse Transcriptase enzyme (1.25 IU/µL) (Applied Biosystems). The mix was aliquoted into individual polymerase chain reaction (PCR) tubes (9µL/tube) and template RNA was added (1 µL/tube of 200ng/µL RNA). A reaction mix devoid of the reverse transcriptase enzyme was included as control for any genomic DNA contamination. The reverse transcription reaction was performed on a GeneAmp® 2700 (Applied Biosytems) PCR machine under the following cycling conditions: 10 min annealing at 25°C, 45 min reverse transcription at 48°C and then denaturation at 95°C for 5min. Thereafter cDNA samples were stored at 4° C.

Table 2.2: Reverse transcription reaction mix; components and concentrations

Component	**Stock concentration**	**Final reaction concentration**
$MgCl_2$	25mM	5.5mmol/L
RT-PCR Reaction Buffer	10×	1mmol/L (1×)
dNTP	10mM	0.5mmol/L
RNase inhibitor	20IU/µL	0.4 IU/µL
Random Primers	3µg/µL	1.5µmol/L
Oligo-dT	50µM	1.25 µmol/L
Reverse Transcriptase enzyme	50U/µL	1.25 IU/µL
RNA	200ng/µL	200ng

2.10 Quantitative real time Polymerase Chain Reaction (RT-PCR)

The advent of quantitative real-time PCR (qRT-PCR) has dramatically changed the field of measuring gene expression [330]. qRT-PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity [331]. qRT-PCR reactions are characterized by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as cycle threshold (C_t), the time at which fluorescence intensity is greater than background fluorescence [332].

2.10.1 Primer design

All primer pairs used in this study for target genes excluding were designed using PRIMER express program (Perkin Elmer, PE Biosystems, Warrington UK) or Primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or ApE- A Plasmid Editor v1.17 software (Wayne Davis, University of Utah, USA) and synthesized at the Molecular and Cellular Biology synthetic DNA laboratory (University of Cape Town, RSA). At least one primer of each pair spanned an exon-exon junction to prevent amplification of genomic DNA during the PCR reaction. The specificity of the designed primers was checked by blasting the sequence using a BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.10.2 Quantitative real-time PCR detection Chemistries

In this study, SYBR® Green dye was mainly used to detect PCR products while the TaqMan® was used at the beginning of the study to validate SYBR® Green dye experiments. Both reactions were carried out on Sequence Detection Systems (SDS) instruments. SYBR Green dye chemistry uses the SYBR Green dye to detect polymerase chain reaction (PCR) products by binding to double-stranded DNA formed during PCR (Figure 2.1). As the double-stranded PCR product accumulates during cycling, more dye can bind and emit fluorescence. Thus, the fluorescence intensity is directly proportional to synthesized dsDNA concentration [333]. TaqMan® chemistry, also known as 5′ nuclease assay, uses fluorogenic probes to detect PCR product accumulation during PCR (Figure 2.1). The sequence-specific probe is labeled with a reporter dye on the 5′ end and a quencher dye on the 3′ end [334], which allows the quencher to reduce the reporter fluorescence intensity by fluorescence resonance energy transfer (FRET) when the probe is intact [335]. FRET reduces fluorescence intensity in hydrolysis probes and increases intensity in hybridization probes. When annealed to the target sequence, the bound and quenched probe will be degraded by the DNA polymerase's 5′ nuclease ability during the extension step of the PCR. Probe degradation allows for separation of the reporter from the quencher dye, resulting in increased fluorescence emission [332,334] (Figure 2.1).

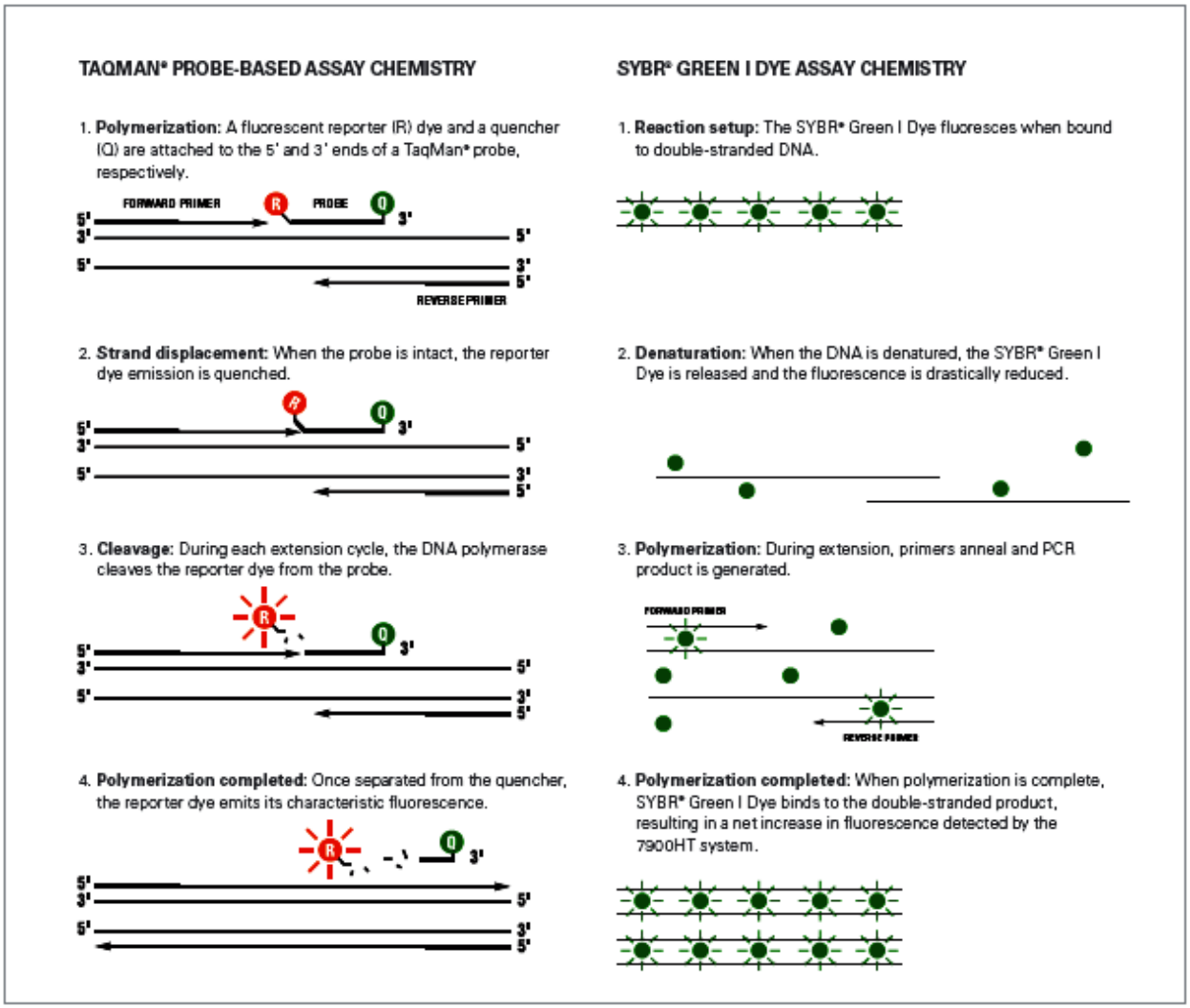


Figure 2.1: Comparison of SYBR® Green dye and TaqMan® qRT-PCR detection chemistries (http://www.lifetechnologies.com).

2.10.3 SYBR Green quantitative real-time PCR reaction

Quantitative real-time PCR was performed on the samples using a SYBR Green 2x SensiMix™ SYBR No-ROX One Step Kit (Bioline, UK). Briefly, a master mix (Table 2.3) containing 1x SensiMix™ SYBR No-ROX, 250nM Forward Primer, 250nM Reverse primer and distilled H_20 was prepared, after which 1µL of cDNA and 9uL of master mix (10µL total reaction volume) were aliquoted in duplicate in each well of the PCR plate (48 wells (Illumina) or 96 wells WHT/WHT Hard Shell® (Biorad). An RT negative, no template control (containing water) and the reference positive control were also added in duplicate. To normalize the gene of interest, a reaction mix containing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or human cyclophilin A (HCYPA) primers and corresponding cDNA samples was included in the experiment to serve as an internal reference control. These genes (GAPDH and HCYPA) were the two most stable genes of all five reference genes (Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), actin, β-actin, GAPDH and HCYPA) analyzed for HeLa S3 and cervical tissue when their average expression stability value M was calculated using geNorm in qbase+ 2.5.1 software (Biogazelle, Belgium). The wells were sealed using plate covers and the qRT-PCR was performed using the following parameters; a holding step of 15 minutes at 95°C followed by 40 cycles polymerase activation at 95°C for 15 seconds and 60°C extension for 60minutes using the Eco™ Real time PCR system (Illumina™) or CFX96™ qRT-PCR detection system (BioRad, South Africa). A melt curve was also performed at 95°C for 15 seconds, 55°C for15 seconds and 95°C for 15 seconds to confirm the purity of the PCR products (Figure 2.2).

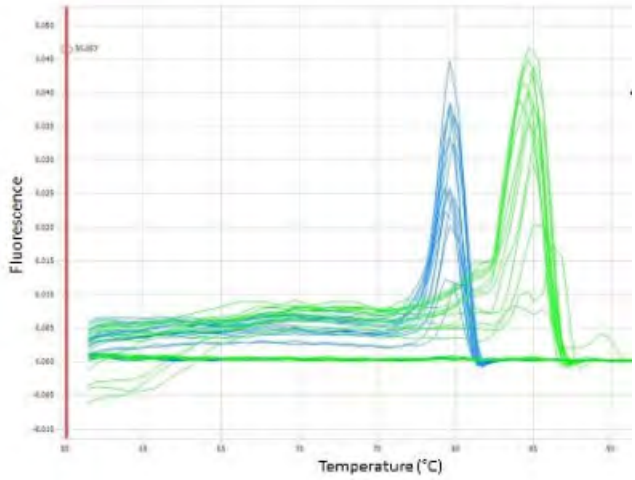


Figure 2.2: Typical melt curve analysis following SYBR green real time qRT-PCR reaction showing two distinct peaks of GAPDH (Green) and IL-1α (blue).

Table 2.3: Real time RT-PCR reaction mix (SYBR Green); components and concentrations

Reagents	Stock concentration	Final reaction concentration
Syber Green 2x SensiMix™	2×	1×
Forward Primer	25µM	250nM
Reverse primer	25µM	250nM

2.10.4 TaqMan quantitative real-time PCR reaction

To validate the results obtained from the SYBR Green qRT-PCR, a Taqman qRT-PCR was also performed for IL-1α, using TaqMan® 2x universal PCR master mix (Applied Biosystems) (Table 2.4). Briefly, a master mix containing 1x TaqMan® 2x universal PCR master mix, 300nM Forward primer, 300nM Reverse primer, 200nM probe (Primer design, UK), and 18.75nM ribosomal18s primers (Primer design, UK) was made. The final reaction mix (9ul of master mix and 1ul of cDNA) was aliquoted in duplicate into 96 well plates (Biorad). The wells were sealed using plate covers and the qRT-PCR was performed using the following parameters; a holding step of 15 minutes at 95°C followed by 40 cycles polymerase activation at 95°C for 15 seconds and 60°C extension for 60minutes using CFX96™ RT-PCR detection system (BioRad,UK). PCR primers and probe for IL-1α was designed using PRIMER express program (Perkin Elmer, PE Biosystems, Warrington UK). The probe had a fluorescent reporter dye, Carboxyfluorescein (FAM™) dye attached to the 5' end and was used to measure the fluorescence of the samples and an internal control 18s (VIC™) dye was included in the wells. Each of the reporters was quenched by tetramethylrhodamine (TAMRA™) dye at the 3´end.

Table 2.4: Real-time RT-PCR reaction mix (TaqMan); components and concentrations

Reagents	Stock concentration	Final reaction concentration
2× TaqMan mix	2×	1×
Forward Primer	25μM	300nM
Reverse primer	25μM	300nM
Ribosomal 18s primers	1.25μM	18.75nM
Target gene probe	5μM	200nM

2.10.5 Calculation of relative mRNA expression using the comparative C_t method

The comparative C_t method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample [330]. In this study, the $\Delta\Delta C_t$ method, also known as the Livak method [336] was used to determine relative mRNA expression of genes assayed. This method normalizes the expression of the target genes relative to a single reference gene and expressed relative to the reference sample [336]. Calculations adapted from Livak et al. (2001) are summarized below;

$\mathbf{\Delta C_t}$ = Average $C_{t\ (\text{target assay})}$ - Average $C_{t\ (\text{Reference assay})}$

$\mathbf{\Delta\Delta C_t}$ = $\Delta C_{t\ (\text{Test sample})}$ - $\Delta C_{t\ (\text{Reference sample})}$

Relative quantification (RQ) = $2^{-\Delta\Delta Ct}$

Table 2.5: Primers and probes used for qRT-PCR

Target gene		Sequence (5'-3')
HCYP-A	Forward primers	CCCACCGTGTTCTTCGACAT
	Reverse primers	CCAGTGCTCAGAGCACGAAA
GAPDH	Forward primers	AATGATTCATAGGGCTTCAG
	Reverse primers	ACAGTCAGCCGCATCTTC
18s	Forward primers	CGGCTACCACATCCAAGGAA
	Reverse primers	GCTGGAATTACCGCGGCT
	Probe (VIC)	TGCTGGCACCAGACTTGCCCTC
IL-1α	Forward primers	TGTATGTGACTGCCCAAGATGAA
	Reverse primers	CTACCTGTGATGGTTTTGGGTATC
	Probe (FAM)	CAGGCATCTCCTTCAGCAAGCACTGGTTG
IL-1RI	Forward primers	TGGTGACTCCCTCCTGAGAAG
	Reverse primers	CAGCCTCCAGAGAAGAAATCAG
IL-8	Forward primers	CTGGCCGTGGCTCTCTTG
	Reverse primers	TTAGCACTCCTTGGCAAAACTG
IL-12α	Forward primers	CGGTCATCTGCCGCAAA
	Reverse primers	TGCCCATTCGCTCCAAGA
COX-1	Forward primers	TGTTCGGTGTCCAGTTCCAATA
	Reverse primers	ACCTTGAAGGAGTCAGGCATGAG
PTGFR	Forward primers	GCAGCTGCGCTTCTTTCAA
	Reverse primers	CACTGTCATGAAGATTACTGAAAAAAATAC

PTGIR	Forward primers	GCCCTCCCCCTCTACCAA
	Reverse primers	TTTTCCAATAACTGTGGTTTTTGT G
CXCR4	Forward primers	CAGTGGCCGACCTCCTCTT
	Reverse primers	CAGTTTGCCACGGCATCA
CD4	Forward primers	CTAAGCTCCAGATGGGCAAG
	Reverse primers	CACCACCAGGTTCACTTCCT
CCR5	Forward primers	AGCTATGCAGGTGACAGAGACTCTT
	Reverse primers	TCCCCGACAAAGGCATAGAT
CCR2b	Forward primers	TGCCTGACTCACACTCAAGG
	Reverse primers	GGCTTCTCAGCAACTGAACC
CXCR6	Forward primers	GGTTCTTCTTGCCACTGCTC
	Reverse primers	CATGAGGTTGAAGGGCATCT
GPR1	Forward primers	TTCTGCCCCTGTACATCTCC
	Reverse primers	AGAAGCCAAAAGCCAGATGA

2.11 Protein extraction from cells

For protein experiment, total protein was extracted from HeLa cells. Briefly, HeLa cells were seeded at a density of 5×10^5 cells in a 3cm TC dishes and cultured in complete media (2mL) overnight. The cells were lysed on ice by an addition of lysis buffer (Table 2.6) (150mM NaCl, 1% Triton X-100 (Sigma), 10mM Tris-HCL pH 7.4, 1mM EDTA, 0.1% sodium diodecyl sulfphate (SDS), and containing cOmplete™, EDTA free protease inhibitor cocktail tablet

(chymotrypsin, thermolysin, papain, pronase, pancreatic extract and trypsin inhibitors) (Roche)) and cells scraped off with cell scraper (Griener Bio-One, Germany). The insoluble material was pelleted by centrifugation at 14000 rpm for 15 minutes at 4°C and the clarified lysate was transferred to a new tube and kept at -20 ^{0}C for further protein quantification.

Table 2.6: Components of protein extraction buffer

Reagents	**Concentration**
NaCl	150mM
Triton X-100	1%
Tris-HCl pH 7.4	10mM
EDTA	1mM
SDS	0.1%

2.12 Protein quantification

Protein concentrations were determined by using the Pierce® BCA Protein Assay Kit (Thermo scientific, Rockford, USA) as directed by the manufacturer's instructions. Bovine serum albumin (BSA) was used as a protein standard and diluted from 2000µg/mL to 0µg/mL in 100µL of distilled water in tubes. Aliquots of 25µL of standards or samples were loaded into a 96-well plate (in triplicate) followed by the addition of 200µL of working reagent (WR) (50 parts of BCA™ Reagent A with 1 part of BCA^{TM} Reagent B [50:1, Reagent A:B]) and plate was mixed thoroughly on a plate shaker for 30 seconds. Thereafter, plate was covered and incubated at 37 ^{0}C for 30 minutes followed by cooling at RT for 15 seconds. Absorbance was then measured at

wavelength of 595nm on Anthos Microplate Reader (Columbia Bioscience Inc, USA). A standard curve was plotted using the program Graphpad Prism® 5.0 and the unknown protein concentrations were determined from the standard curve.

2.13 SDS-PAGE gel

Protein samples were re-suspended in 1× SDS protein sample loading buffer (Table 2.7), loaded in 6% stacking (SDS-PAGE) gel and separated on a 15% sodium diodecylsulfphate poly acrylamide (SDS-PAGE) gel (Table 2.8). The gel is formed by the polymerization of two compounds, acrylamide and N,N-methylenebis-acrylamide (Bis) (BioRad). Briefly, a total of 20-30μg of protein was re-suspended in SDS (1×) protein sample loading buffer (Table 2.7), heated at 95°C for 5 minutes to denature the protein and resolved on the 15% SDS–PAGE gel (Table 2.8) at 100V. The relative sizes of the proteins were determined by a Spectra protein marker (Invitrogen).

Table 2.7: SDS protein sample buffer (reagents and concentration)

Reagents	**Concentration**
Glycerol	10%
Tris-HCl pH 6.8	60mM
SDS	2%
Bromophenol blue	0.01%
Beta-mercaptoethanol	1.25%

Table 2.8: SDS-PAGE (reagents, concentration, and volume pipetted)

Reagents	Concentration	volume
Separating gel (15%)		
Acrylamide (Biorad)	30%	12.5mL
H_2O	-	3.65mL
Tris pH 8.8	1.25M	8.3mL
SDS	10%	0.25mL
Ammonium persulfate (APS)	10%	0.3mL
Tetramethylethylenediamine (TEMED) (Thermo Scientific)	-	25µL
Stacking gel (6%)		
Acrylamide (Biorad)	30%	
H_2O	-	5.075mL
Tris pH 6.8	0.375M	4mL
SDS	10%	0.125mL
Ammonium persulfate (APS)	10%	0.3mL
Tetramethylethylenediamine (TEMED) (Thermo Scientific)	-	20µL

2.14 Western Blot

The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Health Care, Amersham, UK) using a Novex® Semi-Dry Blotter (Invitrogen™). Three layers of chromatography paper (Lasec, Cape Town, RSA) were soaked briefly in transfer buffer (25mM Tris, 192mM glycine, 10% methanol) and stacked onto the anode plate of the Semi Dry blotter. The PVDF membrane cut to the dimension of the gel was activated in methanol for 1 minute, washed in water and equilibrated in transfer buffer before being stacked onto the chromatography paper. The SDS-PAGE gel was removed from the apparatus equilibrated in the transfer buffer and placed over the membrane. Additional three layers of wet (soaked in transfer buffer) chromatography paper were then placed over the gel and the Semi Dry blotter apparatus (Invitrogen™) was covered with the cathode plate, sealed and transferred at 14volts for 90 minutes. After transfer, the PVDF membrane was washed in 20mL PBS-Tween (1× PBS and 0.1%Tween-20) for 5 minutes. The membrane was then incubated in 10mL of blocking buffer (1× PBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) on a shaker for 30 minutes at RT, after which it was washed three times for 10 minutes each with 15mL PBS-Tween followed by an overnight incubation with relevant primary antibody at 4 ^{0}C with gentle shaking. The following day, membrane was washed three times for 10 minutes each with 15mL of PBS-Tween, incubated for 1 hour with relevant horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) at RT and washed again three times with 15mL PBS-Tween as described above. Protein detection was by chemiluminescence; membrane was incubated with SuperSignal® West Pico Chemiluminescent Substrate (ThermoScientific, Rockford, USA) (1mL of SuperSignal West Pico Luminol /Enhancer Solution and 1mL SuperSignal West Pico Stable Peroxide Solution) for 5 minutes at RT; excess developing solution was drained, placed in transparent film

and proteins viewed with BioSpectrum™ 500 Imaging System (Ultra-Violet Products [UVP] Limited, Cambridge, UK).

2.15 Enzyme Linked Immunosorbent Assay (ELISA)

Secreted IL-1α was measured by ELISA using Quantikine® Human IL-1α ELISA kit (R&D Systems, Minneapolis, USA) (Whitehead Scientific, Cape Town, South Africa). HeLa cells were seeded at a density of 5×10^5 in 3cm TC dishes with complete media and allowed to attach and grow overnight. The next day, complete media was aspirated, cells washed twice in PBS and incubated with serum free media for another 24 hours after which they were incubated for 16 and 24 hours in the presence of SP (1:50), vehicle (PBS or DMSO) in the absence/presence of chemical inhibitors. After the incubating period, protein was extracted and quantified as described above using the Pierce® BCA Protein Assay Kit (Thermo scientific, Rockford, USA) (sections 2.11 and 2.12). The secreted IL-1α protein was determined from the total protein in the lysate using Quantikine® Human IL-1α ELISA kit (R&D Systems, Minneapolis, USA) as per the manufacturer's instruction. Five independent experiments were conducted and data expressed as fold change over basal where the amount of IL-1α secreted in treated cells (experiment) is divided by the amount secreted in their respective controls.

2.16 Immunohistochemistry

Immunohistochemistry was done on archival cervical blocks obtained from the Department of Anatomical Pathology, University of Cape Town. Briefly, sections were deparaffinized and rehydrated by immersing in xylene twice for 5 minutes, 100% ethanol twice for 5 minute, 95% ethanol for 5 minutes, 70% ethanol for 5 minutes, 50% ethanol for 5 minutes and rinsed with water. Antigen retrieval was done by pressure cooking for 2 minutes in 0.01M sodium citrate pH 6. Sections were blocked for endogenous peroxidase by incubating with 3% Hydrogen peroxide in methanol on a rocker at RT for 30 minutes and then rinsed with water followed by 1× TBS (tris-buffered saline) (50mM Tris-HCl, 150mM NaCl at pH 7.4). Sections were blocked using 5% normal donkey/goat serum diluted in TBS after which tissue sections were incubated with specific polyclonal primary antibodies at 4 ^{0}C for 18 hours. After incubation, tissue sections were then washed in TBS twice for 5 minutes each followed by incubation with biotinylated secondary IgG antibody at dilution of 1:500 at RT for 30 minutes. Tissue sections were then further incubated with streptavidin-biotin peroxidase complex (1:50) at RT for 30 minutes. Controls were incubated with biotinylated IgG secondary antibody only. Color reaction was developed by incubating with 3.3'-diaminobenzidine. Tissue sections were counterstained in aqueous hematoxylin, before mounting and coverslipping. Fluorescent images were visualized and photographed using a Carl Zeiss laser scanning microscope LSM 510 (Jena, Germany).

2.17 Quantification of immunohistochemical stains

Quantification of immunohistochemical stains was done using image de-convolution (H-DAB) and thresholding analysis on Fiji software (http://fiji.sc/Fiji) as described by Ruifrok and Johnston, 2001 and Viel et al., 2013 [337,338]. Briefly, images were opened on Fiji after which background was subtracted to correct for shadow or color effects. Images were then de-convolved (H-DAB color de-convolution). The resulting blue and brown monochromes were converted to grayscale (8bit) thresholded and % area of staining measured in proportion to the size of the image /tissue. % DAB stained was calculated as follows;

$$\% \text{ DAB stain} = \frac{\% \text{ DAB stain}}{\% \text{ DAB} + \% \text{ H stain}} \times 100$$

2.18 Statistics

All data in this study were analyzed by t-test or one-way ANOVA using Graph Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Paired T-tests were conducted on the untransformed means of the replicates between SP and control. Unpaired T-tests were performed on SP versus SP and inhibitor after conversion to fold increases. One-way ANOVA was used as an additional tool to determine the significant difference between various time points in genes expression by real-time PCR in response to SP.

CHAPTER 3

REGULATION OF INFLAMMATORY PATHWAYS IN NEOPLASTIC CERVICAL EPITHELIAL CELLS BY SEMINAL PLASMA

3.1 Introduction

Inflammation is a complex process that involves a host of resident and recruited immune cell types working together to promote the removal of insult or injury and initiate tissue repair [80]. Many cancers including cancer of the cervix have been shown to develop as a consequence of persistent chronic inflammation [339].

Characteristically regarded as response to tissue injury or pathogenic insult, chronic inflammation is typified by alterations to vascular, epithelial, and immune cell function [287]. These are coordinated by specific molecular inflammatory pathways involving numerous growth factors, cytokines, chemokines, receptors (GPCRs), enzymes, and lipid mediators which orchestrate tissue repair mechanism and alteration of endothelial and vascular function to promote angiogenesis, vascular permeability, and extravasation of immune cells to the inflamed tissue [80]. Over the last decade, numerous experimental studies using gene-disruption and gene over-expression systems in cell lines as well as studies on laboratory animals, and tissue explants have provided evidence to support the role of inflammation and inflammatory pathways such as the COX-PG in cervical cancer pathogenesis and progression [28,312,340-342]. These pro-inflammatory pathways have been discovered to be induced by various stimuli such as endogenous growth factors, cytokines, tumor promoting carcinogens, and lipopolysaccharides (LPS) [130].

Human seminal plasma is a complex organic fluid comprising of numerous endogenous antigenically distinct molecules [307,343]. Some of which have been implicated in the initiation of inflammatory response and activation of specific inflammatory pathways. Furthermore, studies have shown that the exposure of the cervical epithelium to seminal plasma as seen during unprotected sexual intercourse can further modify the inflammatory milieu of the cervix in sexually active women [344-346]. In addition, studies by Sales et al. (2002), Muller et al. (2006), and Sutherland et al. (2012) demonstrated the pro-inflammatory role of seminal plasma on neoplastic cervical epithelium. These studies reveal that seminal plasma can promote cancer cell proliferation by inducing the expression of angiogenic genes and pro-inflammatory cytokines/chemokines via the activation of inflammatory pathways including COX-PGs, EGFR-ERK 1/2, and NF-κB pathways [287,345,346].

The discovery of these pathways led to the hypothesis that the human seminal plasma can augment cervical tumorigenesis by regulating the expression of a wide range of inflammatory pathways that are yet to be identified.

3.2 Aim of the study

This present study was designed to investigate gene arrays of inflammatory pathways that can be regulated by SP in neoplastic cervical epithelial cells, as a model to identify pro-inflammatory genes that exacerbate neoplastic cervical inflammation and promote tumorigenesis in response to SP.

3.3 Materials and Method

3.3 Cell culture, treatment with seminal plasma and RNA extraction

HeLa S3 cells were routinely maintained as previously described in section 2.6. HeLa cells were seeded in complete medium at density of $2x10^5$ cells in 3 cm diameter tissue culture dishes and allowed to attach and grow overnight. The following day, the complete medium was aspirated and cells were serum starved by incubating in serum free medium as described in section 2.6 for 24 hours. Cells were then treated with serum free media containing SP at a dilution of 1:50 or serum free media with PBS (control) for 8hrs (n = 5 individual experiment done in duplicate). RNA was extracted as described in section 2.8 and reverse transcribed as described in section 2.9. For the array, the 5 individual experiments were pooled together.

3.3.2 Quantitative real time PCR and Taqman array analysis.

qRT-PCR was performed to investigate the expression of inflammatory genes and chemokines using the TaqMan® Array 96-Well Plate (Human inflammation) and TaqMan® Array 96-Well Plate (Human Chemokines) (Applied Biosystems, USA). The reaction was conducted on a Bio-Rad CFX96™ quantitative RT-PCR machine (Bio-Rad Laboratories Ltd.) under normal operating conditions. Relative expression was calculated using the comparative C_t method (as described in section 2.10.5) and arrays were normalized for RNA loading using 18s Ribososmal RNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine phosphoribosyltransferase 1(HPRT1), and Glucuronidase beta (GUSB) as internal controls. The array experiment was conducted once on pooled cDNA samples (n = 5).

3.3.3 Bioinformatics analysis of genes regulated by seminal plasma

Approved names and symbols of all genes were obtained from HGNC (HUGO Gene Nomenclature Committee) (www.genenames.org/data/hgnc_data). NCBI (The National Center for Biotechnology Information) Accession number for all genes was obtained from www.ncbi.nlm.nih.gov. Genes were classified into pro and anti-inflammatory based on description obtained from OMIM® (Online Mendelian Inheritance in Man®) (http://www.omim.org). Gene List Analysis, a web tool from PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system (www.pantherdb.org) was assigned to classify seminal plasma regulated genes into molecular functions, biological process, protein class and pathways [347,348].

3.3.4 Verification of the TaqMan Array Data

3.3.4.1 Treatment of Cells with Seminal plasma

With the aim of validating the temporal expression of SP regulated genes as seen in the TaqMan array, 2×10^5 HeLa cells were treated with vehicle (PBS) or SP (1:50) for 4, 8, 16 and 24 hrs as described in section 2.7.1. The cells were then harvested using 1ml Trizol® (Sigma) and their RNA extracted immediately or stored at -80°C until further use. The experiments were repeated individually for n = 5 times.

3.3.4.2 Quantitative real time PCR Analysis

qRT-PCR was performed on an Eco™ Real time PCR system (Illumina™) to detect the expression of IL-1α, IL-8, IL-12α, PTGIR, PTGFR, and CXCR4 genes respectively as described in section 2.10.3. To normalize the gene of interest, a reaction mix containing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and corresponding cDNA samples was included in the experiment to serve as an internal reference control.

To further validate/compare the results obtained from the SYBR Green RT-PCR, a TaqMan RT-PCR was also performed for IL-1α, using TaqMan® on a CFX96™ RT-PCR detection system (BioRad, UK) as described in section 2.10.4. The relative mRNA expression was calculated using the comparative C_t method (section 2.10.5) and data represented on a bar chart as fold above control.

3.3.5 Statistical Analysis

All data are presented as mean ± SEM. Statistical significant differences were determined by t-test (non-parametric) using Graph Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Paired T-test or Wilcoxon matched pair test were conducted on the untransformed means of the replicates between SP and control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). One-way ANOVA was used as an additional tool to determine the significant difference between various time points of IL-1α, IL-8, IL-12α, PTGIR, PTGFR, and CXCR4 gene expression by real-time RT-PCR in response to SP.

3.4 RESULTS

3.4.1 Seminal plasma regulates the expression of inflammatory pathways in cervical cancer cells

TaqMan® 96 well gene expression array (Human inflammation and Chemokines) was used to investigate the role of seminal plasma in the regulation of inflammatory genes/pathways that can augment cancer cell progression. Neoplastic cervical epithelial cells (HeLa) were treated with PBS (control) or 1:50 dilution of seminal plasma for duration of 8 hours and RNA subjected to reverse transcription and TaqMan array analysis. Expression was considered significant based on a fold change of 2 fold or more above control as a cut-off point [349]. Of the 181 (3 genes duplicated in both plates) genes analyzed, seminal plasma regulated the expression of 95 genes. Of these, 44 genes were significantly up-regulated (fold change ≥ 2), 35 genes were down regulated (fold change <1), and 16 genes were not significantly up-regulated (fold change ≥1<2). 86 genes were not detected (ND) (Appendix I). Fold induction of some important pro-inflammatory genes regulated by seminal plasma are as follows: pro-inflammatory enzyme cyclooxygenase-1 (COX-1) (2.43 fold); inflammatory cytokines/chemokines such as IL-6 (fold change 4.19), IL-8 (38.93 fold), CXCL1 (GRO-α) (12.37 fold), TNF (26.55 fold) and IL-1α (68.56 fold); GPCRs such as Prostaglandin F receptor (PTGFR) (20.21 fold), Prostaglandin I2 receptor (PTGIR) (7.22 fold); immune cell chemotactic and activating factors such as CCL2 (monocyte chemotactic and activating factor) (4.43 fold), CCL5 (eosinophil chemotactic cytokine) (5.24 fold); and genes involved in the modulating inflammatory responses/ transcription factor such as Nuclear factor κ B (NF-κB) (2.32 fold) and TLR2 (Toll like receptor 2) (88.47 fold). The list of the genes regulated by seminal plasma, their inflammatory properties (i.e. pro or anti-inflammatory) and the fold increase/decrease are mentioned in table 3.1.

