THE MOLECULAR PATHWAYS MEDIATING THE ROLE OF CYCLOOXYGENASE ENZYMES AND PROSTAGLANDINS IN CERVICAL NEOPLASIAS

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Abstract For Thesis Presented For The Degree Of DOCTOR OF PHILOSOPHY
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Cervical carcinoma is one of the leading causes of cancer-related death in women. The prevalence of this disease is particularly high in South Africa, occurring on average, in 60 out of every 100 000 women. Previous studies have demonstrated over-expression of cyclooxygenase-2 (COX-2) enzyme and enhanced synthesis of prostanoids, such as prostaglandin E_2 (PGE_2), in cervical carcinomas. PGE_2 mediates its effects by interacting with one of four receptors termed EP1-4. Expression and signalling of EP receptors, including EP4, are elevated in cervical carcinomas. It has been proposed that in addition to endogenous PGE_2, EP receptors in cervical carcinomas can be activated by seminal plasma prostaglandins. Prostaglandin concentration in seminal plasma is 10,000 times higher than that found at a site of inflammation, and PGE_2 is the predominant type of prostaglandin detected in semen. This thesis investigates the potential activation of the EP4 receptor by seminal plasma prostaglandins or PGE_2 in wild type (WT) and EP4 receptor over-expressing (EP4S) cervical adenocarcinoma (HeLa) cells. Treatment of EP4S cells with seminal plasma or PGE_2 results in a rapid augmented accumulation of cAMP ($p<0.001$) and phosphorylation of ERK1/2 ($p<0.001$) compared with WT cells. The phosphorylation of ERK1/2 is inhibited by co-treatment of cells with seminal plasma and MEK inhibitor (PD98059), EP4-selective receptor antagonist (ONO-AE2-227) or an inhibitor of epidermal growth factor (EGF) receptor tyrosine kinase (AG1478). Treatment of EP4S cells with PGE_2 or seminal plasma also resulted in elevated expression of COX-2 ($p<0.001$), vascular endothelial growth factor (VEGF; $p<0.001$) and basic fibroblast growth factor (bFGF; $p<0.05$). Expression of COX-2 and VEGF is inhibited by co-treatment of cells with seminal plasma or PGE_2 and the MEK inhibitor, the EP4-selective receptor antagonist or the EGF receptor tyrosine kinase inhibitor. In conclusion, our data demonstrate that seminal plasma prostaglandins can activate signalling and expression of inflammatory and angiogenic genes via the EP4 receptor, in an EGFR- and ERK-dependent manner. These data suggest that the progression of cervical carcinoma in sexually active women may be exacerbated following exposure to seminal plasma and activation of EP-receptor signal transduction pathways. Therefore, COX-enzyme inhibitors on their own may not be sufficient to inhibit prostaglandin action in sexually active women. A more effective therapy may lie in the use of EP receptor antagonists.

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DECLARATION

Except where due acknowledgement 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.

Melissa Muller
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ABBREVIATIONS

μg ………………….. microgram
μl ………………….. microlitre
μM ………………….. micromolar
19-hydroxy PGE …... 19-hydroxyprostaglandin E

AA ………………….. Arachidonic acid
AMP ……………….. adenosine monophosphate
Ang-1 ……………… Angiopoietin-1
Ang-2……………….. Angiopoietin-2
ANOVA ……………. Analysis of variance
Asn ………………… Asparagine
ATP ……………….. adenosine triphosphate

BCP ……………….. 1-bromo-3-chloropropane
bFGF ………………. Basic fibroblast growth factor
bp ………………….. Base pairs
BSA ……………….. bovine serum albumin

cAMP ………………. adenosine3',5'-cyclic monophosphate
cDNA ………………. copy deoxyribose nucleic acid
CIS …………………. Carcinoma in situ
CIN …………………. Cervical intraepithelial neoplasia
COX ………………… cyclooxygenase
Cpm …………………. counts per minute
CRE …………………. cAMP response element
CREB ……………….. cAMP regulatory binding protein

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dATP	2′-deoxyadenosine 5′-triphosphate
dCTP	2′-deoxycytidine 5′-triphosphate
DEPC	diethylpyrocarbonate
dGTP	2′-deoxyguanosine 5′-triphosphate
dH_20	distilled water
DMEM	Dulbecco's modified Eagles medium
DNA	deoxyribonucleic acid
dNTP	2′-deoxynucleoside 5′-triphosphate
dTTP	2′-deoxythymidine 5′-triphosphate
EDTA	ethylene diamine tetra acetic acid
EGF	endothelial growth factor
EGFR	endothial growth factor receptor
EGR	early growth response factor
ELISA	enzyme-linked immunosorbent assay
EP4AS	EP4-antisense HeLa cells
EP4S	EP4-sense HeLa cells
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
ET_AR	Endothelin-A Receptor
FGF	fibroblast growth factor
FIGO	International Federation of Obstetricians and Gynaecologists
g	gravity
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinases
GSK-3	Glycogen synthase kinase-3

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h ……………………..	hour(s)
HB-EGF …………….	Heparin-binding EGF
HIV ………………….	Human Immunodeficiency Virus
HPV …………………	Human papilloma virus
HRP …………………	horseradish peroxidase
IBMX ……………….	3-isobutyl-1-methylxanthine
IgG …………………..	Immunoglobulin G
IHC ………………….	Immunohistochemistry
IL ……………………	Interleukin
IP_3 ……………………	inositol triphosphate
IU ……………………	international units
JNK ………………….	c-Jun amino-terminal kinase
kb …………………….	kilobases
kDa …………………..	kilodalton
L ……………………....	litre
LPS …………………..	Lipopolysaccharide
mA …………………...	miliampere
MAPK ……………….	Mitogen-activated protein kinase
MAPKK ……………..	Mitogen-activated protein kinase kinase
MEK …………………	Mitogen-activated protein kinase/Extracellular signal-regulated kinase
min …………………...	minute(s)
ml …………………….	millilitres
mM …………………..	millimolar
mRNA ………………..	messenger ribonucleic acid

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NFκB ………………...	nuclear factor-kappaB
ng ……………………..	nanogram
nm …………………….	nanometer
nM …………………….	nanomolar
NO …………………….	Nitric oxide
NP40 ………………….	"Nonidet" P40
NREM ………………..	non-rapid eye movement
NSAIDs ………………	non-steroidal anti-inflammatory drugs
PAGE …………………	polyacrylamide gel electrophoresis
PBS …………………...	phosphate buffered saline
PCR …………………...	polymerase chain reaction
PG …………………….	prostaglandin
pg ……………………..	picogram
PGD_2 …………………	prostaglandin D_2
PGE_2 ………………….	prostaglandin E_2
PGES …………………	prostaglandin E synthase
$PGF_{2\alpha}$ …………………	prostaglandin $F_{2\alpha}$
PGG_2 …………………	prostaglandin G_2
PGH_2 …………………	hydroxy cyclic endoperoxide
PGI_2 …………………..	prostacyclin
PGT …………………..	prostaglandin transporter
pH …………………….	negative logarithm of the hydrogen ion concentration
PI3K ………………….	Phosphatidylinositol 3-kinase
PKA ………………….	Protein kinase A
PKC …………………..	Protein kinase C
PLA_2 ………………….	phospholipase A_2
pmol …………………..	picomole
PMSF …………………	phenylmethylsulphonylflouride
PPAR …………………	peroxisome proliferators-activated receptors
PPRE ………………….	peroxisome proliferators-activated response element

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Rb ……………………..	Retinoblastoma
REM …………………..	rapid eye movement
RNA …………………..	ribonucleic acid
RTK …………………..	receptor tyrosine kinases
RT-PCR ………………	reverse transcription-polymerase chain reaction
SAPK …………………	stress-activated protein kinase
SDS …………………..	sodium dodecyl sulphate
SEM …………………..	standard error of the mean
SIL …………………...	Squamous intra-epithelial lesions
Taq …………………...	*Thermis acqaticus*
TBS …………………..	tris-buffered saline
TBST …………………	tris-buffered saline with Tween®20
TEMED ………………	N,N,N',N'-Tetramethylenediamine
TNF …………………..	tumour necrosis factor
Tris …………………...	tris(hydroxymethyl) aminomethane
TXA_2 …………………	Thromboxane A_2
UV ……………………	ultraviolet
V ……………………...	volt
VEGF …………………	vascular endothelial growth factor
W ……………………...	watt
WHO ………………….	World Health Organisation
WT …………………….	wild-type

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ABSTRACT

Cervical Carcinoma is one of the leading causes of cancer-related death in women. The prevalence of this disease is particularly high in South Africa, occurring on average, in 60 out of every 100 000 women. Previous studies have demonstrated over-expression of cyclooxygenase-2 enzyme and enhanced synthesis of prostanoids, such as prostaglandin E_2, in cervical carcinomas. Prostaglandin E_2 mediates its effects by interacting with one of four receptors termed EP1-4. Expression and signalling of EP receptors, including EP4, are elevated in cervical carcinomas.

The initial aim of this study was to localise the site of expression of EP4 receptors in cervical squamous cell- and adenocarcinomas. Immunohistochemical analysis performed on paraffin wax-embedded cervical tissue sections localised the site of EP4 receptor expression to the neoplastic epithelial cells of all squamous cell carcinomas and adenocarcinomas studied. Minimal EP4 receptor immunoreactivity was detected in normal cervix. The site of localisation of the EP4 receptor within the epithelial compartment suggested that prostaglandin E_2 may act in an autocrine/paracrine manner to modulate epithelial cell function and promote tumourigenesis.

In addition to endogenous prostaglandin E_2, EP receptors in cervical carcinomas can be activated by seminal plasma prostaglandins. Prostaglandin concentration in seminal plasma is 10,000 times higher than that found at a site of inflammation, and prostaglandin E_2 is the predominant type of prostaglandin detected in semen. In order to investigate the potential activation of the EP4 receptor by prostaglandin E_2 or seminal plasma prostaglandins, we developed an EP4-overexpressing adenocarcinoma cell model system using HeLa (cervical carcinoma) cells.

Using this model system the signal transduction pathways activated by prostaglandin E_2- or seminal plasma-EP4 receptor interaction in HeLa wild type and EP4 receptor over-expressing (EP4S) HeLa cells were investigated. Treatment of EP4S cells with seminal plasma or prostaglandin E_2 resulted in a rapid accumulation of cAMP ($p<0.001$) and

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phosphorylation of ERK1/2 ($p<0.001$) in EP4S compared with wild-type cells. This elevated phosphorylation of ERK1/2 is inhibited by co-treatment of cells with chemical inhibitors of MEK (PD98059), epidermal growth factor receptor tyrosine kinase (AG1478) or EP4-selective receptor antagonist (ONO-AE2-227). We next investigated the target genes activated by seminal plasma or prostaglandin E_2-EP4 ligand-receptor interaction. Treatment of EP4S cells with seminal plasma or prostaglandin E_2 also resulted in elevated expression of the tumourigenic gene, cyclooxygenase-2 ($p<0.001$), and two genes associated with angiogenesis, vascular endothelial growth factor ($p<0.001$) and basic fibroblast growth factor ($p<0.05$). Expression of these genes was inhibited by co-treatment of cells with seminal plasma or prostaglandin E_2 and the MEK inhibitor, the epidermal growth factor receptor tyrosine kinase inhibitor or the EP4-selective receptor antagonist.

Our data presented in this thesis demonstrate that seminal plasma prostaglandins or prostaglandin E_2 can activate mitogenic signalling and promote the expression of inflammatory and angiogenic genes via the EP4 receptor, by a mechanism involving transactivation of the epidermal growth factor receptor and ERK1/2. These data suggest that in sexually active women, cervical carcinoma may be exacerbated following exposure to seminal plasma and activation of EP-receptor signal transduction pathways. As a result, cycooxygenase enzyme inhibitors on their own may not be sufficient to inhibit prostaglandin action in sexually active women. These data raise the possibility that EP4 receptor antagonists used in combination with cyclooxygenase enzyme inhibitors may be a potential therapy for these women,

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CHAPTER 1 - INTRODUCTION

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Introduction

The development of carcinoma is a multi-factoral process resulting from aberrations in cell regulatory pathways governing homeostasis and cell proliferation. There have been more than 100 different reported cancers occurring in cells and organs throughout the human body. Although the initial triggers of neoplastic cell transformation may vary, different cancers have been shown to have common characteristics. These include a limitless reproductive potential, insensitivity to anti-growth signals, self-sufficiency in growth signals, as well as sustained tissue invasion and metastasis coupled with angiogenesis. Numerous studies have implicated a role for COX-enzymes and COX-enzyme products in the regulation of tumourigenesis in a wide variety of cancers (Chang *et al.* 2000; Jabbour 2001; Kulkarni *et al.* 2001; Sales *et al.* 2001; Denkert *et al.* 2002; Hawk *et al.* 2002; Sales *et al.* 2002; Song *et al.* 2002; Gupta *et al.* 2003). The effect of these COX-enzyme products, or prostaglandins (PG) are mediated by specific receptors, which result in the activation of various signalling pathways ultimately culminating in the transcription of target genes which may enhance tumourigenesis or angiogenesis.

The focus of this work is on the possible regulation of tumourigenesis through the PG EP4 receptor. This introduction will give a brief overview of the epidemiology, histology, risk factors, and staging of cervical carcinomas, and the roles of COX-enzymes, PG and PG receptors (specifically the EP4 receptor) in mediating tumourigenic effects.

1.1. Cervical Carcinoma

Cancer of the uterine cervix is one of the leading causes of cancer-related death in women worldwide, with nearly 500 000 women developing the disease each year (Williams *et al.* 1998; Molina *et al.* 2003; Waggoner 2003). There has been a substantial decrease in incidence and mortality in developed countries, which is though to be as a result of more effective screening. However, in the less developed regions of the world including South-East Asia, South and Central America, and Sub-Saharan Africa, as detailed in Table 1, cervical cancer continues to be a major problem for female

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reproductive health (Munoz *et al.* 1992; Aareleid *et al.* 1993; Moore *et al.* 2004) (Table 1).

Registry	Recording period	Cases	Rate per 100 000 women
Ten highest rates			
Zimbabwe, Harare (African women)	1990-92	295	67.21
Brazil, Belem	1989-91	931	64.78
Peru, Trujillo	1988-90	288	53.48
Uganda, Kyadondo	1991-93	248	40.76
India, Madras	1988-92	2540	38.91
Brazil, Goiania	1990-93	506	37.13
Columbia, Cali	1987-91	1061	34.41
New Zealand (Maori women)	1988-92	193	32.21
Argentina, Concordia	1990-94	108	32.05
Ecuador, Quito	1988-92	697	31.66
Ten lowest rates			
Spain, Navarra	1987-91	82	4.68
USA, Hawaii (Chinese women)	1988-92	10	4.55
China, Tianjin	1988-92	454	4.39
Israel (Jewish women born in USA or Europe)	1988-92	187	4.07
USA, Los Angeles (Japanese women)	1988-92	20	4.05
Finland	1987-92	893	3.62
China, Shanghai	1988-92	860	3.26
Israel (Non-Jewish women)	1988-92	40	2.99
Italy, Macerata	1991-92	12	2.77
China, Qidong	1988-92	97	2.64

Table 1: Registries with the highest and lowest incidence rates of cervical cancer. (Waggoner *et al.* 2003)

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The prevalence of cervical cancer disease is particularly high in South Africa, occurring on average, in 60 out of every 100 000 women (Bailie *et al.* 1996; Sitas *et al.* 1997) (Sales *et al.* 2001). In certain groups of South African women screened at the primary health care clinic in Soweto, Johannesburg, the detection rate of invasive cervical cancers is 1.8 per 1000 women (Leiman 1976). The peak incidence of this disease in women is between 50 and 54 years of age, with more than 80 % presenting with cervical dysplasia at 40 years of age or older (Bailie 1995; Wright *et al.* 2004; Wright *et al.* 2004).

Although cervical carcinoma is generally associated with middle-aged women, several studies have shown an increase in the incidence of both squamous cell carcinoma and adenocarcinoma in younger women (Bulk *et al.* 2004; Smith *et al.* 2004; Smrkolj *et al.* 2004).

While a decline in incidence and mortality from cervical cancer has been documented in first world countries such as the United States, Canada, and Scandinavia (Moodley *et al.* 2003), this trend is not apparent for most developing countries due to lack of, or inefficient screening programs (Williams *et al.* 1998). This results in women being diagnosed with the disease when it has progressed substantially, often with lymph node metastasis, when surgery or clinical treatment is not possible. Cancer of the uterine cervix is thus still regarded as an important clinical problem in many developing countries, especially South Africa.

1.2. Histology of the cervix

The cervix is the cone-shaped neck of the uterus that protrudes into the vagina (Figure 1.1 A). It is about one inch long and is fibromuscular in origin. It surrounds the endocervical canal (the os), which permits the menstrual period and the fetus to pass from the uterus into the vagina, and sperm to pass from the vagina into the uterus.

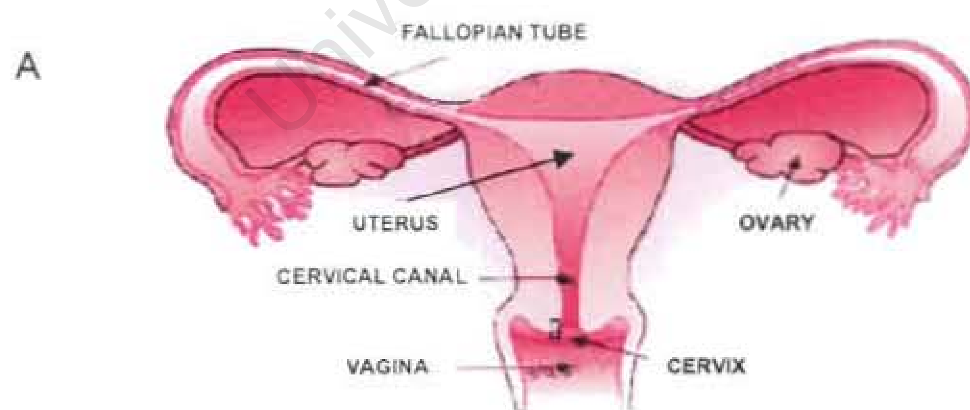

Figure 1.1.A. Female reproductive system
(The Rhode Island Cancer Council, USA)

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B

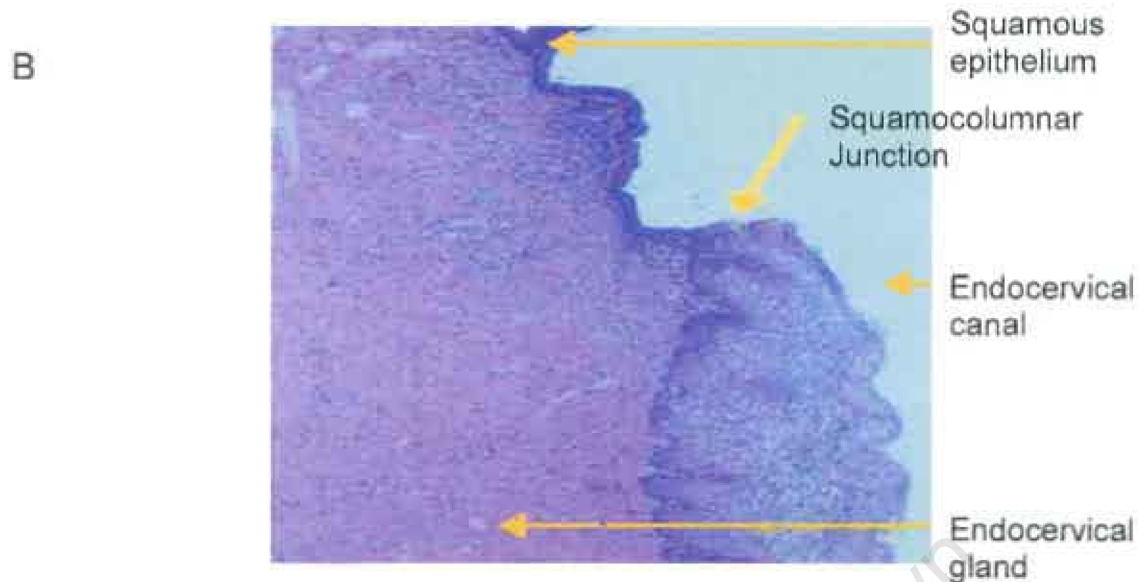

Figure 1.1.B. Representative cross section of Uterine Cervix (Haemotoxylin- and eosin-stained; magnification 400x).

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The epithelial lining of the lower genital tract in women consists of two distinct cell types, namely squamous and columnar epithelial cells (Fichorova *et al.* 1999). The epithelial lining of the vagina and exocervix consists of multiple layers of stratified squamous epithelial cells (Figure 1.1. B); this is a non-sterile environment that tolerates an abundance of vaginal microorganisms as well as diverse seminal antigens. On the other hand, the endocervical canal is lined by a single layer of columnar-type epithelial cells and forms a usually sterile passage into the upper genital tract (Fichorova *et al.* 1999). The junction between the squamous epithelium of the cervix and the columnar epithelium originating from the endocervical glands is referred to as the squamocolumnar junction. During adolescence and pregnancy, the squamous epithelium replaces the columnar epithelium by metastasis to form a new squamocolumnar junction. The area between the new and old squamocolumnar junction is referred to as the transformation zone. Several studies have proposed that carcinogens act at the transformation zone to cause cervical neoplasia (Kristensen *et al.* 1989; Martin-Hirsch *et al.* 1999).

1.3. Histology of pre-invasive lesions

About 90 % of cervical carcinomas arise from flattened or "squamous" cells covering the cervix. Most of the remaining 10 % arise from the glandular, mucus-secreting cells of the cervical canal leading to the uterus (Waggoner 2003). However the incidence of adenocarcinoma is rising in relation to that of squamous cell carcinoma (Young *et al.* 2002). In most cases, adenocarcinoma-in-situ is probably the precursor lesion, but it is detected much less efficiently by Pap smear screening than are pre-invasive squamous lesions. Clear-cell carcinoma is a rare adenocarcinoma subtype, which accounts for fewer than 5 % of adenocarcinomas (Young *et al.* 2002). Previously, many cases developing in young women were associated with *in utero* exposure to diethylstilbestrol (Li *et al.* 2003; Waggoner 2003). Since use of diethylstilbestrol in pregnancy has been prohibited, the number of cases associated with this drug has diminished (Li *et al.* 2003). In the absence of diethylstilbestrol exposure, clear-cell carcinoma most commonly occurs in postmenopausal women. The remaining cases are made up of various subtypes, such as adenosquamous cancers and small-cell (neuroendocrine) carcinomas (Young *et al.* 2002).

The development of cervical cancer is gradual and begins as a pre-cancerous condition called dysplasia. Dysplasia, depending on its severity, can resolve without treatment, particularly in young women. However, it often progresses to actual cancer termed "carcinoma in situ" (CIS) if it has not spread, or "microinvasive" if it has spread only a few millimetres into the surrounding tissue and not into the lymph channels or blood vessels. It may take a few years for dysplasia to turn into CIS or microinvasive cancer, but once this process occurs the cancer can quickly spread deeper into surrounding tissue or other organs, such as the bladder, intestines, liver, or lungs (Juneja *et al.* 2003).

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1.4. Pathology of pre-invasive lesions (Cervical Intraepithelial Neoplasia)

Much controversy still surrounds the histopathologic classification of cervical cancer precursors. The World Health Organisation (WHO) classification system uses the terminology 'dysplasia' and 'carcinoma in situ' to refer to precursor lesions. In the 1960's, Richart introduced the terms cervical intraepithelial neoplasia (CIN) (Richart *et al.* 1993). The nomenclature CIN grades I to III are used to describe pre-invasive epithelial lesions or various categories of dysplasia and carcinoma *in situ* (Wright *et al.* 2003). CIN grade I is equivalent to mild dysplasia in which undifferentiated cells occupy approximately the lower one third of the epithelium. CIN grade II is equivalent to moderate dysplasia where undifferentiated cells replaces two thirds of the thickness of normal epithelium. CIN III denotes severe dysplasia or carcinoma *in situ*. Severe dysplasia describes a condition in which undifferentiated cells replace all but one or two of the most superficial cell layers of the cervical epithelium (Wright *et al.* 2003). All degrees of dysplasia are pre-invasive, meaning that the basement membrane (stromal epithelial junction remains intact).

A newer classification, the Bethesda Classification, has been introduced which designates squamous intra-epithelial lesions (SIL) as either low-grade or high-grade. Low-grade SIL involves mild dysplasia or CIN I, with features of HPV infection. High-grade SIL corresponds to moderate to severe dysplasia, or CIN II and CIN III. In the last stages of neoplasia, before definite invasive carcinoma develops, CIN III is indistinguishable from carcinoma *in situ*. The clinical and biologic utility of the Bethesda Classification has lead to widespread acceptance by both pathological laboratories and clinicians, however there is no widespread consensus as to which terminology should be used.

1.5. Staging and Prognosis of Cervical Cancer

Once a tissue diagnosis of invasive carcinoma has been established, the degree of invasiveness is determined according to the stage of disease set out by The International Federation of Gynecology and Obstetrics (FIGO) as outlined in Table 2.

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Modifications to the FIGO staging system were made in 1994 to clarify the description of microinvasive cervical cancer (stage IA1 and IA2) and to subdivide stage IB into IB1 (tumour < 4 cm) and IB2 (tumour > 4 cm) tumours. For smaller lesions (stage IA and IB1), stage is assigned after measurement of the depth of tumour invasion (on cone biopsy), pelvic examination to assess tumour size clinically, or both. For more advanced tumours, pelvic examination under anaesthesia is occasionally necessary to allow thorough assessment of the parametrial tissues adjacent to the cervix and uterus.