Table 3.1: List of inflammatory genes regulated by seminal plasma in HeLa cells

Accession number	Gene symbol	Gene description	Inflammatory properties	Fold change
		RECEPTORS		
NM_000684	*ADRB1*	Adrenergic, beta-1-, receptor	Pro-inflammatory	1.50
NM_000024	*ADRB2*	Adrenergic, beta-2-, receptor	Pro-inflammatory	0.64
NM_000710	*BDKRB*	Bradykinin receptor B1	Pro-inflammatory	22.39*
NM_000623	*BDKRB2*	Bradykinin receptor B2	Pro-inflammatory	1.63
NM_006639	*CYSLTR1*	Cysteinyl leukotriene receptor 1	Pro-inflammatory	150.14*
NM_001008540	*CXCR4*	Chemokine (C-X-C motif) receptor 4	Pro-inflammatory	1.67
NM_000861	*HRH1*	Histamine receptor H1	Pro-inflammatory (binds to Th1)	1.39
NM_000869	*HTR3A*	5-hydroxytryptamine (serotonin) receptor 3A	Pro-inflammatory	1.82
NM_000416	*IFNGR1*	Interferon gamma receptor 1	Pro-inflammatory	1.03
NM_000877	*IL1R1*	Interleukin 1 receptor, type I	Pro-inflammatory	1.24
NM_016232	*IL1RL1*	Interleukin 1 receptor-like 1	Pro-inflammatory	22.65*
NM_004633	*IL1R2*	Interleukin 1 receptor, type II	Anti-inflammatory (inhibit IL-1 activity)	0.20
NM_000206	*IL2RG*	Interleukin 2 receptor, gamma	Pro-inflammatory (required for T cell maturation)	0.01
NM_001143919	*LTB4R*	Leukotriene B4 receptor	Pro-inflammatory	4.27*
NM_000529	*MC2R*	Melanocortin 2 receptor	Pro-inflammatory	6.52*
NM_000176	*NR3C1*	Nuclear receptor subfamily 3, group C, member 1	Pro-inflammatory	0.84

NM_000952	*PTAFR*	Platelet-activating factor receptor	Pro-inflammatory	0.09
NM_000956	*PTGER2*	Prostaglandin E receptor 2 (subtype EP2)	Pro-inflammatory	0.80
NM_000959	*PTGFR*	Prostaglandin F receptor (FP)	Pro-inflammatory	20.21*
NM_000960	*PTGIR*	Prostaglandin I2 receptor (IP)	Pro-inflammatory	7.22*
NM_001060	*TBXA2R*	Thromboxane A2 receptor	Pro-inflammatory	0.96
NM_001066	*TNFRSF1B*	Tumor necrosis factor receptor superfamily, member 1B	Pro-inflammatory	2.70*
NM_001065	*TNFRSF1A*	Tumor necrosis factor receptor superfamily, member 1A	Pro-inflammatory	3.16*
NM_003264	*TLR2*	Toll-like receptor 2	Pro-inflammatory	88.47*
NM_138554	*TLR4*	Toll-like receptor 4	Pro-inflammatory	1.66
		ENZYMES		
NM_000698	*ALOX5*	Arachidonate 5-lipoxygenase	Pro-inflammatory	0.88
NM_033292	*CASP1*	Caspase 1, apoptosis-related cysteine peptidase	Pro-inflammatory	0.66
NM_001266	*CES1*	Carboxylesterase 1 (monocyte/macrophage serine esterase 1)		3.97*
NM_000860	*HPGD*	Hydroxyprostaglandin dehydrogenase 15-(NAD)	Anti-inflammatory (degrades PGs)	0.32
NM_004972	*JAK2*	Janus kinase 2	Pro-inflammatory	1.72
NM_002257	*KLK1*	Kallikrein-related peptidase 1	Pro-inflammatory	0.64
NM_001002231	*KLK2*	Kallikrein-related peptidase 2	Pro-inflammatory	19.59*
NM_001030047	*KLK3*	Kallikrein-related peptidase 3	Pro-inflammatory	34.58*
NM_022046	*KLK14*	Kallikrein-related peptidase 14	Pro-inflammatory	2.53*
NM_000892	*KLKB1*	Kallikrein B, plasma (Fletcher factor) 1	Pro-inflammatory	1.73
NM_000897	*LTC4S*	Leukotriene C4 synthase	Pro-inflammatory	10.03*

NM_002745	*MAPK1*	Mitogen-activated protein kinase 1	Pro-inflammatory	0.72
NM_001040056	*MAPK3*	Mitogen-activated protein kinase 3	Pro-inflammatory	0.52
NM_002750	*MAPK8*	Mitogen-activated protein kinase 8	Pro-inflammatory	0.83
NM_001315	*MAPK14*	Mitogen-activated protein kinase 14	Pro-inflammatory	0.67
NM_002468	*MYD88*	Myeloid differentiation primary response gene (88)	Pro-inflammatory	9.41*
NM_001111307	*PDE4A*	Phosphodiesterase 4A	Anti-inflammatory (regulates cellular conc. Of cAMP)	0.52
NM_002600	*PDE4B*	Phosphodiesterase 4B	Anti-inflammatory	0.42
NM_001165899	*PDE4D*	Phosphodiesterase 4D	Anti-inflammatory	0.52
NM_000928	*PLA2G1B*	Phospholipase A2, group IB (pancreas)	Pro-inflammatory	0.89
NM_000300	*PLA2G2A*	Phospholipase A2, group IIA	Pro-inflammatory	3.00*
NM_001168357	*PLA2G7*	Phospholipase A2, group VII	Pro-inflammatory	0.005
NM_001159322	*PLA2G4C*	Phospholipase A2, group IVC (cytosolic, calcium-independent)	Pro-inflammatory	0.42
NM_003561	*PLA2G10*	Phospholipase A2, group X	Pro-inflammatory	46.32*
NM_000932	*PLCB3*	Phospholipase C, beta 3 (phosphatidylinositol-specific)	Pro and Anti-inflammatory	0.40
NM_000933	*PLCB4*	Phospholipase C, beta 4	Pro-inflammatory	2.82*
NM_001130964	*PLCD1*	Phospholipase C, delta 1	Pro-inflammatory	0.21
NM_182811	*PLCG1*	Phospholipase C, gamma 1	Pro-inflammatory	29.05*
NM_002661	*PLCG2*	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	Pro-inflammatory	0.85
NM_016341	*PLCE1*	Phospholipase C, epsilon 1	Pro-inflammatory	6.71*
NM_000962	*PTGS1*	Prostaglandin-endoperoxide synthase 1	Pro-inflammatory	2.43*

NM_000963	*PTGS2*	Prostaglandin-endoperoxide synthase 2	Pro-inflammatory	0.53
NM_001061	*TBXAS1*	Thromboxane A synthase 1	Pro-inflammatory	0.63
NM_001953	*TYMP*	Thymidine phosphorylase	Pro-inflammatory	3.22*
		LIGANDS		
NM_000700	*ANXA1*	Annexin A1	Anti-inflammatory (inhibit phospholipase A2)	0.15
NM_005139	*ANXA3*	Annexin A3	Anti-inflammatory	0.29
NM_001154	*ANXA5*	Annexin A5	Anti-inflammatory	0.00031
NM_000074	*CD40LG*	CD40 ligand	Pro-inflammatory	18.69*
NM_013246	*CLCF1*	Cardiotrophin-like cytokine factor 1	Pro-inflammatory	1.46
NM_001735	*C5*	Complement component 5	Pro-inflammatory	2.14*
NM_002982	*CCL2*	Chemokine (C-C motif) ligand 2	Pro-inflammatory	4.43*
NM_002985	*CCL5*	Chemokine (C-C motif) ligand 5	Pro-inflammatory	5.24*
NM_052999	*CMTM1*	CKLF-like MARVEL transmembrane domain containing 1	Pro-inflammatory	3.15*
NM_001511	*CXCL1*	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Pro-inflammatory	12.37*
NM_002089	*CXCL2*	Chemokine (C-X-C motif) ligand 2	Pro-inflammatory	0.49
NM_002090	*CXCL3*	Chemokine (C-X-C motif) ligand 3	Pro-inflammatory	8.19*
NM_005409	*CXCL11*	Chemokine (C-X-C motif) ligand 11	Pro-inflammatory	2.47*
NM_000201	*ICAM1*	Intercellular adhesion molecule 1 (CD54)	Pro-inflammatory (mediates leukocytes adhesion)	3.82*

NM_000632	*ITGAM*	Integrin, alpha M (complement component 3 receptor 3 subunit)	Pro-inflammatory (mediates leukocytes adhesion)	4.78*
NM_002211	*ITGB1*	Integrin, beta 1 (fibronectin receptor, beta polypeptide)	Pro-inflammatory	1.02
NM_000211	*ITGB2*	Integrin, beta 2 (complement component 3)	Pro-inflammatory	0.61
NM_000575	*IL1A*	Interleukin 1, alpha	Pro-inflammatory	68.56*
NM_017416	*IL1RAPL2*	Interleukin 1 receptor accessory protein-like 2	Pro-inflammatory	5.64*
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	Pro-inflammatory	4.19*
NM_000584	*IL8*	Interleukin 8	Pro-inflammatory	38.93*
NM_000882	*IL12A*	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	Anti-inflammatory	2.36*
NM_002188	*IL13*	Interleukin 13	Pro-inflammatory	1.39
NM_001562	*IL18*	Interleukin 18 (interferon-gamma-inducing factor)	Pro-inflammatory	17.84*
NM_002468	*MYD88*	Myeloid differentiation primary response gene (88)	Pro-inflammatory	9.41*
NM_001165412	*NFKB1*	Nuclear factor of kappa B1	Pro-inflammatory	2.32*
NM_004787	*SLIT2*	Slit homolog 2 (Drosophila)	Anti-inflammatory	3.63*
NM_014011	*SOCS5*	Suppressor of cytokine signaling 5	Anti-inflammatory	0.00047
NM_139276	*STAT3*	Signal transducer and activator of transcription 3	Pro-inflammatory	0.86
NM_000594	*TNF*	Tumor necrosis factor (TNF superfamily, member 2)	Pro-inflammatory	26.55*
NM_001190942	*TNFSF10*	Tumor necrosis factor (ligand) superfamily, member 10	Pro-inflammatory	1.91

NM_001145645	*TNFSF13B*	Tumor necrosis factor (ligand) superfamily, member 13b	Pro-inflammatory	4.83*
		CALCIUM CHANNELS		
NM_000722	*CACNA2D1*	Calcium channel, voltage-dependent, alpha 2/delta subunit 1		0.73
NM_000724	*CACNB2*	Calcium channel, voltage-dependent, beta 2 subunit		0.28
NM_000726	*CACNB4*	Calcium channel, voltage-dedependent, beta 4 subunit		0.51

*, Fold change ≥ 2.

3.4.2 Ontologies of genes regulated by seminal plasma

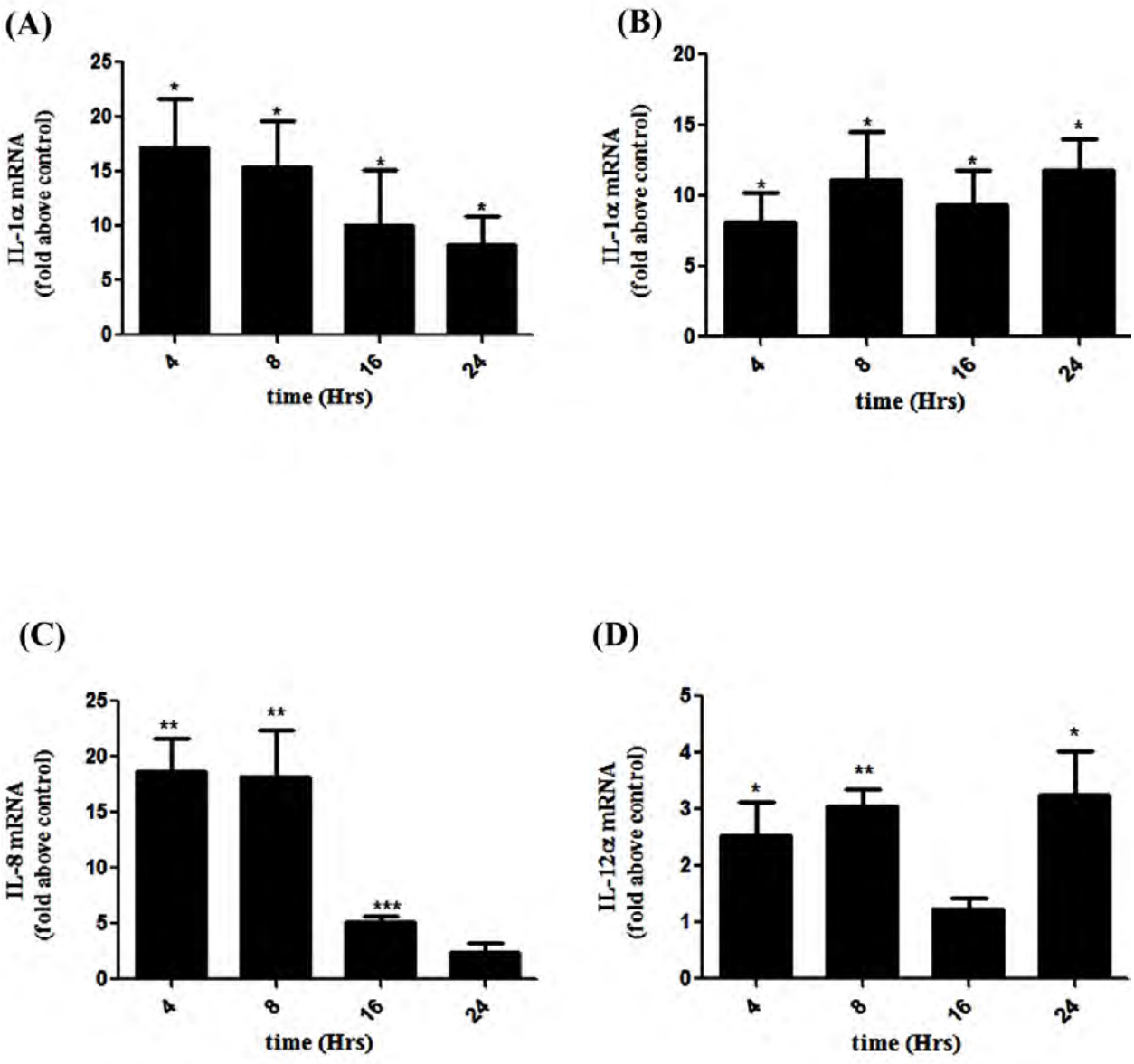
The PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System was designed to classify proteins (and their genes) in order to facilitate high-throughput analysis. PANTHER uses the Gene Ontology™ for classifications by molecular function, biological process and cellular component. The PANTHER Protein Class ontology was adapted from the PANTHER/X molecular function ontology, and includes commonly used classes of protein functions, many of which are not covered by GO molecular function. Molecular function is the function that a protein performs on its direct molecular targets; e.g. the insulin receptor has transmembrane receptor tyrosine protein kinase activity, which means it catalyzes the reaction that adds a phosphate group to a tyrosine in another protein (its target). Cellular component is the location where the protein performs its molecular function; e.g. the insulin receptor is located in the plasma membrane. Biological process covers the biological systems to which a protein

contributes; e.g. the insulin receptor is involved in regulation of carbohydrate metabolic process. Pathway is similar to biological process, but a pathway also explicitly specifies the relationships between the interacting molecules (www.pantherdb.org) (www.geneontology.org). All the 95 genes regulated by seminal plasma were subjected to PANTHER Gene List Analysis. Application of gene ontology annotation for molecular function showed that the top three enriched categories were binding activity (receptor or calcium ion binding) 37 genes (38.9%), receptor activity (GPCRs, cytokine receptor and TNF receptors) 35 genes (36.8%), and enzyme catalytic activity (hydrolases, oxidoreductase or tranferase) 34 genes (35.7%) (Appendix IIA). Similarly, application of gene ontology term "biological processes" revealed that SP-regulated genes regulates three major biological processes namely; cellular process (cell adhesion, cell communication, cell cycle, cell motion, and cellular component organization) 82 genes (86.3%), cell communication (cell-cell signaling and signal transduction) 78 genes (82.1%), and immune system processes (immune response and macrophage activation) 67 genes (70.5%) (Appendix IIB). Other important biological processes regulated by these genes include apoptosis, cell cycle and response to stimulus (Appendix IIB). Further analysis with PANTHER protein classification grouped seminal plasma regulated genes into two major classes; receptor (GPCRs and cytokine receptors) 31 genes (32.6%) and signaling molecule (cytokines) 28 genes (29.4%) (Appendix IIC). PANTHER pathway classification revealed one major enriched pathway for SP-regulated genes, which is inflammation mediated by chemokine and cytokine signaling pathway (comprising of chemokine signaling, cyclooxygenase and lipooxygenase signaling [Eicosanoid signaling], NFκB signaling, cytokine receptor signaling, SOCS5, Janus kinase signaling, and interleukin 2 signaling) 29 genes (30.5%) (Appendix IID).

3.4.3 Real-Time RT-PCR Validation of IL-1α, IL-8, IL-12A, PTGIR, PTGFR, and CXCR4 gene expression in response to SP in HeLa S3 Cells

To validate the results obtained from TaqMan 96 array experiment, qRT-PCR was carried out on cDNA samples from HeLa cells treated with vehicle or SP (1:50) for a duration of 4, 8, 16, and 24 hrs, respectively and the mRNA expression of IL-1α (Figure 3.1 A and B), IL-8 (Figure 3.1 C), IL-12α (Figure 3.1 D), PTGIR (Figure 3.1 E), PTGFR (Figure 3.1 F), and CXCR4 (Figure 3.1G) was determined.

In keeping with the result obtained from the TaqMan 96 array, IL-1α mRNA was found to be significantly induced or up-regulated at all time points investigated in both SYBR Green and TaqMan reporter dye (Figure 3.1 A and B; $P < 0.05$). IL-8 mRNA expression was significantly induced at 4, 8, and 16 hrs (Figure 3.1C; $P < 0.05$, $P < 0.01$, $P < 0.001$), while IL-12α mRNA expression was significantly induced at all-time point investigated except for 16 hrs (Figure 3.1 D; $P < 0.05$, $P < 0.01$). PTGIR mRNA expression displayed a time-dependent increase which peaked at 24 hrs treatment (Figure 3.1 E; $P < 0.05$). PTGFR mRNA expression was significantly up-regulated at 4, 8, and 16 hrs respectively (Figure 3.1 F; $P < 0.05$, $P < 0.01$). While CXCR4 mRNA showed no significant increase in expression at all time point investigated (Figure 3.1 G), this also in keeping with the result obtained from the TaqMan array experiment.


(A)
IL-1α mRNA (fold above control)
25
20
15
10
5
0
4
8
16
24
time (Hrs)
(B)
IL-1α mRNA (fold above control)
20
15
10
5
0
4
8
16
24
time (Hrs)
(C)
IL-8 mRNA (fold above control)
25
20
15
10
5
0
4
8
16
24
time (Hrs)
(D)
IL-12α mRNA (fold above control)
5
4
3
2
1
0
4
8
16
24
time (Hrs)

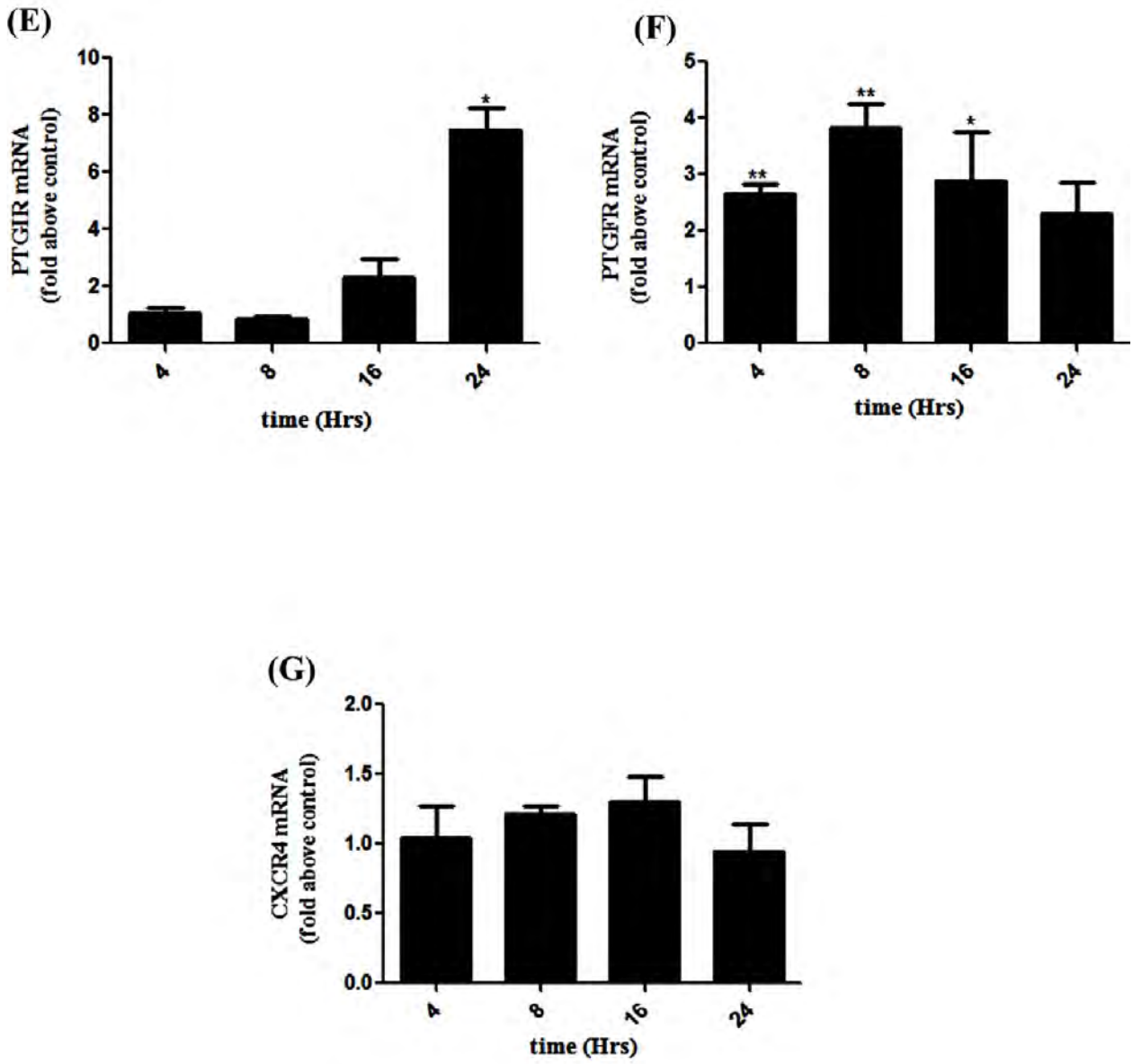


Figure 3.1: Relative mRNA expression of (A) IL-1α (SYBR Green reporter), (B) IL-1α (TaqMan reporter), (C) IL-8, (D) IL-12α, (E) PTGIR, (F) PTGFR, and (G) CXCR4 in HeLa cells treated with PBS (control) or SP 1:50 dilution for 4, 8, 16, or 24 hrs as determined by qRT-PCR. Data are presented as mean ± SEM from five independent experiments. *, **, and *** represents significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

3.5 Discussion

Inflammation involves tissue remodeling events which acts across multiple cellular compartments in the face of infection or injury or insult in order to regulate homeostasis [350]. It has been estimated that chronic inflammation accounts for about more than 25% of all new cancer cases globally [75,351].

Although persistent infection with high risk HPV has been identified as the single most important risk factor for cervical cancer, other factors including multiple sexual partners have been attributed to the etiology of this disease. Several studies have shown that seminal plasma can mount inflammatory response in the cervix [285,352]. Furthermore lipid components of seminal plasma can be reabsorbed into the vaginal fornix or local tissues [353], where they can regulate tissue remodeling processes in an autocrine or paracrine manner [285]. Few studies have reported on the role of seminal plasma in the regulation of inflammatory pathways in cervical cancer cells. Hence I investigated the role of seminal plasma in the regulation of arrays of inflammatory pathways and genes using the TaqMan-Array 96-well plate (human inflammation and human chemokines).

As highlighted in table 3.1, the gene array analysis revealed 95 genes that are regulated by seminal plasma. Analysis of the gene list for Gene Ontology and PANTHER pathway annotations indicated functions in catalytic activity, receptor activity, cellular processes, immune system process and inflammatory pathway.

Interestingly, inflammation mediated by chemokine and cytokine-cytokine receptor signaling pathway was identified as the major pathway significantly regulated by these genes. Further analysis thus reveals that components of this pathway can be grouped into 3 signaling cascades namely; eicosanoid signaling (cyclooxygenase and lipooxygenase), chemokine signaling, and cytokine-cytokine receptor signaling. Other pathways shown to be regulated by SP include toll receptor signaling pathway, kallikrien-kinin-bradykinin receptor signaling pathway, integrin signaling pathway, interferon-gamma signaling pathway, JAK/STAT signaling pathway.

Regulation of eicosanoid signaling

Eicosanoids, including prostaglandins and leukotrienes, are biologically active lipids that have extensively been implicated in inflammation and cancer [354]. It has been documented that altered metabolism of arachidonic acid by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes is a common feature of several epithelial-derived malignancies and it plays a central role in cancer progression [354].

COX exist in two isoforms; COX-1 (PTGS-1) and COX-2 (PTGS-2) and both catalyzes the oxygenation and reduction of arachidonic acid (AA) after its release from glycerophospholipids by phospholipase A_2 (PLA_2) or phospholipase C (PLC) to the intermediate form prostaglandin H_2 (PGH_2). COX-2 is an immediate early response gene that is naturally absent in most cells in health but can be rapidly induced at site of inflammation [133]. COX-1 is considered to be a housekeeping gene responsible for the maintenance of basal prostanoid levels required for tissue homeostasis [355-357]. However, evidence show that both enzymes and their products are up-regulated in numerous cancers including cervical cancer where they play vital roles in cervical

tumorigenesis [28,312,358]. Similarly, COX-1 expression is up-regulated in breast cancer [359], prostate cancer [360], ovarian cancer [361], and murine models of lung tumorigenesis [362]. In addition, COX-1 expression can be induced *in vitro* by tobacco carcinogens [363], VEGF [364], arachidonic acid and PGE_2 [365]. As depicted schematically in figure 3.2, data obtained from this study shows that SP significantly induce the expression of COX enzyme (COX-1) (Figure 3.2).

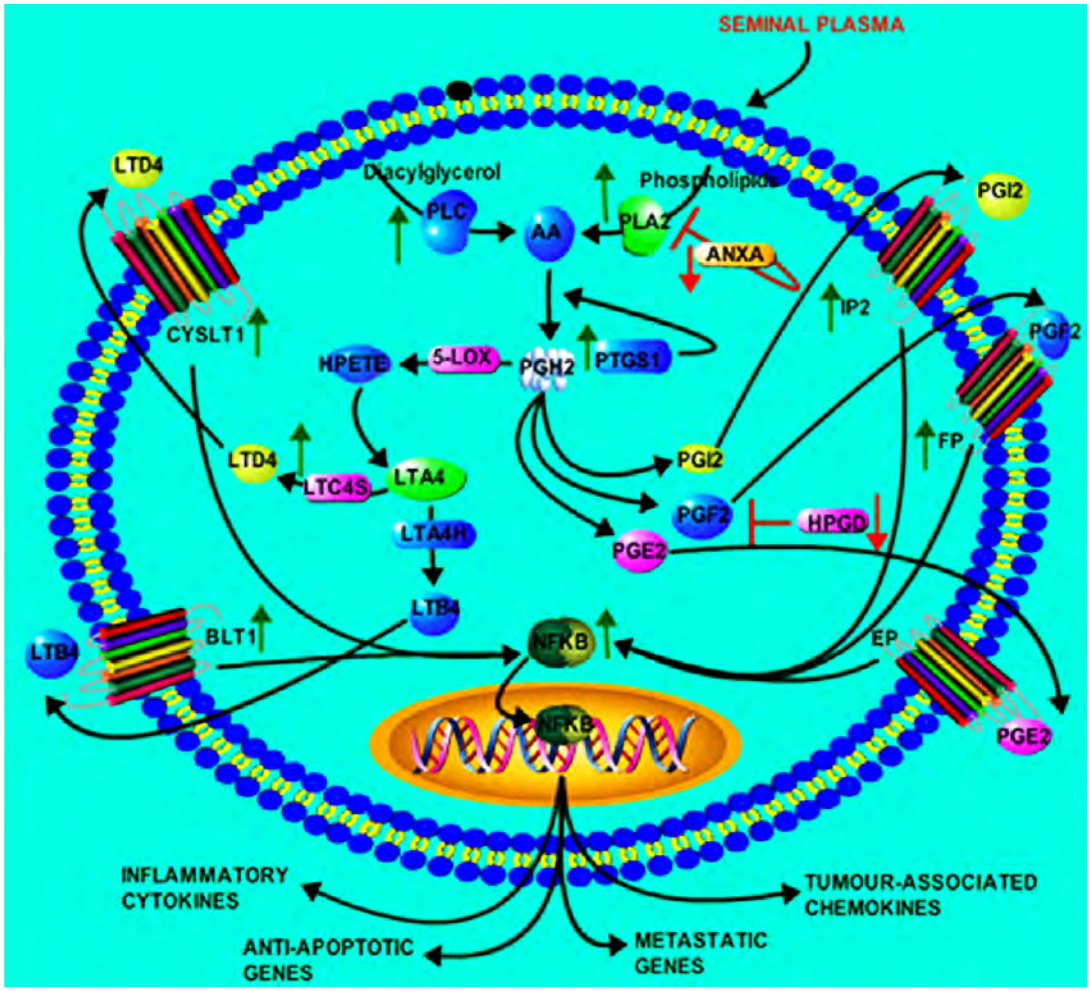


Figure 3.2: Schematic diagram showing the regulation of eicosanoid biosynthesis pathway by SP in neoplastic cervical epithelial cell as means of mediating cervical cancer progression.

↑; Up-regulated genes, ↓; down-regulated genes, ⊤; direct inhibition, ⟶; direct stimulation

This is in agreement with similar study by Sutherland et al. (2012) where it was shown that SP induces the expression of COX-1 in neoplastic cervical epithelial cells (HeLa) [287]. A major metabolite of COX-1 enzyme is PGs. PGs can modulate tumor cell proliferation, differentiation, and apoptosis through multiple signaling pathways in both autocrine and paracrine manner [354]. PGE_2 biosynthesis and signaling are significantly elevated in cervical cancer [287,345,350], and in lung and colon cancer, PGE_2-EP signaling has been shown to promote proliferation by activating the glycogen synthase kinase-3β (GSK3β)-β catenin and Ras-ERK pathways [157,366,367]. In $Apc^{Min/+}$ mice, PGE_2-EP signaling promote colon tumor cell survival by activating the PI3K-Akt-PPARδ cascade [368]. Furthermore, data from this study shows that SP induces FP receptor expression to potentiate PGF2α-FP signaling as shown schematically in figure 3.2. PGF2α-FP signaling has been shown to induce cell proliferation through an FP-ERK-fibroblast growth factor 2(FGF-2)-FGF receptor 1(FGFR1)-ERK cascade in endometrial tumor cell lines [369]. Similarly, Wallace et al. (2009) demonstrated that PGF2α-FP signaling regulates the expression of the chemokine CXCL1 (GRO α) a potent tumor growth factor [370]. Hence in sexually active women with underlying cervical pathology, exposure to seminal plasma can lead to the activation of the COX-PGs signaling pathway consequent of COX-1 induction. Activated COX-PGs pathway can then promote both PGE_2-EP and $PGF_{2\alpha}$-FP signaling which can act in similar manner to enhance cervical inflammation and tumorigenesis.

In addition to regulating inflammatory and pro-tumorigenic pathways, SP can also potentially regulate inflammation and tumor progression by inhibiting negative feedback regulators or inhibitory/anti-inflammatory pathways. The release of AA by phospholipase A_2 is negatively regulated by annexin (encoded by *ANXA 1-5*), a group of cellular proteins known to inhibit $cPLA_2$ activity [371]. The gene array showed that SP inhibited annexin expression (Table 3.1).

By inhibiting annexin expression and up-regulating phospholipase A_2 expression (Figure 3.2), SP ensures steady and continuous release of AA metabolite into the cascade for PGH_2 synthesis, thereby driving inflammatory pathways and potentially enhancing tumor progression, by enhancing biosynthesis of PGE_2, $PGF_{2\alpha}$, PGI_2 [137,138,354].

Furthermore in addition to production of PGs, prolongation of pathways that prevent their destruction or metabolism can also prolong inflammation by enhancing ligand receptor interaction. Prostanoids, such as PGE_2, are metabolized through oxidation of their 15(S)-hydroxyl group by NAD^+-linked 15-hydroxyprostaglandin dehydrogenase (15-PGDH, encoded by HPGD) to inactive 15-keto products [372-374]. 15-PGDH is a cytosolic enzyme that has been reported to act as bladder, breast, gastric, lung and colorectal tumor suppressor [375-380]. In immunodeficient mice, 15-PGDH inhibits the development of murine intestinal neoplasia [380,381]. These findings supported with the fact that 15-PGDH expression is abolished in various cancers [373,382], emphasize the oncogenic potential of the PG biosynthesis pathway. In the array, I found that SP suppressed HPGD expression. This can lead to reduction in PGE_2 degradation and enhanced oncogenic properties of the PGE_2 in cervical tumorigenesis.

Taken together, my data has highlighted that SP can potentially enhance cervical cancers by not only enhancing pro-inflammatory and pro-tumorigenic lipid signaling pathway, but also by concomitantly altering the expression of enzyme systems and pathways that inhibit production and metabolism of these pathways, thereby driving inflammation forward.

The gene array identified not only prostaglandins, but also leukotrienes as being regulated by SP. Leukotrienes are powerful lipid mediators of inflammation in various acute and chronic inflammatory diseases [383,384]. Compared with PGs, much less is known about the role of pro-inflammatory leukotrienes in cancer [354]. However, emerging data suggest that leukotrienes can play vital roles in carcinogenesis [354]. Data from this present study shows that SP induces the expression of leukotriene C_4 synthase (LTC_4S), cysteinyl leukotriene receptor 1 (CysLT1), and leukotriene B_4 receptor 1 (BLT1) (Table 3.1 and figure 3.2).

LTC_4S is a membrane protein that functions as a non-covalent homodimer of two 18-kDa polypeptides. LTC_4S catalyzes the conjugation of leukotriene A_4 (LTA_4) with gluthathione (GSH) to form leukotriene C_4 (LTC_4), the parent compound of the cysteinyl leukotrienes (CysLTs) (LTC_4, LTD_4, LTE_4) [385]. CysLTs exert their biological activity by binding to two distinct CysLTs receptor (CysLT1 or CysLT2), both belonging to the G-protein-coupled receptor (GPCR) superfamily [386,387]. Though, CysLT1 has been shown to have higher affinity to bind to CysLTs than CysLT2 [354].

CysLTs-CysLT1 cascade has been confirmed to be involved in the pathophysiology of inflammatory disease such as bronchial asthma where it activate NF-κB to induce RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) production, causing the migration of eosinophils and activated T lymphocytes into the airway [388]. CysLTs-CysLT1 signaling has been suggested to play an important role in carcinogenesis. For example, in human colorectal and prostate cancer, CysLTs-CysLT1 signaling has been shown to mediate proliferation and inhibit apoptosis, thus conferring poor prognosis [389,390]. It is likely that CysLTs-CysLT1 signaling could regulate cervical cancer via similar mechanisms.

Similarly, in non-transformed human intestinal epithelial cell lines, LTD_4-CysLT1 cascade was shown to promote cell proliferation and survival through multiple parallel pathways such as GSK3β-β catenin, PKC-Raf-ERK1, ERK2, BCL-2, and COX-2 pathways [391-393]. CysLTs (LTD_4) induction of COX-2, as well as PGE_2 production, do suggests that crosstalk exists between the LOX and COX pathways.

Leukotriene B_4 receptor 1 (BLT1); a G-protein coupled receptor (GPCR) is a high-affinity receptor specific for LTB_4 [394]. LTB_4 is a potent chemoattractant and pro-inflammatory mediator in several inflammatory diseases including atherosclerosis, where LTB_4-BLT1 signaling cascade has been shown to phosphorylate MAPKs and stimulate NF-κB dependent inflammation [395]. However, data from several studies have emerged to support the role of LTB_4-BLT1 in tumorigenesis [396,397]. LTB_4-BLT1 cascade has been shown to stimulate cell proliferation and promote cell survival through a LTB_4-BLT1-Erk pathway in colonic tumor cell [398]. Similarly, by activating both Mek-Erk and PI3K-Akt pathways, LTB_4-BLT1 signaling induces cell proliferation in pancreatic cancer cell lines [399]. Taken together, these data suggest that deposition of seminal plasma within the cervical cancer microenvironment can activate the CysLTs-CysLT1 and LTB_4-BLT1 signaling pathways to induce inflammatory reactions which could promote cervical tumorigenesis via similar mechanisms.

Regulation of Kallikrien-kinin-bradykinin

Additional data from this study shows that SP induces the expression of kallikrein-related peptidases (KLKs) which belong to a subgroup of secreted serine proteases [400]. KLKs catalyzes several physiological processes, including tissue remodeling, apoptosis, coagulation and immune responses within the human body [401]. To date, about 15 members KLKs (i.e. KLK 1-15) have been discovered, most of which have been labeled as potential biomarkers for cancer diagnosis and/or prognosis [401]. Despite the significant progress in understanding the biomarker efficacy of the KLKs, their pathophysiological role in inflammation and cancer pathogenesis remains poorly understood. Emerging evidences designate a possible role for KLKs in inflammation and various cancer processes [401].

KLKs have been shown to participate in early neoplastic progression by regulating cancer cell proliferation [400]. KLKs mediate tumor growth mainly via insulin-like growth factors (IGFs) [400]. KLK 2 and KLK 3 can cleave a number of IGF binding proteins (IGFBPs) to indirectly induce tumor growth [402]. Similarly, KLK 14 is also suggested as a potential upstream regulator of IGFBPs thus also participating in tumor growth induction [403-405]. As highlighted in figure 3.3, KLKs can also facilitate tumor metastasis via the remodeling of extracellular matrix (ECM) (Figure 3.3).

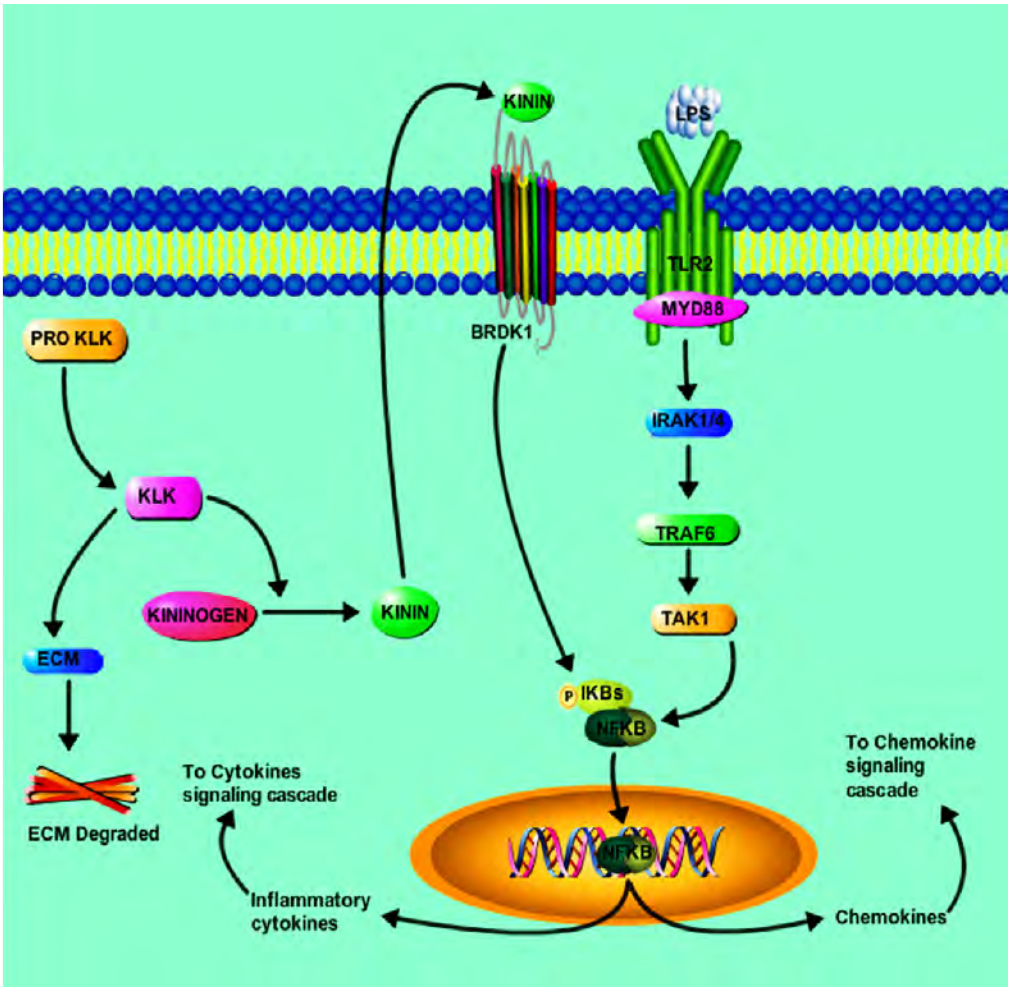


Figure 3.3: Schematic diagram showing the regulation of Kallikrien-Kinin-Bradykinin and TLR2 signaling by SP in neoplastic cervical epithelial cells. ECM (extracellular matrix), LPS (lipopolysaccharide).

In addition, there is compelling evidence that KLKs may stimulate tumor angiogenesis by facilitating blood vessel remodeling and modulating the balance between angiogenic activators and inhibitors [400]. KLKs indirectly facilitate endothelial cell invasion and migration by processing components of the extracellular matrix (ECM) via the matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA), and kinin signaling pathways [401]. Induction

of KLKs by SP does strongly suggest a role for KLKs in SP-mediated cervical tumorigenesis in sexually active women.

More so, activation of KLKs by tissue damage or inflammatory stimuli such as PGs in SP can result in the generation of biologically active kinins by limited proteolysis of kininogens [406,407] (Figure 3.3). Kinins (Bradykinins) are a group of pluripotent peptides implicated in various pathophysiological events [408]. Kinins exert their biological effects on target cells by activating their cognate receptors, BDKR1 and BDKR2 [409], which belongs to the superfamily of GPCRs [410]. BDKR2 is constitutively expressed in all tissues and is thought to mediate most physiological action of kinins [408]. BDKR1 is generally not expressed under physiological conditions, but can be rapidly up-regulated after cell injury and stress [411] or under several inflammatory stimuli [412,413] as orchestrated by SP in this study (Figure 3.3).

The role of kinins-BDKR signaling in inflammation was first demonstrated by Lewis (1964) where kinins-BDKR cascade was shown to evoke cardinal inflammatory signs, such as oedema (*tumor*), increased in vascular permeability and pain (*dolor*) [414]. Thereafter, several studies have emerged supporting the role of both Kinins-BDKR1 and Kinins-BDKR2 cascades in the onset and maintenance of inflammatory and nociceptive functions [408,412,413,415]. Although, little is understood about the mechanism by which kinins mediates its inflammatory function, Pan et al. (1996) showed that kinins-BDKR signaling induces the expression of pro-inflammatory cytokines via the activation of NF-κB in human WI-38 fibroblast cells [416]. Therefore, it is likely that kinins do exert similar mechanism to mediate inflammatory reactions in cervical carcinoma cells (Figure 3.3).

Furthermore, increasing body of evidence has emerged indicating that kinins-BDKR signaling appears to be involved in tumorigenesis [417,418]. Study by Taub et al. (2003) showed that stimulation of BDKR1 in prostatic cancer cells promotes tumor cell growth, migration, and invasion [419]. Though the exact mechanism by which activated BDKR1 mediated these actions was not investigated in that study, the mitogenic properties of kinins and their ability to activate tyrosine and MAP-kinase cascades can explain in part their role in tumor growth and metastasis [408,418,420]. Secondly, BDKR1 can promote tumor cell migration via the activation of focal adhesion kinase and MMPs [421,422]. These data suggests that continuous exposure of neoplastic cervical epithelial cells to SP can activate KLKs-kinin-BDKR1 signaling pathways to mediate chronic inflammatory reactions and augment tumor progression within the cervix.

Regulation of TLR2 signaling

Toll-like 2 receptor (TLRs) belongs to the family of type I transmembrane receptors which are structurally characterized by extracellular leucine rich repeats (LRRs) and an intracellular Toll/IL-1 receptor (TIR) signaling domain [423]. TLRs are activated by a wide range of pathogen-associated molecular patterns (PAMPs) including, lipoproteins, flagellins, and bacterial lipopolysaccharides (LPS) [424]. In addition to PAMPs, TLRs can also be activated by a variety of endogenous molecules released from damaged cells or tissue to initiate a sterile inflammatory process without detectable infection [425]. TLRs initiate series of downstream signaling events that induces the expression of inflammatory mediators such as cytokines, chemokines and adhesion molecules [426].

Upon activation with an appropriate agonist, TLRs recruit proximal cytoplasmic adaptor protein MyD88 (myeloid differentiating antigen 88), in the MyD88 dependent signaling pathway [423]. Recruitment of MyD88 leads to the activation of IRAK1 (IL-1R-associated kinase) by IRAK4, which facilitates the recruitment of TRAF6 (TNF receptor-associated kinase). TRAF6 then recruit MAP3Ks including TAK1, MEKK2/3, and ASK1. Activated TAK1 then activates IKK complex and subsequent IκB degradation and NF-κB translocation to the nucleus. Within the nucleus NF-κB mediate the transcription of pro-inflammatory cytokines and growth factor genes such as IL-1, TNF, IL-6, and EGF [427]; several antiapoptotic genes including *bcl-2, bcl-$_{XL}$, cIAP, xIAP, TRAF1, TRAF2, SOD and A20* [428]; angiogenic and metastatic genes such as COX-2 [429], iNOS [430], uPA [431], MMP-2 [432], IL-8 [287], VEGF [433]; and adhesion molecules (ICAM-1, VCAM-1, and ELAM-1) [434]. In addition to mediating inflammatory response, TLRs have been shown to also regulate cell proliferation and survival [435-437]. No doubt Xie et al. (2009) showed that the activation of TLR2 in breast cancer cells significantly promote cellular invasion via the activation of NF-κB and induced phosphorylation of TAK1 and IκBα [438]. It was also shown that activation of the TLR2/NF-κB signaling induces the expression IL-6, TGF-β, VEGF, and MMP9 in these cells [438].

As seen in this present study, SP significantly induces the expression of TLR2 in HeLa cells (Table 3.1). It is therefore very feasible that induced TLR2 can be activated by the lipoproteins that might be present within SP (Figure 3.3). Activated TLR2 can then recruit MyD88 (also regulated by SP in this study) to activate the TLR2-MyD88-NF-κB signaling pathway (Figure 3.3). Translocated NF-κB can then mediate the release of pro-inflammatory and tumorigenic genes from the neoplastic cells themselves to augment cervical tumorigenesis. In sexually active women, STIs such as gonorrhea are known cause of chronic cervicitis, a persistent inflammation

of the cervix which has been linked to cervical cancer [75,439]. It is plausible that the bacterial LPS present within infective seminal plasma can bind to the induced TLR2 to activate the TLR2-MyD88-NF-κB signaling pathway, releasing pro-inflammatory modulators to mediate cervical inflammation.

Regulation of chemokine signaling

Chemokines (chemotactic cytokines) represent a large family of protein (about 40-50) in man, with structural similarities based on a conserved cystein residues and capacities to bind to particular GPCR [107,440,441]. They are small proteins with molecular weight of about 8-10 kDa [442]. Of the four different families (CC, CXC, XC, and CX_3C) of chemokines that exist, the CC and CXC groups are by far the most common [443]. Chemokines were initially described as soluble factors able to selectively regulate directional migration of leukocytes during infection and inflammation [442,443]. However, their biological effects have been discovered to be far more complex and virtually all cells, including many tumor cell types, can express chemokines and their receptors [441]. In health, chemokines have been shown to be pleiotropic in their effects hence suggesting that in cancer microenvironment chemokines will also exert a wide range of molecular effects [442]. In addition, several chemokines can share same receptor and one chemokine can bind to several receptors, creating multiple combinations ultimately leading to multiple biological outcomes [443].

Most tumors express a broad network of chemokines and chemokine receptors [443,444] which have been extensively studied in cancers of the ovary, breast, cervix and hematological malignancies [442]. Within the tumor microenvironment, tumor-associated chemokines are believed to regulate leukocyte infiltration, angiogenesis, tumor cell metastasis, tumor immune response, and also act as autocrine or paracrine growth and survival factors [445]. Recently chemokines and their receptors have been identified as key mediators of chronic inflammation, which play an important role in the pathogenesis and progression of various human cancers including cancer of the cervix [446-449]. Induction of chemokines in disease state have been shown to be closely regulated by inflammatory cytokines, growth factors and pathogenic stimuli [450]. As seen in this study, SP regulated the expression of chemokines C5, CCL2, CCL5, CXCL1, CXCL2, CXCL3, CXCL8 (IL-8), CXCL11, and CXCR4 (Table 3.1) all of which play vital role in cervical cancer inflammation and tumorigenesis. The induction of tumor-associated chemokines mediated by SP in this present study can be consequent to the effect of the various inflammatory cytokines secreted from some or all of the earlier discussed inflammatory pathways (i.e. COX-1-PGE_2-EP, $PGF_{2\alpha}$-FP, CysLTs-CysLT1, LTB_4-BLT1, KLKs-kinin-BDKR1, and TLR2-MyD88-NF-κB) activated by SP (Figure 3.4).

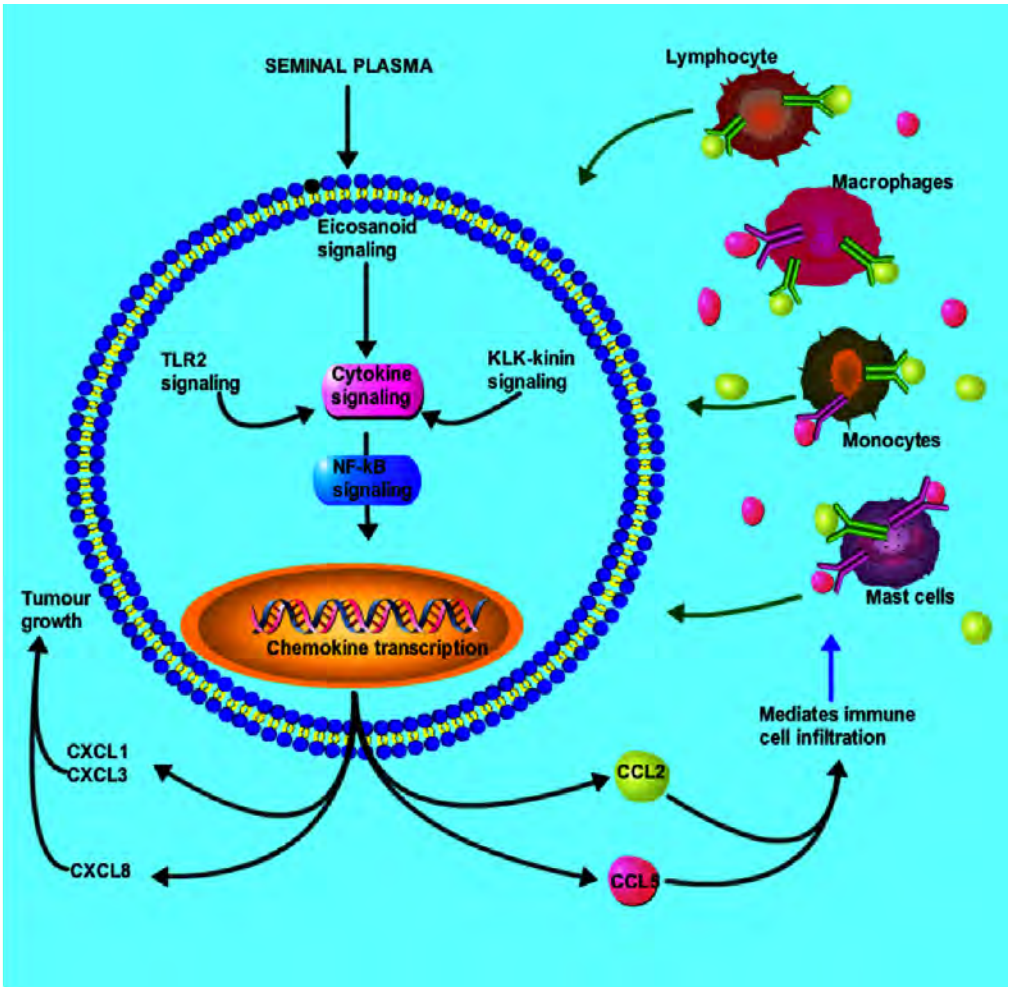


Figure 3.4: Schematic diagram showing the regulation of chemokine signaling by SP in neoplastic cervical epithelial cells.

Alternatively, antigenically distinct signaling molecules including several cytokines and growth factors [280,343,451-453] found in SP can directly stimulate the tumor cells to secrete chemokines.

Chemokines expressed within specific tumor microenvironment can influence the extent and phenotypes of infiltrating leukocytes [442]. For example, the CC chemokines such as CCL2 (MCP-1) are major determinants of macrophage and lymphocyte infiltration [454] expressed in variety of cancers where it modulates the recruitment of macrophages [442]. In a study carried out by Negus et al. (1997) expression of CCL2 by solid ovarian tumor cells correlated with the extent of lymphocyte and macrophage infiltration [87]. In addition CCL2 has been implicated in tumor metastasis [455]. Similarly, RANTES (CCL5) a C-C chemotactic chemokine is also known to promote recruitment and activation of inflammatory cells such as lymphocytes[456], monocytes [457], and mast cells [458]. RANTES has been shown to be a ligand for chemokine receptors CCR1, CCR3, and CCR5 [459], and elevated expression of RANTES has been observed in numerous inflammatory disease [460-462].

Induction of CCL2 (MCP-1) and CCL5 (RANTES) expression by SP in this study suggests a role for both MCP-1 and RANTES in the characteristic post-coital inflammatory response described by Sharkey et al (2012). Furthermore, increased expression of RANTES has also been observed in several human cancers such as melanoma, lung, prostate, pancreatic, and breast cancer [463-465]. However, the most striking finding thus far is in breast cancer where expression of RANTES correlates with disease progression [466,467]. CCL5 has been shown to modulate cell migration and invasion in several cancer cells [468]. No known study have demonstrated the role of SP in the induction of CC-chemokines (CCL2 and CCL5) expression or their role in cervical cancer progression. It is likely that SP-induced CC-chemokines (CCL2 and CCL5) can act in similar manner to enhance cervical cancer cell migration and invasion.

To maintain growth, tumor cells are dependent on angiogenesis [469]. CXC chemokines within tumor microenvironment can positively or negatively regulate angiogenesis depending on the presence or absence of the ELR motif in their amino acid terminus [470]. CXCL1, CXCL2, CXCL3 and CXCL8 (IL-8) are examples of angiogenic (ELR+) chemokines commonly associated with human cancers. CXCL1, CXCL2, and CXCL3 belong to the sub-family Growth-regulated oncogene (GRO) which is a member of the CXC chemokine family that are crucial in recruitment of neutrophils to inflammatory sites [471]. All three ligands together with IL-8 exert their biological action by binding to the CXC chemokine receptor CXCR2, with CXCL1 having the highest affinity [472].

Induction of CXCL1 and IL-8 by SP as seen in this present study is in accordance with similar study by Sales et al. (2012), where it was shown that SP induces the expression of CXCL1 and IL-8 in HeLa cells and regulates vascular function in vitro [350]. Furthermore, study by Wang et al. (2006) showed that PGE_2 induces the expression of CXCL1 in human colorectal cancer cells and that expressed CXCL1 then induces microvascular endothelial cell migration and tube formation in vitro [471]. It is therefore very likely that the abundant PGE_2 found in SP [310] and its ability to regulate the eicosanoid biosynthesis pathway might be responsible for CXCL1 induction in neoplastic cervical cancer cells (Figure 3.4). These data therefore suggests that exposure of neoplastic cervical epithelium to SP can lead to increased tumor angiogenesis and invasiveness/metastasis consequent of CXCL1 and IL-8 expression (Figure 3.4).

Regulation of cytokines signaling

Result obtained from this study showed that SP regulates the expression of inflammatory cytokines IL-1α, TNF, IL-6, IL-12, IL-18 and components of their respective signaling cascade (Table 3.1).

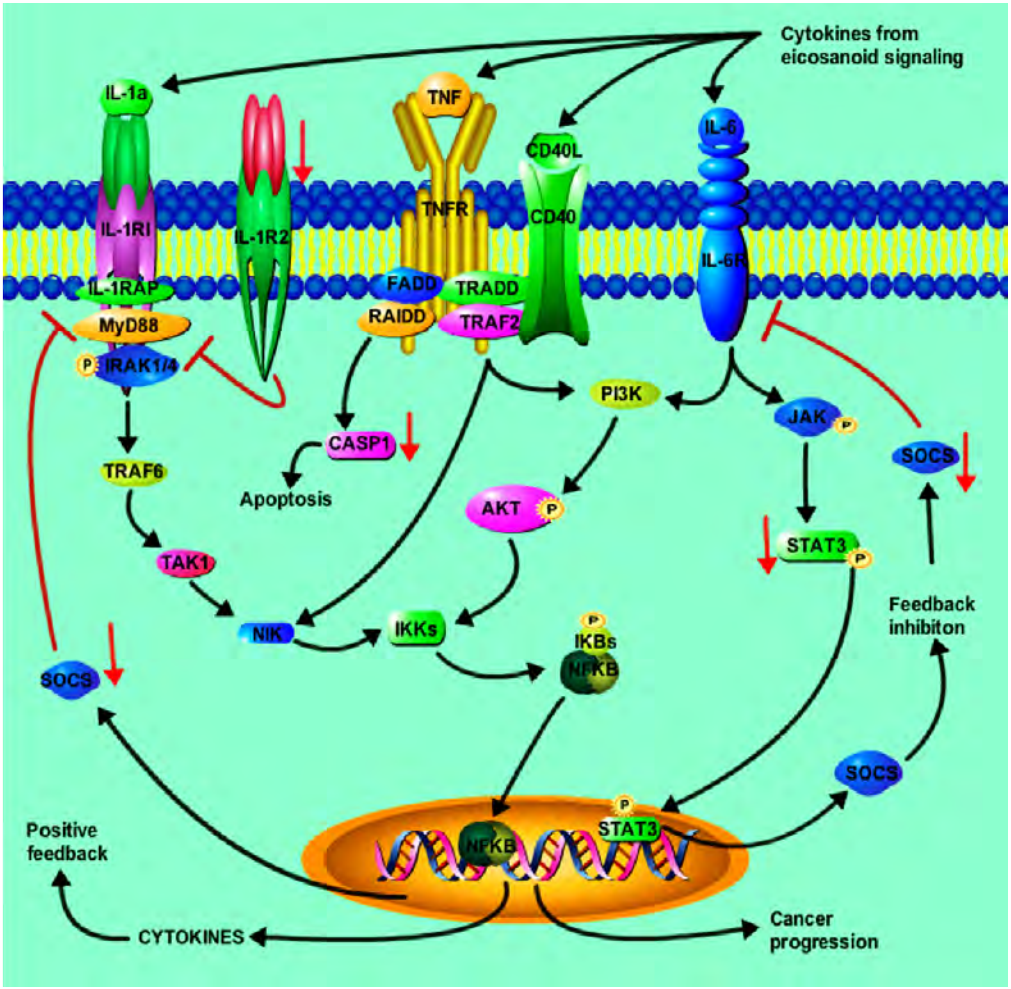


Figure 3.5: Schematic diagram showing the regulation of cytokine signaling by SP in neoplastic cervical epithelial cells.

↑; Up-regulated genes, ↓; down-regulated genes, ⊤; direct inhibition, ⟶, direct stimulation.

IL-1α is a pleotropic pro-inflammatory cytokine belonging to the IL-1 family located in the long arm of chromosome 2 [473]. Studies have emerged demonstrating the role of IL-1α signaling in the pathogenesis of various human cancers including head and neck [474], breast [475], pancreatic [476], and cervical cancer [477] where it induce the activation of immediate-early transcription factors and genes that promote cell survival and proliferation and other cytokines that mediate inflammatory and angiogenic response [478,479]. In this study, SP significantly induces the expression of IL-1α and components of IL-1signaling including IL-1RI and intracellular adaptor molecules MyD88 while down regulating IL-1RII (IL-1 receptor type II), a decoy receptor that inhibits IL-1 signaling [473,479,480]. Hence, it is feasible that SP-mediated activation of IL-1α-IL-1RI signaling can exacerbate inflammatory response within the cervical cancer milieu and promote cervical cancer cell proliferation, growth, and survival via the activation of major transcription factor such as NF-κB (Figure 3.5).

TNF is a potent pro-inflammatory cytokine that has been documented to be secreted by wide variety of tumor cells including cutaneous T cell lymphoma [481], adult T cell leukemia [482], breast cancer [480,483], ovarian carcinoma [484,485], and cervical epithelial cancer [486]. In most of these neoplastic cells, TNF can act as an autocrine growth factor or in some cases promote tumor growth/survival and invasion/metastasis via the activation of NF-κB–dependent genes [487]. It is therefore likely that SP activated TNF-TNFR signaling can act via similar mechanism to mediate cervical cancer progression.

CD40 ligand (CD40L) is a type II transmembrane protein with homology similar to TNF [488]. CD40 has been documented to be highly expressed on many in vitro transformed and carcinoma-derived cell lines including cervical cancer cell lines [488-490]. In these cells, CD40L-CD40 interaction initiates inflammatory responses leading to the synthesis of tumor associated chemokines [488,491,492] and cytokines [493] via the activation and translocation of NF-κB [494]. Hence persistence exposure of neoplastic cervical epithelium to SP can lead to poorer prognosis consequent of chronic inflammatory reactions initiated by SP induced CD40L.

Induction of IL-6 by SP as obtained from this study in keeps with the result of previous study by Sutherland et al. (2012), where it was shown that SP acting via the COX signaling induces the expression of IL-6 in neoplastic cervical epithelial cell (HeLa) [287].

Induction of IL-12α and IL-18 by SP in this study suggests a role for SP in the regulation of cervical immune response. IL-12α and IL-18 are pro-inflammatory cytokines [495,496] capable of inducing the production of interferon-γ (IFN-γ) [497] and cell mediated immunity which are important factors in determining the progression of HPV related cervical lesion including cervical cancer [498-500]. In addition, regulation of SLIT2, a ligand known for its anti-inflammatory and tumor suppressor activity [501] suggest that SP can play a pivotal role in cervical cancer pathogenesis i.e. ability to promote or impede tumorigenesis. Furthermore, induction of cell adhesion molecules ICAM1 and ITGAM1suggest that SP can also play a role in cervical cancer cell metastasis [502].

Finally, SP-regulated signaling cascades and expressed pro-inflammatory cytokines can all act synergistically to modulate disease pattern and outcome by promoting chronic inflammatory process and cervical tumorigenesis.

In conclusion, this study has highlighted that human seminal plasma (SP) can potentially enhance cervical cancer progression by regulating the induction of arrays of inflammatory pathways in neoplastic cervical epithelial cells. Regulation of these inflammatory pathways can play a crucial role in cancer cell angiogenesis, proliferation, invasion, metastasis, and survival. Constant exposure of dysplastic cervical epithelium to seminal plasma as occurs in sexually active women during the act of coitus can mediate early progression to invasive cancer consequent of its molecular effect on the already dysplastic epithelial cells. In view of the above it is of great importance to determine the molecular mechanism by which SP mediate the expression of some of these inflammatory genes/pathways, which form the basis of my next study.

CHAPTER 4

SEMINAL PLASMA INDUCES THE EXPRESSION OF IL-1α IN NORMAL AND NEOPLASTIC CERVICAL CELLS VIA EP_2/EGFR/PI3K/AKT PATHWAYS

4.1 Introduction

The inflammatory milieu of most cancer microenvironment has been shown to consist of tumor cells, surrounding stromal, immune and inflammatory cells which all interact intimately to produce cytokines/chemokines, growth factors, and adhesion molecules in a bid to promote tumorigenesis and metastasis [273]. Of special relevance within this milieu are pro-inflammatory cytokines which are important mediators of chronic inflammatory responses, and have cardinal effects on malignant processes. In the previous chapter, I have shown that SP regulates the expression of arrays of pro-inflammatory cytokines in neoplastic cervical epithelial cells. One of the most robustly regulated cytokines in the array was IL-1α.

Interleukin 1α (IL-1α) is a pleotropic pro-inflammatory cytokine that belongs to the IL-1 family (IL-1α, IL-1β, and IL-1Ra) gene located on the long arm of chromosome 2 [473]. IL-1α possesses a wide range of inflammatory, immunologic and tumorigenic properties [101,503,504]. IL-1α is secreted by a variety of cells including monocytes, tissue macrophages, neutrophils, fibroblasts, smooth muscle cells, dendritic cells, and cervical epithelium [101,480,505]. Accumulative evidence suggests that IL-1α plays a crucial role in tumorigenesis. Within the tumor microenvironment, IL-1α has been shown to induce the expression of metastatic genes such as the matrix metalloproteinases (MMPs) and stimulate the production of angiogenic proteins and growth factors such as IL-8, IL-6, vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGFβ) [503,506]. In

addition, IL-1α is capable of inducing enzymes (COX and iNOS) with carcinogenic properties within inflamed and malignant tissues [507]. Given the importance of IL-1α in regulating inflammation and cancer progression, it was of utmost interest to elucidate the molecular mechanism underlying the role of SP in regulating IL-1α and its potential role in cervical cancer.

4.2 Aim of the study

In the previous chapter, I have demonstrated that SP can induce pro-inflammatory pathways, chemokines and cytokines including IL-1α in neoplastic cervical epithelial cells (HeLa). Hence, this study was aimed at investigating the role of SP in the regulation of IL-1α expression in normal and neoplastic cervical tissue explants and epithelial cells, HeLa and the molecular mechanism underlying this regulation.

4.3 Materials and method

4.3.1 Cervical tissue collection and processing

Cervical cancer tissue (Ca.1-Ca.18) specimens obtained from previously diagnosed (pre or invasive carcinoma of the cervix) patients attending the Gynecologic Oncology Clinic at Groote Schuur Hospital (Cape Town) were processed and treated with SP as previously described in section 2.4.2. Patient age ranged between 29-62 years, with a median age of 41 years. The extent of invasiveness of carcinoma biopsies are presented in Appendix III. Similarly, histologically normal cervical tissues (N1-N12) were collected from women undergoing Wertheim's hysterectomy for benign gynecological malignancies at Groote Schuur Hospital (Cape Town). Tissue explant were also processed and treated with SP as previously described in section 2.4.2. Patient age ranged between 37-73 years with a median age of 50.5 years.

4.3.2 Cell culture, treatment with SP, RNA extraction and cDNA synthesis

HeLa-S3 cells were routinely maintained as previously described in section 2.6. For experiments, HeLa-S3 cells were seeded in medium supplemented with 10% FBS at density of $2x10^5$ cells in 3 cm diameter tissue culture dishes and allowed to attach and grow overnight after which cells were serum starved by incubating in serum free medium for 24 hours. Cells were then treated with vehicle (PBS) or SP at a dilution of 1:50 or butaprost [5μM] or PGE_2 [300nM] or EGF [10ng/ML] for 4, 8, 16, and 24 hours. For receptor blockade and inhibitor experiments, serum starved cells were treated with receptor antagonist/inhibitors alone or SP (1:50 dilution) alone or together for 4 or 16 hours. The antagonist and inhibitors used and their final concentrations were: EP_2 receptor antagonist [AH-6809; 20μM], inhibitors of EGFR [AG-1478; 100nM], PI3 kinase

[LY-294002; 25μM], COX-1 [SC-560; 15μM], and COX-2 [NS-398 8μM]. The concentrations of chemical inhibitors used in this study were determined empirically by titration as described [325-327,508]. At the concentration and time used, the antagonist or inhibitors showed no adverse effects on cell viability when stained with 0.4% trypan blue dye. RNA was extracted individually from each set of experiment by using Trizol (Sigma) and concentrations and purity determined as described in section 2.8. cDNA was synthesized from total mRNA (200ng) by reverse transcription as described in section 2.9. Fold increases was calculated by dividing the values obtained from the SP/SP-inhibitor treatments by the vehicle/vehicle-inhibitor treatments.

4.3.3 Quantitative real time PCR reaction.

Quantitative real time-PCR was performed to determine the expression of IL-1α and IL-1RI in cervical carcinoma biopsies and normal cervical tissue and also to assess the effects of ligands or chemical inhibitors on SP mediated induction of IL-1α in HeLa S3 and normal cervical tissue explants. RNA was extracted from neoplastic cervical tissue (adenocarcinoma Ca.11 and squamous cell carcinoma Ca.1 - Ca.10 and Ca.12 - Ca. 18), normal cervix (N1-N12), and HeLa S3 cells using Trizol-reagent as per the manufacturers' instruction and reversed transcribed as described in section 2.9 [312]. qRT-PCR reaction was carried out on an Illumina ECO^{TM} quantitative RT-PCR machine and detected using SYBR green (Bioline, Celtic Molecular, Cape Town, South Africa) incorporation during PCR reaction as described in section 2.10.3. All relative expressions were calculated using the comparative C_t (section 2.10.5) relative to an endogenous control of HeLa cell cDNA included in each experiment. Data are presented as fold

increase as determined by dividing the relative expression of SP treated group by that of the control. Data are presented as mean ± SEM.

4.3.4 Enzyme linked immunosorbent assay

Quantikine® Human IL-1α ELISA kit was used to assess IL-1α protein expression. The experiment was done on HeLa S3 cells seeded at a density of 5×10^5 in 3 cm tissue culture dishes and serum starved overnight. The cells were then treated with SP (1:50) or PBS (control) for 16 and 24 hrs. Cells were lysed as described previously [312] and total protein quantified using Pierce® BCA Protein Assay Kit. Expressed cellular IL-1α protein was determined from the total protein in the lysate. Data are presented as fold change over control treated, which was calculated by dividing the amount of IL-1α measured in SP treated cells at the different time points by the amount measured in their respective controls. Data are presented as mean ± SEM of six independent experiments

4.3.5 SDS-PAGE and Western blot analysis

Immunoblot analysis was performed as described in section 2.13 and 2.14. Following immunblotting, membranes were incubated overnight with rabbit anti-PKB (AKT) and anti-P-PKB (AKT) (1:1000 dilution) at 4 ^{0}C with gentle shaking. Thereafter, membranes were washed three times for 10 minutes each with 15mL of PBS, 0.1% Tween-20, incubated for 1 hour with anti-Rabbit HRP-conjugated secondary antibody (1:5000 dilution) at RT with gentle shaking and washed again three times with 15mL PBS, 0.1% Tween-20. Protein detection was done as

described in section 2.14. Densitometry on visualized protein bands was done using ImageJ version IJ 1.46r (www.imagej.nih.gov/ij/). Akt phosphorylation was calculated by dividing the value obtained from phosphorylated Akt channel by the value obtained from total Akt channel and expressed as fold above vehicle controls. Data are presented as mean ± SEM of three independent experiments.

4.3.6 Immunohistochemistry

Immunohistochemistry was done on archival cervical blocks (Normal n=5, squamous cell carcinoma n=4 and adenocarcinoma n=4) obtained from the Department of Anatomical Pathology, University of Cape Town. Sections were deparaffinized, rehydrated and antigen retrieval done as described in section 2.16. Blocking for endogenous peroxidase was done as described earlier (section 2.16). Sections were blocked using 5% normal donkey/goat serum diluted in TBS after which tissue sections were incubated with polyclonal goat anti-IL-1α (1:200) antibodies at 4 ^{0}C for 18 hours. After incubation, tissue sections were then washed in TBS twice for 5 minutes each followed by incubation with biotinylated donkey anti-goat secondary IgG antibody at dilution of 1:500 at RT for 30 minutes. Tissue sections were then further incubated with streptavidin-biotin peroxidase complex (1:50) at RT for 30 minutes. Controls were incubated with biotinylated IgG secondary antibody only. Color reaction was developed by incubating with 3.3'-diaminobenzidine. Tissue sections were counterstained in aqueous hematoxylin, before mounting and coverslipping. Images were visualized and photographed using a Carl Zeiss laser scanning microscope LSM 510 (Jena, Germany).

4.3.7 Statistical Analysis

All data in this study were analyzed by t-test or one-way ANOVA using Graph Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Paired T-tests were conducted on the untransformed means of the replicates between SP and control. Unpaired T-tests were performed on SP versus SP and inhibitor after conversion to fold increases. One-way ANOVA was used as an additional tool to determine the significant difference between various time points for IL-1α by real-time PCR in response to SP.

4.4 Results

4.4.1 SP induces expression of IL-1α in HeLa neoplastic cervical epithelial cells

To investigate the potential role of IL-1α in cervical inflammation and tumorigenesis, I used a well-established HPV 18 positive HeLa (adenocarcinoma) cell line as a model system to investigate the regulation of IL-1α expression by SP in neoplastic cervical epithelium. HeLa S3 cells were treated with vehicle or SP (1:50 dilution) for 4, 8, 16, and 24 hours and the expression of IL-1α mRNA assessed using qRT-PCR (Figure 4.1A). SP significantly induced the expression of IL-1α mRNA in HeLa S3 cells at all-time point investigated in keeping with result from the array study. Peak induction of IL-1α was observed after 4 hours of SP exposure and was 17.02 ± 4.43 fold increase, while other inductions were 15.40 ± 4.26, 10.07 ± 5.03, and 8.22 ± 2.68 fold increase for 8, 16 and 24 hours, respectively.

In addition, I investigated IL1-α protein expression in response to SP treatment. Protein lysates extracted from HeLa S3 cells treated with vehicle or SP (1:50) for 16 and 24 hours, respectively were subjected to ELISA. I found that SP treatment of HeLa S3 cells significantly induced the expression of IL-1α protein compared to the control (Figure 4.1 B; 10.91 ± 2.61 and 8.45 ± 2.36 fold increase for 16 and 24 hours, respectively). The ELISA did not detect IL-1α expression in lysate of cells treated with SP at earlier time points (4 and 8 hours).