Stage	Description
Stage 0	Carcinoma-in-situ, intraepithelial carcinoma
Stage I	Invasive carcinoma strictly confined to cervix
Stage IA	Invasive carcinoma identified microscopically (all gross lesions, even with superficial invasion, should be assigned to stage IB)
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 lesions confined to cervix
Stage IB1	Clinical lesions of 4 cm or less in size
Stage IB2	Clinical lesions more than 4 cm in size
Stage II	Carcinoma extending beyond cervix but not to pelvic sidewall; carcinoma involves vagina but not its lower third
Stage IIA	Involvement of upper two-thirds of vagina, no parametrial involvement
Stage IIB	Obvious parametrial involvement
Stage III	Carcinoma extending into pelvic wall; on rectal examination, there is no cancer-free space between tumour and pelvic sidewall. The tumour involves lower third of the vagina. All patients with hydronephrosis or non-functioning kidney are included unless known to be the result of other causes
Stage IIIA	Involvement of lower third of the vagina; no extension to pelvic sidewall
Stage IIIB	Extension to pelvic sidewall and/or hydronephrosis or non-functioning kidney
Stage IV	Carcinoma extends beyond the true pelvis or clinically involves mucus of bladder or rectum. Bullous oedema does not allow a case to be designated as stage IV.
Stage IVA	Spread of growth to adjacent organs
Stage IVB	Spread to distant organs

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Table 2: FIGO staging for cervical cancers.

(Waggoner SE, 2003)

Clinical stage is a reliable prognostic indicator for patients with cervical carcinoma. Five-year survival approaches 100 % for patients with tumours of stage IA and averages 70 – 85 % for those with stage IB1 and smaller IIA lesions (Waggoner 2003). Treatment strategies for stage IB and early stage IIA invasive carcinoma include:

1) a primary surgical approach with radical hysterectomy and pelvic lymphadenectomy or
2) primary radiation therapy with external beam radiation and either high-dose-rate or low-dose-rate brachytherapy (Wright *et al.* 2003).

Survival for more locally advanced tumours (stages IB2 to IV) varies and is influenced significantly by the volume of disease, the patient's age, and co-morbidities. Overall, 5-year disease-free survival is 50 – 70 % for stages IB2 and IIB, 30 - 50 % for stage III, and 5 – 15 % for stage IV. Once cervical cancer has extended beyond the cervix, cure with radical surgery alone is unlikely. Treatment strategies for stage IIB and greater involve external-beam and brachytherapy radiation and concurrent cisplatin-based chemotherapy (Waggoner 2003). Metastasis to pelvic, and especially para-aortic, lymph nodes are associated with poorer survival. Although they comprise less than 5 % of cervical carcinomas, adenosquamous tumours and small-cell carcinomas with neuroendocrine features have a particularly poor prognosis (Waggoner 2003).

Surveillance after primary therapy for invasive carcinoma of the cervix is recommended. Approximately 35 % of patients will have persistent or recurrent disease (Waggoner 2003). The main goal of surveillance is early detection of recurrent disease so that patients may be offered potentially curative salvage therapy. The potential benefit of salvage therapy depends on the stage of disease, type of treatment, and location of the recurrence (i.e. local, regional, or distant). In general, radical radiation therapy is used for recurrent cervical cancer after primary hysterectomy, while salvage surgery is required for patients who relapse after primary radiation therapy.

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1.6. Risk Factors for Cervical Cancer

1.6.1 Human Papilloma Virus (HPV)

Human papilloma virus (HPV) is considered to be the most important risk factor contributing to the development of cervical intraepithelial neoplasia and cervical cancer (Juneja *et al.* 2003; Riley *et al.* 2003; An *et al.* 2004; Silins *et al.* 2004). More than 90% of squamous cell carcinomas contain HPV virus DNA (Waggoner 2003). HPVs are mucosal-trophic viruses infecting basal squamous epithelial cells, with the protective phase of the viral life cycle elaborated in the upper squamous epithelial cell layers (Riley *et al.* 2003). Most HPV infections are transient (Hinchliffe *et al.* 1995), but in the minority of patients, persistent viral disease localises in basal squamous cervical epithelial cells of the cervix, and underlies neoplastic progression and emergence of invasive malignancies.

Although many HPV types have been associated with anogenital neoplasia, types 16, 18, 31, 35, 39, 45, 51, 52, 56, and 58 cause most invasive cancers (Juneja *et al.* 2003; Riley *et al.* 2003; Waggoner 2003). HPV 16 and 18 have two transcriptional units, E6 and E7, which encode proteins essential for viral replication (An *et al.* 2004; Szkaradkiewicz *et al.* 2004). These oncoproteins bind to various tumour-suppressor genes to disrupt the cell-cycle progression (Riley *et al.* 2003). Fortunately, not all women who have had HPV infections, or genital warts develop cervical carcinoma. There are other factors believed to play a role in increasing the risk of developing cervical cancer.

1.6.2. Sexual behaviour

Other risk factors believed to contribute towards cervical carcinoma include sexual activity starting at an early age (<16 years), multiple sexual partners and/or partners who have multiple partners, as the risk of contracting sexually transmitted diseases would be much greater in these individuals (Taylor *et al.* 1959; Martin 1967; Brown *et al.* 1984).

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Domenico Rigoni-Stern in the middle of 19th century, who was an Italian chief physician of Verona Hospital and an instructor at University of Padua, first raised the issue of cervical cancer and marriage. He had observed for the first time that more uterine cervical cancer is found in married than unmarried women, based on mortality reports. The relationship of cervical cancer with sexual behaviour is supported by the fact that disease is rare in nuns (Taylor *et al.* 1959; Martin 1967; Brown *et al.* 1984).

Studies performed by Martin in 1967, based the epidemiology of cervical neoplasm on three fundamentals, which are (i) near absence of neoplasms among nuns (ii) near absence of neoplasms among other species (other than humans), and (iii) extremely low incidence of disease among virgins. The main factors explored were early age at marriage, marital dissolution and remarriage. In all studies, cervical carcinoma cases occur more frequently following early coitus, marital dissolution and remarriage than in controls. This highlighted sexual behaviour as an important contributing factor in the process of cervical tumourigenesis.

1.6.3. Other risk factors associated with cervical cancer development

Patients receiving immunosuppressive agents and those who are HIV-positive are also at an increased risk of developing cervical cancer, as any pre-neoplastic cells would be rendered more susceptible to viral infection (Waggoner 2003). Infections with genital herpes or chronic Chlamydia infections (Anttila *et al.* 2001), both sexually transmitted diseases, may also increase risk rendering the cells more susceptible to neoplastic transformation.

Cigarette smoking (and perhaps even exposure to environmental tobacco smoke) is an independent risk factor for significant cervical dysplasia and invasive cervical (Winkelstein 1990; Szarewski *et al.* 1996). Tobacco-specific carcinogens and polycyclic aromatic hydrocarbons have been identified in the cervical mucus or epithelium of smokers. These compounds can bind to and damage cellular DNA and might act, together with HPV, to facilitate malignant transformation.

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Recent studies have also implicated a role for steroid contraceptive hormones in the pathogenesis of invasive cervical cancer (Moodley *et al.* 2003). Steroid hormones are thought to increase the expression of the E6 and E7 HPV 16 oncogenes, which in turn bind to and degrade pro-apoptotic factors, leading to apoptotic failure and carcinogenesis (Baldus *et al.* 2004). However, to date, the molecular mechanisms through which these agents function is unclear.

1.7. Diagnosis of cervical carcinoma

The symptoms of early cervical carcinoma include watery vaginal discharge, intermittent spotting, and postcoital bleeding and because these signs may apply to a broad range of pathologies, the symptoms often go unrecognised by the patient. Since the introduction of the Pap smear by Papanicolaou and Traut in 1943 to detect precursors of cervical cancer in women, cytological screening has been one of the most successful public health measures introduced for the prevention of cancer.

The Pap smear test samples approximately 500, 000 – 600, 000 superficial surface cells from the epithelium of the cervix (exfoliative cytology). Smear preparations are made from these samples and screened for the presence of precursor malignant (dysplasic) cells by using morphologic criteria (Williams *et al.* 1998). A pap smear can pick up dysplasia and early forms of cervical cancer that have not yet spread. Most women diagnosed today with cervical cancer have either not had regular Pap smear tests or they have not followed up after having an abnormal smear (Misra *et al.* 2004). However, it must be understood that the purpose of Pap smear is just to screen for intraepithelial lesions before they progress to invasive disease, and this is subjected to limitations (Hussein *et al.* 1992). It has reduced the incidence of cervical cancer, but it is not expected to detect all the precursor lesions and it is not appropriate to detect large invasive neoplasias.

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Due to the accessibility of the cervix, accurate diagnosis often can be made with cytologic screening (Pap smear) or visualisation of a lesion on the cervix (Misra *et al.* 2004). A biopsy sample must be taken from any suspicious lesion, because many pap smears are non-diagnostic or falsely negative in the presence of invasive cancer. These may be either a colposcopically directed biopsy, or biopsy of a gross or palpable lesion.

In cases of suspected microinvasion and early stage cervical carcinoma, cone biopsy of the cervix is indicated to evaluate the possibility of invasion or to define the depth and extent of microinvasion. Cold knife cone biopsy provides the most accurate evaluations of the margins (Misra *et al.* 2004).

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1.8. Arachidonic acid metabolism

All mammalian cells except erythrocytes synthesize lipid-derived autocoids, known as eicosanoids (Murakami *et al.* 1999). These molecules are extremely potent, able to cause profound physiological effects at very dilute concentrations. All eicosanoids function locally at the site of synthesis, through receptor-mediated G-protein linked signalling pathways.

Eicosanoid biosynthesis, outlined in Figure 1.2, is controlled by the rate-limited release of the C20 fatty acid, arachidonic acid, from plasma membrane phospholipids by a receptor-activated cytosolic phospholipase A_2 (PLA_2) (Murakami *et al.* 1999). The major source of arachidonic acid is through its release from cellular stores. The immediate dietary precursor of arachidonate is linoleic acid. Following its release into intracellular stores, arachidonic acid is oxidised by either cytochromes P450 to epoxyarachidonic acids, or COX to a hydroperoxide cyclic endoperoxide, prostaglandin G_2 (PGG_2). PGG_2 then acts as the substrate for a glutathione-dependent hydroperoxidase, to yield a hydroxy cyclic endoperoxide (PGH_2). PGH_2 is then the substrate for terminal prostanoid synthase enzymes, leading to the prostaglandins, PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) (Goetzl *et al.* 1995).

A widely used class of drugs, the non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, indomethacin, naproxen, phenylbutazone and aspirin, all inhibit both COX-1 and COX-2, as depicted in Figure 1.2. The mode of action of aspirin involves its acetylation of a Ser residue near the active site, preventing arachidonic acid binding. Because inhibition of COX-1 activity in the gut is associated with NSAID-induced ulcerations, pharmaceutical companies have developed drugs targeted exclusively against the inducible COX-2 activity [e.g. Celebrex (celecoxib), Bextra (valdecoxib), Prexige (lumiracoxib) and the recently removed Vioxx (rofecoxib)]. Another class, the corticosteroidal drugs, act to inhibit phospholipase A2, thereby inhibiting the release of arachidonate from membrane phospholipids and the subsequent synthesis of eicosinoids.

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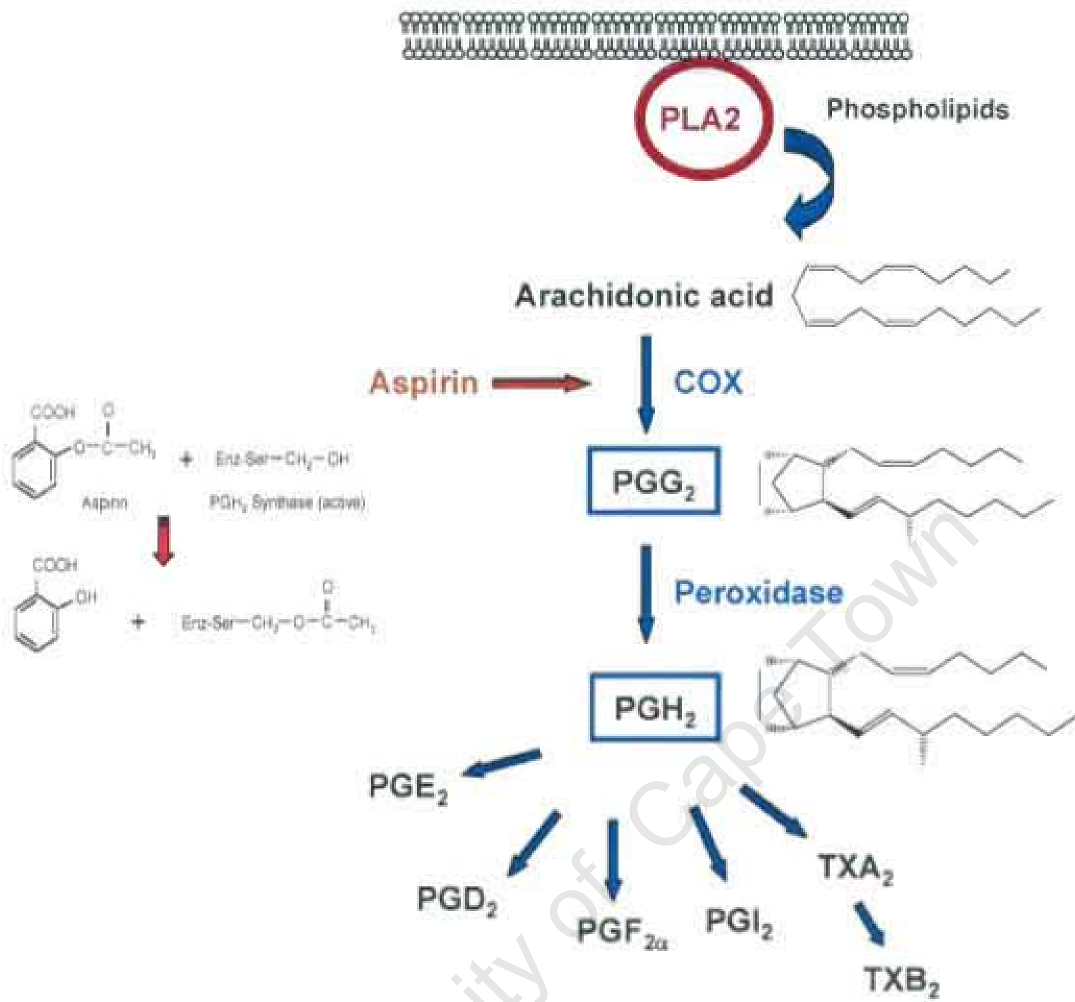

Figure 1.2. Schematic representation of the arachidonic acid cascade.

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1.9. Cyclooxygenase (COX) Enzymes

Three isoforms of the COX-enzyme (COX-1, COX-2 and COX-3) have been identified (Chandrasekharan *et al*. 2002; Morita 2002). The COX-1 cDNA was cloned in 1988 from sheep, mouse, and human sources (Kniss 1999; Marnett 2002). The human COX-1 gene has been mapped to chromosome 9q32-q33.3 and is 25 kb in size. It has 11 exons, produces a 2.8 – 3.0 kb mRNA and 68 kDa protein (Fritsche *et al*. 2001).

The protein has an N-terminal membrane signal sequence, a C-terminal endoplasmic reticulum retention signal, and four potential glycosylation sites. There is no evidence that phosphorylation plays a role in the regulation of COX activity (Turini *et al.* 2002).

COX-2 is an immediate early response gene of 8 kb which is composed of 10 exons and located on human chromosome 1q25.2-q25.3 (Kniss 1999). The mRNA is 4.1 – 4.5 kb and encodes a protein of 68 kDa (Hla *et al.* 1992).

One of the unique differences between the two COX enzymes is the extended C-terminus tail of COX-2, brought about by the insertion of 18 aa, inserted 6 residues from the C-terminus. Post-translational processing of the COX-2 transcript results in the expression of protein homodimers of 72 kDa and 74 kDa, compared with translation of the COX-1 transcript, which results in protein homodimers of 72 kDa subunits (Kniss 1999; Smith *et al.* 2000). Both COX-enzymes exist as dimmers both structurally and functionally. Both isoforms are N-glycosylated at Asn^{68}, Asn^{144} and Asn^{410}. Glycosylation is necessary at Asn^{410} and either Asn^{68} or Asn^{144} for expression of the cyclooxygenase and peroxidase activities. In addition, the COX-2 enzyme is glycosylated at Asn^{580} 50 % of the time, which explains the presence of the 72 kDa and 74 kDa subunits (O'Neill *et al.* 1993; Otto *et al.* 1993; Smith *et al.* 2000).

Both COX isoforms are integral membrane heme-glycoproteins, carry out essentially the same catalytic reaction and have similar tertiary structures (Smith *et al.* 2001). However, the pro-inflammatory role appears to be mediated mainly by COX-2, whereas most of the 'housekeeping' functions appear to be regulated by COX-1 (Smith *et al.* 2001). Both enzymes are present on the luminal surfaces of the endoplasmic reticulum and inner and outer membranes of the nuclear envelope, although COX-2 appears to be more associated with the nuclear envelope than COX-1 (Smith *et al.* 2000). This predominantly nuclear localisation of COX-2 raises the possibility that it may be involved in gene transcription or regulation at a nuclear level.

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More recently, studies have suggested that both COX-enzyme isoforms can be upregulated (Narko *et al.* 1997; Maldve *et al.* 2000; Takeda *et al.* 2003). *In vitro* models have demonstrated that the transcription of both COX-enzymes can be induced by arachidonic acid, prostaglandins, vascular endothelial growth factor (VEGF), forskolin (an activator of adenylate cyclase) and dibutyryl cAMP (a cAMP analogue) (Maldve *et al.* 2000; Prescott *et al.* 2000; Marnett 2002; Iwasaki *et al.* 2003; Takeda *et al.* 2003; Chang *et al.* 2004; Wang *et al.* 2004). These studies have also shown that COX-1 and COX-2 are differently regulated in response to various stimuli, and may be coupled to different prostanoid receptors, which would allow for different prostonoid profiles.

The third isoform of COX, namely COX-3, is a product of COX-1 gene that retains intron 1 in the mRNA (Chandrasekharan *et al.* 2002). In humans, it is expressed as a 5.2 kb transcript in cerebral cortex and heart and selectively inhibited by NSAIDs. However, the role of this isoform in both inflammation and tumourigenesis is unknown.

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1.9.1. Regulation of COX enzymes

In general, COX-1 synthesises prostaglandins that serve noble purposes in housekeeping, such as protecting the stomach from ulcers and regulating renal blood flow (Prescott *et al.* 2000). In selected cells and tissues, including endometrium, monocytes, platelets, renal collecting tubes and seminal vesicles, COX-1 has been reported to be constitutively expressed at high levels (Smith *et al.* 2000; Smith *et al.* 2001). Several studies have also reported expression of COX-1 to be increased in differentiating cells, which suggests that it may be developmentally regulated (Narko *et al.* 1997).

There are several putative transcriptional regulatory elements (Sp1, AP-2, NF-IL-6 and GATA) in the promoter region of the COX-1 gene. However, the COX-1 gene has no TATA or CAAT box and is GC-rich, consistent with the features of a housekeeping gene (Figure 1.3). COX-1 expression is controlled by the Sp1 transcription factor (Taniura *et al.* 2002). The Sp1 *cis*-regulatory element (CRE) on the COX-1 promoter binds the *trans-*

activating Sp1 protein. Deletion of the Sp1 site is associated with significant reduction in basal levels of COX-1 (Smith *et al.* 2000; Smith *et al.* 2001; Subbaramaiah *et al.* 2002).

COX-2 is rapidly induced following stimulation of cells with growth factors and mediators of inflammation, such as the pro-inflammatory cytokine IL-1. IL-1 is upregulated at the site of inflammation and plays a major role in inducing COX-2 by activating the transcription factor NFκB (Kanekura *et al.* 2002; Samad *et al.* 2002). The stimuli necessary for inducing COX-2 expression differ depending on cell type and physiological activity. In the kidney, COX-2 is upregulated in response to increasing salt concentration, whereas in granulosa cells COX-2 is induced in response to follicle-stimulating hormone and luteinizing hormone. Signal transduction pathways linked to the transcriptional activation of COX-2 may differ depending on the stimulus (Gasparini *et al.* 2003). These pathways may be shared or convergent and include the NFκB and MAPK pathways (Kanekura *et al.* 2002; Gasparini *et al.* 2003). COX-2 gene transcription is induced through NFκB by an extracellular-signal-related kinase (ERK), p38, and Jun N-terminal kinase (JNK), through NF-IL6 via p38, and through CRE via ERK2 and JNK pathways. PKC seems to mediate COX-2 transcription through all three promoter sites. COX-2 is transcriptionally downregulated by APC and regulated by c-Myb, and nuclear accumulation of β-catenin, through the *Wnt*-signalling pathway, in human colon and liver carcinogenesis, whereas *k-ras* induces COX-2 mRNA stabilisation (Gasparini *et al.* 2003).

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The molecular regulation of COX-2 gene expression is tightly controlled on transcriptional and post-transcriptional levels. However, dysregulation at either level of COX-2 gene regulation promotes constitutive COX-2 overexpression, which plays a key role in tumourigenesis. Five major regulatory elements flanking the 5′-region of the COX-2 gene have been identified as rigorous regulators of COX-2 transcription. These regulatory elements are: overlapping E-box and ATF/CRE sequences, NF/IL-6 CAAT enhancer binding sites and NFκB binding sites (Figure 1.3.). The NFκB signalling pathway may be activated by various effectors, including hypoxia, endothelin, IL-1β and tumour necrosis factor alpha (TNFα) in various cell types.

Mutations in the NFκB *cis*-regulatory region causes attenuated activation of COX-2 in response to TNFα stimulation as well as reduced binding of NFκB-like proteins, suggesting that NFκB regulates COX-2 expression in response to certain activators (Turini *et al.* 2002). The ATF/CRE site is activated by hetero- and homodimers of the c-fos, c-jun and ATF families and the cAMP regulatory binding protein (CREB) (Hawk *et al.* 2002). Mutations in the ATF/CRE sequence reduces serum-, PDGF-, and Src-stimulated transcription of COX-2 in mice. This suggests a role for cAMP in regulating COX-2 expression. In addition, the COX-2 promoter also contains a peroxisome proliferators-activated response element (PPRE) consensus site suggesting that peroxisome proliferators may modulate transcription of COX-2 (Inoue *et al.* 2000; Han *et al.* 2003; Murakami *et al.* 2004).

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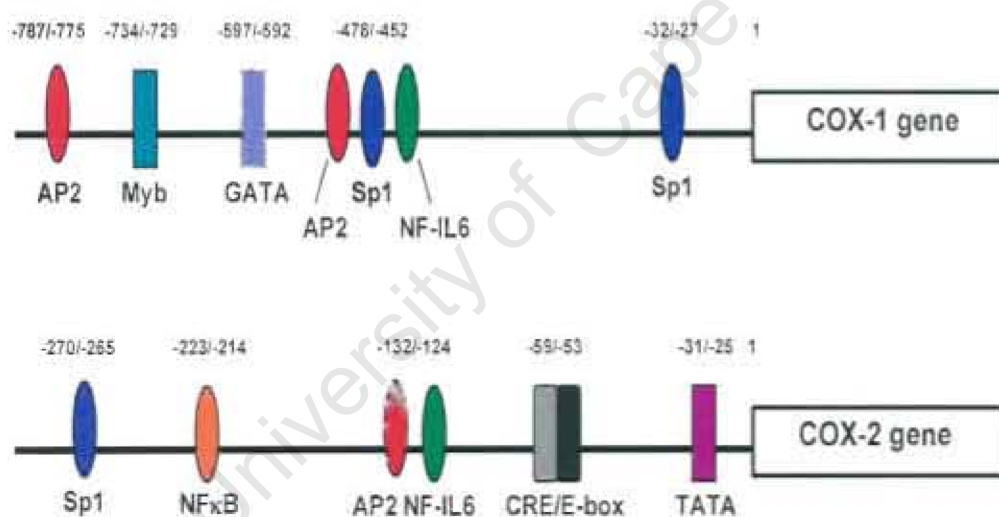

Figure 1.3. Regulatory elements in the promoter region of the human COX-1 and COX-2 genes (Murakami and Kudo, 2004). These promoter regions indicate that COX-1 and COX-2 are housekeeping and immediate early genes, respectively.

Several studies investigating the regulatory mechanisms of COX-2 expression has demonstrated the post-transcriptional regulation to play a role (Dixon 2004). Rapid COX-2 mRNA decay and translational inhibition is mediated through a conserved AU-rich element (ARE) present within the 3'-untranslated region (3'UTR). The COX-2 ARE exerts its control through association with ARE RNA-binding proteins. These trans-acting regulatory factors influence the fate of COX-2 mRNA by controlling mRNA degradation, stabilization, or translation. Recent evidence demonstrates the functional significance rapid mRNA decay and translational inhibition play in controlling COX-2 gene expression and that, if dysregulated, allow for overexpression of COX-2 and other associated angiogenic factors detected in neoplasia (Dixon 2004).