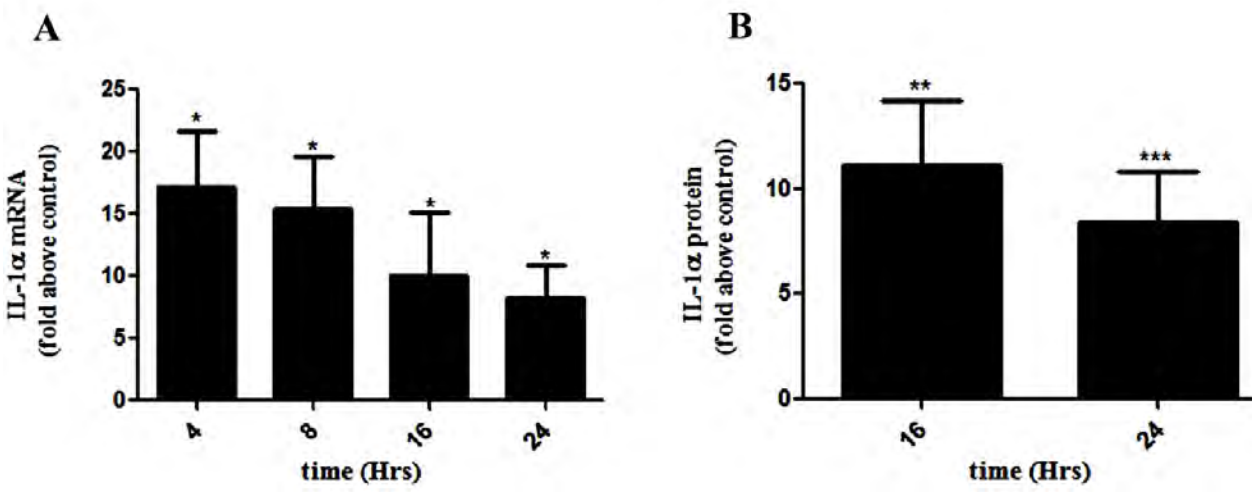


Figure 4.1: Relative expression of IL-1α mRNA (A) and IL-1α protein (B) in HeLa cells treated with SP (1:50 dilution) relative to PBS (control) for 4, 8, 16, or 24 hrs as determined by qRT-PCR and ELISA. Data are presented as mean ± SEM from 5 (A) and 6 (B) independent experiments (n = 5 and 6). Paired T-tests were conducted on the untransformed means of the replicates between SP and control. *, **, and *** indicates significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

4.4.2 SP regulation of IL-1α expression in HeLa cells is dependent on individual semen donor

Induction of IL-1α (mRNA and protein) in HeLa cells seen in figure 4.1 above was mediated by pooled seminal plasma obtained from 10 healthy individual donors. To investigate whether the levels of IL-1α expression was donor dependent, individual ejaculates were assayed for their ability to induce IL-1α mRNA expression in HeLa cells. Result obtained showed that SP from all ejaculates (sperm cells removed by centrifugation, SP1-SP5; full ejaculate with sperm cell, SP6spr and SP7spr; and ejaculates from vasectomized men with no sperm cells present, SP8pv, SP9pv, and SP10pv) significantly induced IL-1α expression in HeLa cells after 4 and 24 hours incubation, albeit with different efficiency (Figure 4.2). Furthermore, I discovered that the presence or absence of sperm cells had no effect on IL-1α mRNA expression (Figure 4.2).

Similarly, the use of centrifugation step to remove spermatozoa had no effect on IL-1α regulation. This data suggests that individual variation in SP potency may also affect cervical inflammation. All subsequent experiments were conducted using pooled SP.

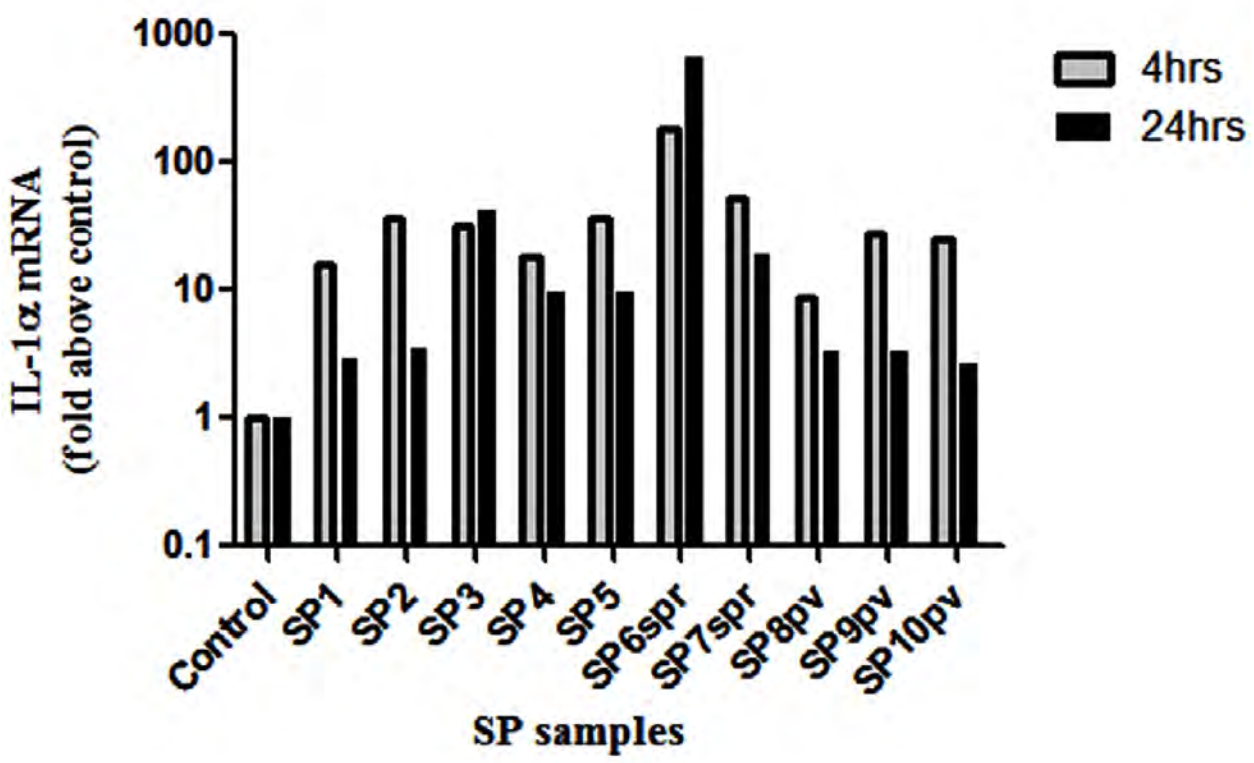


Figure 4.2: Relative expression of IL-1α mRNA in HeLa cells treated with SP1-SP5, SP6spr, SP7spr, and SP8pv-SP10pv (1:50 dilution) relative to PBS (control) for 4 or 24 hrs as determined by qRT-PCR. Data are presented as fold above control.

4.4.3 SP mediated IL-1α induction in HeLa neoplastic cervical epithelial cells via EP2 receptor, EGFR and PI3 kinase pathways.

Next I investigated the signal transduction pathways mediating SP induction of IL-1α mRNA expression, using PGE receptor 2 (EP_2) antagonist and a panel of small molecule chemical inhibitors of signaling proteins. HeLa S3 cells were treated with vehicle or SP (1:50) alone or with EP_2 receptor antagonist (AH-6809) or chemical inhibitors of EGFR (AG-1478), PI3 kinase

(LY-294002), COX-1 (SC-560), and COX-2 (NS-398) for either 4 hours or 16 hours. The expression of IL-1α in HeLa S3 cells following SP treatment in the presence of receptor antagonist or chemical inhibitors was determined by qRT-PCR after 4 hours (Figure 4.3A and B) and ELISA after 16 hours (Figure 4.3C). Figure 4.3A shows that EP_2 receptor antagonist significantly reduced SP-mediated induction of IL-1α mRNA in HeLa S3 cells ($P < 0.05$). SP-mediated induction of IL-1α was also found to be markedly reduced in the presence of chemical inhibitors of EGFR (AG-1478) and PI3 kinase (LY-294002), respectively (Figure 4.3B; $P < 0.001$). Co-incubation of SP with selective COX-1 (SC-560) and COX-2 (NS-398) inhibitors had no inhibitory effect on SP-induced IL-1α mRNA expression (Figure 4.3B; $P = 0.26$ and 0.16). This indicates that SP-mediated induction of IL-1α mRNA expression is via EP_2 and EGF receptors and not via the endogenous COX-PG pathway. In addition, co-treatment with EGTA [calcium chelator; 1.5mM] and PD-98059 [ERK inhibitor; 50μM] did not inhibit SP-mediated induction of IL-1α (Figure 4.3C).

Similarly, data obtained from measuring IL-1α protein with ELISA (Figure 4.3D) revealed that AG-1478 and LY-294002 reduced the expression of SP-induced IL-1α protein in HeLa S3 cells from 20.42 ± 5.16 fold increase to 8.80 ± 1.89 and 2.00 ± 0.32 fold increases, respectively ($P < 0.05$ and $P < 0.001$, respectively). However no significant decrease in IL-1α protein secretion was seen in the presence of NS-398 and SC-560 as compared to SP (1:50) (Figure 4.3D; $P = 0.17$ and $P = 0.11$, respectively). These results together demonstrate that SP-mediated induction of IL-1α transcript and protein expression is via EP_2 and EGF receptors and PI3K and not via the endogenous COX-PG pathway.

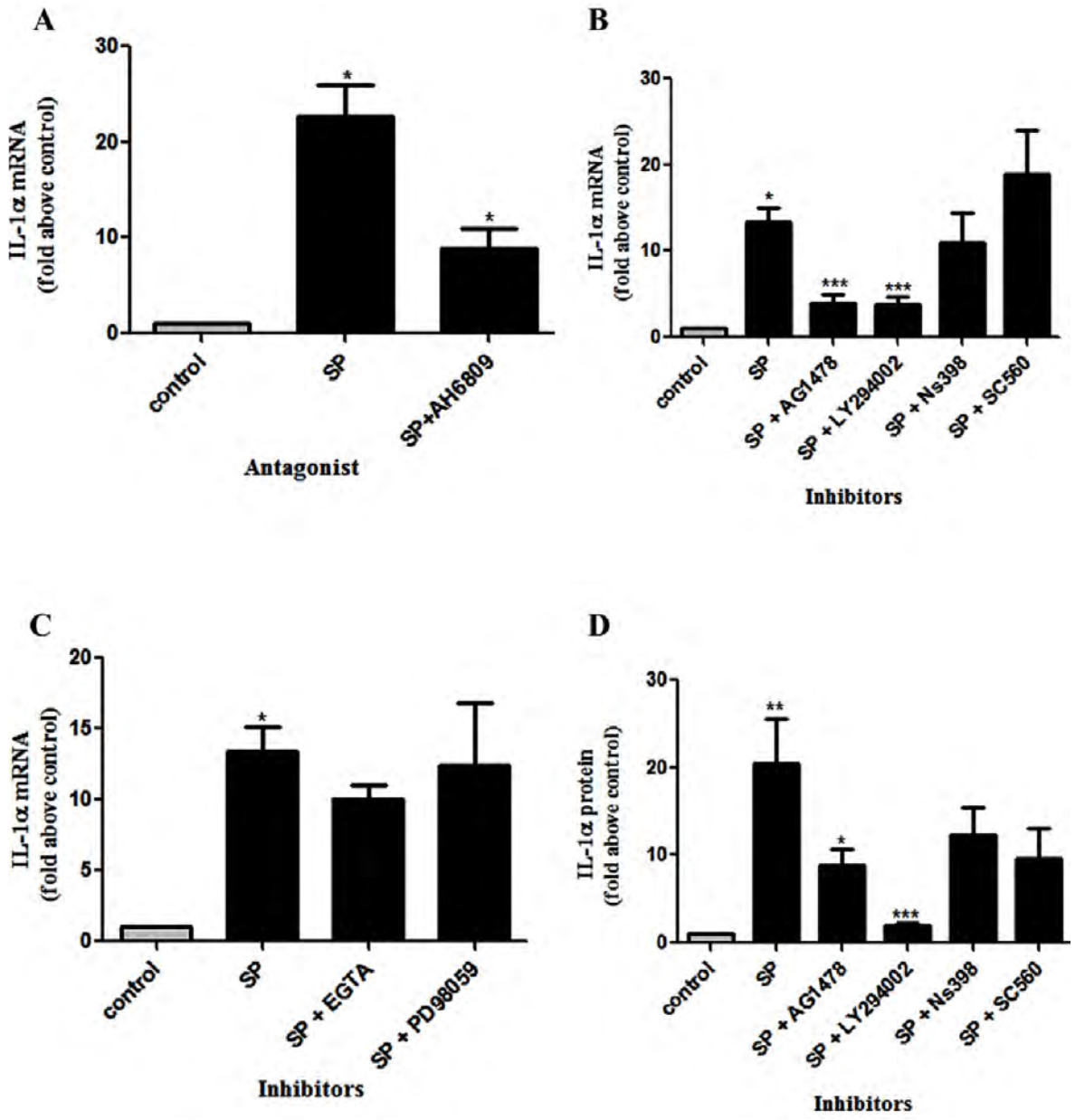


Figure 4.3: IL-1α mRNA and protein expression is regulated by seminal plasma via the EP_2 receptor, EGFR and PI3K pathways. IL-1α mRNA (A, B and C) and protein (D) as determined by qRT-PCR and ELISA analysis, respectively. HeLa cells were treated for 4 (A, B, C) and 16 hours (D) with seminal plasma (1:50) or vehicle in the absence/presence of EP2 receptor antagonist [AH-6809; 20μM] and chemical inhibitors to EGFR kinase [AG-1478; 100nM], PI3 kinase [LY-294002; 25μM], COX-1 [SC-560; 15μM] or COX-2 [NS-398; 8μM], EGTA [calcium chelator; 1.5mM] and PD-98059 [ERK inhibitor; 50μM]. Data are represented as mean ± SEM from 5 independent experiments. Paired T-tests were conducted on the untransformed means of the replicates between SP and control and unpaired T-tests performed on SP versus SP and inhibitor after conversion to fold increases. *, **, and *** indicates significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

4.4.4 PGE_2 and EGF induces IL-1α expression in HeLa neoplastic cervical epithelial cells

Since SP contains a variety of antigenically distinct molecules that include prostaglandins and growth factors [280,307]. I hypothesized that PGE_2 and EGF, present in the SP, can induce IL-1α expression as these molecules have been shown to regulate inflammatory gene expression on their own. To investigate the role of PGE_2 and EGF on IL-1α expression, I treated HeLa cells with vehicle or PGE_2 [300nM] or human recombinant EGF [10ng/mL] alone or together for 4, 8, 16, and 24 hours and determined IL-1α mRNA expression using qRT-PCR. Treatment of HeLa cells with PGE_2 (Figure 4.4A) and EGF (Figure 4.4B) resulted in a 2.49 ± 0.66 and 5.76 ± 0.80 maximum fold increase after 8 and 4 hours, respectively. Treatment of HeLa cells with both PGE_2 and EGF together (Figure 4.4C) resulted in 2.39 ± 0.52, 6.60 ± 0.63, 16.31 ± 1.23 and 10.88 ± 1.52 fold increase after 4, 8, 16, and 24 hours, respectively. With peak IL-1α mRNA induction observed after 16 hours treatment (16.31 ± 1.23 fold increase). Furthermore, the inductions of IL-1α mRNA at 8, 16, and 24 hours by both ligands together was greater than by each ligand on its own and suggesting that PGE_2 and EGF act synergistically in inducing the increase of IL-1α production by HeLa cells.

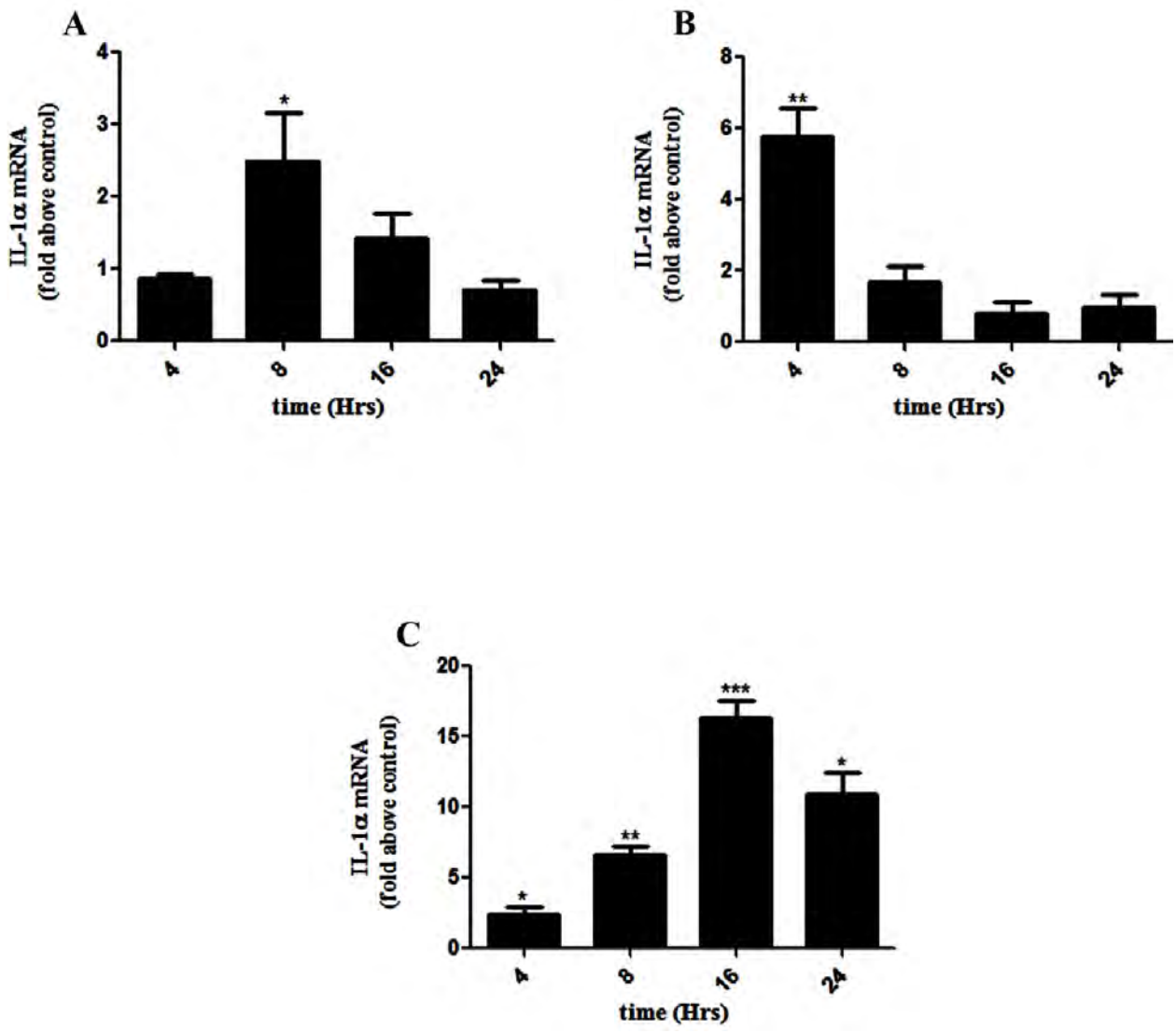


Figure 4.4: Induction of IL-1α mRNA in HeLa cells treated with PGE_2 (A), EGF (B), and PGE_2 plus EGF (C) as determined qRT-PCR. HeLa cells were treated for 4, 8, 16 and 24 hours with PGE_2 [300nM] or human recombinant EGF [10ng/mL] or PGE_2 together with EGF or vehicle as control. Data are represented as mean ± SEM from 5 independent experiments. Paired T-tests were conducted on the untransformed means of the replicates between PGE_2 or EGF or PGE_2 and EGF treated cells and control. *, ** and *** indicates significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

4.4.5 Activation of EP_2 and EGF receptors induces IL-1α expression in HeLa neoplastic cervical epithelial cells

The marked inhibition of SP-mediated induction of IL-1α by the EP_2 receptor antagonist AH-6809 and EGFR kinase inhibitor AG-1478 indicated a role for EP_2 receptor in combination with EGFR in this induction of IL-1α. I next investigated whether IL-1α regulation was mediated by the EP_2 and EGF receptors. In order to confirm that activation of the EP_2 and EGF receptors can regulate IL-1α expression, I treated HeLa cells with vehicle or butaprost which is a specific EP_2 receptor agonist or human recombinant EGF or both agonist together for 4, 8, 16 and 24 hours. Activation of EP_2 (Figure 4.5A) and EGF (Figure 4.5B) receptors significantly induced IL-1α expression after 8 and 4 hours treatment, respectively (2.77 ± 0.23 and 5.76 ± 0.80 fold increases) as shown by qRT-PCR analysis. Co-treatment of HeLa cells with EP_2 and EGF receptor agonists together (Figure 4.5C) significantly induced the expression of IL-1α mRNA at all-time points investigated (Figure 4.5C; 3.56 ± 1.48, 6.29 ± 1.28, 2.84± 0.34, and 2.36 ± 0.75 fold increases). Moreover, the peak IL-1α induction observed after 8 hours of co-treatment was greater than either butaprost or EGF treatment alone suggesting that co-activation of EP_2 and EGF receptors act synergistically in inducing the increase of IL-1α production by HeLa cells (Figure 4.5C).

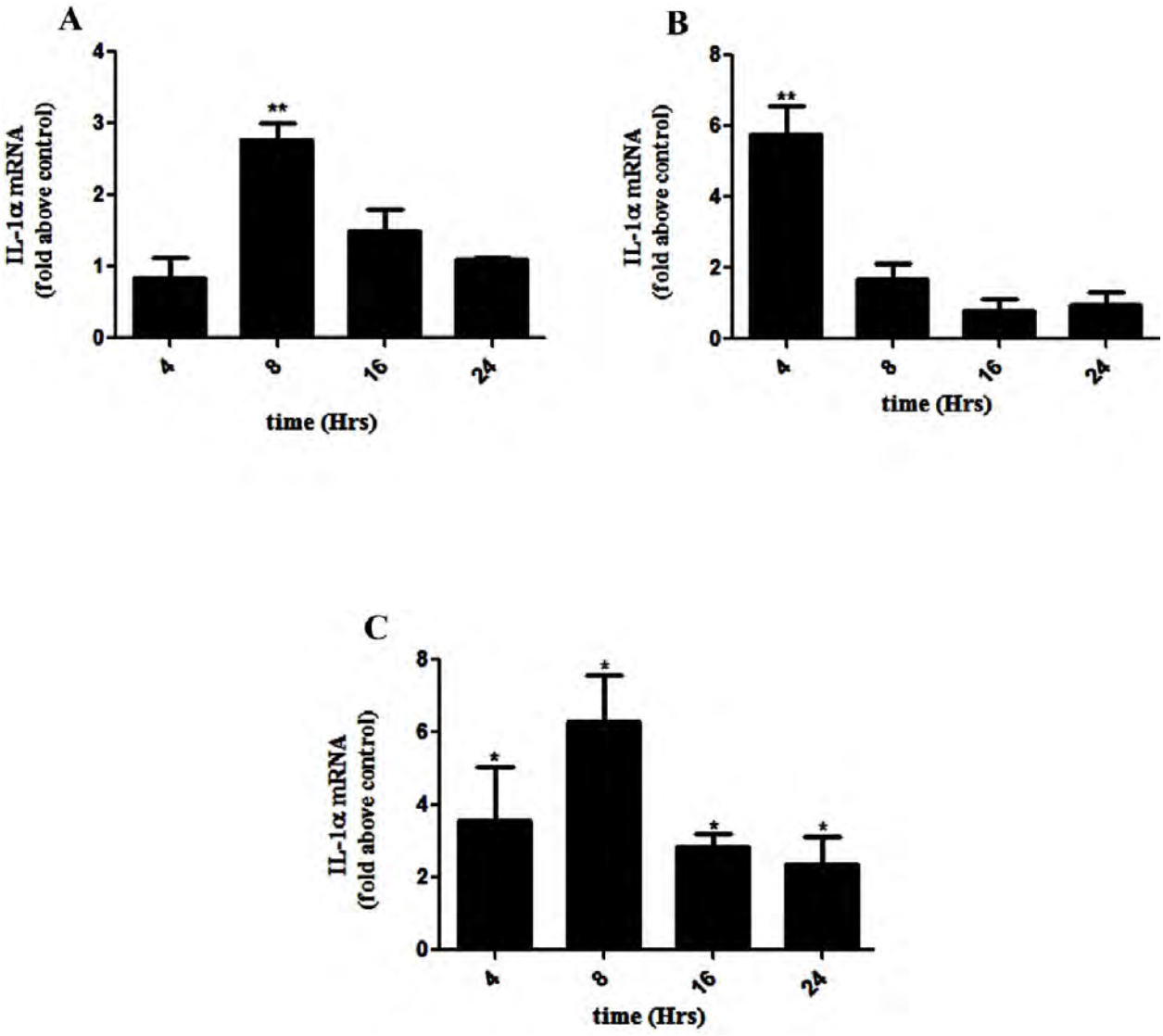


Figure 4.5: Activation of EP_2 receptor (A), EGF receptor (B) and co-activation of EGF and EP2 receptors (C) induces the expression of IL-1α mRNA in HeLa cells as determined by qRT-PCR. HeLa cells were treated for 4, 8, 16 and 24 hours with butaprost [5μM] or EGF [10ng/mL] or butaprost and EGF or vehicle. Data represented as mean ± SEM from 5 independent experiments. Paired T-tests were conducted on the untransformed means of the replicates between butaprost or EGF or butaprost and EGF treated cells and control.* and ** indicates significance at $P < 0.05$ and $P < 0.01$, respectively.

4.4.6 EP_2 receptor antagonist, EGFR and PI3 kinase inhibitors inhibit PGE_2 and EGF mediated induction of IL-1α in HeLa neoplastic cervical epithelial cells.

Having demonstrated that PGE_2 and EGF induced the expression of IL-1α in HeLa S3 cells, I next investigated the transduction pathways by which PGE_2 and EGF induce IL-1α expression in HeLa cells. SP was shown to induce IL-1α expression in HeLa S3 cell via the EP_2/EGFR/PI3 kinase pathways (Figure 4.3 A, B and C), thus I investigated whether PGE_2 and EGF induces the expression of IL-1α in HeLa cells via similar pathways. HeLa S3 cells were treated with vehicle or PGE_2 alone or in the presence of AH-6809 and chemical inhibitors, AG-1478 or LY-294002 for 8 hours and IL-1α mRNA expression was assessed using qRT-PCR (Figure 4.6A). PGE_2-mediated induction of IL-1α (2.49 ± 0.66 fold increase) was significantly reduced in the presence of EP_2 receptor antagonist (0.65 ± 0.38) and chemical inhibitors of EGFR and PI3 kinase (0.91 ± 0.15, and 0.41 ± 0.35 fold increases, respectively; $P < 0.05$ in all cases). Subsequently, HeLa S3 cells were treated with vehicle, or PGE_2 and EGF alone or in the presence of AH-6809 or AG-1478 separately or together or with LY-294002 for 16 hours (Figure 4.6B). Similarly, induction of IL-1α after treatment with PGE_2 and EGF together was markedly reduced in the presence of EP_2 receptor antagonist or EGFR kinase inhibitor, and almost abolished in the presence of EP_2 receptor antagonist together with EGFR inhibitor or in the presence of PI3 kinase inhibitor ($P < 0.01$ in all cases). In addition, HeLa S3 cells were treated with vehicle or EGF alone or in the presence of LY-294002 for 4 hours (Figure 4.6C). The EGF-mediated IL-1α induction was reduced from 5.76 ± 0.80 fold increase to 1.88 ± 0.46 fold increase in the presence of PI3 kinase inhibitor ($P < 0.01$). These findings confirm that EGF regulates IL-1α expression via the activation of PI3 kinase pathways, while PGE_2 regulates IL-1α expression via the activation of EP_2/EGFR/PI3 kinase pathways.

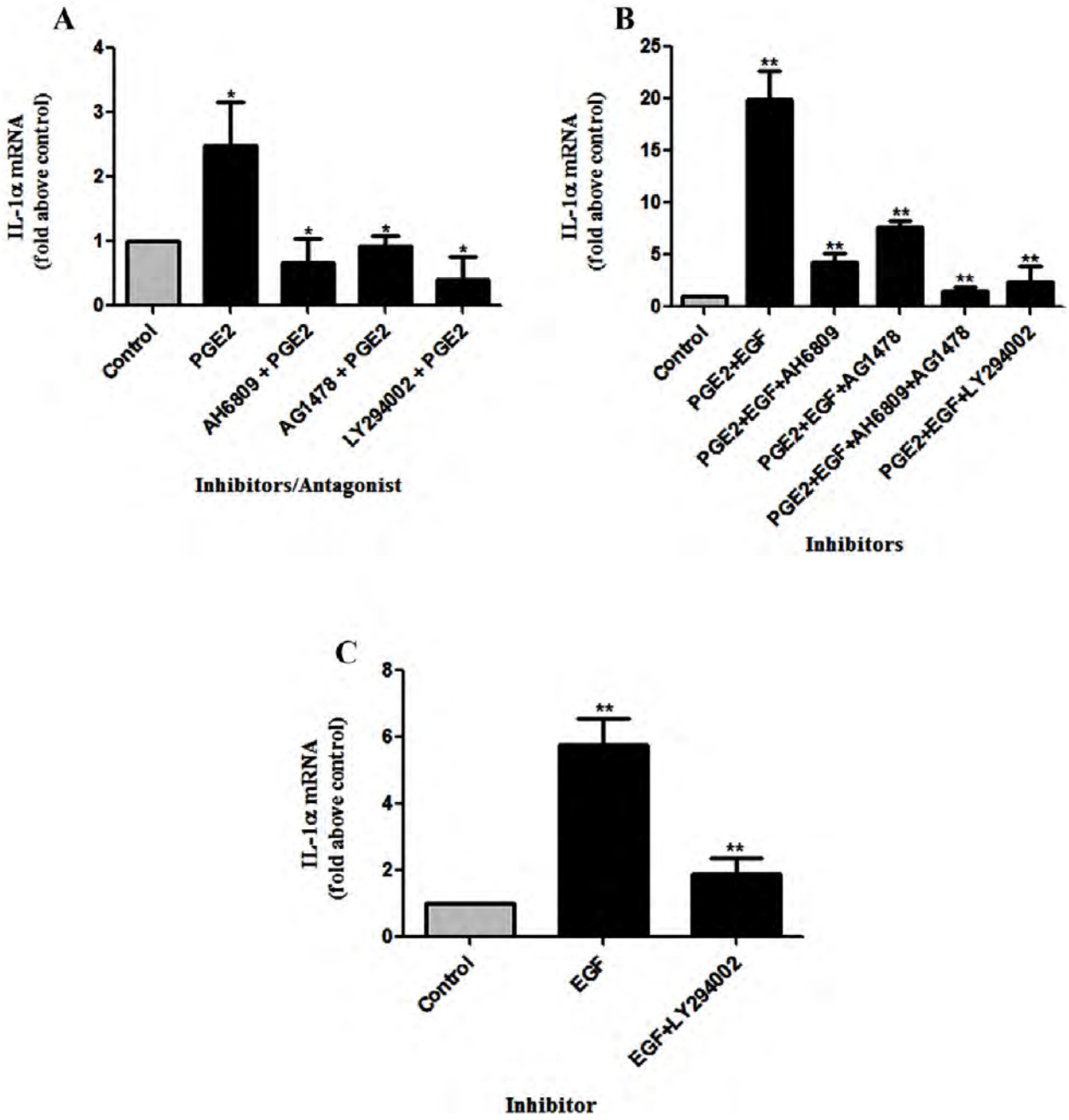


Figure 4.6: EP_2 antagonist, EGFR and PI3 kinase inhibitors inhibit PGE_2 (A), PGE_2 and EGF (B), and EGF (C) mediated induction of IL-1α in HeLa cells as determined by qRT-PCR. HeLa cells were treated for 4, 8 and 16 hours with EGF [10ng/mL], or PGE_2 [300nM] or both or vehicle in the absence/presence of antagonist/inhibitors AH6809 [20μM], AG1478 [100nM], AH6809 together with AG1478, and LY294002 [25μM]. Data are represented as mean ± SEM from 5 independent experiments. Paired T-tests were conducted on the untransformed means of the replicates between ligand/ligands treated cells and control and unpaired T-tests performed on ligand/ligands versus ligand/ligands and inhibitor after conversion to fold increases. * and ** indicates significance at $P < 0.05$ and $P < 0.01$, respectively.

4.4.7 Seminal plasma phosphorylates AKT via EP_2/EGFR/PI3 kinase signaling to induce IL-1α expression in HeLa neoplastic cervical epithelial cells.

Protein kinase B (Akt) is known to be one of the major downstream targets of PI3 kinase and upon activation, Akt moves to the cytoplasm and nucleus where it phosphorylates numerous downstream targets involved in the regulation of various cellular functions [203]. Having shown that SP mediated the expression of IL-1α in HeLa cells via the activation of EP_2/EGFR/PI3 kinase pathways, next was to investigate the role of SP in the phosphorylation of Akt and the position of EP_2, EGFR, and PI3 kinase pathways in relation to Akt signaling using immunoblot analysis. HeLa S3 cells were treated with vehicle or SP (1:50 dilution) for 0, 5, 10, 20, 40, 60, 120 and 240 minutes and Akt phosphorylation was measured by immunoblot analysis. A significant Akt phosphorylation was observed after 40 minutes with a maximum phosphorylation at 60 minutes and remaining until 240 minutes post-stimulation with fold increases of 21.58 ± 5.32, 44.64 ± 9.14 and 9.581 ± 2.475, respectively for these time points (Figure 4.7A) ($P < 0.05$). I next treated HeLa S3 cells with vehicle or SP (1:50 dilution) or in the presence or absence of chemical inhibitors of EGFR kinase (AG 1478), PI3 kinase (LY294002), and EP_2 receptor antagonist (AH6809) for 60 min and measured Akt phosphorylation. SP-mediated phosphorylation of Akt was significantly reduced from fold increase of 8.08 ± 1.688 to fold increases of 1.70 ± 0.23, 3.62 ± 0.08, and 1.00 ± 0.16 in the presence of AH-6809, AG-1478, and LY-294002, respectively ($P < 0.05$) (Figure 4.7B).

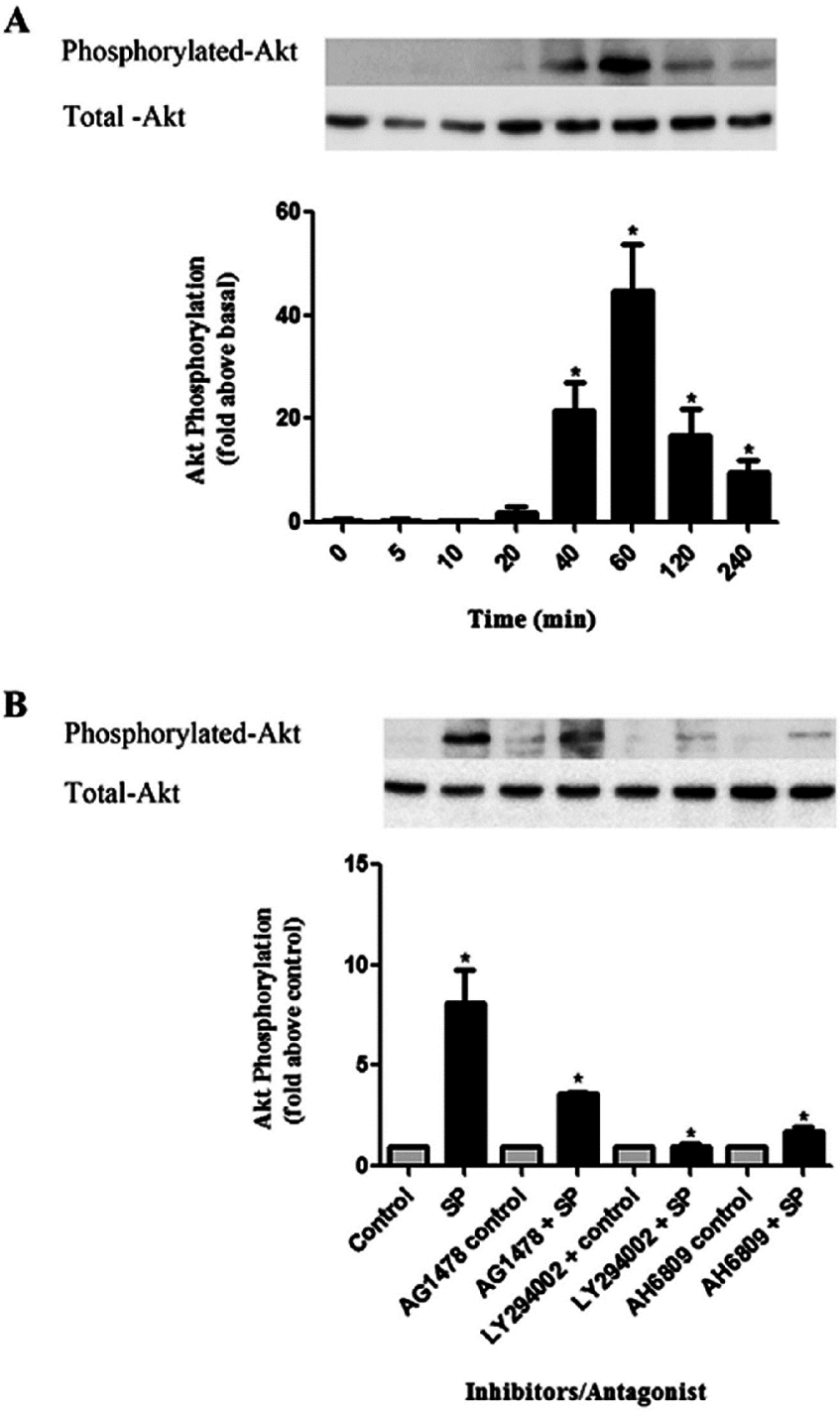
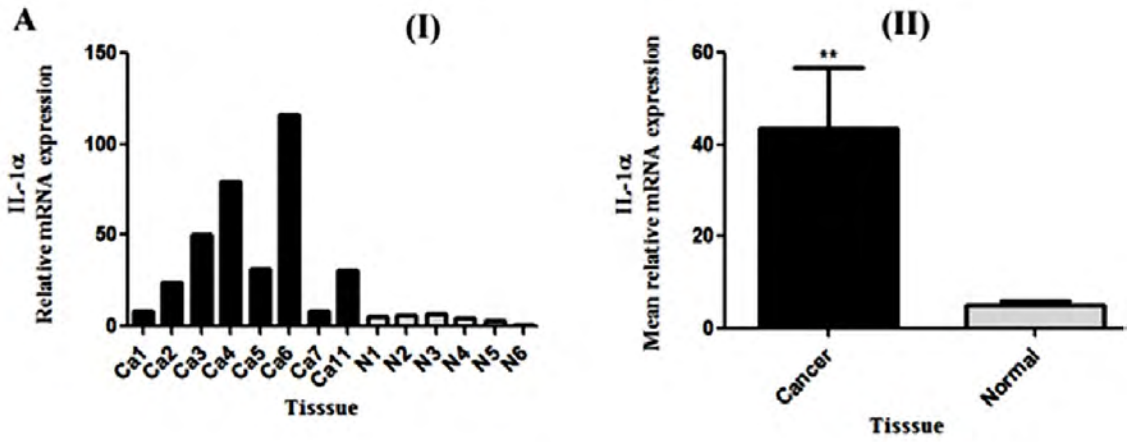


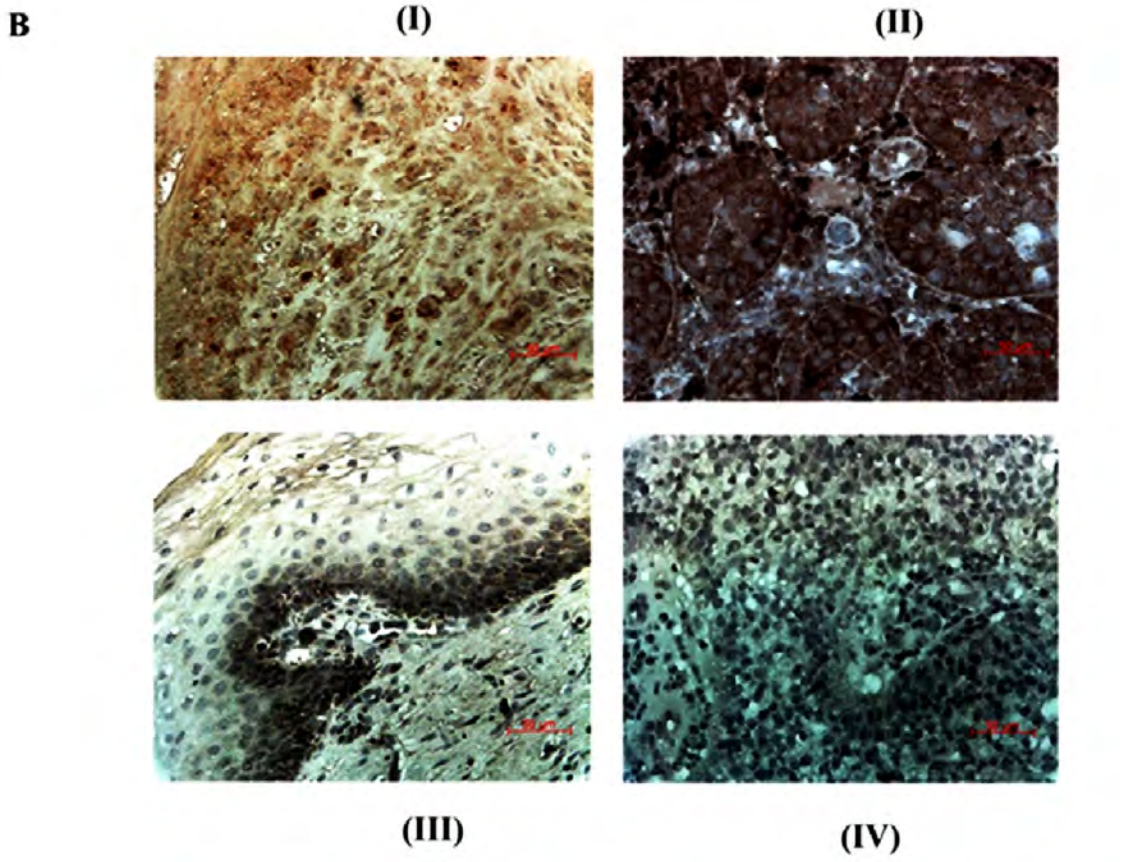
Figure 4.7: Akt phosphorylation in HeLa cells by SP. (A) Akt phosphorylation in HeLa S3 cells treated with SP (1:50) or control for 0, 5, 10, 20, 40, 60, 120, and 240 min. (B) Akt phosphorylation in HeLa cells treated for 60 min with SP (1:50) or control in the presence/absence of chemical inhibitors/antagonist EGFR kinase [AG-1478;100nM], PI3 kinase [LY-294002; 25μM] and EP_2 antagonist [AH-6809; 20μM]. Cell lysate were subjected to

immunoblot analysis. Data are represented as mean ± SEM form 3 independent experiments. Paired T-tests were conducted on the untransformed means of the replicates between SP and control and unpaired T-tests performed on SP versus SP and inhibitor after conversion to fold increases. * indicates $P < 0.05$.

4.4.8 IL-1α is up-regulated in cervical cancer

Having elucidated the molecular pathways underlying SP-mediated induction of IL-1α in cervical epithelial cells using an in vitro HeLa cell line model system next was to investigate whether SP could act via similar pathway to induce IL-1α expression in cervical tissue. Here, I initially investigated the expression of IL-1α in normal and neoplastic cervical tissue explants using quantitative real time PCR (qRT-PCR) (Figure 4.8A I). Expression of IL-1α transcript was significantly elevated in all cancer tissue samples investigated compared with normal cervical tissue sample. IL-1α mean expression as assessed by qRT-PCR was 43.65 ± 13.21 fold greater in neoplastic cervical tissue than in normal cervical tissue (Figure 4.8A II). The site of IL-1α synthesis in the neoplastic tissue was then investigated by immunohistochemistry. IL-1α was localized to the neoplastically transformed squamous epithelium in squamous cell carcinoma (Figure 4.8B I) and to neoplastically transformed columnar epithelium lining the endocervical and the glandular epithelium of the endocervical glands in adenocarcinomas (Figure 4.8B II). In contrast to the high levels of immunoreactive IL-1α observed in cervical cancer tissue, little or no staining for IL-1α was observed in the normal cervical tissues (Figure 4.8B III). Incubation with biotinylated IgG in place of primary antibody as control did not show any immunostaining in carcinoma tissue sections (Figure 4.8B IV). Analysis/quantification of immunohistochemical stains revealed a significant expression of IL-1α in both adenocarcinoma and squamous cell carcinoma tissue compared to normal cervix (Figure 4.8 C and D; $P < 0.05$ in both cases).


A
(I)
IL-1α
Relative mRNA expression
150
100
50
0
Ca1
Ca2
Ca3
Ca4
Ca5
Ca6
Ca7
Ca11
N1
N2
N3
N4
N5
N6
Tisssue
(II)
IL-1α
Mean relative mRNA expression
60
40
20
0
**
Cancer
Normal
Tisssue


B
(I)
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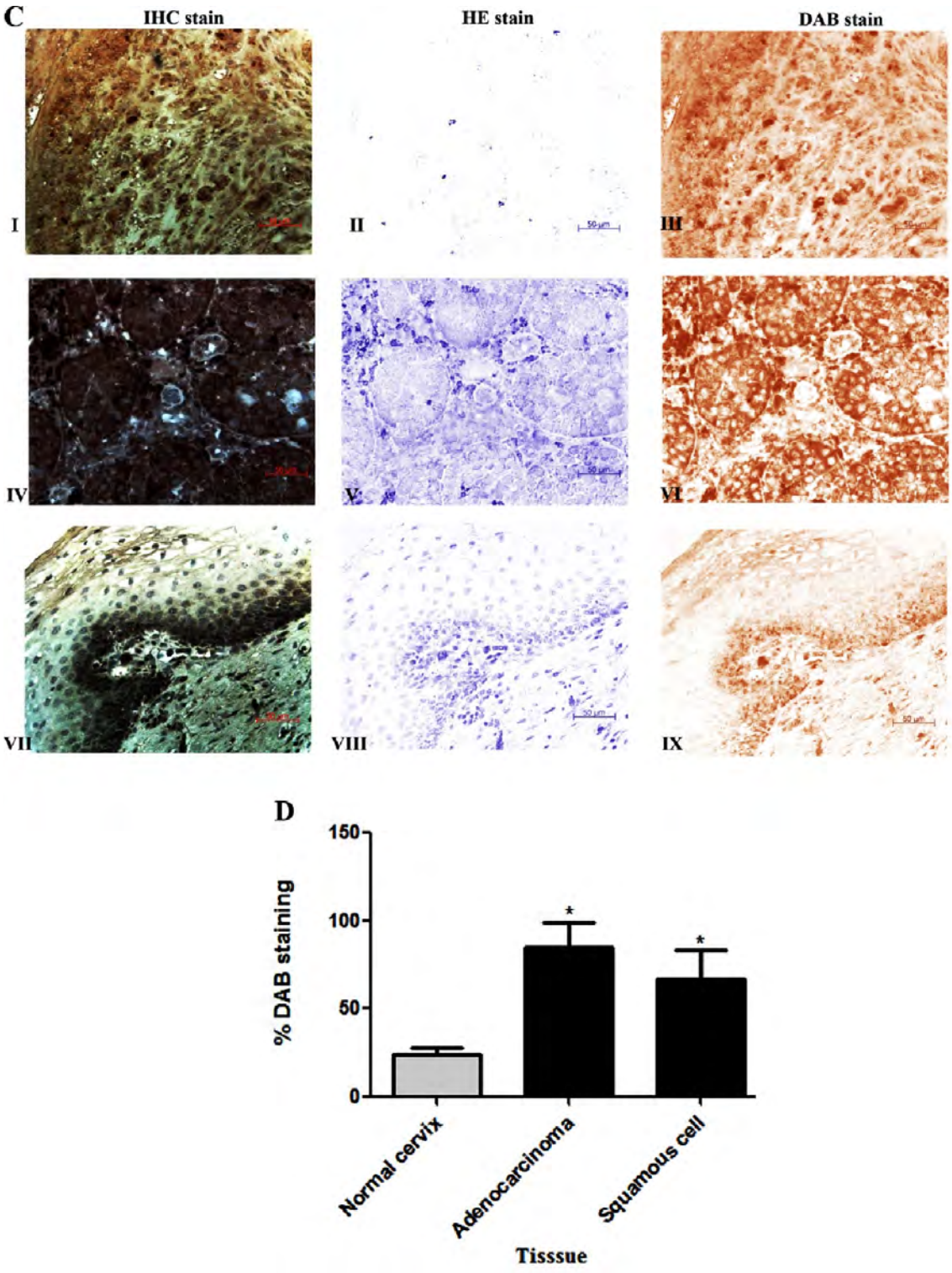


Figure 4.8: A, Relative mRNA expression of IL-1α in (I) cervical squamous cell carcinoma (Ca.1-Ca.7, adenocarcinoma (Ca.11), and normal cervix (N1-N6). (II) Mean relative IL-1α mRNA expression in neoplastic and normal cervical tissue explant as determined by qRT-PCR and shown in (I). B, IL-1α expression by immunostaining in epithelial cells of squamous cell carcinoma (I), and columnar and glandular epithelium of adenocarcinoma (II). Minimal IL-1α

signal was detected in normal cervical tissue (III). IL-1α staining was abolished in sections incubated with the biotinylated secondary antibody alone (IV) (negative control). C, de-convoluted images of squamous cell, adenocarcinoma and normal cervical immunohistochemical (IHC) stain. I-III, IV-VI, and VII-IX; immunohistochemical, hematoxylin (HE) and diaminobenzidine (DAB) stain of squamous cell, adenocarcinoma and normal cervix. D, % DAB stain in immumohistochemical slides. Scale bar, 50μm. Data represented as mean ± SEM. *, and **, indicates statistical significance at $P < 0.05$ and $P < 0.01$, respectively.

4.4.9 IL-1RI is up-regulated in cervical cancer

IL-1R type I (IL-1RI) (80 kD) is a signaling receptor responsible for the initiation of IL-1α signal transduction [509,510]. Overt expression of IL-1RI has been demonstrated in human cancers such as prostate cancer [510]. Having shown that IL-1α is up-regulated in cervical cancer, I next investigated the concurrent expression of IL-1RI in cervical cancer and normal cervix using qRT-PCR (Figure 4.9 A and B). IL-1RI transcript was found to be marginally elevated in all cancer tissue samples investigated compared with normal cervical tissue sample. IL-1RI mean expression as assessed by qRT-PCR was 19.70 ± 5.72 in neoplastic cervical tissue vs 15.33 ± 4.19 in normal cervical tissue (Figure 4.9B), suggesting an enhanced IL-1α/IL-1RI signaling in cervical cancer.

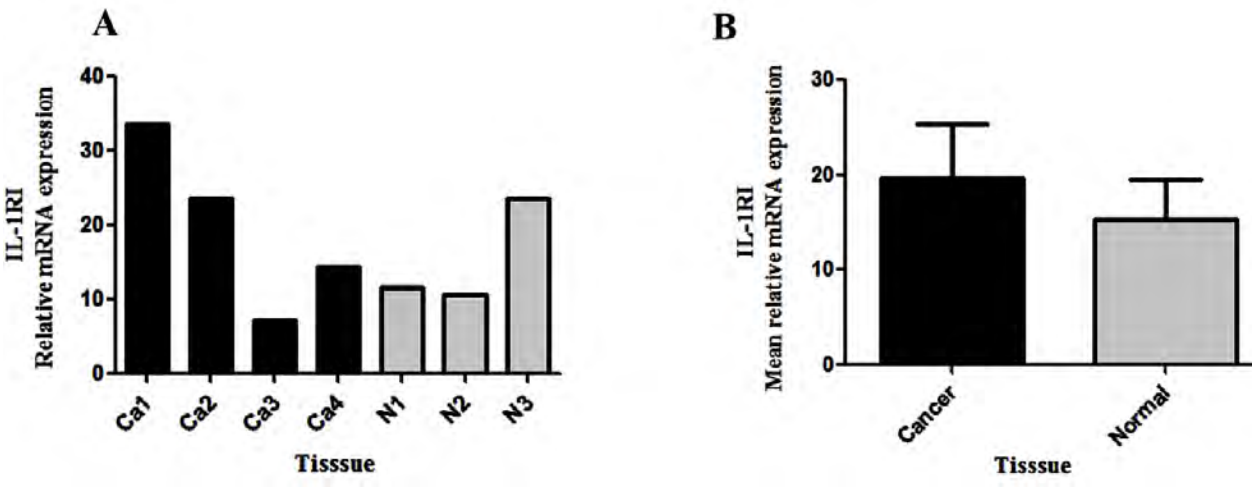


Figure 4.9: A, Relative mRNA expression of IL-1RI in cervical squamous cell carcinoma (Ca.1-Ca. 4) and normal cervix (N1-N3). B, Mean relative IL-1RI mRNA expression in neoplastic and normal cervical tissue explant as determined by qRT-PCR and shown in (A).

4.4.10 SP induces the expression of IL-1α in the cervix via EP2/EGFR/PI3 kinase activation

Finally I investigated the potential role of SP in the regulation of IL-1α expression in the cervix and determined whether SP induces IL-1α expression in the cervix via the same pathways as seen in the HeLa model cell line. Normal and neoplastic (squamous) cervical tissue explant were treated with vehicle or SP (1:50) for 24 hours and IL-1α mRNA expression was determined using qRT-PCR. SP significantly induced the expression of IL-1α in both neoplastic and normal cervical tissue with similar fold induction (1.959 ± 0.3226 and 2.124 ± 0.2673 fold increases) (Figure 4.10A and B). To investigate the signaling pathways by which SP induces IL-1α expression in the cervix, normal cervical tissue explants were incubated with vehicle or SP (1:50) in the presence or absence of AH-6809, AG-1478, and LY-290042. qRT-PCR assessment of IL-1α expression showed a marked reduction in SP-mediated induction of IL-1α in normal cervical tissue explants in the presence of EP_2 receptor antagonist or the chemical inhibitors of

the EGFR (AG-1478) and PI3 kinase (LY-294002) pathways ($P < 0.01$ in all cases) (Figure 4.10C).

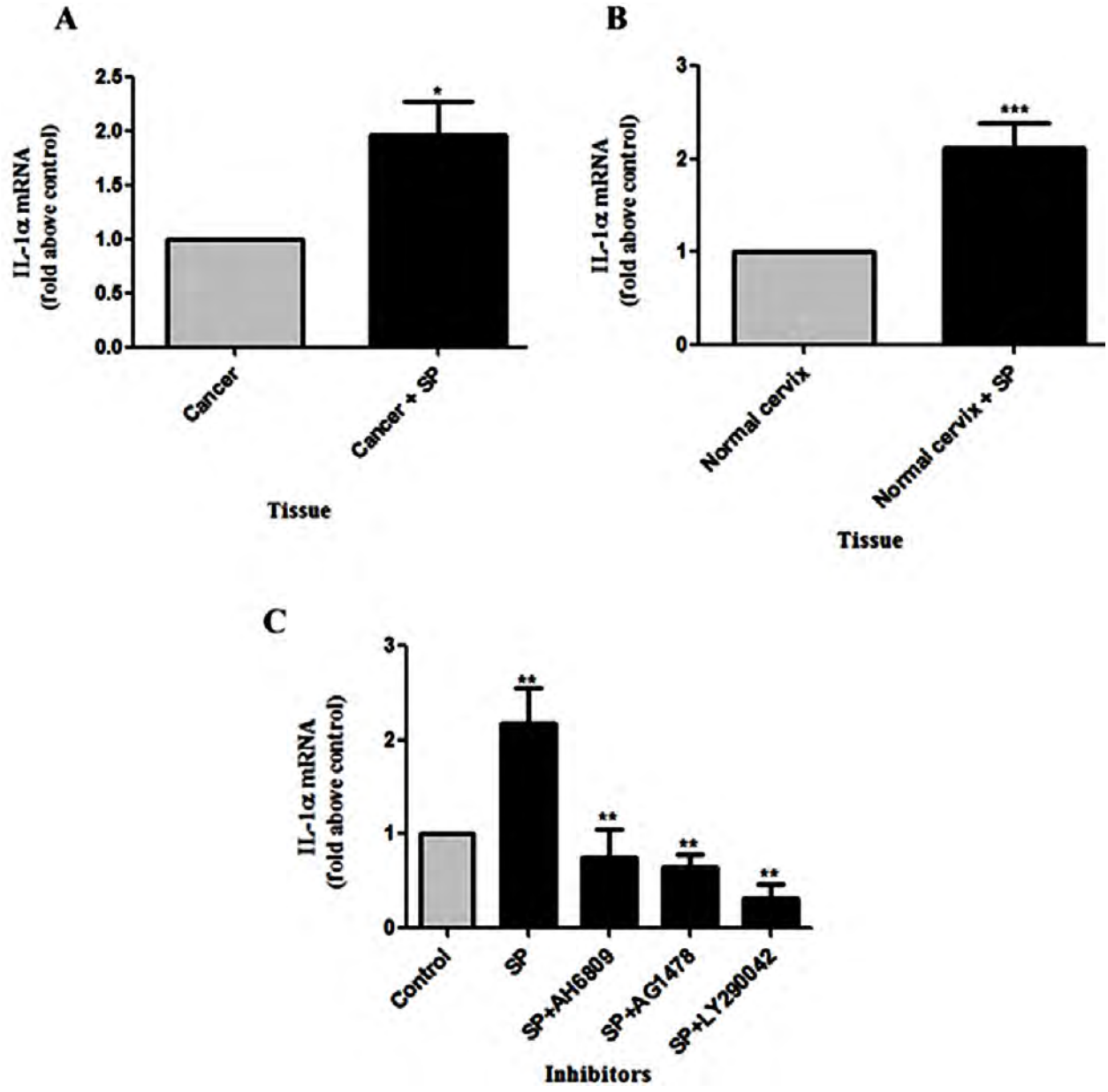


Figure 4.10: SP induce IL-1α mRNA expression in neoplastic (A) and normal (B) cervical tissue explant. SP induces the expression of IL-1α in normal cervical tissue via the activation of EP2/EGFR/PI3 kinase inflammatory pathways as determined by qRT-PCR (C). Data represented as mean ± SEM of (n = 12 (A), n = 10 (B) and n = 7 (c), respectively). Paired T-tests were conducted on the untransformed means of the replicates between SP and control and unpaired T-tests performed on SP versus SP and inhibitor after conversion to fold increases. *, **and *** represent significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

4.5 Discussion

Over the years, the role of inflammation as an etiological factor for cancer has been supported by findings that show that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with reduced incidence of certain cancers [509]. Hence, inflammatory responses within tumor microenvironment are now recognized as a critical component for tumor progression and one of the major hall-marks of cancer [447,511]. Despite experimental and epidemiological evidence that supports the causal relationship between inflammation and cancer, the molecular mechanisms and pathways linking inflammation and cancer remain poorly understood [512]. The inflammatory milieu of most cancer microenvironments consist of tumor, surrounding stromal, immune and inflammatory cells which all interact intimately to produce cytokines/chemokines, growth factors, and adhesion molecules in a bid to promote inflammation, tumorigenesis and metastasis [273]. Of special relevance within this milieu are pro-inflammatory cytokines which are important mediators of chronic inflammatory responses, and have cardinal effects on malignant processes [509] as a result of their direct involvement in carcinogenesis, malignant transformation, tumor growth, invasion, and metastasis [473].

Within the cancer microenvironment, pro-inflammatory cytokines can exert a direct effect on pre-malignant and/or malignant cells. By engaging their cognate receptors they activate downstream kinases to regulate an array of genes involved in cell proliferation, survival, metabolism, angiogenesis and metastasis [513], leading to enhanced tumor growth. Similarly, in epithelial cancer cells, pro-inflammatory cytokines can up-regulate the expression and augment the activity of epithelial NADPH oxidase (Nox) family proteins to increase ROS (reactive oxygen species) production in tumor microenvironment [513]. This leads to damage in genomic

DNA and may produce mutations that can enhance tumor formation in a chronic inflammatory background [513].

Cervical cancer is a chronic inflammatory disease and one of the leading causes of cancer-related death worldwide with a higher incidence rate reported in underdeveloped countries [514]. It is well established that persistent infection with high-risk HPV is crucial to disease pathogenesis [15]. However, only a subset of women infected with high-risk HPV will proceed to develop invasive cervical cancer, thus suggesting that other co-factors must be present for the development of malignancy [515]. Studies have reported an association between the level of cervical inflammation and the development of high grade cervical neoplasia [20] or invasive cervical cancer [516]. It has been reported that cervical inflammation, but not the actual diagnosis of a specific sexually transmitted infection is associated with the development of squamous intraepithelial lesions within the cervix [517]. Direct links between increased pro-inflammatory cytokine levels in patients and increasing grade of cervical intraepithelial neoplasia and invasive cervical cancer have been established [518]. Several in-vitro studies have demonstrated the expression of IL-1α in both normal and neoplastic cervical epithelial cell line [477]. However, few data exist on the expression of IL-1α and IL-1RI in normal and neoplastic cervical tissue. Using immunohistochemistry and qPCR, I investigated the expression of pro-inflammatory cytokine IL-1α and its cognate receptor IL-1RI in normal and neoplastic cervical tissue. Data presented confirmed the elevated expression of IL-1α and IL-1RI in cervical cancer. This is in agreement with similar study by Pao et al. (1995) and Ricote et al. (2004), where it was reported that neoplastic cervical and prostate tissue overtly expresses IL-1α and IL-1RI [510,519]. In addition, these data suggests a similar pattern of IL-1α expression in cervical cancer as demonstrated in other malignancies [520-522].

IL-1α is a pleiotropic pro-inflammatory cytokine and a member of the IL-1 family. Within the human body, IL-1α mediates normal physiological functions ranging from induction of vascular permeability and fever during sepsis to increased secretion of additional cytokines in autoimmune diseases [509]. The production and level of IL-1α and IL-1RI expression is elevated in numerous cancers including head and neck [474], breast [520,523], pancreatic [524], gastric [525] and prostatic cancer [510] and has been associated with virulent tumor phenotype and poorer prognosis via the regulation of inflammatory genes and growth factors to enhance tumor growth and differentiation [477,526,527] and metastatic potential of cells [528]. Indeed Ricote and colleagues reported that high expression levels of IL-1α and IL-1RI in prostate cancer was involved in neoplastic cell proliferation [510]. Similarly, animal studies have further confirmed the role of IL-1α and IL-1RI in tumor development and blood vessel growth [529]. The expression/activation of IL-1α/IL-1RI cytokine family via autocrine and/or paracrine mechanisms leads to the activation of secondary pro-tumorigenic cytokines which can subsequently contribute to angiogenesis, tumor invasion and proliferation [523]. It is therefore probable that expression of IL-1α and IL-1RI in cervical cancers can act via similar manner to confer virulent tumor phenotype and poorer prognosis in these patients.

IL-1α expression can be regulated by a host of inflammatory stimuli and recent studies have shown that seminal plasma can regulate IL-1α expression in the human cervix, post coitus [285,352]. Conventionally, human seminal plasma was regarded primarily as a transport and survival medium for the mammalian spermatozoa traversing the cervix and the uterus during and post coitus [280,451]. However, experimental studies using animal models shows that in addition to its role as a primary transport medium for the spermatozoa, seminal plasma also introduce to the female reproductive tract an array of signaling molecules including prostaglandins, several

cytokines and growth factors [280,343,451,452]. These molecules interact with cognate receptors on the epithelial lining of the female reproductive tract to initiate local cellular and molecular changes reminiscent of an inflammatory response [280]. These changes are required for maternal immune adaptation to pregnancy and for the generation of immune tolerance against fetal antigens [280,530]. However the molecular pathway by which seminal plasma mediates the expression of IL-1α and other cytokines is yet to be fully elucidated. Using HeLa (adenocarcinoma) cells, normal and neoplastic cervical tissue explants, I investigated the role of SP in the regulation of IL-1α expression in the cervix and transduction pathways by which SP induces the expression of IL-1α in neoplastic and normal cervical epithelium. In addition, I further investigated PGE_2 and EGF as possible ligands mediating SP induction of IL-1α in neoplastic cervical cells.

In the present study I have shown that exposure of HeLa neoplastic cervical epithelial cells to SP in vitro increases the expression of IL-1α in a donor dependent manner. The observation that IL-1α is induced by SP as oppose to spermatozoa agrees with study by Robertson et al. (1996) where it was shown that SP from vasectomized mice elicit post-mating inflammatory response characterized by surge in pro-inflammatory cytokine synthesis [283]. In a similar manner, SP was found to increase the expression of IL-1α in both normal and neoplastic cervical tissue explants. This is in agreement with studies by Sutherland et al. (2012) and Sharkey et al. (2007 and 2012) where it was reported that SP mediates pro-inflammatory cytokines expression in HeLa cells in vitro [287] and in the cervix post coitus [285,352] to initiate an inflammatory response. Inflammatory response orchestrated by SP within the female genital tract can impact on both physiological and pathophysiological events within the cervix [285]. Because the epithelial microenvironment is a critical factor in cervical dysplasia and metaplasia [531],

incessant exposure of dysplastic or metaplastic epithelial cells to SP in sexually active women with underlying pathology could influence the incidence and progression of cervical cancer [285] consequent of SP-mediated IL-1α expression. In addition, cytokines influencing the numbers and phenotypes of APCs (Antigen Presenting Cells) could interact with SP-induced expression of tumorigenic and angiogenic factors [345] to favor tumor growth. Having shown that SP regulates IL-1α expression in normal and neoplastic cervical tissue and epithelial cells, I next investigated possible signal transduction pathways by which SP mediates this role.

Employing HeLa cell line as a model, I showed that SP induces the expression of IL-1α via the EP_2 receptor, EGFR and PI3 kinase pathways since EP_2 receptor antagonist (AH6809) and the inhibitors of EGFR kinase (AG1478) and PI3 kinase (LY294002) inhibited SP mediated induction of IL-1α in these neoplastic cells. In contrast, COX-1 and COX-2 were not shown to have a role in the induction of IL-1α by SP, since addition of their inhibitors (SC-560 and NS-398, respectively) did not reduce the induction of IL-1α. In addition, I found that SP-mediated induction of IL-1α in normal cervical tissue explant was also inhibited in the presence of the antagonist and these inhibitors. Similar in vitro studies by Battersby et al. (2006), Muller et al. (2006) and Sales et al. (2012) have shown that SP-mediated expression of pro-inflammatory and angiogenic genes in endometrial and cervical adenocarcinoma cells was significantly inhibited in the presence of AH6809 [344] and AG-1478 (EGFR kinase inhibitor) [345,350]. The inhibition of SP-mediated IL-1α by EP_2 antagonist and EGFR kinase inhibitors suggested that the effects observed were mediated by PGE_2 and EGF present in the SP.

PGE_2 has been established as the predominant PG found in SP [310]. In the present study and as highlighted schematically in figure 4.11, I showed that PGE_2-mediated activation of IL-1α occurs via activation of the EP_2, EGFR and Akt pathways.

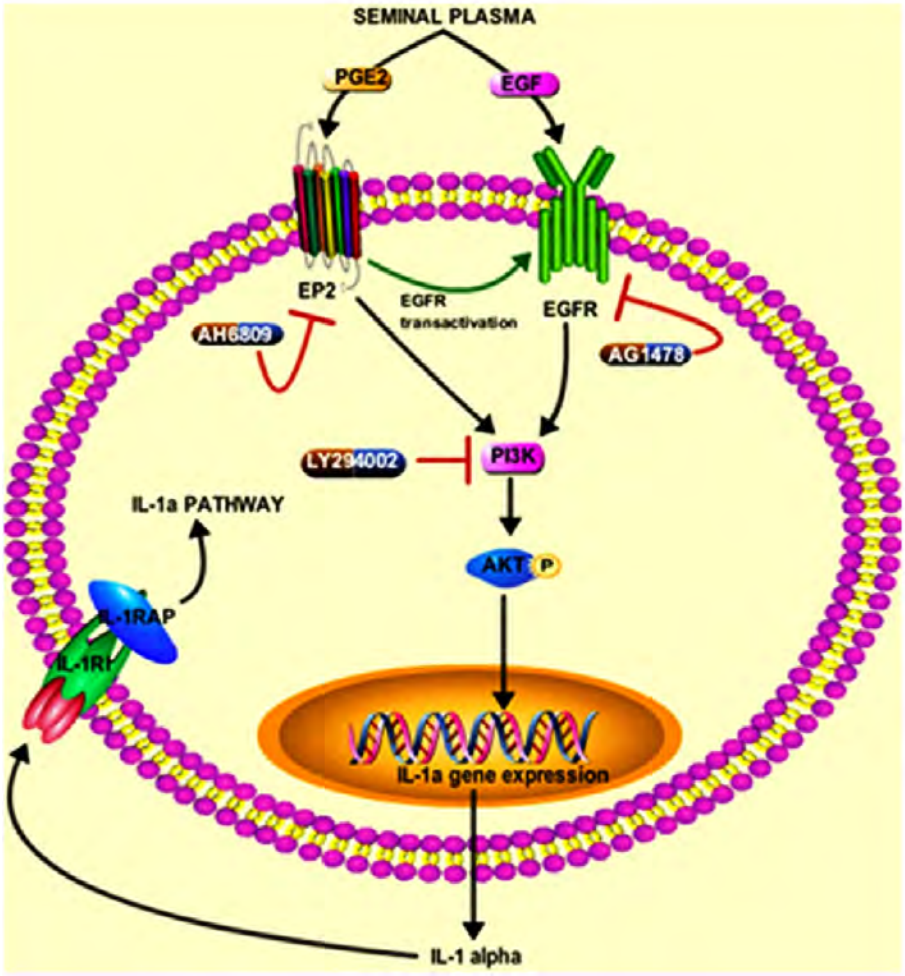


Figure 4.11: Schematic summary highlighting the role of SP and its constituents (PGE_2 and EGF) in the regulation of signaling pathway mediating IL-1α (IL-1 alpha) expression in cervical epithelial cells.

The role of the EP_2 receptor in mediating these effects was further confirmed using the selective EP_2 agonist butaprost. This is consistent with similar study by Shao et al. (2007) where it was shown that PGE_2, acting via EP_2 receptor activate cAMP/PKA pathway to mediate the expression of IL-1α in colon cancer cells in an autocrine/paracrine mechanism [505]. EP_2 has been found to be up-regulated in cervical cancer [28] and its role in the induction of IL-1α in cervical cancer cells explains the greater induction in IL-1α in cervical cancer tissue relative to

normal cervical tissue. These data suggest that PGE_2 in SP [280,532,533] can act via its E-series PGs receptor EP_2 receptor to directly transactivate EGFR via an intracellular signaling mechanism, either by phosphorylation of cSRC or by the MMP-mediated release of heparin-bound EGF tethered to the cell membrane, [344,345] leading to IL-1α induction. In addition to PGE_2, SP has been shown to be rich in growth factors including epidermal growth factor (EGF) [534-536]. Data presented herein showed that EGF can induce IL-1α in neoplastic cervical HeLa cells. This in agreement with similar in vitro study by Hamilton et al. (2003) where it was shown that EGF induces the expression of pro-inflammatory cytokine in lung cancer cells, and the expression of this cytokine was suppressed in the presence of EGFR inhibitor (AG-1478) [537]. It is therefore very feasible that the EGFR expressed on the membrane of these neoplastic cells can be directly activated by EGF in SP to induce IL-1α expression. The evidence that IL-1α induction by SP is due to PGE_2 and EGF present in SP is in agreement with findings of Sharkey et al. (2012) who demonstrated that SP-mediated IL-1α induction in Ect1 cells occurred independently of TGF-β_1, TGF-β_2, and TGF-β_3 which are abundant in SP [286].