1.10. The role of cyclooxygenase (COX) enzymes in cancer

The mechanisms involving PG biosynthesis were initially outlined in 1967 by Hamberg and Samuelsson. The term prostaglandin endoperoxide (PGH) synthase or cyclooxygenase (COX) was coined to describe the enzyme responsible for catalysing the conversion of fatty acids to prostaglandins (Chandrasekharan *et al.* 2004; Zha *et al.* 2004). Several prostaglandins have subsequently been isolated (Marnett *et al.* 1999; Marnett *et al.* 1999). This lead to the purification of COX-1 in 1976 as a key enzyme in the prostaglandin biosynthetic pathway (Marnett *et al.* 1999; Marnett *et al.* 1999). Subsequently the mode of action of non-steroidal anti-inflammatory drugs (NSAIDs) was attributed to inhibition of a single COX-enzyme (Davis *et al.* 2004). The discovery of a second, inducible form of COX enzyme, known as COX-2, dramatically altered the role of NSAID in health and disease (Kniss 1999; Davis *et al.* 2004). Recently, a third isoform of the COX-enzyme (termed COX-3) has been discovered. It is hypothesised that COX-2 functions in resolution of acute inflammatory responses and that COX-3 is "turned on" later in inflammation and may be involved in the biosynthesis of endogenous anti-inflammatory mediators (Davis *et al.* 2004).

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Initial observations by Rolland *et al.* (1980) indicated that PG production was elevated in human breast cancers (Rolland *et al.* 1980). This hypothesis was based on the findings that PG production in human breast carcinomas correlated with neoplastic cell density and active tumour invasion. Further epidemiological studies revealed that long-term continual administration of the NSAID aspirin reduced the risk of colorectal disease by 40 – 50 %. This demonstrated a negative correlation between NSAID use and development of colorectal cancer (Tsujii *et al.* 1998; Tsujii *et al.* 1998). COX inhibitors also exhibit dramatic anti-neoplastic activity in a number of tumour model systems. These include colon cancer cells implanted into nude mice, tumour production in APC mutant mice, and carcinogen-induced tumours in rats (Oshima *et al.* 1996; Sheng *et al.* 1997; Kawamori *et al.* 1998). In one such study, Seed *et al.* (1997), demonstrated that topical administration of non-selective COX inhibitor (diclofenac) reduced the growth of colon-26 cancer cells, which express COX and produce PGE_2, when implanted into nude mice (Seed *et al.* 1997). In this study, the authors made an important observation that the anti-tumour effects of diclofenac were due to an anti-angiogenic effect. This raised the possibility that elevated COX, especially COX-2, plays a role in the growth of colon cancer cells due to its ability to act as a tumour promoter via stimulation of tumourigenesis and angiogenesis (Kakiuchi *et al.* 2002). Such a mechanism might also explain the 90 % inhibition of the growth of COX-2-positive HCA-7 tumours in vivo following COX-2 inhibitor treatment (Sheng *et al.* 1997).

Studies performed by Folkman (1990) confirmed the hypothesis that tumour growth is dependent on angiogenesis (Folkman 1990; Folkman 1990). Any significant increase in tumour mass must be preceded by an increase in the vascular supply to deliver nutrients and oxygen to the tumour. The ability of a tumour to induce angiogenesis represents an essential step for tumour growth beyond 2 –3 mm in size. Data produced by Tsujii *et al.* (1998) employed an experimental model in which endothelial and cancer cells were co-cultured (Tsujii *et al.* 1998). They found that COX-2 overexpressing cells produce high levels of angiogenic factors, which stimulate endothelial tube formation in the co-culture model. A selective COX-2 inhibitor (NS-398) inhibited both the expression of angiogenic

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factors and endothelial tube formation by the COX-2 overexpressing cells (Tsujii *et al.* 1998; Caughey *et al.* 2001).

Since then many studies have implicated a role for COX enzymes in the development of tumourigenesis and angiogenesis in a wide variety of cancers, including ovarian, endometrial, cervical, gallbladder, colon, and many other forms of cancer (Ryu *et al.* 2000; Jabbour *et al.* 2001; Kulkarni *et al.* 2001; Asano *et al.* 2002; Kundu *et al.* 2002; Ristimaki *et al.* 2002; Sales K.J. 2002; Sales *et al.* 2002; Gupta *et al.* 2003; Sales *et al.* 2003; Gately *et al.* 2004; Zha *et al.* 2004).

1.11. Prostaglandins (PGs)

Prostaglandins were first discovered and isolated from human semen in the 1930's by Ulf von Euler of Sweden. Prostanoids are unsaturated carboxylic acids, consisting of a 20-carbon skeleton that also contains a five-member ring. They are biochemically synthesised COX products derived from C20 unsaturated fatty acids such as arachidonic acid. Prostanoids can be divided into two groups, namely prostaglandins (PGs) and thromboxanes (TXs), and include PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 (Narumiya 2003). Each prostanoid is formed by sequential catalysis of COX and specific synthase, and is released outside of the cells immediately after synthesis. Although it has been shown that PGs can cross the membrane by simple diffusion, the estimated flow rate is too low to maintain biological function. Therefore, prostanoids are transported out of the cell by means of a carrier-mediated process through a prostaglandin transporter (PGT) belonging to the organic anion transporter polypeptide family (Nomura *et al.* 2004). It has been proposed that PGT mediates both the efflux of newly synthesised PGs to effect their biological actions as autocrine/paracrine lipid mediators (i.e. they signal at or immediately adjacent to their site of synthesis) (Funk 2001) through their cell surface receptors, and influx of PGs from the extracellular milieu for their inactivation or action through specific nuclear receptors (Banu *et al.* 2003).

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The role prostanoids play in the body has been examined by the use of NSAIDs and by analyzing actions of prostanoids added exogenously. These studies suggest that prostanoids work in a variety of processes such as fever generation, inflammatory swelling and pain, and bleeding and hemostasis. Thromboxane has been found to stimulate constriction and clotting of blood vessels (Caughey *et al.* 2001). Conversely, PGI_2 is produced to have the opposite effect on the walls of blood vessels, where clots should not be forming (Gately *et al.* 2004).

Certain prostanoids have also been associated with the induction of labor and other reproductive processes. PGE_2 has been shown to cause uterine contractions and has been used to induce labor (Kniss 1999). Prostanoids are also involved in several other organs, such as the gastrointestinal tract (inhibit acid synthesis and increase secretion of protective mucus) (Hoshino *et al.* 2003), increase blood flow to kidneys (Breyer *et al.* 2001), and leukotriens promote constriction of bronchi associated with asthma. A few prostanoids have also been implicated in the immune response, for example in studies using EP4-deficient mice, stimulation of the EP4 receptor by PGE_2 in dendritic cells facilitated their migration and maturation, however stimulation in T-cells potently suppresses their activation and proliferation (Narumiya 2003). This latter action is evident in PGE_2-mediated suppression of T cell proliferation in the gut of mice subjected to dextran sodium sulfate-induced colitis, a model of inflammatory bowel disease (Narumiya 2003). These studies have revealed that prostanoids work at various sites or levels of immune responses and exert many, often opposing actions.

1.12. Prostaglandin receptors

The biological actions of PGs are mediated by specific heptahelical G protein-coupled receptors (GPCRs) (Breyer *et al.* 2001) (Figure 1.4). There are at least 9 known prostanoid receptor forms in mouse and man, as well as several additional splice variants with divergent carboxy termini. Four of the receptor subtypes bind PGE_2 (EP1, EP2, EP3 and EP4), two bind PGD_2 (DP1 and DP2), and the receptors that bind $PGF_{2\alpha}$, PGI_2, and TXA_2 (FP, IP, and TP, respectively) each derive from a single gene (Funk 2001). The

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prostaglandin receptors belong to three clusters (on the basis of homology and signalling attributes rather than by ligand binding properties) within a distinct subfamily of the GPCRs. The lone exception is DP2, which is a member of the chemoattractant receptor subgrouping. The "relaxant" receptors IP, DP1, EP2, and EP4 form one cluster, signalling through $G\alpha_s$-mediated increases in intracellular cyclic adenosine monophosphate (cAMP); the "contractile" receptors EP1, FP, and TP form a second group that signals through $G\alpha_q$-mediated increases in intracellular calcium (Funk 2001). The EP3 receptor has several splice variants that may couple to either $G\alpha_s$ or $G\alpha_i$ resulting in either an increase or decrease in intracellular cAMP; or $G\alpha_q$, resulting in an accumulation of inositol triphosphate (IP_3) and an increase in intracellular Ca^{2+} depending on the splice variant and type of cell (Narumiya *et al.* 1993; Sugimoto *et al.* 1993; Narumiya *et al.* 2001).

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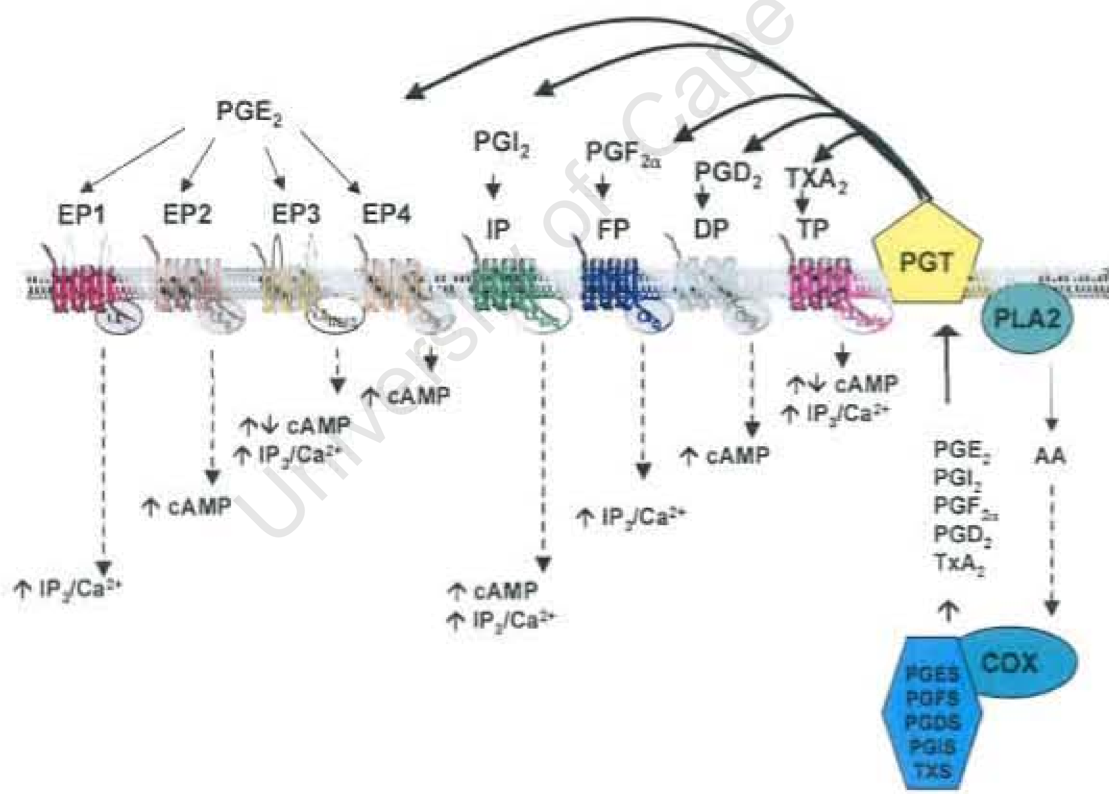

Figure 1.4. Prostanoids and their interaction with specific prostanoid receptors.
(Jabbour and Sales, 2004)

Within the prostanoid family, the receptors share a sequence homology of 20 – 30%, and overall, there are 65-amino-acid residues conserved among the prostanoid receptors (Breyer *et al.* 2001). The majority of these conserved residues lie within the transmembrane regions, although a significant stretch of conserved amino acids exist in the second extracellular loop region. Functionally, there is evidence that both transmembrane and extracellular regions of the prostanoid receptor are involved in ligand binding (Breyer *et al.* 2001). In general, it has been assumed that signal transduction cascades are initiated after ligand-receptor binding at the plasma membrane. However, Bhattacharya *et al.*, (1998, 1999) have ascertained a nuclear localisation for EP receptors, indicating that PGE_2 could directly regulate the transcription of target genes after the release of calcium from nuclear calcium pools or by activation of calcium channels (Bhattacharya *et al.* 1998; Bhattacharya *et al.* 1999). In addition, it is possible the PGE_2 influences transcription of target genes by interacting with nuclear PPARs. Although no role for PGE_2, $PGF_{2\alpha}$ and PPAR in regulation of gene transcription has been described, prostanoids such as PGI_2 and PGJ_2 have been reported as ligands for PPARα and PPARδ, which alter transcription of target genes involved in lipid metabolism and homeostasis (Forman *et al.* 1997; Sales *et al.* 2003). This diversity of receptors with opposing functions may confer a homeostatic control of an autocoid, which is released in high concentrations close to its site of synthesis (Ashby 1998; Sales *et al.* 2003).

1.12.1. The role of prostanoid receptors

The complex biological actions of the prostanoids may be at least partly due to the existence of multiple subtypes and isoforms of prostanoid receptors. Molecular cloning of the individual receptor subtypes and subsequent elucidation of their biochemical properties and expression patterns have been a pivotal step in understanding prostanoid physiology. The importance of the activity of each prostanoid has also been assessed by the analyses of phenotypes of knockout mice for each receptor subtype, under various physiological and pathophysiological settings (Table 3).

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Genotypes	Phenotypes
DP(-/-)	Decreased allergic responses in ovalbumin-induced bronchial asthma
EP1(-/-)	Decreased aberrant foci formation to azoxymethane
EP2(-/-)	Impaired ovulation and fertilization
	Salt-sensitive hypertension
	Vasodepressor or impaired vasodepressor response to intravenous PGE2
	Loss of bronchodilation with PGE2
	Impaired osteoclastogenesis in vitro
EP3(-/-)	Impaired febrile response to pyrogens
	Impaired duodenal bicarbonate secretion and mucosal integrity
	Enhanced vasodepressor response to intravenous infusion of PGE2
	Disappearance of indomethacin-sensitive urine diluting function
EP4(-/-)	Patent ductus arteriosus
	Impaired vasodepressor response to intravenous infusion of PGE2
	Decreased inflammation-dependent bone resorption
FP(-/-)	Loss of parturition
IP(-/-)	Thrombotic tendency
	Decreased inflammatory swelling
	Decreased acetic acid writhing
TP(-/-)	Bleeding tendency and resistance to thromboembolism

Table 3. Major phenotypes of prostanoid receptor knockout mice.
(Kobayashi *et al.* 2002)

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1.12.1.1. Receptors mediating central nervous system actions

Fever

Fever is a representative component of acute phase response to immunological challenge and is elicited by cellular components of infectious organisms, such as LPS, as well as by non-infectious inflammatory insults (Kobayashi 2002). Both infectious and non-infectious insults stimulate the production of cytokines that work as endogenous pyrogens. These cytokines, including IL-1, IL-6, TNF-α, IFN-α, and IFN-γ transmit a signal to the preoptic area, which then stimulates the neural pathways that raise body temperature (Kobayashi 2002). Fever can be suppressed by NSAIDs such as aspirin and indomethacin, indicating that PGs are important in fever generation.
Ushikubi *et al.* (1998) used knockout mice models for each of the EP receptors to investigate their febrile responses to PGE_2, IL-1β and LPS. These studies showed that EP3 receptor-deficient mice failed to show febrile responses to all of these stimuli, implying an important role for this receptor in fever regulation (Ushikubi *et al.* 1998).

Sleep

PGD_2 is a potent endogenous sleep promoting substance in rats and other mammals, including humans (Hayaishi 2000). To clarify the involvement of the DP1 receptor in PGD_2-induced sleep, Mizoguchi *et al.* (2001) infused PGD_2 into the lateral ventricle of wild-type and DP1-deficient mice and determined the number of non-rapid eye movements (NREM) and rapid eye movement (REM) sleep. Results from this study indicated that PGD_2 predominantly increased NREM sleep in wild-type mice and that DP receptors are crucially involved in PGD_2-induced NREM sleep (Mizoguchi *et al.* 2001).

1.12.1.2. Receptors mediating inflammation and pain

Local reddening, heat generation, swelling and pain are classic signs of acute inflammation. Each of these symptoms, except pain, is caused by increased blood flow and vascular permeability with resultant edema (Kobayashi 2002). Previous studies suggested that PGs are primarily involved in vasodilation in the inflammatory process and synergise with other mediators, such as histamine and bradykinin to cause an increase in vascular permeability and edema. These studies have also showed that PGE_2 and PGI_2 are potent prostanoids in causing these effects and that both these PGs are present in high concentrations at sites of inflammation (Davies *et al.* 1984). Murata *et al.* (1997) used IP-deficient mice to test the role of PGI_2 in inflammatory swelling. They employed a carageenan-induced paw swelling as a model. In this model, paw swelling increasedin a time-dependent manner following injection and was decreased by about 50% by treatment with indomethacin. IP-deficient mice developed swelling only to a level comparable to that observed in indomethacin-treated wild-type mice. These data demonstrate that PGI_2 and IP receptor work as the principal PG system mediating vascular changes in this model of inflammation (Murata *et al.* 1997).

The role of prostaglandins in inflammatory pain is also well accepted. This is partly due to the antinociceptive effects of aspirin-like drugs, and also because of documentation in various model systems that PGs added exogenously are able to induce hyperalgesia, an increased sensitivity to painful stimulus, or allodynia, a pain response to a usually non-

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painful stimulus (Kobayashi 2002). These studies using exogenous PGs show that PGE_2 and PGI_2 exert stronger effects than the other types of PGs, indicating the involvement of EP or IP receptors in inducing inflammatory pain (Bley KR *et al.*, 1998). Ueno *et al.* (2001) pretreated EP1-/-, EP2-/-, EP3-/-, EP4-/-, IP-/- receptor knockout and wild-type mice with LPS and then examined their hyperalgesic responses. Results demonstrated that the nociception of the writhing response in non-treated mice is mediated mainly by the IP receptor and the perception of enhanced pain in LPS-pretreated mice can be mediated by both the IP and EP3 receptors (Ueno *et al.* 2001).

In addition to hyperalgesic actions, PGs in the spinal cord are also reported to be involved in elicitation of allodynia. To characterise the PGE receptor subtype(s) involved in PGE_2-induced mechanical allodynia, Minami *et al.* (2001) examined whether PGE_2 could induce allodynia in EP1- and EP3-deficient mice. Intrathecal administration of PGE_2 induced allodynia in wild-type and EP3-deficient mice, but not in EP1-deficient mice (Minami *et al.* 2001). These results clearly demonstrate that the EP1 receptor is involved in PGE_2-induced allodynia.

1.12.1.3. Receptors mediating allergy and immunity

Allergic asthma is caused by the aberrant expansion of Th cells in the lung that produce Th2-cytokines, and is characterised by the infiltration of eosinophils and bronchial hyperreactivity (Kobayashi 2002). This disease is often triggered by mast cells activated by an immuglobulin (Ig) E-mediated allergic challenge. Activated mast cells release various chemical mediators. PGD_2 is the major prostanoid produced by these cells in response to antigen challenge (Murray *et al.* 1986). However, the pathological significance of PGD_2 in allergic asthma remains unclear. Matsuoka *et al.* (2000) used DP-deficient mice to focus on the role of PGD_2 in asthma. These studies demonstrate that PGD_2 produced in response to allergic challenge acts at DP receptors in the lung to recruit lymphocytes to the site of challenge (Matsuoka *et al.* 2001). Thus, PGD_2 functions as a mast cell-derived mediator to trigger asthmatic responses. PGE_2 has also been reported to attenuate some acute inflammatory responses initiated by mast cell degranulation. Raud *et al.* (1988) demonstrated that indomethacin markedly potentiates

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antigen-induced plasma protein extravasation, leukocyte accumulation and histamine release in sensitised hamsters. PGE_2 completely reversed the indomethacin-induced potentiation of plasma extravasation and also effectively reversed the number of emigrating leukocytes after indomethacin treatment (Raud *et al.* 1988). Histamine release was reduced by almost 60 % in the presence of PGE_2. The identity of the EP receptor mediating this action remains to be clarified.

PGs have also been shown to regulate the production or release of pro-inflammatory cytokines. Recently, Shinomiya *et al.* (2001) collected peritoneal macrophages from wild-type, IP-/-, EP2-/- and EP4-/- mice and examined the effects of PGE_2 or the PGI_2 analogue carbacyclin on the production of TNFα and IL-10 by these macrophages stimulated with zymosan (Shinomiya *et al.* 2001). The addition of PGE_2 or carbacyclin to wild-type macrophages reduced the TNFα production to one-half, whereas IL-10 production increased several fold (Shinomiya *et al.* 2001). Macrophages collected from IP-deficient mice showed a down-regulation of TNFα production and up-regulation of IL-10 production only in response to EP2 and EP4 agonists or PGE_2, but not to carbacyclin. Conversely, EP2-/- and EP4-/- macrophages lacked the response to EP2 and EP4 agonists, but not to PGE_2 or carbacyclin (Shinomiya *et al.* 2001). These results demonstrate that PGE_2 and PGI_2 regulate production of pro-inflammatory (TNFα) and anti-inflammatory (IL-10) cytokines redundantly through EP2, EP4 and IP receptors.

1.12.1.4. Receptors mediating vascular homeostasis

Thrombosis and hemostasis

Most PGs elicit contractile and/or relaxant activities on vascular smooth muscles. In particular, PGI_2 and TXA_2 produced abundantly by vascular endothelial cells and platelets, respectively, are a potent vasodilator and vasoconstrictor, respectively. Murata *et al.* (1997) created mice deficient in IP receptor and found that while IP-deficient mice lack the hypotensive response to the synthetic IP agonist cicaprost, their basal blood pressure and heart rate were not different from those of control animals. This result indicates that the PGI_2 and IP system does not work constitutively in regulating the

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systemic circulation but more likely works on demand in response to local stimuli (Murata *et al.* 1997). PGI_2 and TXA_2 also act on platelets to inhibit or induce, respectively, platelet activation and aggregation. Because of their opposite actions on blood vessels and platelets, it has been proposed that a balance between the PGI_2 and TXA_2 systems is important for maintaining vascular homeostasis (i.e. to prevent thrombosis and vasospasm while performing efficient homeostasis) (Murata *et al.* 1997).

PGE_2 also elicits contractile and/or relaxant responses when tested on vascular smooth muscles *in vitro*. Kennedy *et al.* (1999) administered PGE_2 and PGE analogs intravenously into wild-type and EP2-deficient mice and examined the response *in vivo*. They observed that infusion of PGE_2 or an EP2 agonist, butaprost, induces a transient hypotension in wild-type mice, whereas injection of the mixed EP1/3 agonist, sulprostone, resulted in an increase in mean arterial blood pressure. The hypotensive response to butaprost was not observed, and the hypertensive effects of sulprostone persisted in EP2-deficient mice, and, surprisingly, PGE_2 evoked considerable hypertension. Kennedy *et al.* (1999) suggested that the absence of the EP2 receptor abolishes the ability of the mouse vasculature to vasodilate in response to PGE_2 and unmasks the contractile response mediated via the vasoconstrictor EP receptor(s). Interestingly, when fed a high-salt diet, the EP2-deficient mice develop significant hypertension with a concomitant increase in urinary excretion of PGE_2 (Kennedy *et al.* 1999). These results indicate that PGE_2 is produced in the body in response to a high-salt diet and works to decrease blood pressure via the relaxant EP2 receptor and that dysfunction of this pathway may be involved in the development of salt-sensitive hypertension (Kennedy CR *et al.*, 1999). Studies performed by Audoly *et al.* (1999) demonstrated striking differences in the hemodynamic actions of PGE_2 using knockout mice for each of the EP receptors (Audoly *et al.* 1999). In females, EP2 and EP4 were found to mediate the major portion of the vasodepressor response, whereas in males most of the vasodepressor effect was mediated by EP1. The EP3 receptor in males actively opposes the vasodepressor actions of PGE_2. This demonstrated that the hemodynamic actions of PGE_2 are mediated through complex interactions of several EP receptor

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subtypes, and the role of individual EP receptors may differ dramatically in males from that in females (Audoly *et al.* 1999).

Vascular remodelling: closing of ductus arteriosis

At birth, mammals including humans undergo a dramatic change in their circulation with the commencement of respiration, i.e. from the fetal circulation system that shunts blood flow from the main pulmonary artery directly to the aorta via the ductus arteriosus, to the pulmonary circulation system of the neonate (Kobayashi 2002). This adaptive change is caused by the closure of the ductus arteriosus. The patency of the ductus during the fetal period is maintained principally by the dilator effects of PGs, and its closure is induced by withdrawal of the dilator prostaglandins as well as active contraction exerted by an increased oxygen tension (Smith *et al.* 1998). This concept is supported by the fact that administration of aspirin-like drugs or a vasodilator PG, such as PGE_1, suppresses or maintains, respectively, the patency of the ductus in neonates with patent ductus arteiosus (Smith *et al.* 1998). A study using various synthetic PG analogs suggested that both IP and EP4 receptors are present in the ductus and are involved in the dilation of this vessel (Smith *et al.* 1994). Disruption of the mouse IP gene did not appear to cause any abnormality of the ductus (Smith *et al.* 1994). On the other hand, most EP4-deficient mice die within 3 days after birth, due to marked pulmonary congestion and heart failure. Administration of indomethacin into maternal mice during late pregnancy elicited premature closure of the ductus in wild-type fetuses, but not in the ductus of EP4-deficient fetuses, indicating that the dilatory effect of PGE_2 on this vessel is mediated by the EP4 receptor (Smith *et al.* 1994).

These results suggest a critical role for the EP4 receptor in the ductus and can be interpreted to mean that in the absence of the EP4 receptor a compensatory mechanism maintains ductus patency not only in the fetal period but also after birth.