Furthermore, since my data show that concurrent treatment of HeLa S3 cells with PGE_2 and EGF directed a sustained and elevated increase in IL-1α expression compared to either ligand alone, it is likely that the effects of SP on IL-1α induction may be mediated by a combination of PGE_2 and EGF working in synergy. Once released, IL-1α can act in an autocrine/paracrine manner within the site of production to regulate inflammation and tumorigenesis. Indeed, Shao and colleagues showed in their study that IL-1α stimulates the migration of colon cancer cells [505]. It is therefore plausible that in sexually active women with underlying pre-invasive or invasive cervical condition, repeated exposure of the elevated EP_2 receptor expressed on the neoplastic cervical epithelial cells [28] to PGE_2 present in seminal plasma could enhance tumorigenesis

following ligand-receptor binding and activation of similar intracellular signaling pathway to induce IL-1α expression. Expressed IL-1α can then stimulate cervical cancer cell migration to adjacent structures within the pelvis and perineum, hence conferring poor prognosis.

Several studies have shown that PI3 kinase-Akt signaling is deregulated in many cancers including cervical cancer where amplification of the p110α catalytic subunit has been reported [217,218,220,538]. Interestingly in this present study, SP and its constituents (PGE_2 and EGF) have been shown to mediate IL-1α expression in normal and neoplastic cervical epithelial cells via the activation of PGE_2-EP_2-EGFR-PI3 kinase pathways. Once activated Akt phosphorylates proteins on serine and threonine residues resulting in the modulation of multiplicity of downstream substrates, including NF-κB involved in the regulation of cell proliferation and survival [224,230,232]. The SP-mediated Akt phosphorylation seen in this study may act via similar mechanism to induce IL-1α expression. The SP-mediated induction of a pleotropic pro-inflammatory cytokine IL-1α in neoplastic cervical epithelial cells suggests that SP may promote cervical inflammation as well as progression of cervical cancer in sexually active women [350]. This present study is the first to demonstrate that SP regulates pro-inflammatory cytokine IL-1α expression in normal and neoplastic cervical cells via the induction of the EP_2-EGFR-PI3 kinase-Akt pathways.

In conclusion, this study identifies PGE_2 and EGF within SP as possible ligands responsible for SP-mediated induction and secretion of IL-1α in neoplastic cervical cells. Furthermore, this study provides evidence for pathways that are used by SP to induce the production of pleotropic pro-inflammatory cytokine IL-1α in normal and neoplastic cervical epithelial cells following coitus. The secreted IL-1α can in turn regulate in an autocrine or paracrine manner through its cognate receptor the surrounding cells and promote inflammation and tumorigenesis.

CHAPTER 5

C-C CHEMOKINE RECEPTOR 5 IS UP-REGULATED IN CERVICAL CANCER AND IS REGULATED BY SEMINAL PLASMA: POTENTIAL ROLE IN INCREASED RISK OF HIV TRANSMISSION IN CERVICAL CANCER

5.1 Introduction

Human immunodeficiency virus (HIV) infection is a major public health problem in developing countries. In Southern Africa alone, there are more than 5 million people infected with HIV/AIDS, accounting for a staggering 34 % of the global HIV infection burden, with women more affected than men [539]. HIV infection is characterized by persistent immune activation and chronic inflammation which is beneficial for HIV replication but detrimental to the host [540]. Although two decades have now elapsed since the identification of HIV as the etiology of AIDS, knowledge about how pro-inflammatory mediators affect HIV pathogenesis is still unclear [256]. However, it has been established that a main feature of HIV infection is the expression of numerous pro-inflammatory mediators including cytokines and PGs [256,313]. These pro-inflammatory mediators have been shown to enhance HIV-1 transmission [313,541] and viral replication in vitro [313,542].

Mucosal inflammation of the female lower genital tract is regarded as an imperative factor favoring acquisition of HIV infection in receptive vaginal intercourse [543,544]. Cervical cancer, an AIDS-defining illness [545,546] is a characterized chronic inflammation of the cervical epithelium with underlying neoplasia [547]. Histopathologic features of cervical cancer shows a predominance of tumor-infiltrating leukocytes [548] and neoplastic cells interacting to release inflammatory mediators including pro-inflammatory cytokines [519] and PGs [28]. Hence,

women with cervical cancer, where there are already infiltrating leukocytes and elevated cytokines could be at greater risk of HIV infection.

Here I show that CD4 and CCR5 receptors are up-regulated in neoplastic cervical tissue that has high levels of IL-1α [519], PGE_2 and COX enzymes [28,312]. These (PGs and cytokines) have been shown to regulate chemokine receptors including CXCR4, in cervical cancer [276].

5.2 Aim of the study

This study was aimed at investigating the expression and SP regulation of HIV receptors CD4 and CCR5 in cervical cancer.

5.3 Materials and Methods

5.3.1 Cervical cytobrush sample

Cervical cytobrush specimens were collected from women (n=20) attending the Colposcopy Clinic at Groote Schuur Hospital, Cape Town. All women were of reproductive age (median age of 37 years) and reported normal menstrual cycles. Sampling was carried out mid-cycle and any patients that showed signs of bleeding were excluded from the study. Specimens were obtained by cytobrush according to the method described by Musey and colleagues [321] and treated with SP as described in section 2.4.1. After which, cells were pelleted by centrifugation at 15000rpm and RNA extracted using Trizol (Sigma) following the manufacture's guidelines and reverse transcribed as previously described in section 2.9. Data are presented as mean ± SEM.

5.3.2 Cervical tissue collection

Cervical cancer tissue (Ca.1-Ca.7) specimens obtained from previously diagnosed (pre or invasive carcinoma of the cervix) patients attending the Gynecologic Oncology Clinic at Groote Schuur Hospital (Cape Town) were processed and treated with SP as previously described in section 2.4.2. Patient age ranged between 29-62 years, with a median age of 41 years. The extent of invasiveness of carcinoma biopsies are presented in Appendix III. Similarly, histologically normal cervical tissues (N1-N5) were collected from women undergoing Wertheim's hysterectomy for benign gynecological malignancies at Groote Schuur Hospital (Cape Town). Tissue explant were also processed and treated with SP as previously described in section 2.4.2. Patient age ranged between 37-73 years with a median age of 50.5 years.

5.3.3 Construction of HeLa Tet-off cells

HeLa Tet-off cells containing the tetracycline (doxycycline; DOX) transactivator/VP16 domain cloned upstream of the minimal CMV promoter [549,550] were purchased from Clontech. Construction and validation of the COX-1 Tet-Off system is described in Sales et al., 2002 [312]. Briefly, HeLa Tet-off cells were stably transfected with the full length COX-1 cDNA cloned upstream of the tetracycline (DOX) regulatory element fused to the minimal CMV promoter. In the presence of DOX, the TetR/VP16 transcativator protein is inhibited from transactivating the TRE and expression of the transgene is suppressed. Removal of DOX from the culture medium promotes COX-1 transgene expression [312].

5.3.4 Cell culture and treatment with SP

HeLa S3 cells were routinely maintained as previously described in section 2.6. HeLa COX-1 Tet-off cells were maintained under the same culture conditions as HeLa S3 cells, with the addition of 2μg/ml Doxycyline (DOX; Sigma) to maintain the suppression of the COX-1 transgene as previously described by Sales et al. (2002) [312]. For the wild type HeLa cell experiments, HeLa cells were seeded at a density of 2 x 10^5 cells in 3cm dishes and allowed to attach and grow overnight. The following day cells were serum starved for 24 hours in serum-free medium. Cells were then treated with vehicle or 1:50 dilution of SP for 4, 8, 16 and 24 hours, respectively. For inhibitor experiments, cells were serum starved and treated with inhibitor alone or 1:50 dilution of SP and inhibitor of EGFR kinase [AG1478; 200nM], ERK1/2 kinase [PD98059; 50μM], COX-1 [SC560; 10μM], COX-2 [NS398; 10μM] or nuclear factor kappa B [NFκB, SN50; 100μg/ml] or EGTA [1.5mM] for 8 hours or 16 hours. The

concentrations of chemical inhibitors were determined empirically by titration as described previously [325-327]. At the concentrations and time used, the inhibitors showed no adverse effect on cell viability when stained with 0.4% trypan blue dye. For HeLa COX-1 Tet-off experiments, 2 x 10^5 HeLa COX-1 Tet-off cells were incubated in 3cm dishes in the absence (to allow for induction of COX-1 transgene expression) or presence (to switch off transgene expression) of 2µg/ml Doxycyline (DOX; Sigma) in serum free DMEM for 24, 48 and 72 hours. Cells were supplemented daily with DOX. RNA was extracted individually from each set of experiment by using Trizol (Sigma) following the manufacturer's guidelines and concentrations and purity of the RNA determined as described in section 2.8. cDNA was synthesized from total mRNA (200ng) by reverse transcription as described in section 2.9. Fold increase was calculated by dividing the values obtained from the SP only/SP plus inhibitor treatments by the vehicle only/vehicle plus inhibitor treatments. All in vitro cell culture experiments were carried out in duplicate.

5.3.5 Quantitative real time PCR reaction

Quantitative real time PCR was performed to determine the expression of CD4 and CCR5 in normal and neoplastic cervical tissue explant, assess the expression of CD4, CCR5, CCR2b, CXCR4, CXCR6 and GPR1 on SP-treated HeLa cells, and determine the effects of chemical inhibitors on SP-mediated induction of CCR5 in HeLa cells. RNA was extracted from neoplastic cervical tissue (squamous cell carcinoma Ca.1 - Ca.7), normal cervix (N1-N5), and HeLa cells using Trizol reagent as per the manufacturers' instruction and reversed transcribed as described in section 2.9. qRT-PCR reaction was carried out on an Illumina ECO^{TM} quantitative RT-PCR

machine and detected using SYBR green (Bioline, Celtic Molecular, Cape Town, South Africa) incorporation during PCR reaction as described in section 2.10.3. All relative expressions were calculated using the comparative C_t (section 2.10.5) relative to an endogenous control of HeLa cell cDNA included in each experiment. Data are presented as fold increase as determined by dividing the relative expression of SP treated group by that of the control. Data are presented as mean ± SEM.

5.3.6 SDS-PAGE and Western blot analysis

Immunoblot analysis was performed as described in section 2.14. Immunoblots were incubated in 10mL of blocking buffer (PBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) on a shaker for 1 hour at room temperature (RT), after which membranes were washed three times for 10 minutes each with 15mL PBS, 0.1% Tween-20 followed by an overnight incubation with specific β-actin, CCR5, CD4 or COX-1 primary antibody (1:400 dilution) in 5% BSA at 4 ^{0}C with gentle shaking. Thereafter, membranes were washed three times for 10 minutes each with 15mL of PBS, 0.1% Tween-20, incubated for 1 hour with HRP-conjugated secondary antibody (1:5000 dilution) at RT with gentle shaking and washed again three times with 15mL PBS, 0.1% Tween-20. Protein detection was conducted as described in section 2.14. Densitometry on visualized protein bands was done using a UVP Biospectrum 500 Imaging system (UVP, Scientific Group, Cape Town, South Africa). Fold induction was determined in SP-treated cells or HeLa COX-1 Tet-off cells incubated without DOX relative to control-treated cells or HeLa COX-1 Tet-off cells incubated with DOX, after normalizing to β-actin, by dividing the expression in the treated group (HeLa COX-1 Tet-off minus DOX) by the expression in the

control group (HeLa COX-1 Tet-off plus DOX). Data are presented as mean ± SEM from four independent experiments.

5.3.7 Immunohistochemistry

Immunohistochemistry was done on archival cervical blocks (Normal n=4, squamous cell carcinoma n=5 and adenocarcinoma n=5) obtained from the Department of Anatomical Pathology, University of Cape Town. Sections were deparaffinized, rehydrated and antigen retrieval done as described in section 2.16. Blocking for endogenous peroxidase was done as described earlier (section 2.16). Sections were blocked using 5% normal donkey/goat serum diluted in TBS after which tissue sections were incubated with polyclonal goat anti-CCR5 (1:200), mouse anti-CD4 (1:200) or rabbit anti-neutrophil elastase (1:400) antibodies at 4 ^{0}C for 18 hours. After incubation, tissue sections were then washed in TBS twice for 5 minutes each followed by incubation with biotinylated donkey anti-goat or goat anti-mouse secondary IgG antibody at dilution of 1:500 at RT for 30 minutes or donkey anti-rabbit Cy3 at dilution of 1:1000 for neutrophil elastase at RT for 60 minutes. Tissue sections with the CCR5 and CD4 antibodies were then further incubated with streptavidin-biotin peroxidase complex (1:50) at RT for 30 minutes. Controls were incubated with biotinylated IgG secondary antibody only. Color reaction was developed by incubating with 3.3'-diaminobenzidine. Tissue sections were counterstained in aqueous hematoxylin, before mounting and coverslipping. For sections incubated with neutrophil elastase/Cy3, nuclei were counterstained with Dapi (Santa Cruz). Fluorescent images were visualized and photographed using a Carl Zeiss laser scanning microscope LSM 510 (Jena, Germany).

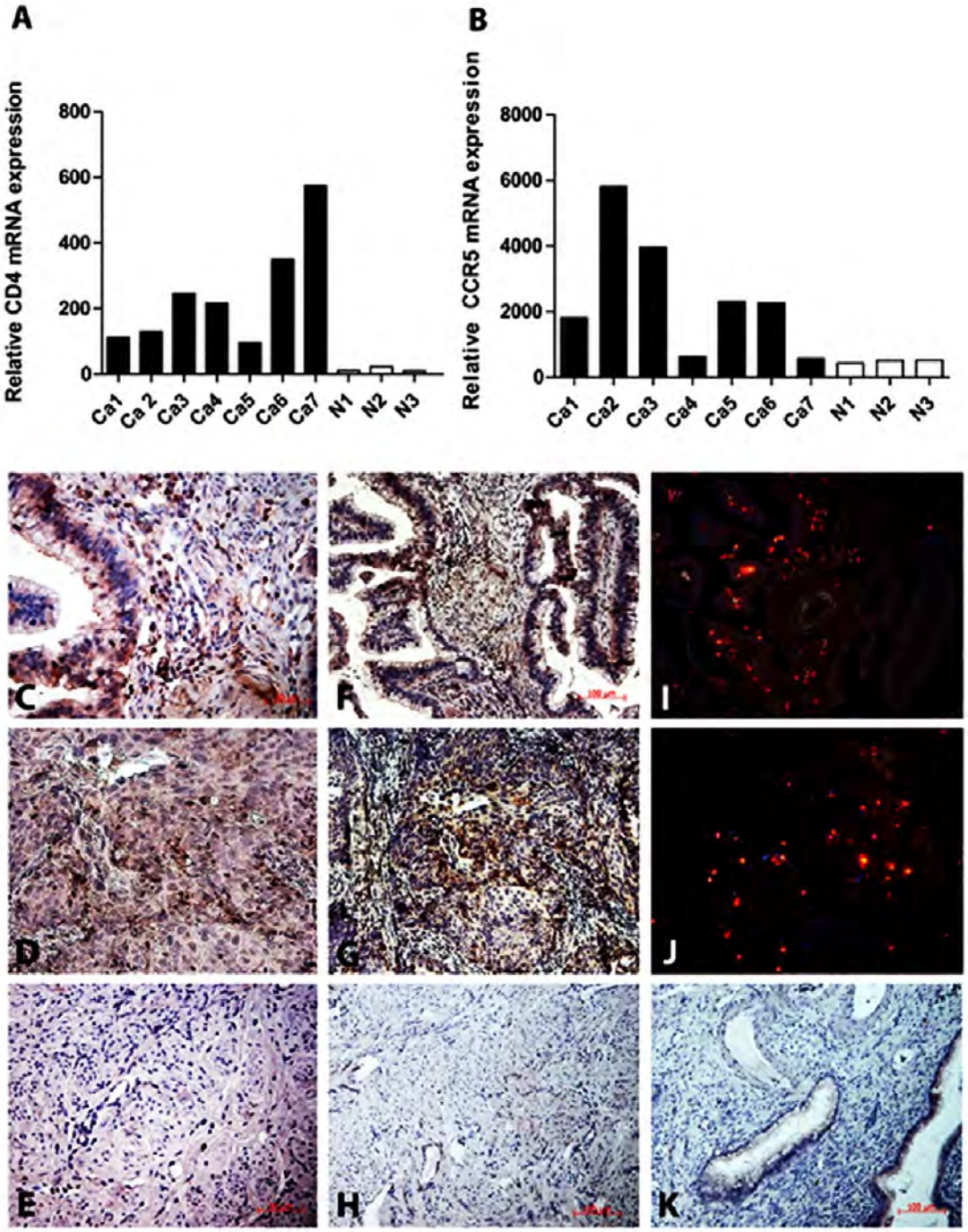
5.3.8 Statistical Analysis

The data in this study was analyzed by t-test or one-way ANOVA using Graph Pad Prism 5.0c (Graph Pad, San Diego, CA). Paired T-Tests were conducted on the untransformed means of the replicates between SP and control or inhibitor and inhibitor and SP or HeLa COX-1 Tet-off cells incubated in the absence vs presence of DOX for each experiment. Unpaired T-Tests were conducted on SP versus SP and inhibitor after conversion to fold/percentage increase. One way ANOVA was used as an additional test to determine significant difference between the various time points for CD4, CCR5, CCR2b, CXCR4, CXCR6 and GPR1 by real-time PCR and/or Western blot analysis in response to SP treatment.

5.4 Results

5.4.1 CD4 and CCR5 expression is elevated in cervical cancer tissue

Quantitative RT-PCR analysis revealed elevated expression of CD4 (Fig. 5.1A) and CCR5 (Fig. 5.1B) in all cervical cancer biopsies (Ca1-Ca7) compared with normal cervical tissues (N1-N3). Using serial sections, the site of CD4 (Figure 5.1C and 5.1D) and CCR5 (Figure 5.1F and 5.1G) expression was localized to the neoplastic epithelial cells in cervical adenocarcinomas (Figure 5.1C and 5.1F) and squamous cell carcinomas (Figure 5.1D and 5.1G) with strong immunoreactivity in immune cells adjacent to the epithelium, in the stromal compartment. Using a specific antibody for leukocyte neutrophil elastase, I confirmed that the immune cells strongly expressing CD4 and CCR5 in the cervical adenocarcinoma (Figure 5.1I) and squamous cell carcinomas (Fig. 5.1J) were infiltrating leukocytes. In contrast to the strong immunoreactive staining for CD4 and CCR5 observed in the cancer tissues, I observed minimal immunoreactivity for CD4 and CCR5 in normal cervix (Figure 5.1E and 5.1H respectively). I also did not detect any infiltrating leukocytes in normal cervical tissues by immunohistochemistry and confocal microscopy. Analysis/quantification of immunohistochemical stains revealed a significant expression of CD4 and CCR5 in both adenocarcinoma and squamous cell carcinoma tissue compared to normal cervix (Figure 5.1L; $P < 0.05$, respectively). The high levels of CD4 and CCR5 expression in the cancer tissues in the same cellular compartment as elevated IL-1α, COX-1 and COX-2 reported in an earlier study conducted in our laboratory [312], suggest that they could be regulated by inflammatory pathways in cervical epithelial cells.


A
Relative CD4 mRNA expression
800
600
400
200
0
Ca1
Ca 2
Ca3
Ca4
Ca5
Ca6
Ca7
N1
N2
N3
B
Relative CCR5 mRNA expression
8000
6000
4000
2000
0
Ca1
Ca2
Ca3
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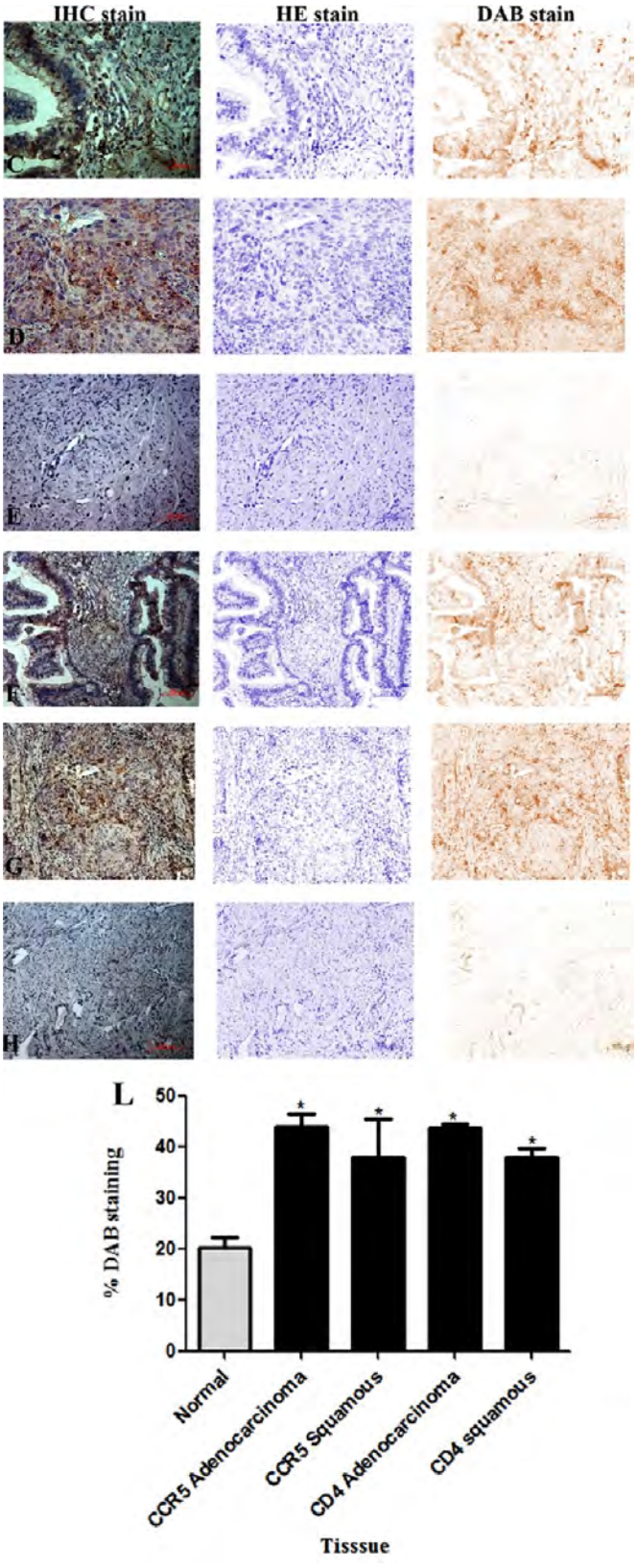

Figure 5.1: Expression and localization of CD4 and CCR5 in cervical tissues. CD4 (A) and CCR5 (B) mRNA expression in cervical cancer (Ca1-Ca7; n=7) and normal cervical tissues (N1-

N3; n=3) as determined by quantitative real time PCR analysis. Localization of CD4 (C, D, E), CCR5 (F, G, H) and neutrophil elastase (I, J) expression was determined in cervical adenocarcinoma (C, F, I), squamous cell carcinoma (D, G, J) and normal cervix (E, H) by immunohistochemistry. A representative section of de-convoluted (HE and DAB) IHC stain is shown for each. Controls incubated with IgG from host species (K; representative section shown) were negative for immunoreactivity. % DAB stain for CD4 and CCR5 in normal, squamous and adenocarcinoma tissues (L). Scale bar, 100μm. Data represented as mean ± SEM. *, represent significance at $P < 0.05$.

5.4.2 SP regulates expression of CD4 and CCR5 in the cervix

SP has been shown to induce inflammatory and tumorigenic pathways in the vagina [315] and cervix [287,346,350]. I investigated whether SP could regulate expression of CD4 and CCR5, the main receptor-co-receptor pair involved in HIV infection, in the cervical mucosa using heterogeneous cervical epithelial cells (cytobrush samples), neoplastic and normal cervical tissue explants. Samples were treated for 24 hours with vehicle or 1:50 dilution of SP and expression of CD4 and CCR5 determined by qRT-PCR. SP was found to significantly elevate the expression of CD4 (Figure 5.2A; 5.2C and 5.2E; $P < 0.05$) and CCR5 (Figure 5.2B; $P<0.01$, 5.2D; $P < 0.01$, and 5.2F; $P < 0.05$) in the cervical specimens obtained from the cervical mucosa by cytobrush sampling (Figure 5.2A and 5.2B), normal cervical biopsy (Figure 5.2C and 5.2D), and cervical cancer biopsies (Figure 5.2E and 5.2F) compared with vehicle treated controls.

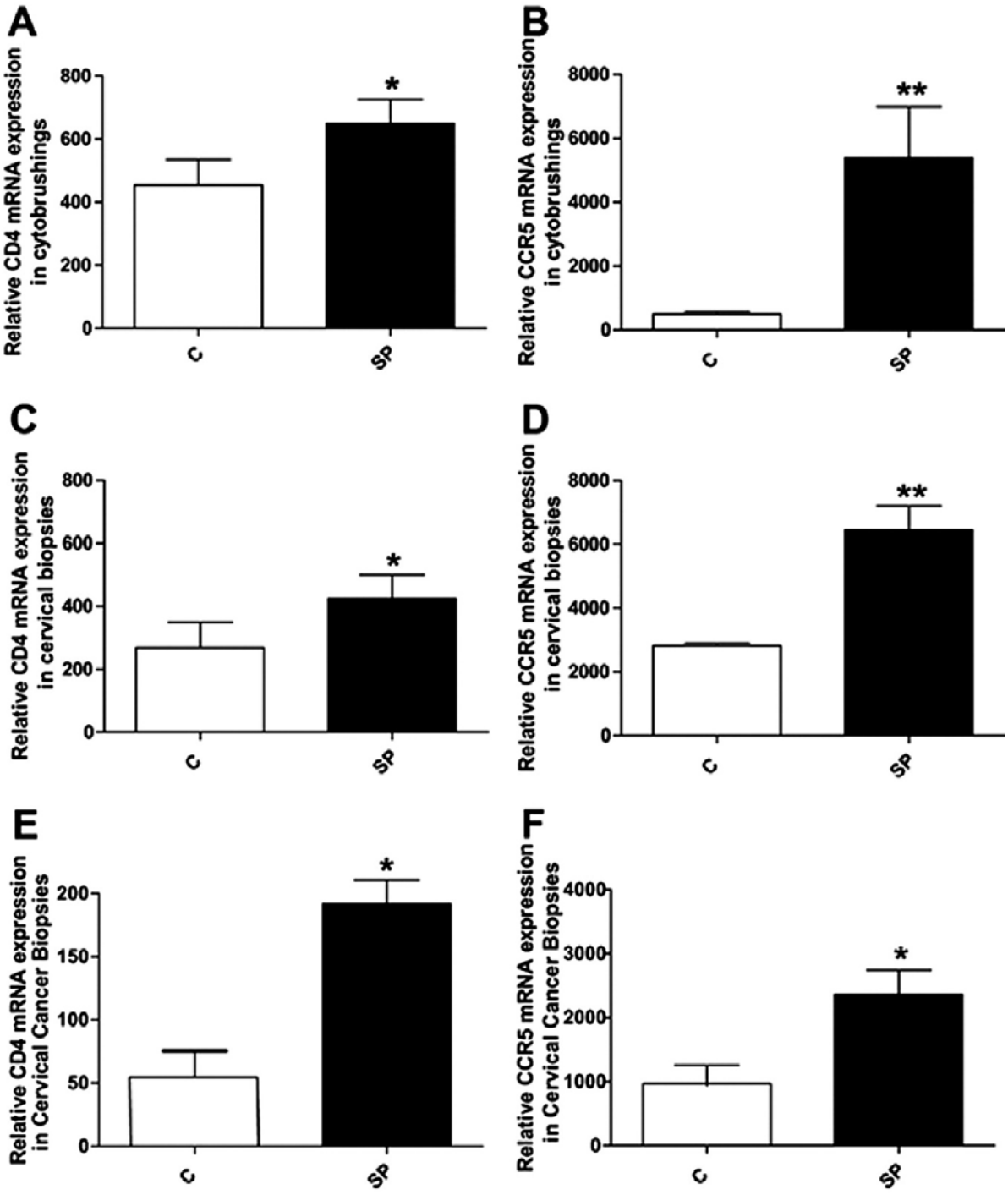

Figure 5.2: Seminal plasma induces expression of CD4 and CCR5 in the cervix. CD4 (A, C, E) and CCR5 (B, D, F) mRNA expression was determined by qRT-PCR analysis of cervical tissues obtained from the cervix with a cervical cytobrush (A and B), normal cervical biopsy from the ectocervix-transformational zone (C and D), cervical cancer biopsy (E and F). Cervical

cytobrush samples (n=20) and biopsies (n=5) were treated with control (PBS) or 1:50 dilution of seminal plasma for 24 hours. Data are presented as mean ± SEM. *, ** indicate significance at $P<0.05$ and $P<0.01$ respectively.

5.4.3 SP regulates the expression of HIV chemokine co-receptors in HeLa neoplastic cervical epithelial cells

The role of SP in the regulation of pro-inflammatory chemokines and their receptors within the epithelial and stroma compartments of the cervix after coitus has been demonstrated in vivo [285]. Here, I hypothesized that SP can regulate HIV chemokine co-receptors expression in neoplastic cervical epithelial cells in vitro, using a well-established HeLa (adenocarcinoma) cell line as a model system. HeLa cells were treated with vehicle or SP (1:50 dilution) for 4, 8, 16, and 24 hours and the expression of CD4, CCR5, CCR2b, CXCR4, CXCR6 and GPR1 mRNA expression assessed using qRT-PCR (Figure 5.3A, 5.3B, 5.3C, 5.3D, 5.3E, and 5.3F). SP significantly induces the expression of CD4, CCR5, and CCR2b mRNA at all-time points investigated (Figure 5.3A, B, and C, respectively; $P < 0.01$). SP treatment of HeLa cells significantly induce CXCR4 expression after 24 hours, indicating that longer exposure of neoplastic epithelial cells is required to induce CXCR4 (Figure 5.3D; $P < 0.01$). This is in agreement with prior result from the Taqman array, where no significant expression of CXCR4 was observed after 8 hours treatment with SP. CXCR6 expression was found to be increased significantly in a biphasic manner at 4 hours ($P < 0.01$) and 24 hours ($P < 0.05$), with minimal expression observed at 8 and 16 hours after SP treatment (Figure 5.3E). I found no increase in GPR1 mRNA expression in response to SP treatment (Figure 5.3F). These results suggest that

SP-mediated inflammation can regulate the expression of HIV chemokine co-receptors in neoplastic cervical epithelial cells.

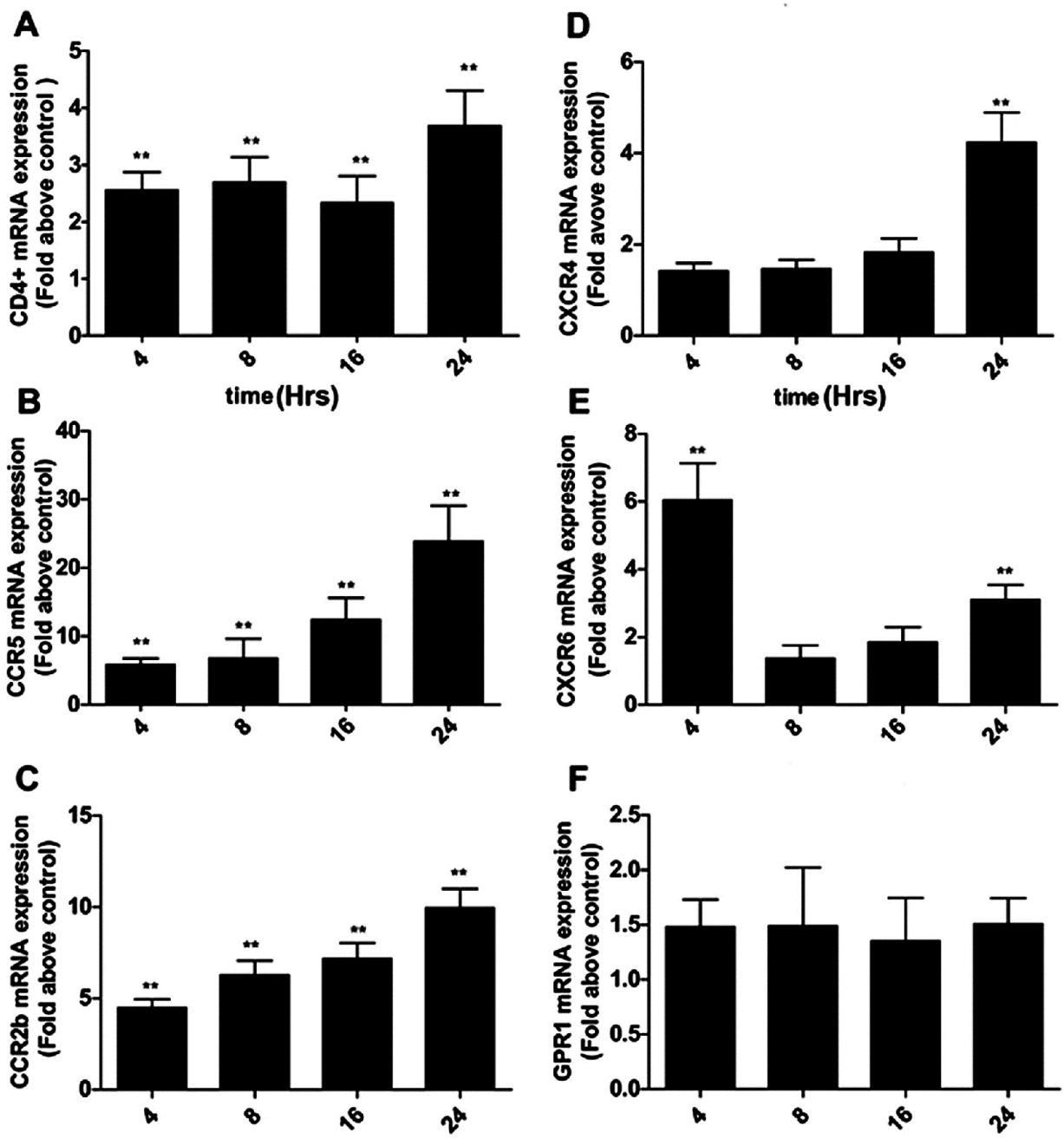

Figure 5.3: Seminal plasma induces expression of HIV chemokine co-receptors in HeLa cells. (A) CD4, (B) CCR5, (C) CCR2b, (D) CXCR4, (E) CXCR6 and (F) GPR1 mRNA expression as determined by qRT-PCR analysis. HeLa cells were treated with control (PBS) or 1:50 dilution of

seminal plasma for 4, 8, 16 or 24 hours. Data are presented as mean ± SEM from 8 individual experiments. ** indicates significance at $P < 0.01$.