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1.12.1.5. Receptors mediating reproduction

Ovulation and fertilization

Recent studies on COX-2-deficient mice showed multiple reproductive failures in early pregnancy, such as in ovulation, fertilization, implantation, and decidualization, suggesting that PGs play essential roles in these processes (Hizaki *et al.* 1999; Kennedy *et al.* 1999; Tilley *et al.* 1999; Kobayashi 2002). Because IP-, EP1-, EP3-, EP4- and TP-deficient females are fertile, these receptors may be dispensable in female reproduction (Kobayashi 2002). Recently, three groups reported reproductive failure in early pregnancy in EP2-deficient female mice (Hizaki *et al.* 1999; Kennedy *et al.* 1999; Tilley *et al.* 1999). Kennedy *et al.* (1999) and Tilley *et al.* (1999) reported that EP2-deficient female mice consistently deliver fewer pups than their wild-type counterparts irrespective of the genotypes of mating males. They detected slightly impaired ovulation and a dramatic reduction in fertilization in EP2-deficient mice and concluded that the reproductive failure during early pregnancy in COX-2-deficient mice is due to dysfunction of the EP2 receptor.

As described above, it has been suggested that COX-2 and its products contribute to implantation. The EP2 receptor may play a role in this process, since its mRNA is highly induced in luminal epithelial cells during the pre-implantation period via a steroid-dependent pathway (Katsuyama *et al.* 1998). However, the uteri of EP2-deficient females appear normal in their ability to support implantation of wild-type embryos. One possibility is that EP4 expression in the luminal epithelium can compensate for the EP2 receptor in implantation (Kobayashi 2002). Recently, Lim H *et al.* (1999) reported that the impaired ability for implantation in COX-2-deficient mice is reversed by both PGI_2 analogs and an agonist for PPARδ. They proposed that COX-2-derived PGI_2 may participate in implantation through PPARδ (Lim *et al.* 1999).

Leuteolysis and parturition

$PGF_{2\alpha}$ is accepted as an inducer of leuteolysisin domestic animals such as the sheep and cow, and has been implicated in parturition via its action as a strong uterotonic substance (Kobayashi 2002). However, FP-deficient mice do not show any abnormalities in early

pregnancy, and there were no changes in the estrous cycle. Sugimoto *et al.* (1997) found that, despite no alteration in the estrous cycle, FP-deficient female mice do not undergo parturition, apparently due to lack of labor. They further found that FP-deficient mice do not undergo parturition even when given exogenous oxytocin and that they show no prepartum decline in progesterone (Sugimoto *et al.* 1998). These experiments show that the luteolytic action of $PGF_{2\alpha}$ is required in mice to diminish progesterone levels and thus permit the initiation of labor. Administration of exogenous $PGF_{2\alpha}$ to these mice can furthermore rescue parturition and result in normal birth. It has been shown in many species that a large amount of PGs are produced in intrauterine tissues during labor, but the exact roles of these PGs remain to be fully elucidated.

1.12.1.6. Receptors mediating gastrointestinal functions

The PG are widely distributed in the digestive system and are involved in a number of physiological processes including motility, blood flow, water and electrolyte absorption, and mucus secretion (Kobayashi 2002). In addition, treatment with aspirin-like drugs reduce the risk of colorectal neoplasia, and the involvement of PGE_2 in the proliferative activity of the colonic epithelium has been suggested (Kobayashi 2002). In spite of the accumulating evidence for the involvement of PGs in these physiological and pathophysiological processes, little is known about the receptor types involved in each of the processes occurring in the gastrointestinal tract. Takeuchi *et al.* (1999) showed that EP3, but not EP1, is involved in acid-induced duodenal bicarbonate secretion, which is physiologically important in mucosal defence against acid injury (Takeuchi *et al.* 1999). Studies performed by Boku *et al.* (2001) reported that endogenous PGI_2, but not PGE_2, had a role in adaptive cytoprotection of gastric mucosa (Boku *et al.* 2001). The rationale for COX-isoforms and their products having roles in the development of colon cancer are based on both human epidemiological data and experiments on rodents (Rao *et al.* 1995; Kobayashi 2002). Epidemiological studies have shown that aspirin reduces colon cancer mortality in humans (Thun *et al.* 1991; Thun *et al.* 1993). Further support of a role for COX-2 in intestinal neoplasia has been presented by Oshima *et al.* (1996), who showed that genetic disruption of COX-2 reduces the number and size of the intestinal polyps

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dramatically in *Apc*-knockout mice. Sonoshita *et al.* (2001) demonstrated that homozygous deletion of the EP2 receptor in *Apc* knockout mice caused significant decreases in the number and size of the intestinal polyps, effects similar to those induced by COX-2 gene disruption (Sonoshita *et al.* 2001). Studies performed by Watanabe *et al.*, (1999) used EP1 and EP3 knockout mice in a model of colon carcinogenesis. Treatment of EP3-deficient mice with the colon carcinogen, azoxymethane caused aberrant crypt foci, putative pre-neoplastic lesions of the colon with an incidence similar to that of wild-type mice (Watanabe *et al.* 2000). In contrast, foci formation was decreased in EP1-deficient mice to ~60 % of the level of wild-type mice. Furthermore, partial reduction of foci formation was observed following the administration of an EP1-specific antagonist (ONO-8711) in the diet of azoxymethane-treated mice (Watanabe *et al.* 2000). A similar treatment also reduced the number of polyps in Min mice (Watanabe *et al.* 1999). These results suggest that PGE_2 may contribute to colon carcinogenesis to some extent through its action on the EP1 receptor.

1.12.1.7. Receptors mediating bone metabolism

Bones undergo continuous resorption and renewal, a process termed bone remodelling. Bone resorption is carried out by osteoclasts, and bone formation, by osteoblasts (Kobayashi 2002). These events are controlled by systemic humoral factors such as parathyroid hormone, estradiol, and Vitamin D as well as by local cytokines such as IL-1β, IL-6 and insulin-like growth factor (Kobayashi 2002). PGs, particularly PGEs, can also affect bone remodelling, in both bone formation and resorption. The bone resorptive activity of PGE is associated with an increase in the number of osteoclasts. Studies performed by Sakuma *et al.* (2000) and Miyaura *et al.* (2000) reported impaired osteoclast formation in cells cultured from EP4-deficient mice. PGE_2 was added to cultures of parietal bone from mice deficient in each of the EP receptors, as well as wild-type mice, and the bone resorptive activity was examined. Bone resorption was much decreased in bones from EP4-deficient mice. These studies confirm the role of the EP4 receptor in PGE_2-mediated bone resorption (Miyaura *et al.* 2000; Sakuma *et al.* 2000).

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1.12.1.8. The role of EP receptors in cancer development

Some EP receptors are also reported to be involved in tumourigenesis (Table 4). Although the efficacy of selective blockade of prostanoid receptor signalling in angiogenesis has not been investigated fully, an anti-PGE_2 antibody is reported to have an anti-tumour effect (Zweifel *et al.* 2002) and other results indicate that EP receptor subtypes promote tumour-associated angiogenesis (Seno *et al.* 2002; Yang *et al.* 2003).
As discussed previously, PGE_2 has been shown to promote the growth of colorectal carcinomas, in part through activation of EP1 and EP4 receptors. Studies performed by Watanabe *et al.* (1999) and Mutoh *et al.* (2002), treated several lines of knockout mice with a colon carcinogen, azoxymethane, and found that aberrant crypt foci were significantly suppressed in both EP1- and EP4-deficient mice. This is particularly important as aberrant crypt formation represents an initial step in colon tumourigenesis and illustrates an important role for both the EP1 and EP4 receptors in this process. Further studies performed by Pozzi *et al.* (2004), used mouse colon adenocarcinoma (CT26) cells to investigate the role of PGE_2 in cell proliferation. CT26 cells express both COX-1 and COX-2 and metabolize arachidonic acid to PGE_2. Treatment with indomethacin, or COX-selective inhibitors, prevented PGE_2 biosynthesis and CT26 cell proliferation. The anti-proliferative effects of COX inhibition were rescued specifically by treatment with PGE_2 or the EP4 receptor-selective agonist via PI3K/ERK activation, thus providing a functional link between PGE_2-induced cell proliferation and EP4-mediated signalling (Pozzi *et al.* 2004).

An important factor in tumour development is angiogenesis, and tumour-associated angiogenesis is mediated by the migration and proliferation of host endothelial cells. Substantial increases in tumour mass must be preceded by an increase in blood supply to provide the nutrients and oxygen required for tumour growth and it has been suggested that the mechanisms that promote angiogenesis are activated in the early stages of tumour development (Majima *et al.* 2003). Studies performed by Yang *et al.* (2003) using isolated synovial cells demonstrated *in vitro* that EP2 receptor signalling is related to VEGF concentration. The sponge models in prostanoid receptor knockout mice have

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clarified the prostanoid receptor signalling responsible for angiogenesis *in vivo* (Amano *et al.* 2003). Proliferative granulation tissues formed around the sponge implants exhibit extensive angiogenesis in a COX-2 dependent manner, which mimics the stromal angiogenic response around tumours. Topical injections of wild-type mice with specific EP agonists revealed that only the EP3 receptor agonist markedly increased the extent of angiogenesis (Amano *et al.* 2003). Data published by Spinella *et al.* (2004) implicate a role for PGE_2-EP4 receptor interaction in the expression and secretion of VEGF in ovarian carcinoma cells. Their data suggest that targeting the PGE_2- and EP4-related signalling cascade in ovarian carcinoma could effectively impair the transcription of target genes associated with angiogenesis and invasiveness (Spinella *et al.* 2004).

Prostanoid	Receptor	Relationship to cancer development	References
PGE_2	EP1	EP1 receptor antagonist decreases the incidence of aberrant crypt foci in azoxymethane-treated mice	Watanabe K *et al*., 2000
		EP1 receptor -/- mice are resistant to azoxymethane-induced aberrent crypt foci	Watanabe K, 1999
PGE_2	EP2	In EP2 receptor -/- mice, the number of *Apc* $^{(delta)716}$ intestinal polyps and the intensity of angiogenesis and VEGF expression are decreased	Sonoshita M *et al.*, 2001; Seno H *et al.*, 2002
		EP2 receptor -/- miceexhibit cancer-associated immunodeficiency and defective dendritic-cell differentiation	Yang L *et al*., 2003
		EP2 receptor -/- mice exhibit reduced tumour growth but normal tumour-associated angiogenesis and VEGF induction	Yang L et al., 2003
PGE_2	EP3	EP3 -/- mice have reduced tumour-associated angiogenesis and tumour growth because induction of VEGF is reduced	Amano H *et al*., 2003
PGE_2	EP4	EP4 receptor mediates cell proliferation and motility of colorectal carcinoma cells	Sheng H *et al*., 2001
		EP4 receptor -/- mice are resistant to azoxymethane-induced aberrent crypt foci	Mutoh M *et al*., 2002

Table 4: PGE_2 signalling and cancer development.
(Majima *et al.* 2003)

1.13. Prostaglandin E_2 (PGE_2) and its receptors

As discussed previously, PGE_2 is a major product of cyclooxygenase-initiated arachidonic acid metabiolism. PGE_2 may have multiple and at times apparently opposing functional effects on a given target tissue. For example, the vasodilator effects of PGE_2 have long been recognised in both arterial and venous beds (Lawrence *et al.* 1992; Coleman *et al.* 1994; Lydford *et al.* 1996; Davis *et al.* 2004).

PGE_2 has also been shown to be a potent constrictor in other smooth muscle beds, including trachea, gastric fundus, and ileum (Breyer *et al.* 2001). Renal PGE_2 synthesis is critical for the maintenance of normal renal function (Schweda *et al.* 2004). PGE_2 not only dilates the glomerular microcirculation and vasa recta, supplying the renal medulla, it also modulates salt and water transport in the distal tubule (Breyer *et al.* 2001). PGE_2 has also associated with the induction of labor (causing uterine contractions) and other reproductive processes, such as menstruation (Rees *et al.* 1984).

PGE_2 elicits its autocrine/paracrine effects on target cells through interaction with transmembrane GPCRs as mentioned in Section 1.12. To date four main sub-types of PGE_2 receptors have been identified based on responses to agonists and antagonists and are pharmacologically divided into EP1, EP2, EP3 and EP4, which utilise alternative and in some cases opposing intracellular signalling pathways (Tsuboi *et al.* 2002).

1.13.1. EP1 receptors

The EP1 receptor was originally described as a smooth muscle constrictor. The cloned human EP1 receptor cDNA encodes a 402-amino acid polypeptide. EP1 receptor mRNA is expressed most highly in the kidney, followed by gastric muscularis mucosae and then adrenal tissue. PGE_2 interaction with the EP1 receptor increases intracellular calcium via G_q, leading to signals via IP_3 generation and increased Ca^{2+}.

Okuda-Ashitaka E *et al.*, 1996, described distinct signalling for an EP1 receptor mRNA variant (Okuda-Ashitaka *et al.* 1996). Although this variant binds ligand in a manner similar to that of the rat EP1 (rEP1) receptor, it does not elicit detectable signal transduction. When the rEP1-variant was stably co-transfected with the longer rEP1 receptor in CHO cells, the Ca^{2+} mobilisation mediated by the EP1 receptor was significantly suppressed, suggesting that the shorter variant antagonised rEP1 receptor signalling. Furthermore, when the rEP1-variant receptor was expressed in CHO cells, cAMP formation by activation of endogenous EP4 receptor was blocked. These authors suggest that the rEP1-variant receptor may affect the efficiency of signal coupling of

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PGE receptors and attenuate the action of PGE_2 on tissues (Okuda-Ashitaka *et al.* 1996). Although the mechanism of action of this alternative variant of the EP1 receptor is unclear, recent evidence suggests that GPCRs may form dimers (Jordan *et al.* 1999). If this EP1 variant associated with other EP receptors, it might form non-productive dimers and act as a dominant-negative regulator of signal transduction (Breyer 2001; Wise *et al.* 2002).

1.13.2. EP2 receptors

EP2 receptor cDNA, originally reported in Genbank as EP4 receptor in 1995 (Bastien *et al.* 1994), encodes a 358-amino acid polypeptide that signals through an increase in intracellular cAMP. The tissue distribution of EP2 receptor has been only partially characterised, using northern blot analysis of mRNA distribution, which revealed a mRNA species of ~3.1 kb, which is most abundantly expressed in uterus, lung, and spleen (Smock *et al.* 1999; Desai *et al.* 2000). In general, EP2 mRNA is expressed at much lower levels in tissue than EP4 mRNA (Desai *et al.* 2000).

The EP2 receptor was originally characterised by its ability to cause smooth muscle relaxation in cat trachea (Gardiner 1986). Moreover, relaxation of the trachea could be stimulated with the EP2 specific agonist, known as butaprost. The use of butaprost has allowed functional distinguishing between the EP2 and EP4 receptors.

Although they have similar affinities for PGE_2, and function similarly to activate cAMP, the EP2 and EP4 receptors have distinct structural characteristics, and only share approximately 38 % identity in their transmembrane domains. This is not appreciably different from the aa identity they share with the EP1 (37 %) and EP3 (34 %) receptors. EP2 is a 358-amino acid protein with a relatively short intracellular loop and C-terminal domains (Regan *et al.* 1994). On the other hand, the EP4 receptor consists of 488 aa, and has a long third intracellular loop and a long cytoplasmic tail (Slipetz *et al.* 2001). The EP2 receptor does not undergo agonist-induced, short-term desensitisation, whereas EP4 desensitises rapidly (Bastepe 1997; Bastepe *et al.* 1999; Slipetz *et al.* 2001). Hence,

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although boundaries between the roles of the EP2 and EP4 receptors remain to be clarified, agonist-induced short-term desensitisation is a significant regulatory element in EP4-mediated signalling, but not in EP2-mediated signalling. This suggests that EP2 may be involved in mediating sustained actions of PGE_2, whereas EP4 mediates rapidly waning events (Desai *et al.* 2000).

Although initial studies of the EP2 and EP4 receptors indicated that these receptors did not appear to activate IP_3 or inhibit cAMP formation, it now appears that they are capable of activating other secondary messenger pathways, which in the case of the EP4 receptors may be cAMP independent. Studies performed by Fujino H *et al.* (2002) demonstrated that by using a T-cell factor/lymphoid enhancer factor (Tcf/Lef) responsive luciferase reporter gene, PGE_2 could stimulate promoter activity in HEK cells stably expressing these receptors (Fujino *et al.* 2002). This stimulation of reporter gene activity was associated with an agonist dependent phosphorylation of glycogen synthase kinase-3 (GSK-3), which is known to inhibit activity of this enzyme. Inhibition of GSK-3 decreases the phosphorylation of cytosolic β-catenin to the nucleus where it can alter gene expression via interactions with the Tcf/Lef family of transcription factors (Cadigan *et al.* 1997). Interestingly, this stimulation of Tcf/Lef reporter gene activity by the EP2 and EP4 receptors occurs through different mechanisms. EP2 receptor-mediated activation of Tcf transcriptional activity is primarily through a cAMP/protein kinase A (PKA) dependent mechanism; whereas EP4 receptor mediated activation occurs primarily through a phosphatidylinositol 3-kinase (PI3K) dependent pathway (Fujino *et al.* 2002; Fujino *et al.* 2003).

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1.13.3. EP3 receptors

The EP3 receptor generally acts as a constrictor of smooth muscle. Nuclease protection and Northern blot assays have demonstrated high levels of EP3 receptor expression in several tissues, including kidney, uterus, adrenal, and stomach. This receptor is unique in that it has several splice variants defined by unique C-terminal cytoplasmic tails (Breyer *et al.* 2001; Bockaert *et al.* 2003). These splice variants encode proteins of a predicted

molecular mass between 40 and 45 kDa. Proposed functional differences between the splice variants of EP3 include alternate signal transduction pathway usage, receptor phosphorylation and desensitisation, and intracellular trafficking (Breyer 2001). Several of the phenotypes may be interrelated, for example, the intracellular localisation may determine the signal transduction pathway activated. Although these variants generally inhibit cAMP generation via pertussis toxin-sensitive G_i-coupled mechanism, additional signalling mechanisms including G_s and Ca^{2+} release appear to be differentially activated by C-terminal tails (Breyer *et al.* 2001). Recent studies suggest that the EP3 receptor signals through the small G-protein Rho (Aoki *et al.* 1999; Hatae *et al.* 2002). Studies performed by Yano *et al.*, 2002, have also implicated the EP3 receptor in activation of the Ras signal pathway (Yano *et al.* 2002). The EP3 receptor also activates protein kinase C (PKC) and cAMP-independent CRE-mediated gene transcription in HEK293-transfected cells (Audoly *et al.* 1999).

Despite the extensive characterisation of EP3 receptor splice variants in cell culture systems, physiologic significance of these different C-terminal splice variants remains uncertain.

1.13.4. EP4 receptors

As mentioned previously, EP4 receptor signals through increased cAMP. The human EP4 receptor cDNA encodes a 488-amino-acid polypeptide with a predicted molecular mass of ~53 kDa. In addition to the human EP4 receptor, EP4 receptors for the mouse, rat, rabbit, and cow have been cloned (Honda *et al.* 1993; Bastien *et al.* 1994; Nishigaki *et al.* 1995; Breyer *et al.* 1996; Breyer *et al.* 1996; Boie *et al.* 1997; Arosh *et al.* 2003). EP4 receptor mRNA is widely distributed in many human tissues, including thymus, ileum, lung, spleen, adrenal, and kidney. PGE_2 is a potent mediator of the immune system, and activation of EP4 receptor has been shown to stimulate isotype switching to IgE and inhibit B-cell activation (Fedyk *et al.* 1996). Recent evidence also suggests that the potent anabolic effects of PGE_2 in mouse and rat bone marrow are mediated via the EP4 receptor (Ono *et al.* 1998; Weinreb *et al.* 1999). In support of this hypothesis, a potent

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and selective EP4 antagonist suppressed PGE_2-mediated increases in trabecular bone volume in young rats (Machwate *et al.* 2001).

Important vasodilator effects of EP4 receptor activation have been described in venous and arterial beds (Coleman 1994). As mentioned previously, an important role for the EP4 receptor in regulating the peri-natal closure of the pulmonary ductus arteriosis has also been suggested by the recent studies of mice with targeted disruption of the EP4 receptor gene (Nguyen *et al.* 1997; Segi *et al.* 1998). Thus, EP4 receptor ligands may prove useful in promoting closure or maintaining patency of the ductus arteriosus in newborns with congenital heart disease (Kajino *et al.* 2004). Other roles for the EP4 receptor in controlling blood pressure have been suggested, including the ability to stimulate the release of aldosterone from zona glomerulosa cells (Csukas *et al.* 1998). These reports illustrate the emerging importance of EP4 and the potential for therapeutic intervention with EP4 agonists in many human diseases.

Table 4 (Section 1.12) illustrates a role for the EP4 receptor in carcinogenesis. The use of gene knockout mice studies has contributed significantly to our understanding of the potential physiological and pathophysiological role of prostanoid receptors. Some of these gene knockout studies are now being complemented by pharmacological studies using selective agonists and antagonists that have been developed to these receptors (Narumiya *et al.* 2001). Gene knockout studies have also been used to study the potential involvement of the EP4 receptor in colon cancer. Homozygous deletion of the EP4 receptor decreased the formation of aberrant crypt foci (putative preneoplastic lesions) in animals that had been treated with azoxymethane, a known colon carcinogen (Mutoh *et al.* 2002). Further evidence of the potential involvement of the EP4 receptor in colon cancer was obtained in pharmacological studies in which an EP4-selective antagonist decreased the number of aberrant crypt foci in azoxymethane-treated mice (Mutoh *et al.* 2002). This EP4 selective antagonist also decreased intestinal polyp formation in *Min* mice (Mutoh *et al.* 2002). The *Min* mouse model for human familial adenomatous polyposis, whereby a mutation in a tumour suppressor gene results in a nearly 100 % incidence in colon cancer.

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Recent studies by Fujino H and Regan JW, (2003) have also proposed a mechanism that could explain how the EP4 receptor could induce the expression of COX-2 and PGE_2 synthase, which are both known to be upregulated in cancer and inflammation (Fujino *et al.* 2003). PGE_2 stimulation of the EP4 receptor can activate a PI3K/ERK signalling pathway resulting in the induction of functional EGR-1 expression. This involvement of PI3K with EP4 receptor signalling has been further strengthened by a recent study showing that PGE_2 stimulation of HEK cells stably expressing EP4 receptors leads to the phosphorylation of extracellular signal-regulated kinases (ERKs) by a PI3K dependent mechanism (Fujino *et al.* 2003; Fujino *et al.* 2003). Furthermore, this activation of PI3K/ERK signalling by the EP4 receptor induced the functional expression of early growth response factor (EGR-1) and it was not observed in HEK cells stably transfected with the EP2 receptor. This induction of EGR-1 is potentially significant since a number of genes, including PGE_2 synthase, cyclin D1 and TNFα are known to be regulated by EGR-1 (Fujino *et al.* 2003; Fujino *et al.* 2003). These interactions of the EP4 receptor with various signalling pathways could also help explain the recent gene knockout studies that demonstrate a role for EP4 receptor in cancer and inflammation. Likewise, EP4 receptor stimulation can activate a PI3K dependent pathway resulting in *Tcf* transcriptional activation (Fujino *et al.* 2002) and COX-2 expression has been shown to be upregulated by nuclear β-catenin accumulation and *Tcf*-mediated transcriptional activation (Araki *et al.* 2003). β-Catenin is a transcriptional activator and has been implicated in embryonic development and cancer (Moon *et al.* 2002). GSK-3 phosphorylates β-catenin leading to cytosolic sequestration and degradation; phosphorylation of GSK-3 inhibits its activity and allows β-catenin to translocate to the nucleus.

Thus, stimulation of the EP4 receptor has the potential to induce expression of both PGES and COX-2, thereby setting up a positive feedback loop in which the increased synthesis of PGE_2 would further stimulate the receptor (Regan 2003).

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1.14. Other prostaglandins and their receptors

PGD_2

PGD_2 is the major prostanoid released from mast cells after challenge with IgE, and it has also been shown to affect the sleep-wake cycle and body temperature (Lewis *et al.* 1982; Lewis *et al.* 1982; Urade *et al.* 1999). Peripherally, PGD_2 has been shown to mediate vasodilation and vasoconstriction, as well as inhibition of platelet aggregation (Leff *et al.* 1992). PGD_2 exerts its effects by binding and activating two distinct GPCRs – the DP receptor (DP1) and the recently discovered CRTH2 receptor (DP2) (Hata *et al.* 2004). The DP1 receptor shows significant sequence homology with the other members of the prostanoid GPCRs, such as the IP and EP2 receptors (Hata *et al.* 2004). DP1 receptor mRNA is expressed at low levels in bronchial epithelium and has been proposed to mediate production of chemokines and cytokines that recruit inflammatory lymphocytes and eosinophils, leading to airway inflammation and hyperreactivity seen in asthma. Activation of the DP1 receptor leads to mobilisation of intracellular calcium, but not production of inositol phosphate (Boie *et al.* 1995). DP1 receptor knockout mice display decreased allergic responses towards ovalbumin-induced bronchial asthma suggesting that PGD_2 may act as a mediator of allergic asthma (Narumiya *et al.* 2001).