5.4.4 SP regulation of CD4 and CCR5 expression in the cervix is dependent on individual semen donor.

In the present study, prior experiments describing SP regulation of CD4 and CCR5 in HeLa cells, neoplastic and normal cervical tissue (Figure 5.2 and 5.3) was conducted with pooled SP obtained from 10 healthy individual donors. To assess whether SP-mediated regulation of CD4 and CCR5 in the cervix was donor-dependent, I analyzed the effect of SP from individual donors on CD4 and CCR5 mRNA expression in HeLa cells. Results as shown in figure 5.4 A and B revealed that SP from all ejaculates (SP1-SP5; spermatozoa removed by centrifugation, SP6spr and SP7spr; full ejaculates containing spermatozoa, SP8pv-SP10pv; ejaculates from vasectomized men i.e. no spermatozoa present) all increased CD4 and CCR5 expression in HeLa cells after 4 and 24 hours incubation, respectively with different efficacies (Figure 5.4 A and B). Thus suggesting that individual variation in SP potency can affect HIV susceptibility and transmission in sexually active women. All subsequent experiments were conducted using pooled SP.

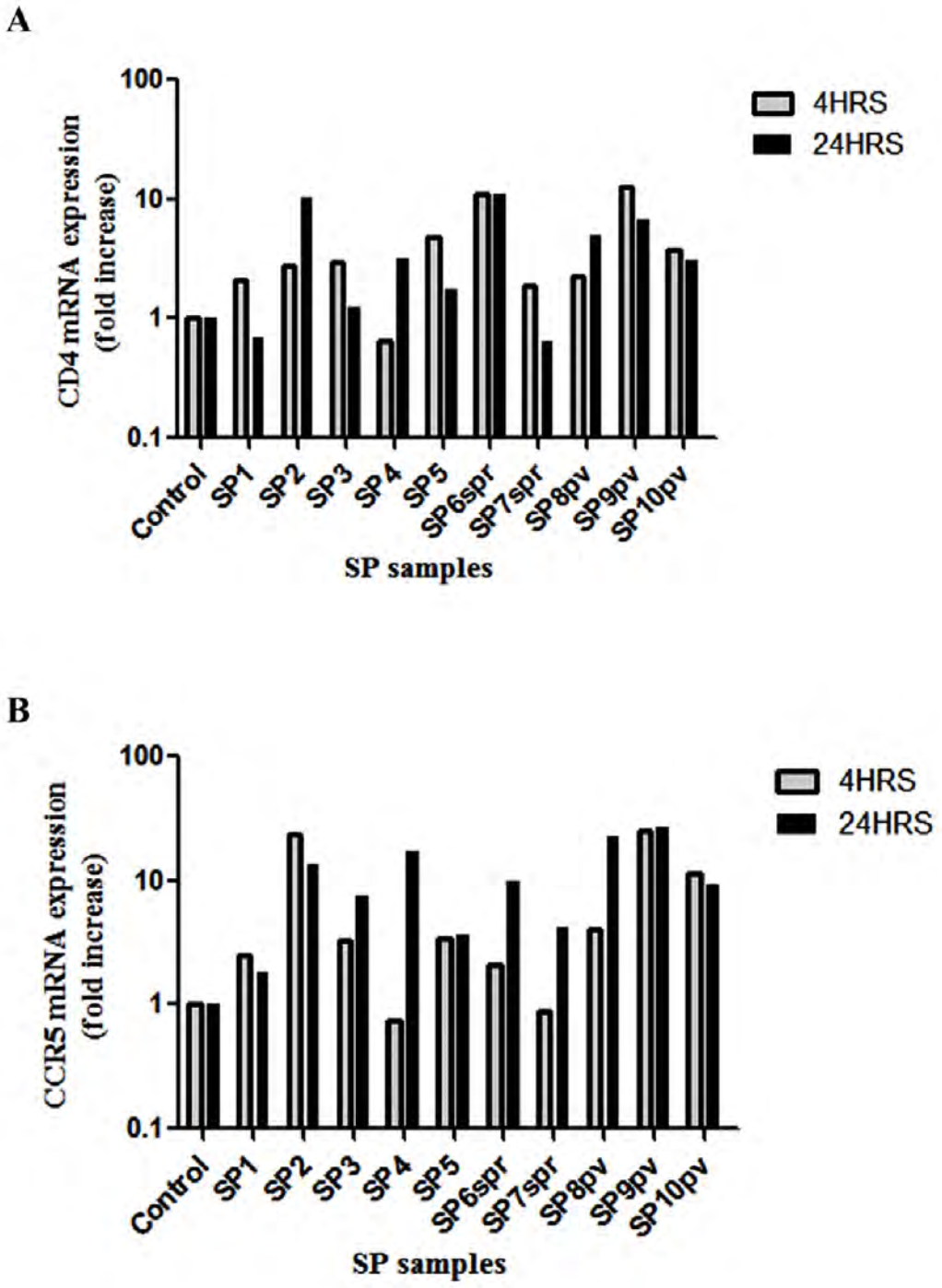

Figure 5.4: Relative expression of CD4 (A) and CCR5 (B) mRNA in HeLa cells treated with SP1-SP5, SP6spr, SP7spr, and SP8pv-SP10pv (1:50 dilution) relative to PBS (control) for 4 or 24 hrs as determined by qRT-PCR. Data are presented as fold above control

5.4.5 SP induces CCR5 protein expression of in HeLa cells

To further investigate protein expression of CD4 and CCR5 in response to SP treatment, Western blot analysis was carried out on HeLa cell lysates treated with SP for 8, 16, and 24 hours. I found no significant change in HeLa CD4 protein levels in response to SP treatment in contrast to observed fold increase (2.5, 2.2, and 3.5 fold increase at 8, 16, and 24 hours, respectively) in mRNA level expression (Figure 5.5A). CCR5 protein on the other hand was robustly induced at 8, 16 and 24 hours in HeLa cells treated with 1:50 dilution of SP, compared with control treated cells (Fig. 5.5B; $P < 0.05$), similar to my observations for mRNA.

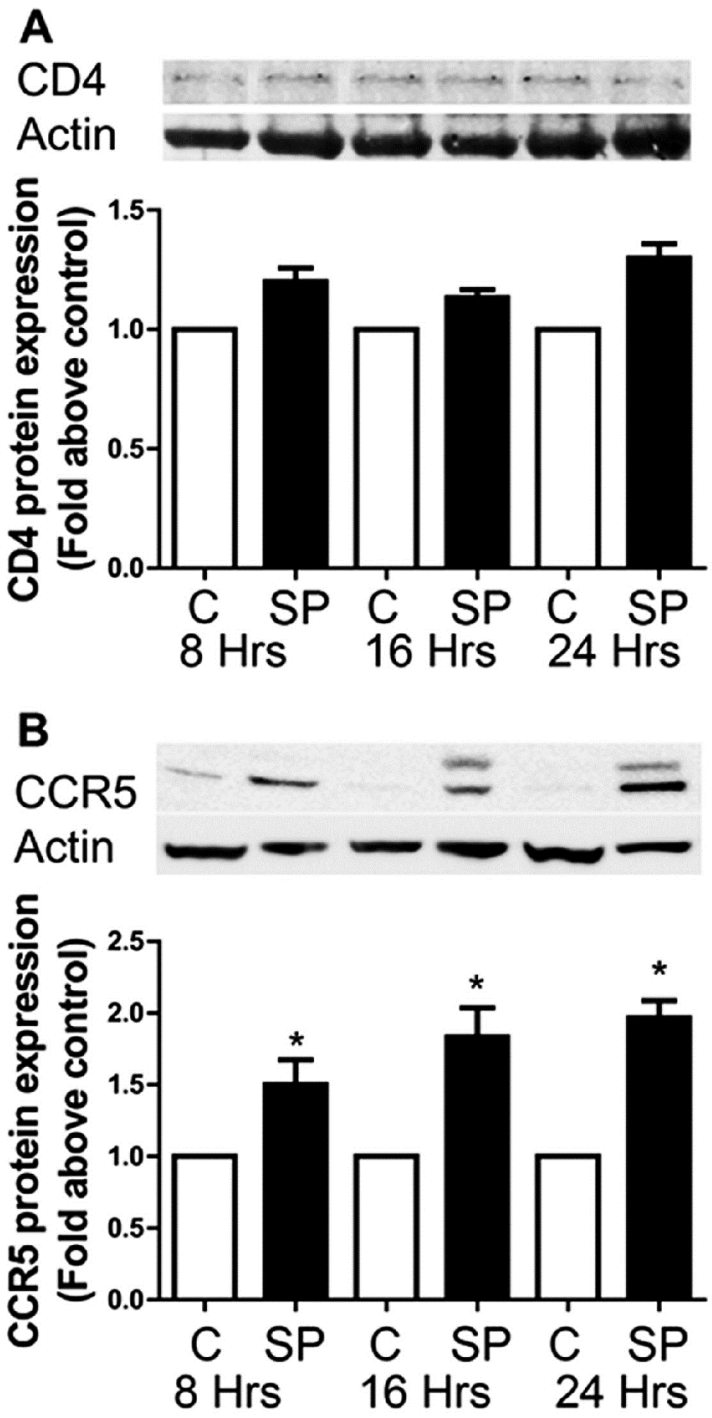

Figure 5.5: Seminal plasma induces expression of CCR5 protein in HeLa cells. (A) CD4 and (B) CCR5 protein expression as determined by Western blot analysis. HeLa cells were treated with control (PBS) or 1:50 dilution of seminal plasma for 8, 16 or 24 hours. Immunoblots were revealed and quantified as described above. A representative immunoblot is shown for each with quantification from 4 individual experiments shown beneath. Data are presented as mean ± SEM.* indicates significance at $P < 0.05$.

5.4.6 Seminal plasma regulates CCR5 expression in HeLa cells via the EGFR and COX-1 pathways

Having shown that SP regulates CCR5 expression next was to investigate the signal transduction pathways underlying SP-mediated induction of CCR5 expression, using a panel of small molecule chemical inhibitors of cell signaling. HeLa cells were treated with vehicle or chemical inhibitor or 1:50 dilution of SP or SP and inhibitor for 8 hours (mRNA) (Fig. 5.6A) or 16 hours (protein) (Fig. 5.6B). SP treatment of HeLa cells induced a 6.5 ± 0.8 fold increase in CCR5 mRNA (Fig. 5.6A; $P < 0.01$) and a 1.8 ± 0.5 fold increase in CCR5 protein (Fig. 5.6B; $P < 0.05$) expression respectively compared with control treated cells. Co-treatment of cells with SP and inhibitor of epidermal growth factor (EGF) receptor tyrosine kinase (AG1478) and COX-1 (SC560) significantly inhibited SP-mediated increase in CCR5 mRNA expression (Fig.5.6A; $P < 0.001$) and protein (Fig. 5.6B; $P < 0.001$) respectively. However, co-treatment of cells with SP and inhibitors of NFκB (SN50), extracellular signal-regulated kinase (PD98059), COX-2 (NS398) or calcium (EGTA) had no significant effect on SP-induced CCR5 mRNA or protein expression.

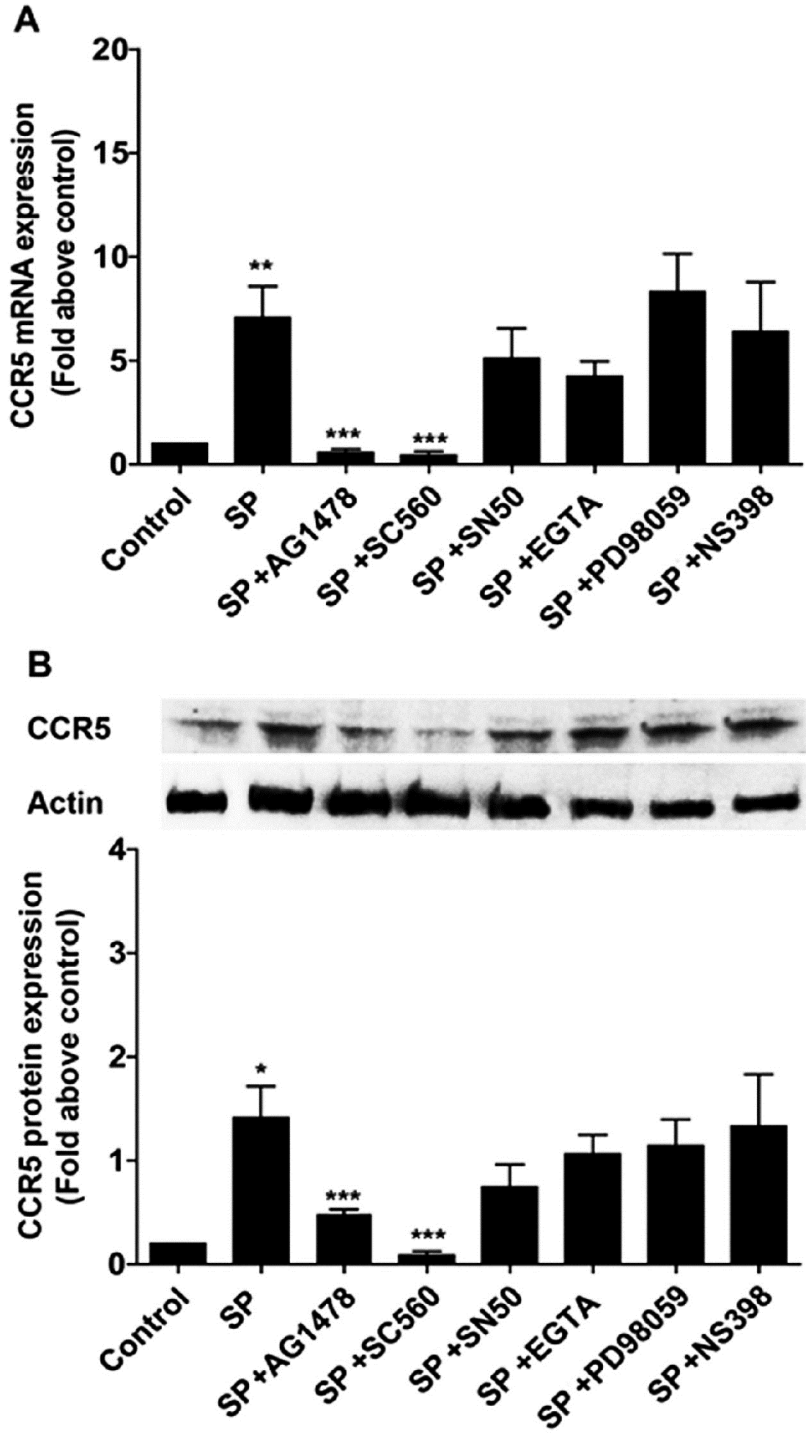

Figure 5.6: SP induces CCR5 expression in HeLa cells via the activation of EGFR and COX-1 pathways. (A) CCR5 mRNA and (B) CCR5 protein expression as determined by qRT-PCR and Western blot analysis respectively. HeLa cells were treated for 8 hours (A) or 16 hours (B) with SP (1:50) or vehicle (PBS) in the absence/presence of inhibitors of EGFR kinase [AG1478; 200nM], COX-1 [SC560; 10μM], NFκB [SN50; 100μg/ml], calcium [EGTA; 1.5mM], ERK1/2

kinase [PD98059; 50μM] or COX-2 [NS398; 10μM]. Data are represented as mean ± SEM from 4 independent experiments. *, **, *** indicate significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

5.4.7 EGF and PGE_2 induces the expression of CCR5 in HeLa cells

PGE_2 is abundant in SP [315,533]. Prior studies have shown a role for PGE_2 and the E-series prostaglandin receptors (EP_2 and EP_4 receptors) in mediating the effects of SP in cervical and endometrial cancer cells via transactivation of the EGFR [344,345]. Here I sought to determine whether EGF or PGE_2 could be the active fraction in SP which was responsible for mediating the elevation of CCR5 in HeLa cells. HeLa cells were treated with 10ng/mL human recombinant EGF (Figure 5.7A) or 300nM PGE_2 (Figure 5.7B) for 4, 8, 16 and 24 hours and subjected the cDNA to quantitative RT-PCR analysis. EGF treatment of HeLa cells was found to marginally increase CCR5 expression only at 16 hours of stimulation (Figure 5.7A; $P < 0.05$), however PGE_2 treatment rapidly and significantly elevated expression of CCR5 at 4 and 8 hours of stimulation (Figure 5.7B; $P < 0.05$). Furthermore, I found that PGE_2-mediated CCR5 increase occurred via the transactivation of the EGFR, since treatment of HeLa cells with the EGFR tyrosine kinase inhibitor AG1478 inhibited the PGE_2-induction of CCR5 (Figure 5.7C; $P < 0.05$). These data suggest that the SP-mediated induction of HIV chemokine co-receptors in the cervix is mediated via the inflammatory PGE_2-E-series PG receptor axis.

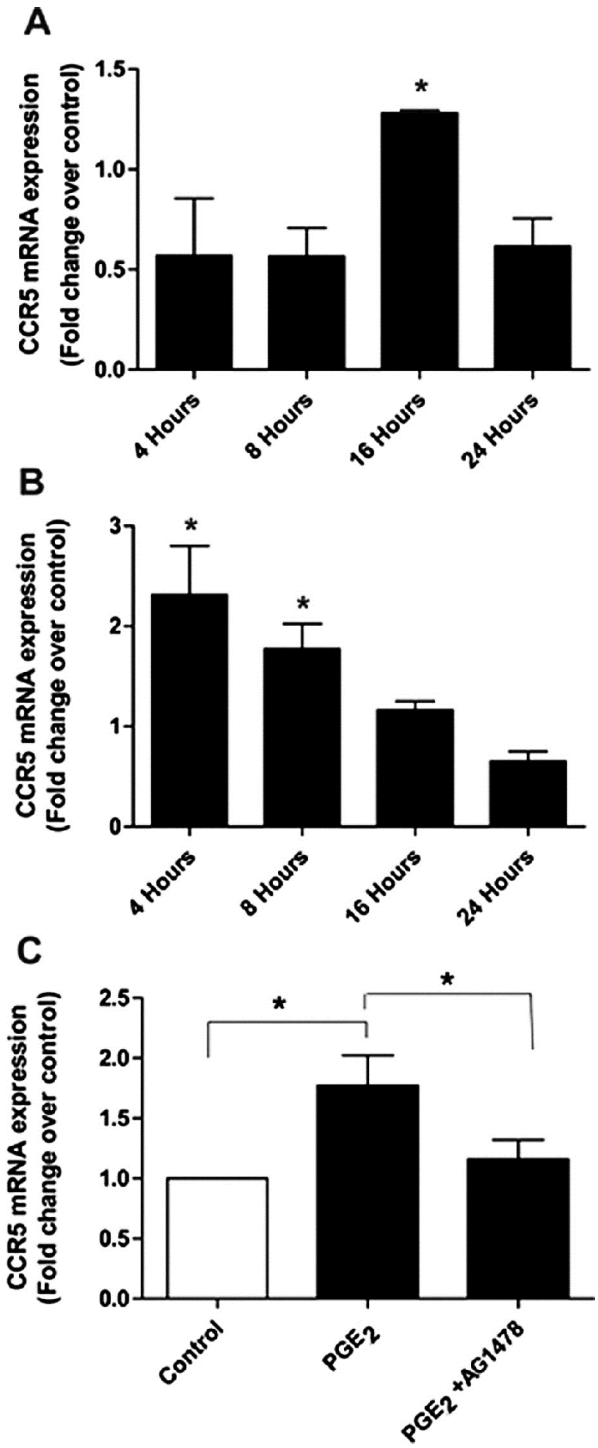

Figure 5.7: Regulation of CCR5 mRNA expression by EGF and PGE_2 in HeLa cells. CCR5 mRNA expression in HeLa cells treated for 4, 8, 16 or 24 hours with vehicle or 10ng/mL recombinant EGF (A) or 300nM PGE_2 (B). CCR5 mRNA expression in HeLa cells treated for 8 hours with vehicle or 300nM PGE_2 in the absence or presence of the EGFR tyrosine kinase inhibitor AG1478 (C). mRNA expression was determined by qRT-PCR analysis. Data are presented as mean ± SEM from 4 independent experiments done in duplicate.

5.4.8 Expression of CCR5 in HeLa cells can be regulated by an inducible COX-1 enzyme

Having shown that PGE_2 could induce CCR5 expression to similar levels observed for SP. Next was to confirm that the COX-1-PGE_2 axis can regulate expression of CCR5, using a doxycycline (DOX)-inducible expression system in HeLa cells (COX-1 Tet-off system) [312]. In HeLaCOX-1 Tet-off cells, the COX-1 transgene is under the control of a DOX-inducible promoter, which contains a tetracycline (DOX) response element (TRE) upstream of the immediate early promoter of cytomegalovirus (as outlined schematically in Figure 5.8A). When HeLa COX-1 Tet-off cells are maintained in culture medium containing 2μg/mL DOX, the tetracycline transactivator protein (TetR/VP16) is prevented from binding to the TRE. Under these conditions, induction of the COX-1 transgene is prevented. Removal of DOX from the culture medium results in activation of the COX-1 transgene and biosynthesis of PGE_2 [312]. HeLa COX-1 Tet-off cells were cultured in serum free medium with or without DOX for 24, 48 or 72 hours. Removal of DOX from the culture medium induced a time dependent increase in COX-1 mRNA and protein levels (Figure 5.8B) reaching a maximum after 72 hours (3.8 ± 0.7 and 4.5 ± 0.8 fold increase in COX-1 mRNA and protein expression, respectively; Figure 5.8B; $P < 0.001$), consistent with previous study [312]. Coincident with the induction of COX-1 expression in HeLa cells, I observed a 3.8 ± 0.9 and 2.9 ± 0.8 fold increase in expression of CCR5 mRNA and protein respectively (Fig. 5.8C) in HeLa COX-1 Tet-off cells cultured in the absence of DOX for 72 hours, compared with control HeLa COX-1 Tet-off cells cultured in the presence of DOX for 72 hours, which had the COX-1 transgene switched off (Fig. 5.8C; $P < 0.01$).

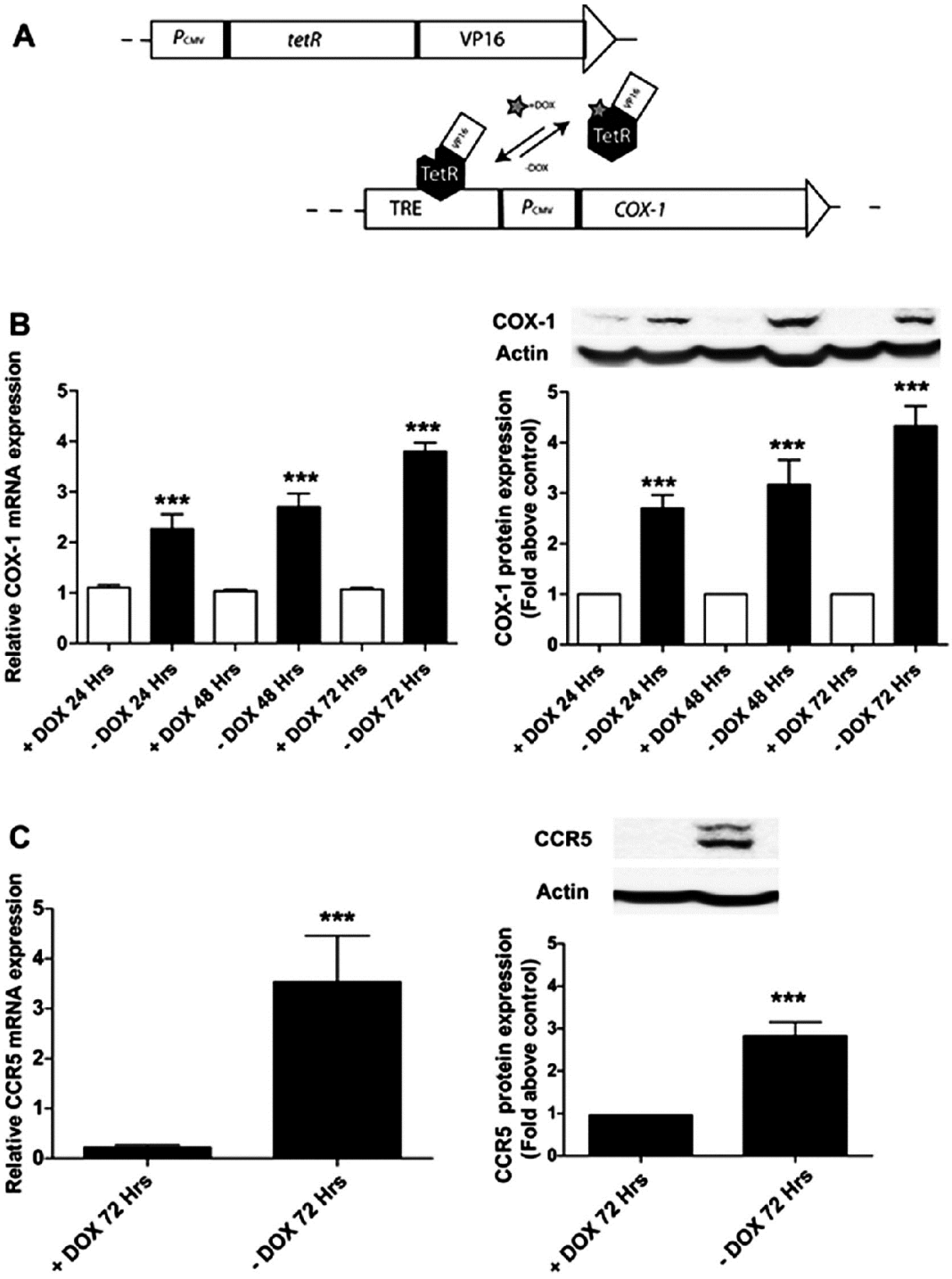

Figure 5.8: CCR5 expression is regulated by COX-1. (A) A schematic overview of the HeLa COX-1 Tet-off system. (B) COX-1 mRNA and protein expression in HeLa COX-1 Tet-off cells incubated in the presence or absence of DOX for 24, 48 or 72 hours. (C) CCR5 mRNA and protein expression in HeLa COX-1 Tet-off cells incubated in the presence or absence of DOX for 72 Hours. Data are represented as mean ± SEM from 4 independent experiments. *** indicates significance at $P < 0.001$ respectively.

5.5 Discussion

Cervical cancer displays all the hallmarks of a chronic inflammatory disease and over the past two decades, the COX-PG pathway has emerged as a central regulator of inflammatory and tumorigenic events [80,129]. In cervical cancers, the expression both COX enzymes (COX-1 and COX-2) is elevated [340,551], and they have been shown to mediate the induction of inflammatory chemokines, cytokines and angiogenic genes via the autocrine/paracrine regulation of PGE_2 and the E-series prostaglandin receptors [312] within the cancer microenvironment. Moreover COX-1 and COX 2 can be regulated by SP and inflammatory cytokines and PGs in a positive feedback manner [346,552]. In this study, the high levels of CD4 and CCR5 detected in cervical cancer tissues was found to be localized in the same cellular compartment in which high levels of IL-1α and COX-1 had previously been detected [312], raising the possibility that CD4 and CCR5 could be regulated by inflammatory pathways. In addition, these pro-inflammatory mediators COX-1 [313] and IL-1α have been shown to enhance HIV-1 transmission [541] and viral replication in vitro [542].

Recent studies have shown that in addition to endogenous regulation of inflammatory pathways by the COX-PG axis, SP and SP prostaglandins can directly regulate inflammatory and tumorigenic pathways in cervical [287,346,350] and vaginal cells [315]. Inflammation of the cervical mucosa is considered a significant risk factor for HIV infection, however the role of inflammatory mediators and SP on pathways involved in HIV infection in the cervix have yet to be fully elucidated. I hypothesized that SP and inflammatory pathways could regulate chemokine receptors and pathways with known roles in HIV infection, in cervical epithelial cells and cervical cancer.

I found that SP significantly elevated the expression of the HIV receptor CD4 and chemokine co-receptor CCR5 in heterogeneous cervical epithelial cells obtained by cytobrush sampling of the cervical os and both normal and neoplastic cervical tissue explants. Similarly, SP also elevated the expression of CD4, CCR5 and other chemokine receptors that have been shown in vitro, to mediate HIV infection such as CCR2b, CXCR4 and CXCR6 [253,254,553] in neoplastic cervical epithelial cells HeLa. Although pooled SP was used in the present study, I show that though the potency in CD4 and CCR5 induction varies between individual semen samples, all samples irrespective of whether they were whole ejaculates, centrifuged to remove sperm cells or ejaculate from vasectomized patients, with no detectable sperm cells, all had ability to induce CD4 and CCR5 expression in the cervix.

The epithelial compartment of several tissues in the human body, including the gastrointestinal tract, prostate and cervix have all been implicated in the uptake and transport of HIV to submucosal leukocytes [554,555]. Cervical epithelial cells can behave as viral reservoirs, to sequester and transfer virus to activated peripheral blood mononuclear cells in the submucosa [555-557]. Furthermore, several studies have shown that levels of CCR5 expression in cells positively correlate with HIV infectivity and levels of cellular activation in vivo [558-560]. It is therefore plausible that any mechanism that enhances CCR5 expression, or expression of other HIV co-receptors, could enhance HIV susceptibility. In sexually active women, this could be enhanced by exposure of the cervix to SP.

The SP-mediated regulation of CCR5 was further explored in more detail. In accordance with my observation for SP regulation of CCR5 mRNA expression, I found a similar robust increase

in CCR5 protein in HeLa cells in response to SP, indicating a good correlation between SP-mediated CCR5 mRNA and protein expression. I explored the molecular mechanism whereby SP regulates CCR5 expression using a panel of small molecule chemical inhibitors. I found that SP regulated CCR5 expression via the EGFR and COX-1 pathways. These pathways have been shown to regulate inflammatory and angiogenic genes in cervical and endometrial cancers and are regulated by SP and SP prostaglandins [344,345]. SP is rich in a variety of cytokines and inflammatory mediators, including epidermal growth factor (EGF), transforming growth factor beta (TGFβ) and PGE_2 [280,532,533,536,561]. In this study, I found that although EGF could modestly induce CCR5 expression, PGE_2 which is present at high concentrations in SP [315,533] robustly enhanced CCR5 expression to similar levels observed for SP. Recently the PGE_2 fraction of SP has been shown to be the main fraction responsible for inducing inflammatory gene expression in vaginal cells [315]. Furthermore, in this study, I confirmed the importance of the EGFR in mediating CCR5 expression, since both SP and PGE_2 induction of CCR5 could be abolished with the EGFR tyrosine kinase inhibitor AG1478. It is likely that SP and PGE_2 induces intracellular second messenger scaffolds to transactivate the EGFR in HeLa cells in a similar manner reported previously, via ligand-receptor activation of the E-series PG receptors (EP_2 and EP_4 receptors) [344,345].

To confirm the role of COX-1 in regulating CCR5 expression, I used an inducible COX-1 model system in HeLa COX-1 Tet-off cells [312]. I show here that induction of COX-1 induces expression of CCR5 in HeLa cells to a similar level observed for SP, indicating that activation of this inflammatory pathway can regulate CCR5 expression directly, and further confirms that it is an intermediate step in the SP-mediated induction of CCR5 in HeLa cells. Furthermore, inhibition of COX-1 with the COX-1 inhibitor SC560 or maintaining HeLa COX-1 Tet-off cells

in DOX, to maintain the transgene in its inactive state, abolished induction of CCR5 expression in HeLa cells. Inhibition of the inflammatory COX enzyme pathway over the past 2 decades has elicited significant attention in terms of therapeutic intervention strategy for a host of inflammatory diseases. My observations for the role of COX-1 in regulating HIV receptor expression, suggests that administration of non-steroidal anti-inflammatory drugs (NSAIDs) to suppress COX-enzyme expression in sexually active women might also suppress inflammatory pathways that regulate HIV receptor expression and susceptibility to HIV infection.

In conclusion, these data show for the first time a potential role for seminal plasma in regulating HIV chemokine co-receptors in cervical epithelial cells, via the inflammatory cyclooxygenase pathway. These findings implicate seminal plasma in the regulation of the mucosal epithelium in sexually active women and may enhance susceptibility to HIV infection.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion

In sub-Saharan Africa, cervical cancer is the most common cancer among women, and also the leading cause of cancer related deaths in this region [3]. Disease prevalence is gradually having a decline trend in the developed countries due to prophylactic vaccination and regular screening programs. However, in many countries in sub-Saharan Africa the incidence rate is on the rise because of inadequate screening programs and inaccessibility to medical intervention and vaccination [4]. As highlighted in section 1.2 the main causal factor for cervical cancer is infection of the cervix with HPV, present in seminal fluid [562] or infected skin during intercourse. HPV triggers the development of cervical neoplasia by its direct effects on epithelial cell DNA and subsequent dysregulation of intracellular signal transduction pathways [27,563]. However, the finding that only a subset of women infected with high-risk HPV will proceed to develop invasive cervical cancer pose the likelihood that other factors are involved in the development of malignant transformation. It has been reported that cervical inflammation, but not the actual diagnosis of a specific sexually transmitted infection is associated with the development of squamous intraepithelial lesions within the cervix [517].

Inflammation is a normal host response to tissue damage caused by infectious agents or other stimuli [513]. Most pathogenic infection are known to incite an acute inflammatory response that results in complete clearance of the irritants in suitable host, however inadequate resolution of inflammation and unabated inflammatory reactions can evoke chronic inflammation predisposing the host to various diseases including cancer. Chronic inflammation enhance tumor

initiation and progression by inducing DNA damage, enhancing neoplastic cell proliferation, and stimulating angiogenesis and tissue remodeling to mediate cell invasion and metastasis [104,564,565]. A main feature of chronic inflammation includes the predominance of inflammatory mediators or cytokines that amplify immune reactivity and presence of immune cells in the tissue. Cervical cancer is regarded as a chronic inflammatory disease [514] and exhibit all the hallmark of chronic inflammation. One of the major cytokines responsible for regulating inflammation is IL-1α [507], which I have found to be up-regulated in cervical cancers in the current study.

The association of HIV infection and cervical cancer has long been established [566,567]. It has been shown that in many areas with high incidence of cervical cancer, large numbers of women are also likely to be HIV infected, raising a theoretical possibility that HIV infection may increase the incidence of cervical cancer. Moreover, cervical cancer and premalignant cervical lesions may increase transmission and acquisition of HIV infection [5]. Mucosal inflammation of the female lower genital tract is regarded as an imperative factor favoring acquisition of HIV infection in receptive vaginal intercourse [543,544]. It has been demonstrated that chronic inflammation of the cervical mucosa as seen in cervical cancer increases the risk of HIV transmission [568]. The site of inflammation are enriched with HIV target immune cells and may have breaches in mucosal epithelium both of which facilitate HIV acquisition [293].

HIV infects cells by binding to receptors on the host cell surface. Initially, the viral cell surface glycoprotein gp120 attaches to host cells. This can occur via heparin sulphate proteoglycans [569]. The initial step in membrane fusion begins with binding of the viral envelope protein (Env, consisting of a trimer of gp120-gp41 heterodimers) to the CD4 cell surface protein and a chemokine co-receptor present on the host cell [570]. Most HIV-1 variants use CCR5 and

CXCR4 as the main co-receptor in vivo, however up to 12 other chemokine co-receptors for HIV infection have been identified in vitro [252,253].

Findings generated in this study confirm the up-regulation of CD4 and CCR5 in cervical cancer. Cells expressing this receptor-co-receptor complex CD4/CCR5 in cervical cancer were confirmed to be infiltrating immune cells. Hence, in women with cervical cancer, breach in cervical mucosal epithelium and chronic inflammation characterized by infiltration with HIV target immune cells would predispose them to greater risk of HIV infection. This in keeping with findings by Gichangi et al. (2003) where higher incidence of HIV infection was reported in women with cervical cancer as compared to women of the same age group with uterine fibroids [5]. This was associated with younger age at presentation, poor histological differentiation of the tumor, accelerated clinical progression of premalignant cervical lesions and poorer prognosis.