The CRTH2 (DP2) receptor shows little similarity with the DP1 receptor, despite the fact that it possesses similar affinity for PGD_2; instead it is more closely related to other chemoattractant receptors (Hata *et al.* 2004). This receptor binds an overlapping but distinct set of ligands compared with the DP1 receptor. This raises the possibility that certain metabolites of PGD_2 may exert effects through CRTH2 but not the DP1 receptor. Similar to many chemoattractant receptors, CRTH2 receptor activation leads to increases in intracellular calcium in a variety of cell types. CRTH2 receptor is expressed on Th2 lymphocytes, eosinophils, and basophils. CRTH2 receptor activation has been reported to lead to pertussis toxin-insensitive activation of PI3-kinase, PLC, and MAP kinases in eosinophils. These pathways mediate eosinophil shape change, actin polymerisation, and CD11 up-regulation (Hata *et al.* 2004).

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Therefore, PGD_2 acting through both DP1 and CRTH2 receptors likely contributes to the eosinophilic infiltration that is a hallmark of allergic asthma. Characterization of mice deficient in the CRTH2 receptor will begin to clarify the exact role that each receptor plays in mediating the effects of PGD_2 in allergic inflammation.

$PGF_{2\alpha}$

$PGF_{2\alpha}$ is produced during the menstrual cycle by secretory endometrium and plays a crucial role in mammalian reproduction. $PGF_{2\alpha}$ also plays a role in renal function (Breyer *et al.* 2001), cardiac hypertrophy, and regulation of intraocular pressure (Hata *et al.* 2004). $PGF_{2\alpha}$ exerts its action via FP receptors, which are expressed most abundantly in the corpus luteum. It is critical for normal birth, and homozygous disruption of the murine FP receptor gene results in failure of parturition, apparently due to failure of the normal preterm decline in progesterone levels (Sugimoto *et al.* 1994). $PGF_{2\alpha}$-FP interaction results in tyrosine phosphorylation and subsequent increase in Ca^{2+}, PLC activation and DNA synthesis (Narumiya *et al.* 2001). Recent studies have ascertained a role for $PGF_{2\alpha}$-FP receptor interaction in endometrial cancer (Sales *et al.* 2004).

PGI_2

Prostacyclin (PGI_2) is a primary prostaglandin produced by endothelial cells and plays an important role in vascular homeostasis as a result of its potent vasodilatory and antithrombotic effects (Caughey *et al.* 2001). PGI_2 exerts its physiological functions via IP receptors. IP receptors are widely distributed throughout the dorsal root ganglia suggesting it is also a mediator of acute inflammation and inflammatory pain transmission (Sales *et al.* 2004). In the kidney, IP receptors are localised to the glomerulus suggesting a role in glomerular filtration (Breyer *et al.* 2001; Breyer *et al.* 2001). The IP receptor usually couples to a G_s-type protein leading to an increase in cAMP, although differential coupling to multiple signalling pathways may be modulated by C-terminal modification (Sales *et al.* 2004). IP knockout mice display sensitivity towards thrombosis as well as exhibiting decreased inflammatory swelling (Narumiya *et al.* 2001).

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TXA_2

Thromboxane A_2 (TXA_2) has been most extensively characterized for its role in modulating hemodynamics and cardiovascular function (Caughey *et al.* 2001). It is a potent mediator of platelet shape change and aggregation. TXA_2 or thromboxane exerts its functions via TP receptors, which are expressed abundantly in the vasculature as well as the heart, lung and kidney (Breyer *et al.* 2001). TXA_2 receptors signal via G_q G-protein activating Ca^{2+}/DAG signalling pathways (Breyer *et al.* 2001). TP null mutations in mice are associated with bleeding tendency and resistance to thromboembolism (Narumiya *et al.* 2001).

1.15. Seminal plasma composition and prostaglandins

PGE_2 and 19-hydroxyprostaglandin E (19-hydroxy PGE) present in mM concentrations within semen (Figure 1.5.) (Taylor *et al.* 1974; Templeton *et al.* 1978) are thought to be the principal effectors of the immunosuppresssion. These levels of prostaglandins are 5 orders of magnitude (10 000 times) greater than those found locally at a site of inflammation (Figure 1.5.). They are potent stimulators of cAMP, thus inhibiting lymphocyte proliferation and natural killer cell activity, and are likely to modify cytokine release from antigen-presenting cells (Kelly 1995). Human seminal plasma has powerful immunosuppressive properties containing high concentrations of the soluble p55 tumour necrosis factor-α (TNF-α) receptor (Liabakk *et al.* 1993), receptors for the Fc portion of γ-globulin, transforming growth factorβ (TGFβ) (Nocera *et al.* 1993), spermine (Evans *et al.* 1995), complement inhibitors (Kelly 1995), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) (Gutsche *et al.* 2003).

Studies performed by Gutsche S *et al.* (2003) demonstrated that seminal plasma stimulates expression of pro-inflammatory cytokines in endometrial epithelial cells *in vitro* (Gutsche *et al.* 2003). Robertson *et al.* (2002) suggested that seminal plasma might elicit the production of pro-inflammatory cytokines by endometrial epithelial cells in the outer endocervical canal or cervical ectropion, thereby leading to the formation of a post-coital inflammatory response (Robertson *et al.* 2002). The cervical inflammatory cells

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may then alter the local uterine immune response, which in turn indirectly affects blastocyst function (Robertson *et al.* 2002).

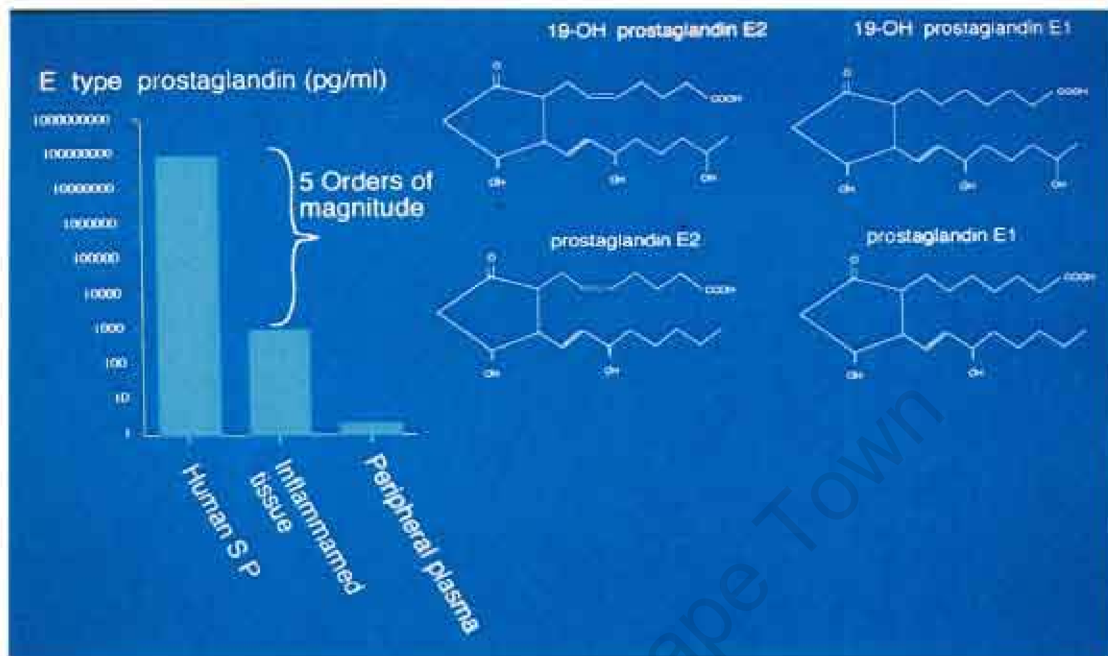

Figure 1.5. Levels of prostaglandins in seminal plasma.
(Kelly RW)

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Aims and Objectives of the Thesis

As discussed in Section 1.1, cervical carcinoma is an important clinical problem in South African women. Gynaecologic malignancies of this nature have an enormous socio-economic impact on health care, resources and morale of a country. Research into the molecular mechanisms governing cervical cancer and its precursors could lead to improved therapies for women suffering from this condition.

Several researchers have implied an important role for prostaglandins and their receptors in the development of tumourigenesis. However, in the case of cervical tumourigenesis, pre-neoplastic cervical epithelial cells will potentially be under the direct influence from PGE available in seminal plasma. As discussed previously, levels of PGs in seminal plasma are 10 000 times higher than those found at a site of inflammation. Cervical tumourigenesis may thus be regulated in an autocrine/paracrine manner by PGE present in seminal plasma via PGE-EP receptor interaction.

Previous studies have confirmed that seminal plasma and PGE_2 induce expression of COX-2 and cAMP linked PGE receptors (namely EP2 and EP4) in the HeLa cervical epithelial cell line (Sales *et al.* 2001). These findings are coincident with enhanced intracellular signalling of the EP2/EP4 receptors in response to stimulation with seminal plasma or exogenous PGE (Sales *et al.* 2002; Sales *et al.* 2002).

Taken together, these data suggest that endogenously and exogenously (via seminal plasma) induced COX/PGE biosynthetic pathways may play a critical role in the regulation of cervical tumourigenesis and highlight the risk of seminal plasma in enhancing cervical tumourigenesis in sexually active women with dysplasias.

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The specific aims of research were to:

i) Determine the localisation of the site of expression of EP4 receptor in cervical carcinomas and normal cervix. *This was conducted using immunohistochemical analysis on cervical tissue sections as outlined in Chapter 3.*

ii) Investigate the molecular signal transduction pathways mediating the role of elevated EP4 receptor in cervical carcinoma cells. *This was achieved using a stably transfected EP4-overexpressing HeLa cell line, which was established as described in Chapter 4.*

iii) Investigate the interaction between seminal plasma and cervical neoplastic epithelial cells. *This was conducted by investigating possible signal transduction pathways, genes and receptors regulated by PGE_2-EP4 receptor activation as outlined in Chapter 5 and 6.*

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CHAPTER 2 - GENERAL MATERIALS AND METHODS

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2.1. Chemicals and Suppliers

All chemicals used were molecular biology grade, and were obtained from Sigma Chemical Company (Dorset, UK or Cape Town, South Africa) and IBI (Cambridge, UK) unless otherwise stated. Enzymes were purchased from Boehringer Mannheim (Buckinghamshire, UK) or Promega (Southhampton, UK or Cape Town, South Africa). Phenol/Chloroform, pre-buffered with Tris pH 8.0, was purchased from Camlab (Cambridge, UK) and all photographic film was purchased from Eastman Kodak (Rochester, NY, USA) and supplied by Sigma Chemical Company.

Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 was purchased from Life Technologies (Gibco, Life Technologies, Paisly, UK), penicillin-streptomycin was purchased from PAA (PAA Laboratories Ltd., Middlesex, UK).
Samples and synthetic standards for the PGE_2 ELISA assays were purchased from Applied Therapeutics (Applied Therapeutics, Paisly, UK). ECF and ECLplus chemiluminescence systems were purchased from Amersham (Amersham, Little Chalfont, Bucks, UK). cAMP kits were purchased from Biomol (Biomol Affiniti, Exeter, UK). VEGF ELISA assay kits were purchased from Oncogene (Oncogene, Beeston, Nottingham, UK).

Seeblue™ pre-stained molecular weight marker was purchased from Novex (Novex, UK). Whatman no. 3 paper was purchased from Whatman (Whatman, UK) and polyvinylidene difluoride membrane (PVDF) membrane was purchased from Millipore (Millipore, Watford, UK). G418 and indomethacin were purchased from Sigma (Sigma Chemical Co., Dorset, UK). NS-398 was purchased from Calbiochem (Calbiochem, Beeston, Nottingham, UK). The MEK inhibitor (PD98059) (18.7mM stock in dimethylsulfoxide, DMSO) and an inhibitor of EGF receptor tyrosine kinase (AG 1478) (10mM stock in DMSO) were purchased from Calbiochem (Nottingham, UK).
EP4 antagonist (ONO-AE2-227) was chemically synthesised by Charnwood Molecular Ltd., Leics, UK (See Appendix II).

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The following antibodies used for Western blotting were purchased from Santa Cruz Biotechnology, inc. (Autogenbioclear, Wiltshire, UK): COX-2 goat polyclonal (sc-1745); ß-actin goat polyclonal (sc-1616); VEGF rabbit polyclonal (sc-152); c-Myc (9E10) (sc-40); Total 42/44 MAPK antibody (ERK) (sc-93). EP4 receptor rabbit antibody (101775) was purchased from Cayman Chemical Company (Caymen Chemical, Cheshire, UK). Phospho-p42/44 MAPK antibody (9101); phospho-AKT antibody (9271); Total AKT antibody (9272); phosphorylated JNK antibody (9251); Total JNK antibody (9252); Phospho-p38MAPK antibody (9211) and p38MAPK antibody (9212) were purchased from Cell Signalling Technology (Cell Signalling Technology, New England Biolabs (UK) Ltd., Herts, UK). Anti-goat-alkaline phosphatase, anti-rabbit-alkaline phosphatase, were purchased from Sigma (Sigma Chemical Co., Dorset, UK).

2.2. Tissue Collection and processing

Cervical specimens were obtained at the time of surgery or biopsy from patients that were attending the Gynaecologic Oncology Clinic at Groote Schuur Hospital, Cape Town and that had previously been diagnosed with invasive carcinoma of the cervix. Punch biopsies were taken from the lesion by an experienced gynaecologist with a special interest in oncology. Histologically normal cervical samples were obtained from patients undergoing Wertheims hysterectomy for non-malignant conditions. Informed consent was obtained from all patients before tissue collection. This study was approved by the University of Cape Town Research Ethics Committee.

Tissue sections for immunohistochemical analysis were obtained from biopsy material, which had been fixed in formalin. The tissue was placed in disposable embedding moulds (Polysciences) followed by paraffin wax-embedding. Glass slides to be used for immunohistochemistry were washed in a 0.25 % solution of 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone, followed by a wash in acetone and finally a rinse in filtered double-distilled water and dried. Paraffin wax-embedded tissue was sectioned to a thinkness of 5 μM using a hand-operated "820" Spencer Microtome

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(American Optical Corporation) and a D-profile knife. Sections were floated on water, transferred to coated slides and dried overnight before use.

2.3. Seminal plasma collection

Semen was collected from healthy male volunteers. The collected ejaculates were pooled and incubated at room temperature for 30 minutes prior to overlaying on a 100-50 % percoll gradient. Seminal plasma was isolated from the pooled ejaculate by percoll density gradient centrifugation at 500 g for 20 minutes. The seminal plasma was then pooled and stored at –70°C. The seminal plasma was used on the cells at various concentrations of 1:50 up to 1:5000. At these dilutions, seminal fluid has been reported to exert no effect on HeLa cell viability (Jeremias *et al.* 1999).

2.4. Maintenance of HeLa cell lines

The HeLa-S3 wild-type cells were purchased from BioWhittaker (BioWhittaker, Berkshire, UK). Stable EP4-overexpressing HeLa (EP4S) cells and stable antisense (EP4AS) cells were routinely cultured under sterile conditions in culture flasks with a surface area of 170 cm^2 (Corning Science Products, UK). Cells were grown at 37°C and 5 % CO_2 (v/v) in 50 mls of complete DMEM (Dulbecco's Modified Eagle's medium nutrient F-12 (Gibco) with glutamax and pyroxidine), supplemented with 10 % Foetal Calf Serum (Clontech), and 1 % antibiotics (stock 500 IU/ml penicillin and 500 μg/ml streptomycin). Cells grew as a monolayer on the bottom of the flask. Cells were observed using an inverted light microscope (Olympus CK40), to determine the level of confluence and were passaged at about 80 % confluency. The growth medium was removed by vacuum suction (Dymax 30, Charles Austen Pumps, UK), and cells were washed twice with PBS, thereafter 1 ml of trypsin-EDTA (0.1 % trypsin and 0.04 % EDTA in PBS) was added to each flask. The trypsin was allowed to flow over the monolayer surface, and the flask was then left at 37°C for 3 minutes. Flasks were then tapped to loosen cells. Trypsin was inhibited by the addition of 9 ml of complete DMEM into the flasks. Cells were re-suspended by pipette action and 1 ml cell suspension was transferred into a new

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170 cm^2 flask. Cells were then transferred into the incubator and incubated at 37°C in humidified 5 % CO_2 (v/v). The EP4S and EP4AS cells were maintained under the same conditions with the addition of 200 µg/ml of G418.

2.5. Plasmid Preparation and Transfections into HeLa cells

2.5.1. Plasmids

The cDNA (1.5kb) for human prostanoid receptor EP4 was ligated into the *EcoRV* site of the mammalian expression vector pcDNA3 (Invitrogen) (see Appendix I). These pcDNA3 vectors containing the EP4 cDNA in both the sense and antisense directions were kindly supplied by Dr. Mark Abramovitz in the Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, Quebec, Canada.
Other plasmids used in this study are listed as follows: pcDNA3 containing dominant negative cDNA EGFR insert (see Appendix I); pcDNA3 containing dominant negative cDNA MEK insert (see Appendix I); pcDNA3 containing dominant negative cDNA RAF insert (see Appendix I) were kindly supplied by Prof. Zvi Noor, Department of Biochemistry, Tel Aviv University, Israel.

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2.5.2. Transformation of competent cells with plasmid DNA

Plasmids were transformed into competent TOP10 cells using TOP10 One Shot kit (Invitrogen, UK). Two µl of 0.5 M ß-mercaptoethanol was added to a vial of competent cells together with 2 µl of TOPO cloning reaction and 100 µg of DNA and mixed prior to incubation on ice for 30 minutes. Cells were then heat shocked at 42°C for 30 seconds and transferred to ice for 2 minutes. To this mixture, 250 µl SOC medium (supplied with the kit) was added and the tube incubated under vigorous shaking at 37°C for 30 minutes. Thereafter, 50 µl and 100 µl aliquots were streaked out and grown on LB agar plates containing 10 mg/ml ampicillin at 37°C overnight. Single colonies were picked, and inoculated into 10 ml of LB broth containing 10 mg/ml ampicillin and grown at 37°C

under constant agitation overnight. Plasmid DNA was recovered using Qiagen endofree plasmid isolation kit (Qiagen, GmbH, Crawley, UK).

2.5.3. Large Scale Plasmid DNA Recovery – Maxiprep

Large-scale plasmid recovery was carried out using the Endofree™ plasmid Maxi kit (Qiagen), an alkaline lysis plasmid recovery system, according to manufacturers instructions. Briefly, 100 ml of an overnight bacterial culture was pelleted by centrifugation at 4°C for 15 minutes at 6000 g. The pelleted cells were resuspended in 10 mls of buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 100 µg/ml RNAse A). The cells were then incubated for 5 minutes at room temperature with 10 ml of buffer P2 (200 mM NaOH and 1 % SDS), which facilitates cell lysis. The cell lysate was neutralised by the addition of 10 ml of 3 M potassium acetate pH 5.5, transferred to a QIAfilter, incubated for 10 minutes and then filtered. Thereafter, 2.5 ml of buffer ER (Qiagen) was added and the filtered cell lysate was incubated for 30 minutes on ice. Subsequently, the cell lysate was applied to the QIAGEN-tip and then washed twice with 30 ml of buffer QC (1 mM NaCl, 50 mM MOPS pH 7.0 and 15 % isopropanol). The plasmid DNA was then eluted with 15 ml of buffer QN (1.6 mM NaCl, 50 mM MOPS pH 7.0 and 15 % isopropanol) and precipitated with 10.5 ml of isopropanol. The sample was then centrifuged at 4°C for 30 minutes at 15 000 g. The supernatant was removed, the DNA pellet was washed with 15 ml of 70 % ethanol and then centrifuged at 4°C for 15 minutes at 15 000 g. Thereafter, the pellet was air dried and resuspended in 1.5 ml of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The concentration and quality of the DNA was determined using spectrophotometry at 260 nm and 280 nm. The concentration of the DNA was calculated from the 260 nm value obtained, given that an optical density of 1.0 is equal to 50 µg/ml for double-stranded DNA and 33 µg/ml for single-stranded DNA. DNA quality was determined by dividing the 260 nm reading by the 280 nm reading; a ratio of 1.6 to 1.9 was taken to be of sufficient quality containing minimal protein contamination. Purified DNA was stored at –20°C.

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2.6. cAMP assays

EP4 receptor signalling was assessed by measuring cAMP accumulation following stimulation of cells with either PGE_2 (300 nm) or seminal plasma (various dilutions of 1:250 up to 1:5 000). Cells ($2x10^5$) were seeded in 6 well dishes and allowed to attach overnight. The next day, cells were washed twice with PBS followed by incubation with serum-free DMEM for a minimum of 16 hours to allow synchronization. Cells were then stimulated for the time periods indicated in the figure legends. Following treatments, the medium was removed and the cells washed in ice-cold PBS before being lysed in 0.1 M HCl. cAMP concentration was quantified by ELISA using a cAMP kit (Biomol Affiniti, Exeter, UK) as per the manufacturer's protocol and normalized to the protein concentration of the lysate.

Protein concentrations were determined using protein assay kits (Bio-Rad) as described in Section 2.8.2. The ELISA was performed using a 96 well microtitre plate provided in the assay kit. The wells of the plate are pre-coated with goat anti-rabbit IgG. cAMP standards (100 μl of each), ranging from 200 pmol/ml up to 0.78 pmol/ml, were added to the plate to produce a standard curve. A 100 μl volume of sample was then added to the plate in duplicate. Alkaline phosphatase-cAMP conjugate (50 μl) and 50 μl of polyclonal rabbit anti-cAMP antibody (both provided with the kit) were added to each well of the plate.
The plate was then incubated at room temperature for 2 hours on a plate shaker at ~500 rpm. Thereafter, the wells were aspirated and washed three times with the wash buffer provided (TBST; 50 mM Tris-HCl, 150 mM NaCl, 0.5% Tween®20, pH 7.4; containing sodium azide). The assay was developed by the addition of 200 μl/well of p-nitrophenyl phosphate (provided with the kit). The colour reaction was allowed to develop for 20 minutes and was stopped by the addition of 200 μl/well of stop solution provided (trisodium phosphate in water). Colour reaction was measured at 405 nm by spectrophotometry. The concentration of cAMP per sample was calculated by extrapolation from the standard curve using the Assay Zap computer program (Biosoft, UK). Results are presented as Mean ± SEM from 3 independent experiments.

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2.7. RNA

2.7.1. Total RNA extraction

Total RNA was isolated from HeLa cells, which had been routinely maintained as described in Section 2.2. RNA was isolated using a commercially available guanidinium thiocyanate-based extraction reagent Tri-reagent (Sigma-Aldrich, Dorset, UK) according to the manufacturer's protocol. Volumes were adjusted appropriately, allowing approximately 1 ml Tri-reagent per $1x10^6$ cells. Cells were lysed for 1 minute in Tri-Reagent until completely dissociated and then supplemented with 0.1 volume of 1-bromo-3-chloropropane (BCP) per volume of Tri-reagent used. After vigorous shaking for 20 seconds, the mixture was allowed to stand at room temperature for 5 minutes before centrifuging at 14 000 g for 20 minutes at 4°C. Thereafter the upper aqueous layer, containing the RNA was transferred to a fresh RNAse-free eppendorf tube and the RNA was precipitated with 1 volume of isopropanol (Sigma-Aldrich). The RNA was pelleted by centrifugation at 14 000 g for 20 minutes at 4°C. Following removal of the supernatant, the RNA pellet was washed in 75 % ethanol and then dissolved in RNAse-free water at 65°C for 5 minutes. RNA was quantified using spectrophotometrically.

2.7.2. Determination of RNA concentration

The concentration and quality of RNA was determined by spectrophotometry at 260 nm and 280 nm. The concentration of the RNA was calculated from the 260 nm value obtained, given that an optical density of 1.0 is equal to 40 µg/ml for RNA. RNA quality was determined by dividing the 260 nm reading by the 280 nm reading; a ratio of 1.6 to 1.9 was taken to be of sufficient quality containing minimal protein contamination. Purified RNA was stored at –70°C.

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2.7.3. Reverse-Transcription (RT) Reaction

Total RNA was extracted from HeLa cells using Tri-reagent (Sigma-Aldrich) following the manufacturer's instructions as described in Section 2.7.1. For reverse transcription reactions, 200 μg of RNA was reverse transcribed using a TaqMan GeneAMP RNA PCR kit (Perkin Elmer, PE Biosystems, Warrington, UK). After incubation for 10 minutes at room temperature, polymerase chain reaction (PCR) was performed for 1 cycle (1 hour at 42°C, 5 minutes at 99°C and 5 min at 5°C) and contained 5 mM $MgCl_2$, PCR buffer, 1 mM of each deoxynucleoside triphosphate, 1 U/μl RNAse inhibitor, 2.5 U/μl MuLV reverse transcriptase, random hexamers (1.25 μM), oligo-dT (1.25 μM) each (all from PE Biosystems) and RNAse-free water to a final reaction volume of 10 μl. The resultant cDNA was stored at –20°C

2.7.4. Real-time quantitative polymerase chain reaction (RT-PCR)

Real-time quantitative RT-PCR was performed to assess the relative expression of COX-2, VEGF, FGF and prostaglandin receptor subtypes EP1, EP2, EP3 and EP4 in HeLa wild-type and EP4 over-expressing sense cells (EP4S). RNA samples were extracted from treated WT and EP4S HeLa cells using Tri-reagent as described in Section 2.7.1 and were reverse transcribed as described in Section 2.7.3. A reaction mix was made containing *Taq*man buffer (5.5 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP), ribosomal 18s forward and reverse primers and probe (all at 50 nM), forward and reverse primers for all target genes were used at 250 nM (See Table 5: Sequences of Primers and Probes), AmpErase UNG (0.01 U/μl) and AmpliTaq Gold DNA Polymerase (0.025 U/μl; all from PE Biosystems, Warrington, UK). A volume of 48 μl of reaction mix was aliquoted into separate tubes for each cDNA sample and 2 μl/replicate of cDNA was added. After mixing 23 μl of sample were added to the wells on a PCR plate. Each sample was added in duplicate. A no template control (containing water) was included. Wells were sealed with optical caps and the PCR reaction run on an ABI Prism 7700 Quantitative PCR machine. Primers were designed using the PRIMER express program (PE Biosystems).

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Gene	Sequence of primers and probes
COX-2	Forward 5'-CCTTCCTCCTGTGCCTGATG-3' Reverse 5'-ACAATCTCATTTGAATCAGGAAGCT-3' Probe (FAM labelled, 6-carboxyfluorescein) 5'-TGCCCGACTCCCTTGGGTGTCA-3'
VEGF	Forward 5'-TAGCTGCGCTGATAGACAT-3' Reverse 5'-TACCTCCACCATGCCAAGT-3' Probe (FAM labelled, 6-carboxyfluorescein) 5'-ACTTCGTGATGATTCTGCC-3'
FGF	Forward 5'-CCGACGGCCGCGTTGAC-3' Reverse 5'-GACACAACTCCTCTCTCTT-3' Probe (FAM labelled, 6-carboxyfluorescein) 5'-AGAAGAGCGACCCTCACA-3'
EP1	Forward 5'-AGATGGTGGGCCAGCTTGT-3' Reverse 5'-GCCACCAACAGCATTG-3' Probe (FAM labelled, 6-carboxyfluorescein) 5'-CAGCAGATGCACGACACCACCATG-3'
EP2	Forward 5'-GACCGCTTACCTGCAGCTGTAC-3' Reverse 5'-TGAAGTTGCAGGCGAGCA-3' Probe (FAM labelled, 6-carboxyfluorescein) 5'-CCACCCTGCTGCTGCTTCTCATTGTCT-3'
EP3	Forward 5'-GACGGCCATTCAGCTTATGG-3' Reverse 5'-TTGAAGATCATTTTCAACATCATTATCA-3' Probe (FAM labelled, 6-carboxyfluorescein) 5'-CTGTCGGTCTGCTGGTCTCCGCTC-3'
EP4	Forward 5'-ACGCCGCCTACTCCTACATG-3' Reverse 5'-AGAGGACGGTGGCGAGAAT-3' Probe (FAM labelled, 6-carboxyfluorescein) 5'-ACGCGGGCTTCAGCTCCTTCCT-3'
Ribosomal 18s	Forward 5'-CGGCTACCACATCCAAGGAA-3' Reverse 5'-GCTGGAATTACCGCGGCT-3' Probe (VIC®-labelled, PE Biosystems) 5'-TGCTGGCACCAGACTTGCCCTC-3'

Table 5: Sequence of Primers and Probes

Data were analysed and processed using Sequence Detector v1.6.3 (PE Biosystems). Expression of each gene was normalised to RNA loading for each sample using the 18s ribosomal RNA as an internal standard. Fold induction was calculated as fold increase in mRNA expression of cells treated with PGE_2 (300 nM) or seminal plasma (1:500) above vehicle-treated cells at the same time point.

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2.8. Protein

2.8.1. Protein extraction

HeLa cells were grown until the desired confluency was reached in 5 cm dishes. Cells were lysed by addition of 250 μl protein lysis buffer (1 % Triton X-100, 150 mM NaCl, 10 mM Tris/HCl pH7.4, 1 mM EDTA, 0.1 % SDS containing 2 mM PMSF). Proteins were extracted by allowing the dishes to sit on ice for 10 minutes and the cells were then scraped off using a plate scraper. Thereafter insoluble material was pelleted by centrifugation at 14 000 g for 15 minutes at 4°C. The clarified lysate was then transferred to a new eppendorf tube for protein quantification and SDS-PAGE.

2.8.2. Protein quantification

Proteins were quantified according to the BIO-RAD DC Protein microassay (Biorad, UK) as per manufacturer's instructions. Samples were diluted in distilled water to a ratio of 1:30. A concentration range of bovine serum albumin (BSA, supplied with the assay) ranging from 0 μg/ml to 200 μg/ml was made up in distilled water to achieve an OD_{595} response from 0.1 to 1.0 OD units. For the assay, 25 μl standards or samples were added to each well (in duplicate) of a 96 well plate. To this, 25 μl Reagent A, followed by 100 μl Reagent B was added and the plate incubated at room temperature for 10 minutes. Thereafter, the colour reaction was assayed at an absorbance of 690 nm using a Multiscan® MCC/340 plate reader. A standard curve was produced using the Assay Zap computer software program (Biosoft) and used to determine the average protein concentration of each sample.

2.8.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using pre-cast 4 % - 20 % Tris-Glycine gels (NOVEX, Invitrogen). Gels were set up in the gel running tank with running buffer (25 mM Tris-HCl, 0.2 M glycine, 0.1 % SDS). A total of 40 μg protein was resuspended in a total

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volume of 25 μl sample loading buffer (125 mM Tris-HCl pH 6.8, 4 % SDS, 20 % glycerol, 5 % 2-mercaptoethanol and 0.05 % bromophenol blue), boiled for 5 minutes at 95°C and loaded into separate wells of the gel. 10 μl of Seeblue™ (Novex) pre-stained molecular weight markers were loaded into a separate well. Gels were run at 4 mA constant current for about 90 minutes prior to immunoblotting.

2.8.4. Western Blotting

Following electrophoresis, the gel was transferred to a protein-free tray and equilibrated with transfer buffer (25 mM Tris/HCl, 0.192 M glycine, 20 % methanol) for 5 minutes. Whatman no. 3 paper (Whatman, UK) and polyvinylidene difluoride membrane (PVDF) membrane (Millipore, Watford, UK were cut to the dimensions of the gel and equilibrated in transfer buffer. The PVDF membrane was pre-soaked in methanol for 1 minute followed by a rinse in distilled water prior to equilibration with transfer buffer. The blot was assembled by overlaying three pieces of pre-soaked Whatman paper with the PVDF membrane followed by the gel and three layers of Whatman paper to form a sandwich. Air bubbles were then removed by rolling a clean glass pipette over each surface. Protein was transferred to the membrane for 1 hour 45 minutes at 14 V constant voltage using a semi-dry blotter (BIO-RAD, UK) assembled as per the manufacturer's instructions. Pre-stained molecular weight markers run in parallel on SDS-PAGE were used to determine whether transfer was successful. Following transfer, membranes were blocked for 1 hour at 25°C using 4 % BSA made up in TBS-Tween (50 mM Tris-HCl, 150 mM NaCl and 0.05 % v/v Tween-20). Thereafter, membranes were incubated with the relevant primary antibody at 4°C for 18 hours. After washing 3 times with TBS-Tween, membranes were subsequently incubated for 1 hour respectively with the relevant secondary antibody. Proteins were either revealed on photographic film by chemiluminescence (ECLplus kit) following the manufacturers instructions and quantified by scanning densitometry or by the ECF chemiluminescence system following the manufacturers instructions. Proteins developed by the ECF system were revealed and quantified by PhosphorImager analysis using the STORM 860 system (Molecular

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Dynamics, UK). The molecular weights of the proteins were determined relative to the mobility of the pre-stained markers on SDS-PAGE.

2.9. Immunohistochemistry (IHC) of cervical sections and cells

2.9.1. IHC of cervical sections

Levels of EP4 expression in cervical sections was carried out using archival cervical blocks obtained from the Department of Anatomical Pathology, University of Cape Town, South Africa. Tissue samples were prepared and mounted onto coated slides. Sections were dewaxed in xylene for 15 minutes, rehydrated in graded ethanol (100 %, 96 % and 70 % respectively), washed in water followed by Tris buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl pH 7.4). Thereafter the tissue sections were blocked for endogenous endoperoxidase for 30 minutes in 1 % hydrogen peroxide (H_2O_2) in methanol to reduce endogenous peroxidase activity as a peroxidase detection method was used for immunodetection. The slides were then washed in TBS for 5 minutes. Antigen retrieval was performed by pressure cooking for 2 minutes in 0.01 M sodium citrate buffer (pH 6.0). Sections were blocked for 30 minutes at 25°C by incubation with 5 % normal rabbit serum diluted in TBS. The tissue sections were then incubated with polyclonal EP4 specific antibody (Cayman Chemical Company Ltd.) at a dilution of 1:250. The slides were covered with Gelbond film (Flowgen, Rockland, ME, USA), hydrophobic side down and incubated in a humidified chamber at 4°C for 18 hours. The following day, the tissue sections were washed three times for 5 minutes each with TBS to remove excess primary antibody. Tissue sections were then subjected to secondary antibody and Horseradish Peroxidase detection. The tissue sections were incubated 25°C for 40 minutes with swine anti-rabbit secondary IgG antibody (Dako) at a dilution of 1:500. Excess secondary antibody was washed from slides by three washes of 5 minutes with TBS. Thereafter the tissue sections were incubated with streptavidin-peroxidase complex (Dako) for 20 minutes at 25°C and then washed thoroughly three times for 5 minutes each with TBS. The horseradish peroxidase complex was prepared according to suppliers protocol (Dako) in 0.05 M Tris/HCl pH 7.6 at least 20 minutes before use.

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Bound antibody was visualised by incubating the tissue sections with a solution of 225 μM 3.3'-diaminobenzidine in 0.05 M Tris/HCl pH 7.6 containing 0.01 % H_2O_2. After the colour reaction had developed, the reaction was stopped by washing the slides in distilled water. The tissue sections were then counterstained with haemotoxylin and eosin, rinsed in acid-alcohol and blued in Scotts tap water. Finally sections were dehydrated, cleared in xylene and coverslipped with pertex (Cellpath, Hemel Hampstead, UK).

2.9.2. Immunofluorescent microscopy of cells

The site of EP4 receptor expression was localized in wild-type and EP4S HeLa cells by immunofluorescence microscopy. Approximately 10 000 wild-type and sense cells were seeded in chamber slides, allowed to attach and grow overnight, before being fixed in 100 % ice-cold methanol. Following fixing, cells were washed in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and blocked using 5 % normal swine serum diluted in TBS. Subsequently the cells were incubated with polyclonal rabbit anti-EP4 receptor antibody at a dilution of 1:50 at 4°C for 18 hours. Control cells were incubated with rabbit immunoglobulin (IgG). Thereafter the cells were incubated with secondary swine anti-rabbit tetramethyl rhodamine isothyocyanate (TRITC; Dako Corp, High Wycombe, UK) at 25°C for 20 minutes and counterstained with To-Pro at a dilution of 1:2000 for 2 minutes. Cells were then mounted in Permafluor (Immunotech-Coulter, Buckinghamshire, UK) and coverslipped. Fluorescent images were visualised and photographed using a Carl Zeiss (Jena, Germany) laser scanning microscope LM510. The Alexafluor 546 and 488 was captured using the helium/neon 1 laser (excitation beam, 543 nm) and an emission band pass filter 560-615 nm.

2.10. Vascular Endothelial Growth Factor Enzyme-linked Immunosorbent Assay (VEGF ELISA)

Secreted VEGF was measured using an ELISA kit. Cells were seeded out in 6 well plates at $2x10^5$ cells per well and allowed to adhere overnight. Cells were first synchronized by serum withdrawal for at least 16 hours in serum-free medium. Thereafter, cells were pre-

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treated with vehicle, specific inhibitors or the EP4 antagonist (ONO-AE2-227) for 1 hour prior to stimulation with 300 nm PGE_2 or 1:500 dilution of seminal plasma (for the time periods specified in the figure legends). Culture medium was removed and VEGF protein was measured using a Human VEGF ELISA kit (Oncogene) as per manufacturers instruction. Serial dilutions of VEGF standards (15.6 pg/ml up to 1000 pg/ml) were made up in Calibrator diluent RD5K (supplied with kit). Samples were diluted 1:10 with Calibrator diluent RD5K to ensure that VEGF detection levels fall within those on the standard curve. A 96-well plate coated in monoclonal antibody specific for the VEGF protein (supplied with the kit) was loaded with samples and standards. The plate was then incubated at room temperature for 2 hours, this allows any VEGF present in the samples to bind to the capture antibody. Following this incubation, the plate was washed thoroughly 3 times with wash buffer (supplied with kit) to remove any unbound material. Polyclonal, horseradish peroxidase (HRP)-conjugated anti-VEGF antibody (200 μl) is added to each of the wells. Following an incubation of 2 hours at room temperature, the plate is washed again three times using the wash buffer provided in the kit. A chromogenic substrate (200 μl) is added to the wells and the plate incubated for 20 minutes at room temperature. The horseradish peroxidase catalyses the conversion of the chromogenic substrate tetra-methylbenzidine from a colourless solution to a blue substrate. Stop solution (50 μl) (2 N sulphuric acid) (supplied with the kit) is added to each well, causing the colour to turn yellow. The intensity of each well is proportional to the amount of human VEGF protein in the test samples. The coloured reaction product is measured on a spectrophotometer at 450/540 nm. Quantification is achieved by the construction of a standard curve using known concentrations of human VEGF protein. By comparing the absorbance obtained from a sample containing an unknown amount of human VEGF protein with that obtained from the standards, the concentration of human VEGF protein in the test samples can be determined. Data are expressed as fold change in comparison to basal where the amount of VEGF secreted in treated cells is divided by the amount secreted in cells treated with the vehicle.

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2.11. Prostaglandin E_2 (PGE_2) Assay

The PGE_2 levels in seminal plasma samples were measured using PGE_2 assays. The ELISA was performed using 96 well plates (Amine-binding plates; Cosar; High Wycombe, UK) coated with donkey anti-rabbit antibody. Plates were then coated with rabbit immunoglobulin G (1 mg/ml diluted in PBS with 1 % carbonate buffer, pH 9.6) at 200 μl/well for 16 hours at 4°C. The solution was aspirated and blocking solution (50 mM glycine, 10 mg/ml bovine serum albumin) added at 25 μl/well for 2 hours at 23°C. The plates were then washed and donkey anti-rabbit serum (Scottish Antibody Production Unit, Carluke, UK) added to a final volume of 150 μl/well, before washing, air-drying and storage with dessicant at 4°C. The link was prepared by ether extraction and reverse phase chromatography using 20 mg of synthetic PGE_2, 320 μl dry dimethylformamide, 3 μl butylchloroformate and 0.05 mM biocytin. Samples and synthetic standards were diluted in ELISA buffer (150 mM NaCl, 100 mM Tris-HCl, 0.05 % Tween-20, 50 mM phenol red, 1 mM 2-methylisothiazolone, 1 mM bromonitrodioxane, 2 mM EDTA, 2 mg/ml bovine serum albumin to a final pH 7.2), and 100 μl of each added in duplicate to the plate. The link was diluted $1:1.5x10^6$ in ELISA buffer and 50 μl added to each well. Antisera, diluted 1:50 000 in ELISA buffer, was added to a final volume of 50 μl to all wells except those used for measuring non-specific binding. Plates were incubated at 4°C for 16 hours, washed and 100 μl/well of 0.2 unit/ml streptavidin-peroxidase (Boehringer Mannheim) was added. Plates were then incubated for 20 minutes at 23°C on an orbital shaker, washed and substrate (0.3 g/L urea-hydrogen peroxide, 0.1 g/L tetramethyl benzine in 100 mM sodium acetate, pH 6.0) added to a final volume of 200 μl/well for 10 minutes before quenching with 50 μl/well 1M sulphuric acid. Colour reaction was measured at 450 nm by spectrophotometry. The rabbit antiserum that was raised against PGE_2-complexed keyhole limpet hemocyanin has been previously characterised (Kelly *et al.* 1989).

2.12. Statistical Analysis

The data in this study were analysed by ANOVA and Fishers PLSD tests using Statview 5.0 (Abacus Concepts Inc., Berkeley, CA, USA). The data is presented as Mean ± SEM.

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CHAPTER 3 - EXPRESSION AND LOCALISATION OF EP4 RECEPTOR IN CERVICAL TISSUE SECTIONS.

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3.1. Introduction

Prostaglandin E_2 (PGE_2) is a major cyclooxygenase (COX) product in a number of physiological settings. In the gastrointestinal tract, COX-1-derived PGE_2 plays a protective role in maintaining the integrity of the gastric mucosa (Hoshino *et al.* 2003). PGE_2 production in the kidney is crucial for normal renal function (Breyer *et al.* 1996; Breyer *et al.* 1996), and it has also been shown to play a role in the maintenance of blood pressure (Audoly *et al.* 1999). Prostaglandins (PGs) have also been recognised for many years as potent mediators of female reproductive tract physiology, including ovulation, implantation, cervical ripening, cervical dilation, menstruation, luteolysis, myometrial contractility, placental vascular tone and parturition (Kniss 1999; Matsumoto *et al.* 2001; Sales *et al.* 2003). In addition, COX-enzymes and PGs, such as PGE_2, are elevated in various carcinomas, including cervical carcinoma (Prescott *et al.* 2000; Sales K.J. 2001; Sales *et al.* 2001; Marnett *et al.* 2002; Mann *et al.* 2004). Cervical cancer is an important problem in sub-Saharan Africa and has a major impact on morbidity and health care costs (Leiman 1976; Bailie *et al.* 1996). There is much evidence indicating Human Papilloma Virus (HPV) to be an initiator of cervical neoplastic transformation, however numerous studies have suggested that development and progression of cervical carcinomas may be regulated by COX-enzyme products, including PGE_2 (Kulkarni *et al.* 2001; Sales *et al.* 2001; Riley *et al.* 2003; Szkaradkiewicz *et al.* 2004). In addition, numerous studies have shown elevated COX-enzymes in cervical carcinoma (Ryu *et al.* 2000; Kulkarni *et al.* 2001; Sales *et al.* 2001; Han *et al.* 2003).

The diverse effects of PGE_2 may be accounted for in part by the existence of four G-protein coupled receptors (GPCRs) termed EP1, EP2, EP3, and EP4 receptors (Breyer *et al.* 1996; Boie *et al.* 1997). Interaction of PGE_2 with the EP1 receptor mobilizes intracellular calcium and inositol triphosphate (IP_3) via $G\alpha q$ (Samad *et al.* 2002). EP2 and EP4 are both coupled to $G\alpha s$, and signal via stimulation of adenylyl cyclase resulting in an increase in cAMP (Samad *et al.* 2002). There are several splice variants of the EP3 receptor, which are coupled to different signalling pathways that result in either a positive or negative cAMP response to PGE_2 administration or an increase in intracellular calcium

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mobilization and accumulation of IP_3, depending on the splice variant and cell type (Hatae *et al.* 2002).

Functional roles for prostaglandin EP receptors have been determined by studies in knockout mice systems, deficient for each of the receptors as described in Section 1.4.12.8. (Table 4) (Audoly *et al.* 1999). These data have ascertained an important role for the EP2 and FP receptors in normal reproductive function. Recent data, however, have shown an association between aberrant expression and signalling of prostanoid receptors (EP2/EP4 and FP receptors) and pathologies of the reproductive tract, including endometrial adenocarcinoma and cervical carcinoma (Sales *et al.* 2004; Sales *et al.* 2004), suggesting that the EP2/EP4 receptor may play a role in cervical tumourigenesis. In support of this latter observation, Sheng *et al.* (2001) and Sonoshita *et al.* (2001) have demonstrated a role for EP2/EP4 receptors in enhancing colon tumourigenesis. In these model systems, PGE_2, via the EP2 receptor has been shown to accelerate intestinal polyp formation (Sonoshita *et al.* 2001) as well as enhance proliferation and tumourigenic effects via the EP4 receptor (Sheng *et al.* 2001). Similarly, Fujino *et al.*, 2003 have ascertained a role for EP4 receptor in mediating mitogenic signalling, which may lead to activation of target molecules implicated in cancer (Fujino *et al.* 2003). Taken together, these findings and that of elevated EP2/4 receptors in cervical carcinoma strongly suggest a role for PGE_2 signalling via EP4 receptor in modulating tumourigenesis, however, the signal transduction pathways and target genes mediating this effect remain to be fully elucidated.

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3.2. Aim

Previous studies have demonstrated elevated expression and signalling of EP4 receptor in cervical carcinoma, indicating a potential autocrine/paracrine regulation of neoplastic cervical cell function by PGE_2 via the EP4 receptor (Sales *et al.* 2001). The aim of this section was to determine the localisation of the site of EP4 receptor expression in cervical carcinomas.

3.3. Materials and Methods

Informed consent was obtained from patients prior to tissue collection. Five squamous cell carcinoma, 4 adenocarcinoma and 5 normal cervical tissues were collected and processed as described in Section 2.2. The localisation of the site of expression of EP4 receptor in the cervical tissue was investigated by immunohistochemistry using EP4-specific antibodies as described in Section 2.9. Briefly, cervical sections were dewaxed and subsequentially rehydrated as described and incubated with specific primary and secondary antibodies prior to detection with DAB. Ethical approval for this study was granted by University of Cape Town Research Ethics Committee.

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3.4. Results

Levels of immunoreactive EP4 receptor were upregulated in all cervical carcinoma samples investigated compared with the expression in normal cervix.

The site of EP4 receptor expression was localised by immunohistochemistry to the neoplastically transformed squamous epithelium in squamous cell carcinoma (n=5; arrowhead, Figure 3.1. A), and to neoplastically transformed columnar epithelium lining the endocervical canal and the glandular epithelium of the endocervical glands in adenocarcinomas (n=4; arrowhead, Figure 3.1. B). One representative squamous carcinoma and one adenocarcinoma are shown respectively. Normal cervical tissue sections showed no elevated EP4 immunoreactivity above basal levels (n=5; Figure 3.1. C). Sections probed with IgG in place of primary antibody (EP4 negative control; Figure 3.1. D) abolished the EP4 immuno-staining of the EP4 antibody.

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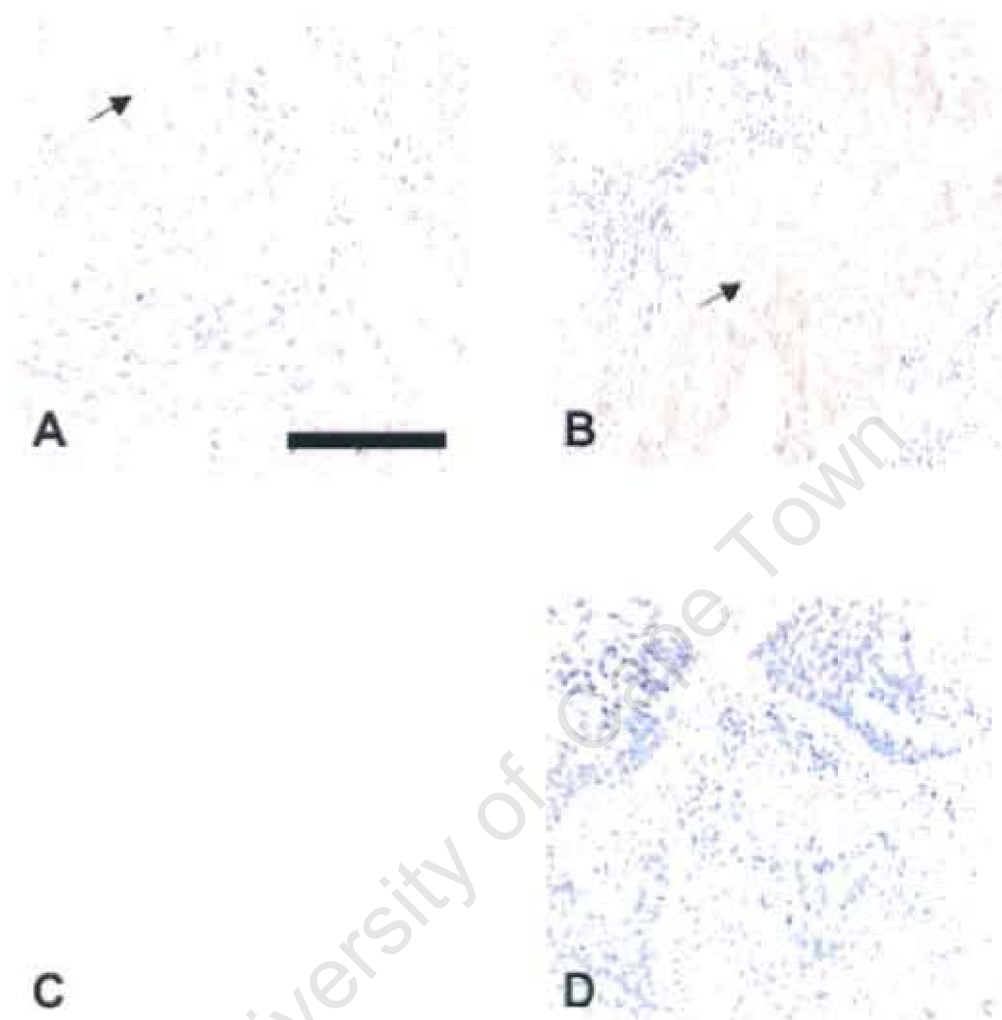

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Figure 3.1. Localisation of the site of EP4 receptor expression in epithelial cells of squamous cell carcinoma (A), adenocarcinoma (B) and normal cervix (C). Section of tissue incubated with IgG (negative control) are shown for adenocarcinoma (D). Scale bar is 100 μm.

3.5. Discussion

This study confirms the expression of EP4 receptor in cervical squamous cell carcinomas and adenocarcinomas. EP4 receptor expression was localised to the neoplastically transformed epithelial cells in all carcinomas investigated, compared with normal cervix. This pattern of expression of EP4 is similar to that observed for COX-2 and PGE_2 in cervical squamous cell carcinoma and adenocarcinoma cases reported previously by Sales *et al.*, 2001. The role that PGE_2 plays in cancer development and progression is not fully elucidated, though numerous mechanisms have been outlined in various model systems. Enhanced synthesis of PGE_2 resulting from upregulated COX-2 can induce malignant change in epithelial cells through immunosuppression (Wang *et al.* 2004), inhibiting apoptosis (Song *et al.* 2002), increasing metastatic potential of cells (Chang *et al.* 2000; Kundu *et al.* 2002), and promoting angiogenesis (Tsujii *et al.* 1998; Jones *et al.* 1999; Dormond *et al.* 2001; Chang *et al.* 2004).