Little is known about the association between HIV and HPV-mediated cervical neoplasia. However, the natural course of HPV is altered in the presence of HIV infection [571]. For example, HIV mediated immunosuppression can facilitate inadequate clearance of HPV in infected individuals, enhancing HPV persistence or re-infection, and increases the likelihood that premalignant lesions will develop into cancer. Furthermore, HIV may modify HPV related carcinogenesis by altering the expression of inflammatory components (cytokines) in the cervix and diminution of local cervical cellular immunity, thus altering HPV regulation [249]. In addition, HIV *Tat* gene has been shown to activate the early and late HPV genes leading to increased virulence of HPV [27]. Taken together, this suggests that in women with cervical cancer co-infected with HIV, HIV could act via similar manner to mediate HPV virulence leading to advanced disease and poor prognosis.

Cervical inflammation can be mediated by a variety of effectors, including exposure to SP [572]. In addition to being the primary means of transport and survival medium for the mammalian spermatozoa [280,451] or the major dissemination vector of HIV [294,573], seminal plasma contains arrays of antigenically distinct signaling molecules [343]. When these signaling molecules are introduced in to the female reproductive tracts, they initiate local cellular and molecular changes typical of an inflammatory response [280]. During inflammation, a wide array of intracellular signaling pathways are often activated leading to the expression of cell surface receptors, kinases, transcription factors and pro-inflammatory genes involved in malignant transformation [513].

Using TaqMan-Array 96-well plate, this study initially confirmed the role of SP in the regulation of arrays of inflammatory pathways, cytokines and chemokines in neoplastic cervical epithelial cells. SP was found to significantly regulate COX-1-PGs, LTC_4S-CysLTs-CysLT1 and LTB_4-BLT1 signaling pathways in HeLa cells. It is well documented that altered expression/activation of cyclooxygenase (COX) and lipooxygenase (LOX) enzymes is a common feature of several epithelial-derived malignancies where they mediate the induction of inflammatory chemokines, cytokines and angiogenic genes leading to cell proliferation, inhibition of apoptosis, and poor prognosis [389,390]. Hence, this study suggests that in sexually active women with cervical pathology, incessant exposure to SP can confer poorer prognosis consequent of SP-mediated activation of eicosanoid signaling pathways. In addition, SP-mediated activation of TLR-MyD88-NFκB signaling suggests that SP can transcriptionally regulate the expression of pro-inflammatory mediators within the cervical milieu [513]. Upon nuclear localization, NFκB encodes genes such as pro-inflammatory chemokines/cytokines [574] that are involved in inflammation associated carcinogenesis.

More so, I show that SP activated EP_2-EGFR-PI3K-Akt and EGFR-COX-1 signaling pathways up-regulate in particular the expression of pleotropic pro-inflammatory cytokine IL-1α and HIV chemokine co-receptor CCR5, respectively in neoplastic cervical cells exacerbating the already up-regulated levels of these inflammatory mediators within the cancer microenvironment. Furthermore, this study suggests that PGE_2 and EGF could be the active component in SP mediating inflammatory cytokines and chemokines expression in the cervix.

Interleukin 1α is a pro-inflammatory cytokine that possess a wide range of inflammatory and tumorigenic properties [101,503,504]. In addition to its tumorigenic effects, IL-1α-mediated NFκB activation has been shown to enhance HIV transmission [541] and viral replication in vitro [542]. Similarly, studies have shown that levels of CCR5 expression in cells positively correlate with HIV infectivity and levels of cellular activation in vivo [558-560]. Taken together, these findings suggest that in women with pre-invasive or invasive cervical cancer, repeated exposure to SP can exacerbate the overall inflammatory process within the cancer microenvironment consequent of IL-1α expression. In these women, IL-1α-mediated inflammatory response can alter local tissue remodeling to facilitate immune cell extravasation and trafficking. This can lead to increase in inflammatory and/or immune cell infiltrates and can alter receptor expression of epithelial cells, including CD4 and CCR5 to further increase their risk of HIV acquisition. Aggravated inflammatory process in the presence of HIV can then further augment the course of cervical neoplasia conferring poorer prognosis (Figure 6.1).

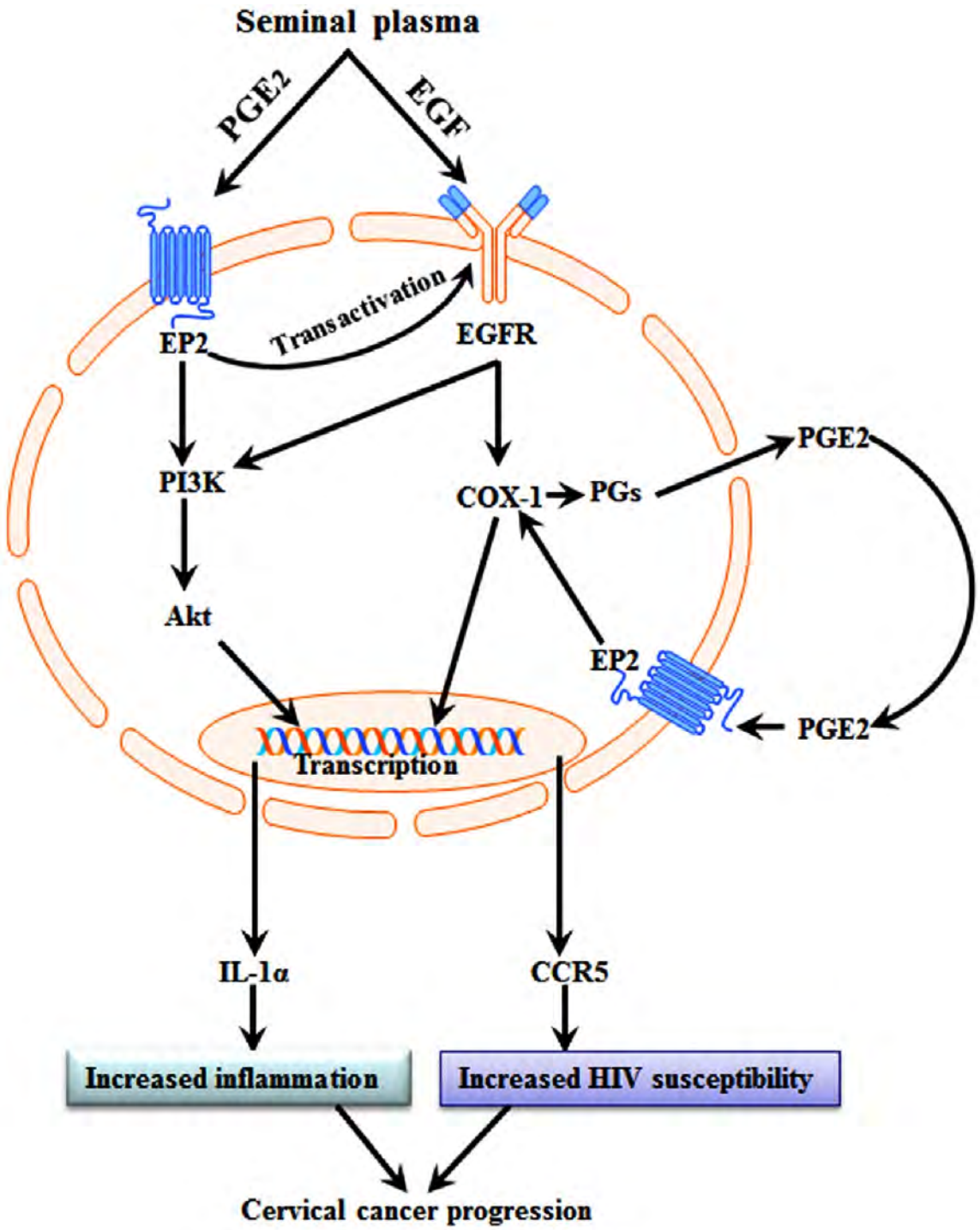

Figure 6.1: Schematic diagram showing the role of seminal plasma in the regulation of IL-1α and CCR5 in neoplastic cervical epithelial cells and their role in cervical cancer progression.

6.2 Conclusion

From the findings generated in this study, it can be concluded that SP regulates the activation of arrays of inflammatory pathways in neoplastic cervical epithelial cells. Induction of these inflammatory pathways plays a crucial role in cervical inflammation and cancer progression by regulating the expression of a pleiotropic cancer associated pro-inflammatory cytokine IL-1α. Moreover, this study demonstrates that SP-mediated inflammatory response could enhance HIV transmission in women with cervical cancer by inducing the expression of HIV chemokine co-receptor CCR5 in the cervix.

Taken together, this study suggests that repeated exposure of neoplastic cervical epithelium to seminal plasma as seen in sexually active women with cervical cancer could mediate advanced disease and poorer patient outlook consequent of its molecular effect on inflammatory pathway activation, pro-inflammatory cytokine expression, and promotion of HIV infection in the cervical cancer milieu.

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APPENDIX I

Inflammatory pathways and genes regulated by seminal plasma in HeLa cells.

Accession number	Gene	Gene description	Fold change
NM_000014	*A2M*	Alpha-2-macroglobulin	ND
NM_000684	*ADRB1*	Adrenergic, beta-1-, receptor	1.50
NM_000024	*ADRB2*	Adrenergic, beta-2-, receptor, surface	0.64
NM_000697	*ALOX12*	Arachidonate 12-lipoxygenase	ND
NM_000698	*ALOX5*	Arachidonate 5-lipoxygenase	0.88
NM_000700	*ANXA1*	Annexin A1	0.15
NM_005139	*ANXA3*	Annexin A3	0.29
NM_001154	*ANXA5*	Annexin A5	0.00031
NM_001030047	*KLK3*	Kallikrein-related peptidase 3	34.58*
NM_000710	*BDKRB1*	Bradykinin receptor B1	22.39*
NM_000623	*BDKRB2*	Bradykinin receptor B2	1.63
NM_000719	*CACNA1C*	Calcium channel, voltage-dependent, L type, alpha 1C subunit	ND
NM_000720	*CACNA1D*	Calcium channel, voltage-dependent, L type, alpha 1D subunit	ND
NM_000722	*CACNA2D1*	Calcium channel, voltage-dependent, alpha 2/delta subunit 1	0.73
NM_000724	*CACNB2*	Calcium channel, voltage-dependent, beta 2 subunit	0.28
NM_000726	*CACNB4*	Calcium channel, voltage-dependent, beta 4 subunit	0.51
NM_033292	*CASP1*	Caspase 1, apoptosis-related cysteine peptidase	0.66
NM_001250	*CD40*	CD40 molecule, TNF receptor superfamily member 5	ND
NM_000074	*CD40LG*	CD40 ligand (TNF superfamily, member 5, hyper-IgM syndrome)	18.69*
NM_001266	*CES1*	Carboxylesterase 1 (monocyte/macrophage serine esterase 1)	3.97*

NM_001143919	*LTB4R*	Leukotriene B4 receptor	4.27*
NM_001315	*MAPK14*	Mitogen-activated protein kinase 14	0.67
NM_000176	*NR3C1*	Nuclear receptor subfamily 3, group C, member 1	0.84
NM_000860	*HPGD*	Hydroxyprostaglandin dehydrogenase 15-(NAD)	0.32
NM_000861	*HRH1*	Histamine receptor H1	1.39
NM_001131055	*HRH2*	Histamine receptor H2	0.32
NM_000869	*HTR3A*	5-hydroxytryptamine (serotonin) receptor 3A	1.82
NM_000201	*ICAM1*	Intercellular adhesion molecule 1 (CD54)	3.82*
NM_000877	*IL1R1*	Interleukin 1 receptor, type I	1.24
NM_000417	*IL2RA*	Interleukin 2 receptor, alpha	ND
NM_000878	*IL2RB*	Interleukin 2 receptor, beta	ND
NM_000206	*IL2RG*	Interleukin 2 receptor, gamma	0.018
NM_002188	*IL13*	Interleukin 13	1.39
NM_001114380	*ITGAL*	Integrin, alpha L (antigen CD11A (p180)	ND
NM_000632	*ITGAM*	Integrin, alpha M (complement component 3 receptor 3 subunit)	4.78*
NM_002211	*ITGB1*	Integrin, beta 1 (fibronectin receptor, beta polypeptide)	1.02
NM_000211	*ITGB2*	Integrin, beta 2 (complement component 3)	0.61
NM_002257	*KLK1*	Kallikrein 1	0.64
NM_001002231	*KLK2*	Kallikrein-related peptidase 2	19.59*
NM_000892	*KLKB1*	Kallikrein B, plasma (Fletcher factor) 1	1.73
NM_001102416	*KNG1*	Kininogen 1	ND
NM_000895	*LTA4H*	Leukotriene A4 hydrolase	ND
NM_000897	*LTC4S*	Leukotriene C4 synthase	10.03*
NM_000529	*MC2R*	Melanocortin 2 receptor (adrenocorticotropic hormone)	6.52*
NM_001165412	*NFKB1*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	2.32*

		(p105)	
NM_000625	*NOS2A*	Nitric oxide synthase 2A (inducible, hepatocytes)	ND
NM_001111307	*PDE4A*	Phosphodiesterase 4A	0.52
NM_002600	*PDE4B*	Phosphodiesterase 4B	0.42
NM_000923	*PDE4C*	Phosphodiesterase 4C	ND
NM_001165899	*PDE4D*	Phosphodiesterase 4D	0.52
NM_000928	*PLA2G1B*	Phospholipase A2, group IB (pancreas)	0.89
NM_000300	*PLA2G2A*	Phospholipase A2, group IIA	3.00*
NM_000929	*PLA2G5*	Phospholipase A2, group V	ND
NM_004573	*PLCB2*	Phospholipase C, beta 2	ND
NM_000932	*PLCB3*	Phospholipase C, beta 3 (phosphatidylinositol-specific)	0.40
NM_000933	*PLCB4*	Phospholipase C, beta 4	2.82*
NM_001130964	*PLCD1*	Phospholipase C, delta 1	0.21
NM_182811	*PLCG1*	Phospholipase C, gamma 1	29.05*
NM_002661	*PLCG2*	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	0.85
NM_002745	*MAPK1*	Mitogen-activated protein kinase 1	0.72
NM_001040056	*MAPK3*	Mitogen-activated protein kinase 3	0.52
NM_002750	*MAPK8*	Mitogen-activated protein kinase 8	0.83
NM_000952	*PTAFR*	Platelet-activating factor receptor	0.09
NM_000953	*PTGDR*	Prostaglandin D2 receptor (DP)	ND
NM_000956	*PTGER2*	Prostaglandin E receptor 2 (subtype EP2), 53kDa	0.80
NM_000957	*PTGER3*	Prostaglandin E receptor 3 (subtype EP3)	ND
NM_000959	*PTGFR*	Prostaglandin F receptor (FP)	20.21*
NM_000960	*PTGIR*	Prostaglandin I2 (prostacyclin) receptor (IP)	7.22*
NM_000961	*PTGIS*	Prostaglandin I2 (prostacyclin) synthase	ND

NM_000962	*PTGS1*	Prostaglandin-endoperoxide synthase 1	2.43*
NM_000963	*PTGS2*	Prostaglandin-endoperoxide synthase 2	0.53
NM_001060	*TBXA2R*	Thromboxane A2 receptor	0.96
NM_001061	*TBXAS1*	Thromboxane A synthase 1	0.63
NM_001066	*TNFRSF1B*	Tumor necrosis factor receptor superfamily, member 1B	2.70*
NM_001078	*VCAM1*	Vascular cell adhesion molecule 1	ND
NM_004633	*IL1R2*	Interleukin 1 receptor, type II	0.20
NM_001168357	*PLA2G7*	Phospholipase A2, group VII	0.005
NM_003561	*PLA2G10*	Phospholipase A2, group X	46.32*
NM_001159322	*PLA2G4C*	Phospholipase A2, group IVC (cytosolic, calcium-independent)	0.42
NM_016232	*IL1RL1*	Interleukin 1 receptor-like 1	22.65*
NM_006028	*HTR3B*	5-hydroxytryptamine (serotonin) receptor 3B	ND
NM_001145645	*TNFSF13B*	Tumor necrosis factor (ligand) superfamily, member 13b	4.83*
NM_006639	*CYSLTR1*	Cysteinyl leukotriene receptor 1	150.14*
NM_007232	*HRH3*	Histamine receptor H3	ND
NM_001271814	*PLA2G2D*	Phospholipase A2, group IID	ND
NM_017416	*IL1RAPL2*	Interleukin 1 receptor accessory protein-like 2	5.67*
NM_022046	*KLK14*	Kallikrein-related peptidase 14	2.53*
NM_016341	*PLCE1*	Phospholipase C, epsilon 1	6.71*
NM_017509	*KLK15*	Kallikrein-related peptidase 15	ND
NM_001164693	*LTB4R2*	Leukotriene B4 receptor 2	ND
NM_005161	*AGTRL1*	Angiotensin II receptor-like 1	ND
NM_001716	*CXCR5*	Chemokine (C-X-C motif) receptor 5	ND
NM_001735	*C5*	Complement component 5	2.14*
NM_002981	*CCL1*	Chemokine (C-C motif) ligand 1	ND

NM_002986	*CCL11*	Chemokine (C-C motif) ligand 11	ND
NM_005408	*CCL13*	Chemokine (C-C motif) ligand 13	ND
NM_004590	*CCL16*	Chemokine (C-C motif) ligand 16	ND
NM_002987	*CCL17*	Chemokine (C-C motif) ligand 17	ND
NM_002988	*CCL18*	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	ND
NM_006274	*CCL19*	Chemokine (C-C motif) ligand 19	ND
NM_002982	*CCL2*	Chemokine (C-C motif) ligand 2	4.43*
NM_004591	*CCL20*	Chemokine (C-C motif) ligand 20	ND
NM_002989	*CCL21*	Chemokine (C-C motif) ligand 21	ND
NM_002990	*CCL22*	Chemokine (C-C motif) ligand 22	ND
NM_005064	*CCL23*	Chemokine (C-C motif) ligand 23	ND
NM_002991	*CCL24*	Chemokine (C-C motif) ligand 24	ND
NM_005624	*CCL25*	Chemokine (C-C motif) ligand 25	ND
NM_006072	*CCL26*	Chemokine (C-C motif) ligand 26	ND
NM_006664	*CCL27*	Chemokine (C-C motif) ligand 27	ND
NM_148672	*CCL28*	Chemokine (C-C motif) ligand 28	ND
NM_002983	*CCL3*	Chemokine (C-C motif) ligand 3	ND
NM_002984	*CCL4*	Chemokine (C-C motif) ligand 4	ND
NM_002985	*CCL5*	Chemokine (C-C motif) ligand 5	5.24*
NM_006273	*CCL7*	Chemokine (C-C motif) ligand 7	ND
NM_005623	*CCL8*	Chemokine (C-C motif) ligand 8	ND
NM_016602	*CCR10*	Chemokine (C-C motif) receptor 10	ND
NM_001123041	*CCR2*	Chemokine (C-C motif) receptor 2	ND
NM_001164680	*CCR3*	Chemokine (C-C motif) receptor 3	ND
NM_005508	*CCR4*	Chemokine (C-C motif) receptor 4	ND

NM_016557	*CCRL1*	Chemokine (C-C motif) receptor-like 1	ND
NM_001130910	*CCRL2*	Chemokine (C-C motif) receptor-like 2	ND
NM_016326	*CKLF*	Chemokine-like factor	ND
NM_013246	*CLCF1*	Cardiotrophin-like cytokine factor 1	1.46
NM_052999	*CMTM1*	CKLF-like MARVEL transmembrane domain containing 1	3.15*
NM_000758	*CSF2*	Colony stimulating factor 2 (granulocyte-macrophage)	ND
NM_172220	*CSF3*	Colony stimulating factor 3 (granulocyte)	ND
NM_002996	*CX3CL1*	Chemokine (C-X3-C motif) ligand 1	ND
NM_001511	*CXCL1*	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	12.37*
NM_001565	*CXCL10*	Chemokine (C-X-C motif) ligand 10	ND
NM_005409	*CXCL11*	Chemokine (C-X-C motif) ligand 11	2.47*
NM_000609	*CXCL12*	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	ND
NM_006419	*CXCL13*	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	ND
NM_004887	*CXCL14*	Chemokine (C-X-C motif) ligand 14	ND
NM_022059	*CXCL16*	Chemokine (C-X-C motif) ligand 16	ND
NM_002089	*CXCL2*	Chemokine (C-X-C motif) ligand 2	0.49
NM_002090	*CXCL3*	Chemokine (C-X-C motif) ligand 3	8.19*
NM_002994	*CXCL5*	Chemokine (C-X-C motif) ligand 5	ND
NM_002993	*CXCL6*	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	ND
NM_002416	*CXCL9*	Chemokine (C-X-C motif) ligand 9	ND
NM_001008540	*CXCR4*	Chemokine (C-X-C motif) receptor 4	1.67
NM_002036	*DARC*	Duffy blood group, chemokine receptor	ND
NM_001953	*TYMP*	Thymidine phosphorylase	3.22*
NM_000557	*GDF5*	Growth differentiation factor 5	ND

NM_032554	*GPR81*	G protein-coupled receptor 81	ND
NM_002086	*GRB2*	Growth factor receptor-bound protein 2	ND
NM_001530	*HIF1A*	Hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	ND
NM_000619	*IFNG*	Interferon, gamma	ND
NM_000416	*IFNGR1*	Interferon gamma receptor 1	1.03
NM_020124	*IFNK*	Interferon, kappa	ND
NM_000572	*IL10*	Interleukin 10	ND
NM_000882	*IL12A*	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	2.36*
NM_002187	*IL12B*	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	ND
NM_002188	*IL13*	Interleukin 13	ND
NM_172217	*IL16*	Interleukin 16 (lymphocyte chemoattractant factor)	ND
NM_001562	*IL18*	Interleukin 18 (interferon-gamma-inducing factor)	17.84*
NM_000575	*IL1A*	Interleukin 1, alpha	68.56*
NM_000576	*IL1B*	Interleukin 1, beta	ND
NM_001167928	*IL1RAP*	Interleukin 1 receptor accessory protein	ND
NM_000586	*IL2*	Interleukin 2	ND
NM_000589	*IL4*	Interleukin 4	ND
NM_000879	*IL5*	Interleukin 5 (colony-stimulating factor, eosinophil)	ND
NM_000600	*IL6*	Interleukin 6 (interferon, beta 2)	4.19*
NM_000584	*IL8*	Interleukin 8	38.93*
NM_000590	*IL9*	Interleukin 9	ND
NM_004972	*JAK2*	Janus kinase 2 (a protein tyrosine kinase)	1.72

NM_002468	*MYD88*	Myeloid differentiation primary response gene (88)	9.41*
NM_002619	*PF4*	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	ND
NM_002704	*PPBP*	Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	ND
NM_004757	*SCYE1*	Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	ND
NM_004787	*SLIT2*	Slit homolog 2 (Drosophila)	3.63*
NM_014011	*SOCS5*	Suppressor of cytokine signaling 5	0.00047
NM_139276	*STAT3*	Signal transducer and activator of transcription 3 (acute-phase response factor)	0.86
NM_003151	*STAT4*	Signal transducer and activator of transcription 4	ND
NM_003264	*TLR2*	Toll-like receptor 2	88.47*
NM_138554	*TLR4*	Toll-like receptor 4	1.66
NM_000594	*TNF*	Tumor necrosis factor (TNF superfamily, member 2)	26.55*
NM_001065	*TNFRSF1A*	Tumor necrosis factor receptor superfamily, member 1A	3.16*
NM_001190942	*TNFSF10*	Tumor necrosis factor (ligand) superfamily, member 10	1.91*
NM_003807	*TNFSF14*	Tumor necrosis factor (ligand) superfamily, member 14	ND
NM_002995	*XCL1*	Chemokine (C motif) ligand 1,chemokine (C motif) ligand 2	ND
NM_001024644	*XCR1*	Chemokine (C motif) receptor 1	ND

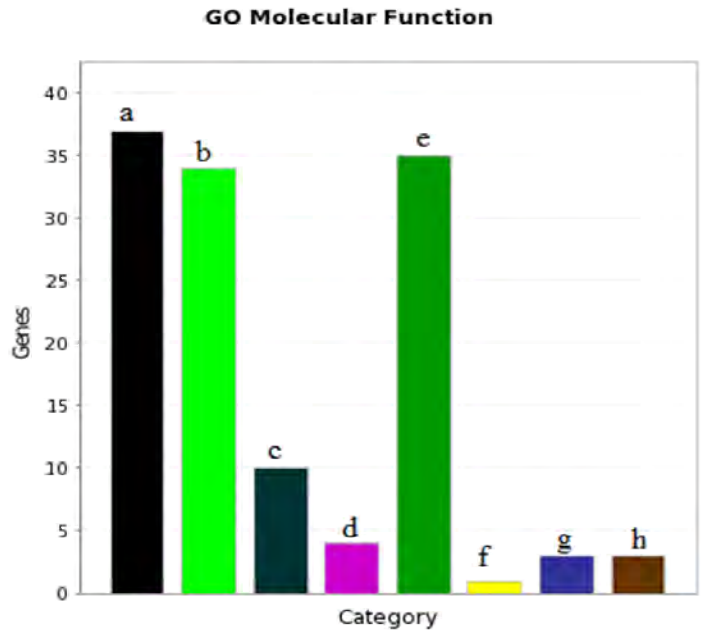
*, Fold change ≥ 2.

ND, Not detected.

APPENDIX II

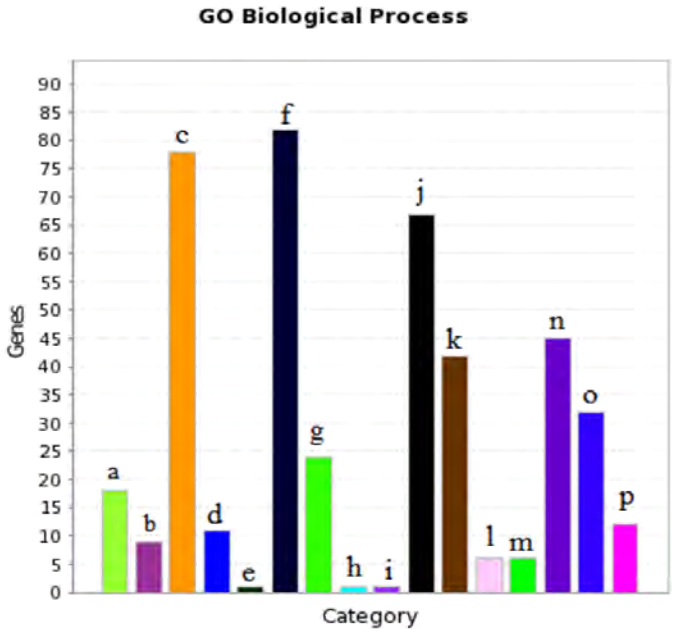
Gene ontology and PANTHER classification of genes regulated by seminal plasma in to (A) molecular function, (B) biological processes, (C) protein class, and (D) pathways regulated by the genes.

(A)



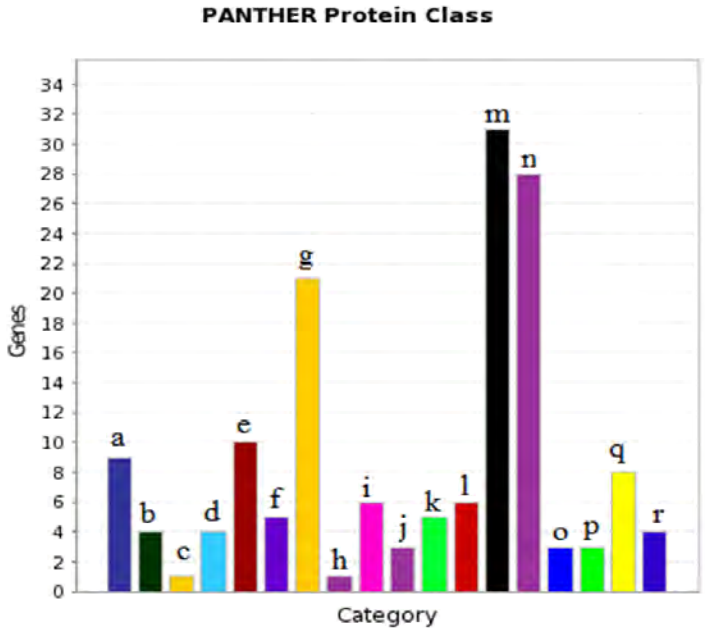
(a) Binding, (b) Catalytic activity, (C) Enzyme regulator activity, (d) Ion channel activity, (e) Receptor activity, (f) Structural molecule activity, (g) Transcription regulator activity, (h) Transporter activity.

(B)



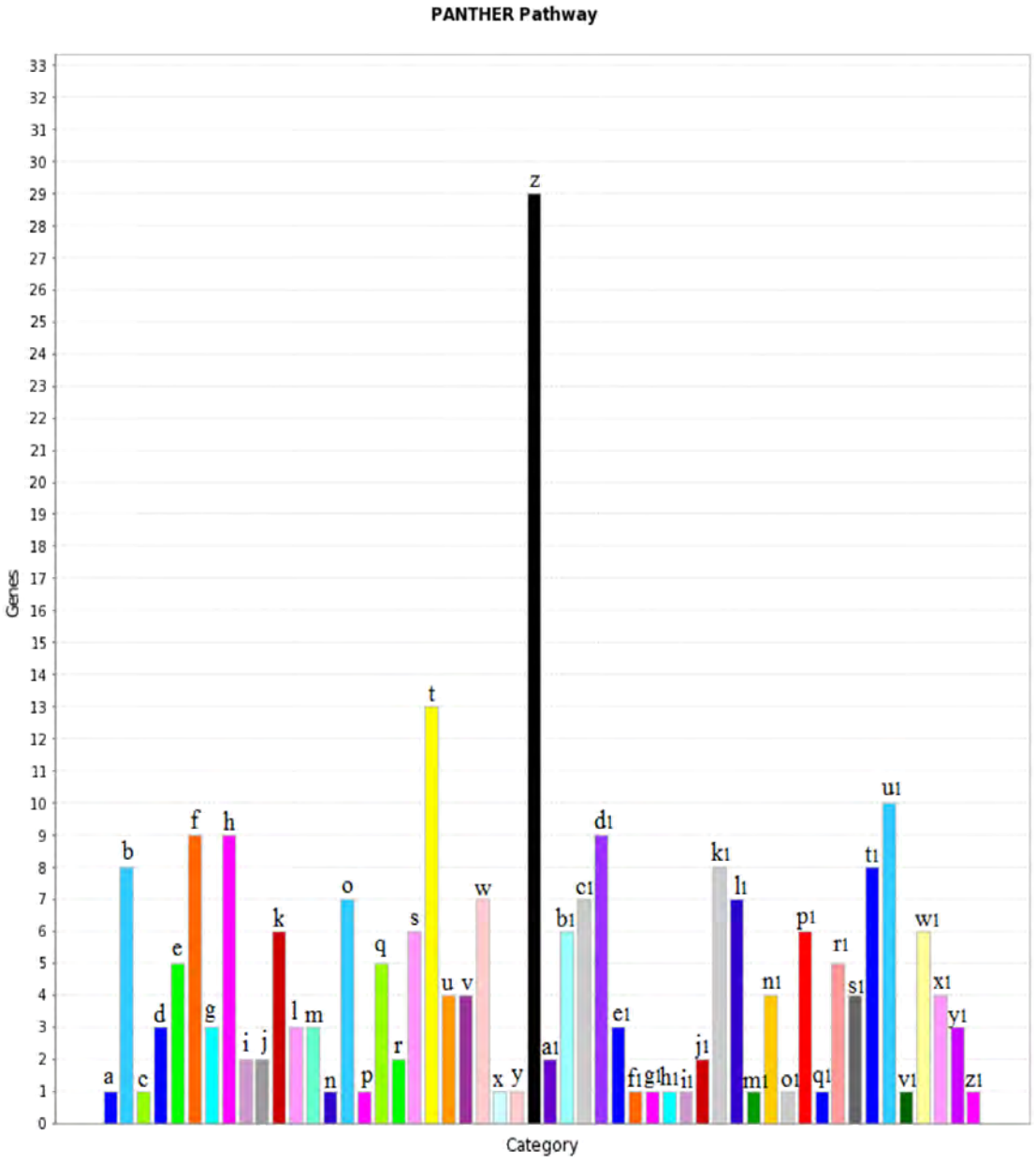
(a) Apoptosis, (b) Cell adhesion, (c) Cell communication, (d) Cell cycle, (e) Cellular component organization, (f) Cellular process, (g) Developmental process, (h) Generation of precursor metabolites and energy, (i) Homeostatic process, (j) Immune system process, (k) Metabolic process, (l) Regulation of biological process, (m) Reproduction, (n) Response to stimulus, (o) System process, (p) Transport.

(c)



(a) Calcium-binding protein, (b) Cell adhesion molecule, (c) Cytoskeletal protein, (d) Defense/immunity protein, (e) Enzyme modulator, (f) Extracellular matrix protein, (g) Hydrolase, (h) Isomerase, (i) Kinase, (j) Nucleic acid binding, (k) Oxidoreductase, (l) Protease, (m) Receptor, (n) Signaling molecule, (o) Transcription factor, (p) Transfer/carrier protein, (q) Transferase, (r) Transporter.

(D)



(a) 2-arachidonoylglycerol biosynthesis, (b) 5HT2 type receptor mediated signaling pathway, (c) 5HT3 type receptor mediated signaling pathway, (d) Alpha adrenergic receptor signaling pathway, (e) Alzheimer disease-amyloid secretase pathway, (f) Angiogenesis, (g) Angiotensin II-stimulated signaling through G proteins and beta-arrestin, (h) Apoptosis signaling pathway, (i) Axon guidance mediated by Slit/Robo, (j) Axon guidance mediated by netrin, (k) B cell

activation, (l) Beta1 adrenergic receptor signaling pathway, (m) Beta2 adrenergic receptor signaling pathway, (n) Blood coagulation, (o) EGF receptor signaling pathway, (p) Endogenous cannabinoid signaling, (q) Endothelin signaling pathway, (r) FAS signaling pathway, (s) FGF signaling pathway, (t) Gonadotropin releasing hormone receptor pathway, (u) Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway, (v) Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway, (w) Histamine H1 receptor mediated signaling pathway, (x) Histamine H2 receptor mediated signaling pathway, (y) Huntington disease, (z) Inflammation mediated by chemokine and cytokine signaling pathway, (a1) Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade, (b1) Integrin signalling pathway, (c1) Interferon-gamma signaling pathway, (d1) Interleukin signaling pathway, (e1) JAK/STAT signaling pathway, (f1) Metabotropic glutamate receptor group I pathway, (g1) Muscarinic acetylcholine receptor 1 and 3 signaling pathway, (h1) Muscarinic acetylcholine receptor 2 and 4 signaling pathway, (i1) Nicotinic acetylcholine receptor signaling pathway, (j1) Oxidative stress response, (k1) Oxytocin receptor mediated signaling pathway, (l1) PDGF signaling pathway, (m1) PI3 kinase pathway, (n1) Parkinson disease, (o1) Pyrimidine Metabolism, (p1) Ras Pathway, (q1) Salvage pyrimidine deoxyribonucleotides, (r1) T cell activation, (s1) TGF-beta signaling pathway, (t1) Thyrotropin-releasing hormone receptor signaling pathway, (u1) Toll receptor signaling pathway, (v1) Toll_pathway_drosophila, (w1) VEGF signaling pathway, (x1) Wnt signaling pathway, (y1) p38 MAPK pathway, (z1) p53 pathway feedback loops 2.

APPENDIX III

Histological typing, extent of invasiveness, and FIGO staging of carcinoma biopsies

Sample identification	**Histological type**	**FIGO staging**
Ca.2-Ca.10, Ca.12-Ca.15	Squamous carcinoma	0; carcinoma in-situ
Ca.5,Ca.16	Squamous carcinoma	IA; moderately differentiated
Ca.1,Ca.17	Squamous carcinoma	IA_1; moderately differentiated
Ca.11	Adenocarcinoma	IB1; well differentiated
Ca.18	Squamous carcinoma	IB1; moderately differentiated
Ca.4	Squamous carcinoma	IIIB; moderately differentiated