These data show that the site of EP4 expression is in close proximity to the site of PGE_2 biosynthesis, suggesting an autocrine/paracrine control of neoplastic cell function by PGE_2 via the EP4 receptors. Such an interaction has been proposed for several other model systems (Regan 2003; Pozzi *et al.* 2004; Spinella *et al.* 2004; Timoshenko *et al.* 2004). Studies performed by Pozzi *et al.*, 2004, using mouse colon adenocarcinoma (CT26) cells to investigate the role of PGE_2 in cell proliferation showed that CT26 cells express both COX-1 and COX-2 and metabolize arachidonic acid to PGE_2. Treatment of these cells with indomethacin, or COX-selective inhibitors, prevented PGE_2 biosynthesis and CT26 cell proliferation. These anti-proliferative effects of COX inhibition were abolished specifically by treatment with PGE_2 or the EP4 receptor-selective agonist (ONO-AE2-227) via PI3-kinase/ERK activation, thus providing a functional link between PGE_2-induced cell proliferation and EP4-mediated ERK signalling (Pozzi *et al.* 2004). The role of EP4 receptor in tumourigenesis has been further strengthened following data published by Spinella *et al.*, 2004. These data associated EP4 receptors in the induction of VEGF expression and cell invasiveness via PGE_2-dependent machinery, which resulted in increased angiogenesis and invasive phenotype in ovarian carcinoma cells

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(Spinella *et al.* 2004). Other data published by Timoshenko *et al.*, 2004, reported that endogenous PGE_2, resulting from COX-2 expression in a metastatic murine breast cancer cell line C3L5 upregulates IFN-γ and LPS-induced nitric oxide synthase expression and nitric oxide production. This action of PGE_2 was mediated through the EP4 receptor in a cAMP-dependent manner (Timoshenko *et al.* 2004).

The reduction of PGE_2 biosynthesis by the action of NSAIDs on COX-enzymes, has been shown to down-regulate the survival, metastatic, and angiogenic potentials of cancerous tissue (Grosch *et al.* 2001; Turini *et al.* 2002; Zweifel *et al.* 2002; Zha *et al.* 2004). Thus, treatment of cervical carcinoma with NSAIDs will suppress endogenous expression of COX-2 and synthesis of PGE_2, which may be acting in an autocrine/paracrine manner via various EP receptors, including the EP4 receptor. It is important to emphasize however, that in addition to endogenously synthesied PGE_2, neoplastically transformed cervical epithelial cells in sexually active women will potentially be under the direct stimulation of prostaglandins (PGs) from seminal plasma. PG levels in seminal plasma are 10 000 times higher than that found at a site of inflammation, and PGE is the predominant type of prostaglandin detected (Kelly 1995). Thus, in sexually active women with pre-neoplastic lesions, up-regulation of expression of genes that may modulate mitogenesis and invasiveness of neoplastically transformed cervical epithelial cells, may be regulated in part by endogenous expression of PGE, as well as PGE available in seminal plasma. Enhanced PGE-EP4 interaction as a consequence of elevated EP4 receptor expression in cervical carcinomas, may lead to enhanced or sustained cervical tumourigenesis. Hence the application of NSAIDs on their own may not be sufficient to treat cervical carcinoma, a more effective treatment may lie in the use of prostanoid receptor antagonists, such as those targeting the EP4 receptor, coupled with NSAIDs.

Following the observation of elevated EP4 receptor in neoplastic epithelial cells of the cervix (outlined in this chapter), I proceeded to investigate the effects of seminal plasma prostaglandins on cervical epithelial cells using a HeLa cell line stably overexpressing the EP4 receptor (outlined in Chapter 4).

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CHAPTER 4 - CONSTRUCTION OF A HELA EP4 RECEPTOR OVEREXPRESSING CELL MODEL SYSTEM

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4.1. Introduction

In the previous chapter, the site of expression of the EP4 receptor was localised to the neoplastic epithelial cells in all cervical carcinomas. These cells have previously been reported to be the site of synthesis of PGE_2 in cervical carcinoma.

PGE_2 is a major product of COX-initiated arachidonic acid metabolism. The mode of action of PGE_2 is brought about by coupling to multiple PGE_2 receptors (EP1, EP2, EP3, and EP4 receptors), which are often co-expressed together in the same cell type, indicating that PGE_2 may have multiple and at times opposing functional effects on a given target tissue (Breyer 2001). Roles for each of the EP receptors have been ascertained (as described in Chapter 1) (Breyer *et al.* 1996; Boie *et al.* 1997; Audoly *et al.* 1999; Hatae *et al.* 2002; Kawamori *et al.* 2003), and several studies in *in vitro* model systems and animal models have implicated an important role for EP4 receptor in tumourigenesis (Sales *et al.* 2001; Sales *et al.* 2002; Fujino *et al.* 2003; Regan 2003; Pozzi *et al.* 2004; Spinella *et al.* 2004; Timoshenko *et al.* 2004). The EP4 receptor is a seven transmembrane-segment GPCR, which signals through an increase in cAMP accumulation. The human EP4 receptor cDNA encodes a 488 amino acid polypeptide with a predicted molecular mass of ~53 kDa (Breyer 2001). In addition to the human receptor, EP4 receptors for the mouse, rat, rabbit, and cow have been cloned (Honda *et al.* 1993; Nishigaki *et al.* 1995; Breyer *et al.* 1996; Boie *et al.* 1997). EP4 receptor mRNA expression is detected in a variety of tissues including, thymus, ileum, lung, spleen, adrenal, uterus, and kidney tissue (Regan *et al.* 1994; Katsuyama *et al.* 1998; Desai *et al.* 2000). The EP4 receptor protein has a long cytoplasmic tail (156 amino acid residues). This C-terminal sequence contains 38 serine and threonine residues that might serve as multiple phosphorylation sites (Breyer *et al.* 2001). Several studies have implicated this region in agonist-induced desensitisation (Desai *et al.* 2001) and EP4 receptors were found to undergo rapid internalisation in response to PGE_2 stimulation (Desai *et al.* 2000). Among the elements that are uniquely present in the carboxyl terminus of the EP4 receptor are sites for potential phosphorylation by cAMP-dependent protein kinase (PKA) and G-protein coupled receptor kinases (Breyer 2001). Following

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PGE-EP4 receptor interaction, various signalling pathways, including MAPK or PI3kinase may be activated (Breyer 2001).

Recent studies have demonstrated a role for the EP4 receptor in mediating events associated with tumourigenesis. One such study, reported by Spinella *et al.*, 2004, has ascertained a role for the EP4 receptor in the promotion of angiogenesis and invasion. In this study, activation of the EP4 receptor in ovarian carcinoma cell enhanced VEGF expression and promoted cell invasiveness by endothelin-1. Similarly, in a separate study Timoshenko *et al.*, 2004 demonstrated the involvement of the EP4 receptor in enhancing nitric oxide production mediated by PGE_2 in breast cancer cells. Recent work reported by Pozzi *et al.*, 2004 has shown that PGE_2–EP4 receptor interaction can enhance cell growth in colon carcinomas. Moreover, data from our laboratory have demonstrated significantly increased expression of EP4 receptor mRNA in reproductive tract carcinomas, including cervical carcinoma tissues, compared with normal cervix (Sales *et al.* 2001). Taken together, these data suggest that cervical tumourigenesis could be regulated in an autocrine/paracrine manner by PGE_2 via activation of the EP4 receptor and initiation of intracellular signal transduction cascades.

In order to elucidate the molecular signal transduction pathways activated following PGE-EP4 receptor interaction and the role of EP4 receptor in cervical epithelial cells, we constructed a stable cell line over-expressing the EP4 receptor in HeLa (cervical adenocarcinoma cells). Since 9 prostanoid receptors have been reported to date, any attempt to dissect out the signalling of a given receptor is made difficult due to the multiplicity of different signalling pathways and the complex network of crosstalk between them. The EP4 model system thus provides a unique opportunity to dissect out the signalling of a single receptor, namely the EP4 receptor, and elucidate its potential role in cervical tumourigenesis.

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4.2. Aim

The initial aims of this study were to establish a HeLa cervical adenocarcinoma cell model system overexpressing the EP4 receptor. This model system would subsequently be used as a tool to investigate:

1. Intracellular signal transduction pathways mediating the role of the EP4 receptor in cervical adenocarcinoma cells (discussed in Chapter 5).
2. The potential role of EP4 receptor signalling in mediating events associated with cervical tumourigenesis by investigating the target genes activated by PGE_2 or seminal plasma in our HeLa EP4 model system (discussed in Chapter 6).

4.3. Materials and Methods

The HeLa cell line was chosen for this study, because of its cervical carcinoma origin, immortality and homogeneity. The HeLa cell model system provides a useful tool in studying the *in vitro* biological properties of cervical carcinoma. HeLa cells were the first aneuploid epithelial-like cell line to be derived from human tissue and maintained continuously in serial cell culture. HeLa was derived from cervical adenocarcinoma origin, from the carcinoma of a 31 year old female by GO Gey, WD Coffman and MT Kubicek in February 1951 (Gey 1958). Since its origin, the HeLa cell line has been one of the most extensively studied cell lines. HeLa cells were maintained as described in Section 2.4.

4.3.1. Transfection of EP4 containing plasmids into HeLa-S3 cells

HeLa-S3 cells were purchased for the purpose of creating stable EP4-overexpressing HeLa cell line (EP4S) and a stable antisense cell line (EP4AS). Approximately 5000 cells were seeded in a final volume of 1 ml per well in each well of a 12 well plate in complete medium. Cells were allowed to attach and grow overnight. Either the pcDNA3 vector containing EP4 cDNA (2 μg) in the sense direction, or pcDNA3 vector containing EP4 cDNA (2 μg) in the anti-sense direction was transfected into each well using Superfect

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reagent (Qiagen, GmbH, Crawley, UK) diluted in Opti-MEM (Gibco, Invitrogen, Paisley, UK). Transfected and control cells were incubated for 4 hours at 37°C in 5 % humidified CO_2 (v/v). Thereafter, the medium was replaced with fresh complete medium. Cells were allowed to grow for 72 hours. Transfected cells were then seeded together with wild-type cells using a ration of transfected:wild-type of 1:10. Clones were then selected against 200 µg/ml G418. Once control wild-type cells had died, at least 80 G418 resistant clones of both EP4S and EP4AS were picked. Clones were allowed to grow under continuous selection with G418, and then screened for the level of expression of EP4 receptor by Western Blot analysis (Section 4.3.2) and Real-time quantitative RT-PCR (Section 4.3.3). Clones were routinely maintained in 200 µg/ml G418.

4.3.2. Western blot analysis of clones

Western blot analysis was conducted as detailed in Section 2.8. Briefly, HeLa cells were grown until the desired confluency was reached in 5 cm dishes. Cells were lysed by addition of 250 µl protein lysis buffer (1 % Triton X-100, 150 mM NaCl, 10 mM Tris/HCl pH7.4, 1 mM EDTA, 0.1 % SDS containing 2 mM PMSF). Proteins were extracted by allowing the dishes to sit on ice for 10 minutes and the cells were then scraped off using a plate scraper. Thereafter, insoluble material was pelleted by centrifugation at 14 000 g for 15 minutes at 4°C. The clarified lysate was then transferred to a new eppendorf tube for protein quantification and SDS-PAGE. Proteins were quantified according to the BIO-RAD DC Protein microassay as described in Section 2.8.2. SDS-PAGE was performed using pre-cast 4 % - 20 % Tris-Glycine gels. A total of 40 µg protein was resuspended in a total volume of 25 µl sample loading buffer (125 mM Tris-HCl pH 6.8, 4 % SDS, 20 % glycerol, 5 % 2-mercaptoethanol and 0.05 % bromophenol blue), boiled for 5 minutes at 95°C and loaded into separate wells of the gel. 10 µl ofpre-stained molecular weight markers were loaded into a separate well. Gels were run at 4 mA constant current for about 90 minutes prior to immunoblotting. Following electrophoresis, protein was transferred to the PVDF membrane for 1 hour 45 minutes at 14 V using a BIO-RAD semi-dry blotter. Following transfer, membranes were blocked for 1 hour at 25°C using 4 % BSA made up in TBS-Tween. Thereafter,

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membranes were incubated with the EP4-specific primary antibody at 4°C for 18 hours. After washing 3 times with TBS-Tween, membranes were subsequently incubated for 1 hour respectively with the relevant secondary antibody. Proteins were revealed on photographic film by chemiluminescence (ECLplus kit) and quantified by scanning densitometry. The molecular weights of the EP4 receptor proteins were determined relative to the mobility of the pre-stained markers on SDS-PAGE. Data was presented as Mean ± SEM from 3 independent experiments.

4.3.3. Quantitative real-time RT-PCR analysis of clones

Real-time quantitative RT-PCR was performed to assess the relative expression of prostaglandin receptor subtype EP4 in HeLa wild-type and EP4 over-expressing sense cells (EP4S). RNA samples were extracted from WT and EP4S HeLa cells using Tri-reagent as described in Section 2.7.1 and were reverse transcribed as described in Section 2.7.3. Quantitative real-time PCR was then performed on samples using a reaction mix containing *Taq*man buffer (5.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP), ribosomal 18s forward and reverse primers and probe (all at 50 nM), forward and reverse primers specific for the EP4 gene were used at 250 nM (See Table 5: Sequences of Primers and Probes), AmpErase UNG (0.01 U/µl) and AmpliTaq Gold DNA Polymerase (0.025 U/µl). The PCR reaction was run on an ABI Prism 7700 Quantitative PCR machine. Expression of the EP4 gene was normalised to RNA loading for each sample using the 18s ribosomal RNA as an internal standard. Fold increase was determined by dividing the relative expression of EP4 receptor mRNA in EP4S cells by the relative expression of EP4 receptor in HeLa wild-type cells and presented as Mean ± SEM from 3 independent experiments.

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4.3.4. cAMP assay analysis of clones

In order to determine the functionality of the EP4 receptors, cAMP assays were performed on PGE_2-treated cells as described in Section 2.6. Cells ($2x10^5$) were seeded in 6 well dishes and allowed to attach overnight. The next day, cells were washed twice with PBS followed by incubation with serum-free DMEM for a minimum of 16 hours to allow synchronization. Cells were then stimulated with 300 nM PGE_2 for the time periods of 0, 5 and 10 minutes. Following stimulations, the medium was removed and the cells washed in ice-cold PBS before being lysed in 0.1 M HCl. cAMP concentration was quantified by ELISA using a cAMP kit and normalized to the protein concentration of the lysate. Protein concentrations were determined using protein assay kits (Bio-Rad) as described in Section 2.8.2. Results are presented as Mean ± SEM from 3 independent experiments.

4.3.5. Immunofluorescence microscopy

The site of EP4 receptor expression was localized in wild-type and EP4S HeLa cells by immunofluorescence microscopy as described in Section 2.9.2. Approximately 10 000 wild-type and sense cells were seeded in chamber slides, allowed to attach and grow overnight, before being fixed in 100 % ice-cold methanol. Following fixing, cells were washed in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and blocked using 5 % normal swine serum diluted in TBS. Subsequently the cells were incubated with polyclonal rabbit anti-EP4 receptor antibody at a dilution of 1:50 at 4°C for 18 hours. Control cells were incubated with rabbit immunoglobulin (IgG). Thereafter the cells were incubated with secondary swine anti-rabbit tetramethyl rhodamine isothyocyanate (TRITC) at 25°C for 20 minutes and counterstained with To-Pro at a dilution of 1:2000 for 2 minutes. Cells were then mounted in Permafluor and coverslipped. Fluorescent images were visualised and photographed using a Carl Zeiss laser scanning microscope LM510. The Alexafluor 546 and 488 was captured using the helium/neon 1 laser (excitation beam, 543 nm) and an emission band pass filter 560-615 nm.

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4.4. Results

4.4.1. Western blot analysis of clones

Western blot analysis performed on cellular extracts from 45 sense and 30 antisense clones revealed 4 sense and 2 antisense clones that demonstrated the highest and lowest levels of EP4 receptor expression when compared with wild-type cells, respectively. As shown in Figure 4.1, an immunoreactive band migrating at approximately 52 kDa, corresponding to the relative known molecular weight of the EP4 receptor protein, was observed for all clones. The lower panel of Figure 4.1. shows fold increase in levels of EP4 receptor expression of various clones, as determined by scanning densitometry, compared with wild-type cells. Levels of EP4 receptor protein were normalised for loading against β-actin on the same blots.

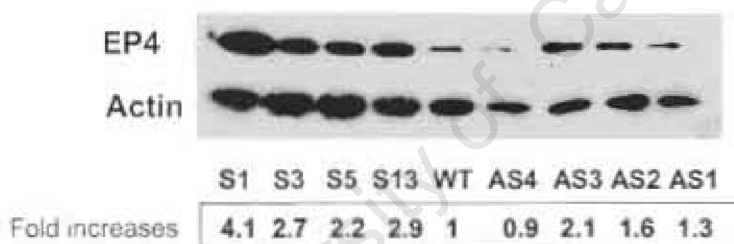

Figure 4.1. Immunoblot showing EP4 protein expression of HeLa EP4S (S1, S3, S5, S13) clones, HeLa wild-type (WT), and HeLa EP4AS (AS4, AS3, AS2, AS1) clones. A significant increase in protein expression was observed in all EP4S clones when compared to WT cells. EP4AS clone AS4 showed a decrease in EP4 receptor expression when compared to wild-type cells. Lower panel shows fold increases in EP4 protein following phosphorimager analysis and quantification. Proteins were normalised for loading against β-actin.

EP4S clones with the greatest level of EP4 receptor protein expression (S1, S3, S5, S13) and EP4AS clone exhibiting the lowest level of EP4 receptor expression (AS4) were selected for further studies. Subsequently, the level of EP4 mRNA expression of these clones was confirmed by Quantitative real-time RT-PCR. Moreover, the functionality of the EP4 receptors was examined by analysing the cAMP response of the clones to treatment with 300 nM PGE_2.

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4.4.2. Quantitative real-time RT-PCR analysis of clones

Quantitative real time RT-PCR analysis was performed to confirm the level of EP4 mRNA expression of the clones selected by Western Blot analysis. As mentioned previously, sense clones S1, S3, S5 and S13 demonstrated significant increases in EP4 receptor protein expression compared with wild-type HeLa cells. Following quantitative real-time PCR analysis, EP4S clones S1 and S5 demonstrate highest levels of EP4 receptor mRNA expression (Figure 4.2), with an increase of 7.4 fold and 7.0 fold, respectively above wild-type HeLa cells (WT).

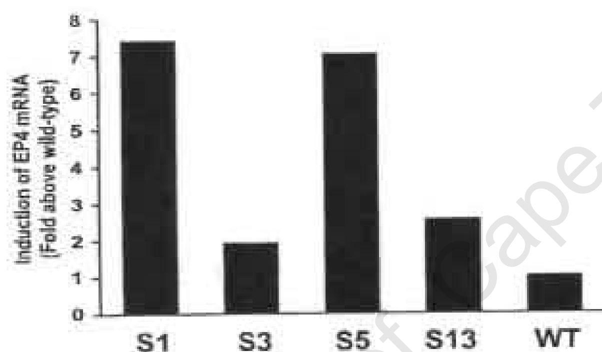

Figure 4.2. Fold increase in expression of EP4 receptor mRNA in HeLa EP4S clones (S1, S3, S5, S13) and HeLa wild-type cells (WT) as determined by real-time quantitative RT-PCR. Fold increase was determined by dividing the relative expression in EP4S cells by the relative expression in HeLa wild-type cells.

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4.4.3. cAMP analysis of clones

The EP4 receptor is a $G\alpha_s$-coupled receptor, which upon activation leads to an accumulation of cAMP in the cell (Regan 2003). In order to determine the functionality of the transfected EP4 receptor cDNA expressed in the EP4 clones, the accumulation of intracellular cAMP was measured in WT, EP4S and EP4AS clones following stimulation for 0, 5 and 10 minutes with 300 nM PGE_2.

The accumulation of intracellular cAMP was determined in EP4S clones S1, S5, and S13 to be 17.01; 16.24 and 15.14 pmol cAMP/mg protein, following 5 minutes of PGE_2 stimulation respectively and 18.33; 14.58 and 11.51 pmol cAMP/mg protein, following 10 minutes of PGE_2 stimulation respectively. The relative accumulation of cAMP in clones S1 was elevated at 5 and 10 minutes following PGE_2 challenge, compared with the cAMP accumulation in WT cells (12.49 and 11.53 pmol cAMP/mg protein for 5 and 10 minutes respectively) (Figure 4.3). However, upon closer evaluation, the basal levels of cAMP accumulation in clones S5 and S13 (15.76 and 14.10 pmol cAMP/mg protein) was observed to be elevated compared with clone S1 and WT cells (7.27 and 7.45 pmol cAMP/mg protein respectively).

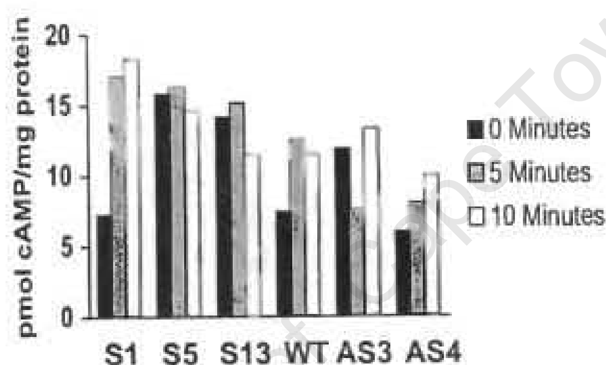

Figure 4.3. cAMP levels (pmol/mg protein) in HeLa EP4S clones (S1, S5, S13), HeLa wild-type cells (WT) and EP4AS clones (AS3, AS4) following stimulation of cells with 300 nM PGE_2 for time periods of 0, 5 and 10 minutes.

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The accumulation of cAMP was determined in EP4AS clones AS3 and AS4 to be 7.58 and 8.04 pmol cAMP/mg protein, following 5 minutes of PGE_2 stimulation respectively and 13.27 and 9.91 pmol cAMP/mg protein, following 10 minutes of PGE_2 stimulation respectively. The relative accumulation of cAMP in clone AS4 was reduced at 5 and 10 minutes following PGE_2 challenge, compared with the cAMP accumulation in WT cells (12.49 and 11.53 pmol cAMP/mg protein for 5 and 10 minutes respectively) (Figure 4.3). However, the basal levels of cAMP accumulation in clone AS3 (11.86 pmol cAMP/mg protein) was observed to be elevated compared with clone AS4 and WT cells (5.98 and 7.45 pmol cAMP/mg protein respectively) (Figure 4.3.).

4.4.4. Selection of EP4S and EP4AS clones

Western blot analysis (Section 4.4.1), Quantitative real-time RT-PCR (Section 4.4.2) and cAMP assays (Section 4.4.3) confirmed an elevated EP4 receptor expression in clone S1. Moreover, the basal cAMP accumulation and growth characteristics of clone S1 were not significantly different to WT cells. Based on these criteria, clone S1 was thus chosen for further studies. Western blot analysis showed similar levels of EP4 receptor protein expression for antisense clone AS4 and WT cells (Figure 4.1), moreover antisense clone 4 (AS4) exhibited significantly less cAMP accumulation following 300 nM PGE_2 stimulation compared with WT cells and was thus chosen for further studies. Clones S1 and AS4 clones (termed EP4S and EP4AS, respectively) were expanded and characterised by Western blot analysis, Quantitative real-time RT-PCR analysis and cAMP assay analysis a further 3 times in order to determine statistical significance.

Western blot analysis of protein collected from HeLa wild-type cells (WT), EP4 sense clone 1 (EP4S), and EP4 antisense clone 4 (EP4AS) demonstrated a 4.10 ± 0.57 fold increase in EP4 protein expression in the sense (EP4S) ($p < 0.05$), and 1.0 ± 0.32 fold in the antisense clone relative to wild-type cells (Figure 4.4.).

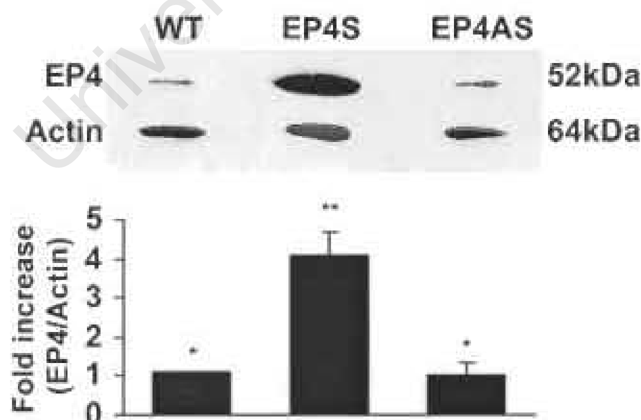

Figure 4.4. Immunoblot of HeLa wild-type (WT), EP4S clone and EP4AS clone showing fold increase in EP4 receptor protein expression of cells. Proteins were normalised for loading against β-actin. Data is expressed as Mean ± SEM from 3 independent experiments (** is significantly different from *, $p < 0.05$)

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4.4.5. Immunofluorescence microscopy of cells

EP4 receptor protein expression was localised in HeLa wild-type (WT), sense clone 1 (EP4S) and antisense clone 4 (EP4AS) cells by immunofluorescence microscopy (as described in Section 2.8.) to determine whether all the EP4 receptor was trafficked to the plasma membrane compartment. We performed immunofluorescence microscopy using an EP4-specific antibody as shown in Figure 4.5.

Immunoflourescence microscopy showed elevated EP4 receptor immunoreactivity in EP4S cells and reduced immunoreactivity in EP4AS cells compared with WT cells. EP4 receptor localised to the plasma membrane in all cells. Incubating cells with rabbit IgG abolished the immunoreactivity (Figure 4.5, control representative of EP4 receptor sense cells; C).

Figure 4.5. Immunofluorescent microscopy using EP4-specific antibodies on HeLa wild-type (WT), EP4S, EP4AS and negative control (C) cells.

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4.5. Discussion

In this chapter, a HeLa EP4 receptor overexpressing model system was established in human cervical adenocarcinoma cells. EP4 receptor cDNA was introduced in the sense or antisense orientations and cells were made stable by antibiotic selection. Various sense and antisense clones were selected, expanded and screened by Western blot, Quantitative real-time RT-PCR and cAMP assay analysis. Western blot analysis revealed a band, which corresponded to the EP4 receptor protein (Figure 4.4.). Following phosphorimager analysis and quantification, data confirmed that levels of EP4 receptor protein expression in sense clone 1 were significantly higher, when compared to levels of EP4 receptor protein in HeLa WT cells. The increase in EP4 protein expression was consistent with findings from quantitative real-time PCR analysis (Figure 4.5), which confirmed increased EP4 receptor mRNA in sense clone 1, indicating that an increase in gene transcription of the EP4 construct preceded an increase in translation of the EP4 protein.

As mentioned previously, PGE-EP4 receptor interaction couples to $G\alpha_s$ resulting in stimulation of adenylyl cyclase and increased cAMP levels (Regan 2003). Therefore, functionality of the EP4 receptors was confirmed by cAMP assay analysis (Figure 4.3). These data showed increased cAMP in sense clone 1 compared with both antisense clone 4 or WT cells, confirming functionality of the transfected EP4 receptor. A reduction in accumulation of intracellular cAMP was observed in antisense clone 4 when compared to WT cells, which confirms lower levels of EP4 signalling in this antisense clone. In addition, the fold accumulation of cAMP in sense clone 1 compared favourably with that observed in cervical tissue explants treated with PGE_2 previously reported by Sales *et al.*, 2002.

It is important to consider that previous studies have demonstrated the presence of both EP2 and EP4 receptors in WT HeLa cells, both of which were upregulated in cervical carcinoma tissues when compared with normal cervical tissue (Sales *et al.* 2001). Although both EP2 and EP4 receptors couple to $G\alpha_s$, direct comparison of their relative abilities to increase cAMP in HEK293 cells demonstrated much weaker $G\alpha_s$ coupling by

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EP4 compared with the EP2 receptor (Fujino *et al.* 2002). Recent studies have also revealed important functional differences that suggest unique roles for each receptor in different cell types. For instance, PGE_2 stimulation of the EP4 but not EP2 receptor leads to PI3-kinase-dependent phosphorylation of ERK and expression of EGF-1 in COS-7 cells (Fujino *et al.* 2003). PI3-kinase has also been reported to play a role in EP4-induced activation of the GSK-3/β-catenin signalling pathway in HEK293 cells (Fujino *et al.* 2002). β-Catenin is a transcriptional activator and has been implicated in embryonic development and cancer (Fujino *et al.* 2002). Stimulation of the EP2 receptor also leads to GSK-3 phosphorylation and subsequent activation of β-catenin, but in a PKA-dependent, PI3-kinase–independent manner in HEK293 cells (Fujino *et al.* 2002). Taken together, these studies raise the possibility that $G\alpha_s$-mediated increases in cAMP play a less important role for EP4 receptor signalling in some cell types compared with the EP2 receptor, a possibility further supported by observations that the EP4 receptor mediates PGE_2-stimulated proliferation of colon carcinoma cells in the absence of detectable increases in cAMP (Pozzi *et al.* 2004). In the HeLa EP4 overexpressing cervical adenocarcinoma cell model system cumulative roles of EP2 and EP4 receptors may be contributing to cAMP signalling. This issue was addressed with the use of a specific EP4 antagonist (ONO-AE2-227), which ensured specificity of signalling and gene responses via the EP4 receptor. In addition, it is important to consider that the role of the EP4 receptor may be different in HeLa cells when compared to the studies performed on HEK293 or COS-7 cells. EP4 receptor signalling may be cell-type specific and result in differential physiological effects.

In general, it has been assumed that signal transduction cascades are initiated after ligand-receptor binding at the plasma membrane (Sales *et al.* 2003). However, recent data implies that prostanoids may act intracellularly. Studies performed by Bhattacharya *et al.*, 1999, using radioligand binding studies on isolated nuclear membrane fractions localized EP4 receptors in the nuclear envelope of endothelial cells in neonatal porcine brain and adult rat liver and in transfected HEK 293 cells that stably overexpress these receptors. Data also revealed that nuclear EP receptors are functional as they affect transcription of genes such as inducible nitric-oxide synthase and intranuclear calcium transients

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(Bhattacharya *et al.* 1999). Therefore, in order to confirm increased EP4 receptor protein in the HeLa EP4S cells, immunofluorescence microscopy was performed (Figure 4.5). In the HeLa EP4 over-expressing model system, the transfected EP4 receptor levels were increased, suggesting that in ligand-receptor binding and intracellular signalling may be increased in this cell line.

The data presented in this chapter confirm the establishment of a HeLa cell line overexpressing the EP4 receptor. This is based on elevated RNA and protein expression of EP4, as well as elevated cAMP generation in response to PGE_2 in the EP4S clone 1 compared with WT cells. In addition, the levels of cAMP accumulation observed in the EP4S clone 1 compared favourably with those reported in cervical tissue explants treated with PGE_2 (Sales *et al.* 2001). This clone was used in subsequent studies assess signalling pathways and target genes that may be activated by EP4 receptor in cervical epithelial cells which may potentiate cervical tumourigenesis. In addition, EP4AS clone was established, which demonstrate a comparable EP4 receptor protein expression (as determined by Western blotting) but reduced cAMP response to treatment with PGE_2.

During the course of my PhD, an EP4 receptor antagonist (ONO-AE2-227) became available. It was deemed that specificity of EP4-mediated signalling on target gene transcription in EP4S cells treated with the antagonist is a better control treatment than cells transfected with antisense cDNA. All subsequent studies made comparisons of responses to seminal plasma or PGE_2 in wild-type cells, EP4S (clone 1) cells and EP4S (clone 1) cells treated with the EP4 antagonist.

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CHAPTER 5 - THE INTRACELLULAR SIGNALLING PATHWAYS MEDIATING THE ROLE OF EP4 RECEPTOR IN HELA EP4-OVEREXPRESSING CELLS

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5.1. Introduction

EP4 receptor activation by PGE_2, is coupled to $G\alpha_s$, and results in an increase of intracellular cAMP. Following PGE_2 stimulation, EP4 receptors have been shown to undergo rapid agonist-mediated desensitisation and internalisation (Desai *et al.* 2000). The role of the carboxyl tail in rapid agonist-induced desensitisation and internalisation has been demonstrated for the EP4 receptor using studies of truncated receptor and site-directed mutagenesis (Desai *et al.* 2000). Deletion of successive sequences of the human EP4 receptor C terminus identified a stretch of six serine residues in the tail, one or more of which might serve as a target for phosphorylation and subsequent desensitisation (Bastepe *et al.* 1999). Receptor phosphorylation by a variety of serine/threonine kinases is one of the most rapid events to occur and is critical in affecting receptor function by ultimately uncoupling the agonist-occupied form of the receptor from the G-protein, thereby limiting receptor function (Slipetz *et al.* 2001). Both G-protein coupled receptor kinases (GRK) and the second messenger kinases, such as PKA or PKC, are principle regulators of GPCR phosphorylation and uncoupling.

Crosstalk between GPCR and growth factor signalling results a complex myriad of pathways being sequentially activated, with mitogen-activated protein kinases (MAPKs) playing an integral role (Desai *et al.* 2001). The MAPK signalling cascades consist of several individual signalling pathways: extracellular signal-regulated kinase (ERK)-1/2, Jun amino-terminal kinases (JNK/SAPK) and p38 MAPK (Herlaar *et al.* 1999; Chang *et al.* 2001; Yeh *et al.* 2002). The MAPK pathways are key signalling mechanisms that regulate many cellular functions such as growth, differentiation and transformation (Smith *et al.* 2000). ERK is mainly activated by mitogenic stimuli such as growth factors and hormones, while JNK and p38 are predominantly activated by stress stimuli (Yang *et al.* 2003).

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MAPKs are activated by phosphorylation on Thr and Tyr by dual-specicificity MAP kinase kinases (MAPKK), which in turn are activated by Ser/Thr phosphorylation by MAP kinase kinase kinases (MAPKKK) (Tanoue *et al.* 2003). Upstream of MAPKKKs, additional protein kinases may participate, for example members of the Ras and Rho families of GTPases. The small GTPase Ras is one of the key components in the pathway which activates the serine/threonine kinase Raf, which in turn phosphorylates and activates extracellular signal regulated kinase (ERK) (Kranenburg *et al.* 2001; Chong *et al.* 2003; O'Neill *et al.* 2004). Once activated, MAPKs can directly phosphorylate proteins containing the minimal phosphoaccepter motif Ser/Thr-Pro (Yang *et al.* 2003). Phosphorylation can take place in both the cytoplasm or the nucleus and many of their targets are transcription factors or transcriptional coregulators. In addition, MAPKs can phosphorylate and activate downstream protein kinases (Yang *et al.* 2003). Many of these regulate gene expression through phosphorylation of histones and transcriptional regulatory proteins (Belcheva *et al.* 2002). Activation of these pathways is often complex and may lead to a vast array of signalling molecules being sequentially activated.

Studies performed by Fujino *et al.*, 2003 have implicated the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in EP4 receptor activation. PGE_2 stimulation of HEK 293 cells stably transfected with EP4 receptor was shown to phosphorylate extracellular signal-regulated kinases (ERKs) by a PI3K dependent mechanism, followed by induction of the functional expression of early growth response factor (EGR-1) (Fujino *et al.* 2003). It has been reported that cyclin D1, a key regulator of cell cycle progression, is under the control of EGR-1 through a PI3K- and ERK-dependent pathway (Fujino *et al.* 2003). These findings of a PGE_2-mediated induction of EGR-1 expression by the EP4 receptor is interesting in light of the recent studies with knockout mice that show a potential involvement of the EP4 receptor in colon carcinogenesis and rheumatoid arthritis (Mutoh *et al.* 2002; Fujino *et al.* 2003). EP4 knockout mice showed a reduced formation of preneoplastic lesions following treatment with azoxymethane, a known colon carcinogen (Mutoh *et al.* 2002). EP4 knockout mice also showed a significantly decreased incidence and severity of collagen antibody induced arthritis, an animal model of rheumatoid arthritis (McCoy *et al.* 2002). In addition, both colon cancer and rheumatoid arthritis

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prostaglandin levels are elevated and both conditions benefit to some extent by treatment with NSAIDs (Fujino *et al.* 2003). In colon cancer, it has been reported that the expression of cyclin D1, an important regulator of cell cycle progression, is regulated by Tcf signalling (Fujino *et al.* 2002). However it has also been reported that the expression of cyclin Dl is regulated by EGR-1 through a PI3K- and ERK-dependent pathway (Guillemot *et al.* 2001). Furthermore data shows that PGE_2 synthase is upregulated by the binding of EGR-1 to the promoter region of the mouse gene encoding PGE_2 synthase (Naraba *et al.* 2002). Signalling through an EP4 receptor would have the potential, therefore, to increase the expression of cyclin Dl and PGE_2 synthase through a PGE_2-mediated induction of EGR-1 expression. Since the product of PGE_2 synthase is PGE_2 itself, this would have the potential to set up a positive feedback loop in which increased PGE_2 synthesis would further drive EP4 receptor activation.

In sexually active women, growth and invasiveness of neoplastic epithelial cells may be also under the direct influence of endogenously synthesised PGE_2 and PGE present in seminal plasma. Prostaglandins are present in seminal plasma at 10 000-fold greater concentrations than those detected at a site of inflammation, and PGE_2 is one of the predominant types detected (Templeton *et al.* 1978). Little is known of the effect of seminal plasma and seminal plasma prostaglandins, including PGE_2, on neoplastic cervical epithelium of sexually active women. Hence the studies reported in this chapter were designed to investigate signalling pathways that may be activated by interaction of PGE_2 and seminal plasma prostaglandins with EP4 receptor in neoplastic cervical epithelial cells.

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5.2. Aims

The initial aim of this section was to determine the optimal dilution of seminal plasma needed to produce a cAMP response in the cells. Once this had been determined, we could proceed to investigate the interaction between seminal plasma or PGE_2 and cervical neoplastic epithelial cells overexpressing the EP4 receptor. This interaction may result in activation of various signalling pathways, such as cAMP, downstream MAPK signalling pathways (ERK1/2, p38 and cJun N-terminal kinase; JNK), or activation of the PI3kinase/AKT signalling pathway, all of which were investigated.

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5.3. Materials and Methods

5.3.1. Seminal plasma collection.

Semen was collected from healthy male volunteers. The collected ejaculates were pooled. Seminal plasma was isolated from the pooled ejaculate by percoll density gradient centrifugation at 500 g for 20 minutes (Section 2.3). The seminal plasma was stored at –70°C. Seminal plasma was used on the cells at various concentrations of 1:50 up to 1:5000. At these dilutions, seminal fluid has been reported to exert no effect on HeLa cell viability (Jeremias *et al.* 1999).

5.3.2. PGE_2 Assay analysis

PGE_2 assays were performed on pooled seminal plasma samples as described in Section 2.11. The ELISA was performed using 96 well plates (Amine-binding plates) coated with donkey anti-rabbit antibody. The link was prepared by ether extraction and reverse phase chromatography using 20 mg of synthetic PGE_2, 320 µl dry dimethylformamide, 3 µl butylchloroformate and 0.05 mM biocytin. Samples and synthetic standards were diluted in ELISA buffer, and 100 µl of each added in duplicate to the plate. The link was diluted $1:1.5 \times 10^6$ in ELISA buffer and 50 µl added to each well. Antisera, diluted 1:50 000 in ELISA buffer, was added to a final volume of 50 µl to all wells except those used for measuring non-specific binding. Plates were incubated at 4°C for 16 hours, washed and 100 µl/well of 0.2 unit/ml streptavidin-peroxidase was added. Plates were then incubated for 20 minutes at 23°C on an orbital shaker, washed and substrate added to a final volume of 200 µl/well for 10 minutes before quenching with 50 µl/well 1M sulphuric acid. Colour reaction was measured at 450 nm by spectrophotometry.

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5.3.3. EP4 receptor antagonist

The specificity of EP4 receptor activation was ensured by the use of an EP4-specific antagonist (ONO-AE2-227) (Appendix II). It was deemed that EP4S treated with specific EP4 antagonist is a better control than EP4AS. This negated the need for the antisense clone, which was no longer used in further experiments. For this, EP4S cells were pre-treated with 1 μM ONO-AE2-227 for 1 hour prior to stimulations with seminal plasma (1:500) or 300 nM PGE_2. Thereafter WT, EP4S and pre-treated EP4S cells were stimulated with dilutions of seminal plasma or 300 nM PGE_2 for time periods indicated in figure legends.

5.3.4. cAMP assay analysis

In order to determine cAMP accumulation following seminal plasma- or PGE_2-EP4 receptor interaction, cAMP assays were performed on PGE_2 or seminal plasma stimulated cells as described in Section 2.6. Cells ($2x10^5$) were seeded in 6 well dishes and allowed to attach overnight. The next day, cells were washed twice with PBS followed by incubation with serum-free DMEM for a minimum of 16 hours to allow synchronization. Cells were then stimulated with either seminal plasma (1:250 up to 1:5000) or 300 nM PGE_2 for the time periods of 0, 5 and 10 minutes. In addition, EP4S cells were pre-treated with 1 μM of EP4 antagonist for 1 hour followed by stimulation with seminal plasma (1:250 up to 1:5000) or 300 nM PGE_2 for the time periods of 0, 5, and 10 minutes. Following stimulations, the medium was removed and the cells washed in ice-cold PBS before being lysed in 0.1 M HCl. cAMP concentration was quantified by ELISA using a cAMP kit and normalized to the protein concentration of the lysate. Protein concentrations were determined using protein assay kits (Bio-Rad) as described in Section 2.8.2. Results are presented as Mean ± SEM from 3 independent experiments.

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5.3.5. Western blot analysis

HeLa WT or EP4S cells were grown until the desired confluency was reached in 5 cm dishes. Thereafter, cells were synchronised and stimulated with either seminal plasma (1:500) or 300 nM PGE_2 for time periods of 0, 1, 5 or 10 minutes. In addition, EP4S cells were pre-treated with 1 µM of EP4 antagonist followed by stimulation with seminal plasma (1:500) or 300 nM PGE_2 for 0, 1, 5 or 10 minutes. Cells were lysed by addition of 250 µl protein lysis buffer. Proteins were extracted by allowing the dishes to sit on ice for 10 minutes and the cells were then scraped off using a plate scraper. Thereafter, insoluble material was pelleted by centrifugation at 14 000 g for 15 minutes at 4°C. The clarified lysate was then transferred to a new eppendorf tube for protein quantification and SDS-PAGE.

Proteins were quantified according to the BIO-RAD DC Protein microassay as described in Section 2.8.2. SDS-PAGE was performed using pre-cast 4 % - 20 % Tris-Glycine gels. A total of 40 µg protein was resuspended in a total volume of 25 µl sample loading buffer, boiled for 5 minutes at 95°C and loaded into separate wells of the gel. 10 µl of pre-stained molecular weight markers were loaded into a separate well. Gels were run at 4 mA for about 90 minutes prior to immunoblotting. Following electrophoresis, protein was transferred to the PVDF membrane for 1 hour 45 minutes at 14 V using a BIO-RAD semi-dry blotter. Following transfer, membranes were blocked for 1 hour at 25°C using 4 % BSA made up in TBS-Tween. Thereafter, membranes were incubated with the specific primary antibodies at 4°C for 18 hours. Specific primary antibodies used are listed in Section 2.1. After washing 3 times with TBS-Tween, membranes were subsequently incubated for 1 hour respectively with the relevant secondary antibody. Proteins were revealed and quantified by the ECF chemiluminescence system following the manufacturers instructions. Proteins developed by the ECF system were revealed and quantified by PhosphorImager analysis using the STORM 860 system. The molecular weights of the proteins were determined relative to the mobility of the pre-stained markers on SDS-PAGE.

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5.4. Results

PGE_2 assay analysis of the pooled seminal plasma aliquots used in this study showed that the concentration of PGE_2 in these aliquots was 43.6 μg/ml.

We optimised the EP4-seminal plasma mediated cAMP response by performing a dilution curve of seminal plasma. Dilutions of seminal plasma ranging from 1:250 to 1:5000 were investigated. EP4-overexpressing sense (EP4S) cells were pre-treated for 1 hour with 1 μM ONO-AE2-227 prior to seminal plasma stimulations. These pre-treated cells are designated EP4S+Ant. Stimulations were then carried out on HeLa wild-type (WT), EP4S, and EP4S+Ant cells for time periods of 0, 5, and 10 minutes with seminal plasma dilutions ranging from 1:250 up to 1:5000 (Figure 5.1).

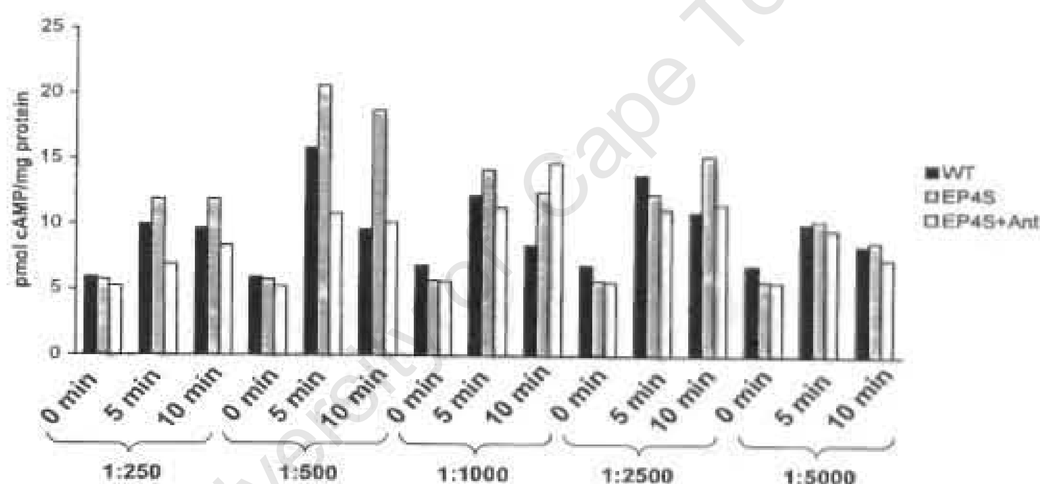

Figure 5.1. cAMP accumulation in response to treatment with serial dilutions of seminal plasma (1:250, 1:500, 1:1000, 1:2500, 1:5000) for 0, 5 and 10 minutes in HeLa wild-type (WT), EP4S and EP4S cells pre-treated for 1 hour with EP4 antagonist (EP4S+Ant).

The highest levels of cAMP accumulation were observed in EP4S cells stimulated with 1:500 dilution of seminal plasma (Figure 5.1). It was therefore decided to proceed with all further experiments using the pooled seminal plasma aliquots at a dilution of 1:500. At a dilution of 1:500 the seminal plasma contains 247 nM PGE_2. At this dilution, seminal plasma has been reported to exert no effect on HeLa cell viability (Jeremias *et al.* 1999).

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5.4.1. cAMP accumulation in response to seminal plasma or PGE_2

cAMP signalling in HeLa cells was determined following stimulation of cells with seminal plasma (1:500) (Figure 5.2. A) or exogenous PGE_2 (300 nM) (Figure 5.2. B) in the absence or presence of 1μM specific EP4 receptor antagonist (ONO-AE2-227).

A rapid accumulation of cAMP was observed after 5 and 10 minutes of stimulation with seminal plasma (1:500) in EP4S cells (18.65 ± 1.04 pmol cAMP/mg protein and 15.42 ± 1.72 pmol cAMP/mg protein, respectively), which was greater than that observed in HeLa wild-type cells (WT) (13.27 ± 1.38 pmol cAMP/mg protein and 9.63 ± 0.06 pmol cAMP/mg protein, respectively) ($p < 0.05$) (Figure 5.2. A). Pre-treatment of EP4S cells for 1 hour with ONO-AE2-227 (EP4S+Ant) resulted in a significant decrease in cAMP accumulation at both 5 and 10 minutes (10.84 ± 0.27 pmol cAMP/mg protein and 9.25 ± 0.55 pmol cAMP/mg protein, respectively) when compared to EP4S cells treated with seminal plasma alone at the same time points ($p < 0.05$) (Figure 5.2. A).

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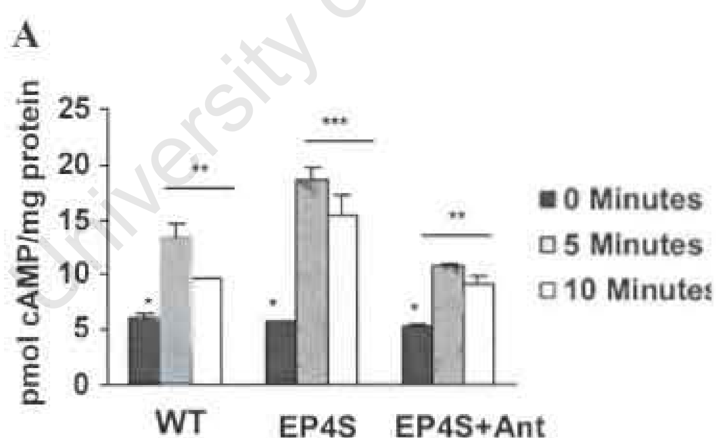

Figure 5.2. A. cAMP accumulation in response to treatment with a 1:500 dilution of seminal plasma for 0, 5 and 10 minutes in HeLa wild-type (WT), EP4S and EP4S cells pre-treated for 1 hour with EP4 antagonist (ONO-AE2-227) (EP4S+Ant). Data shown as Mean ± SEM from 3 independent experiments (** is significantly different from * ($p < 0.05$); *** is significantly different from * and ** ($p < 0.05$).

A rapid accumulation of cAMP was also observed after 5 and 10 minutes of stimulation with 300 nM PGE_2 in EP4S cells (17.01 ± 1.15 and 18.33 ± 2.37 pmol cAMP/mg protein, respectively), which was significantly greater when compared to HeLa WT cells at the same time points (12.49 ± 1.20 and 11.52 ± 0.53 pmol cAMP/mg protein, respectively) ($p < 0.05$) (Figure 5.2. B). Pre-treatment of EP4S cells with 1μM ONO-AE2-227 (EP4S+Ant) resulted in a significant decrease in cAMP accumulation at 5 and 10 minutes (11.25 ± 0.68 and 13.55 ± 0.79 pmol cAMP/mg protein, respectively) when compared to EP4S cells treated with 300 nM PGE_2 alone at the same time points alone ($p < 0.05$) (Figure 5.2. B).

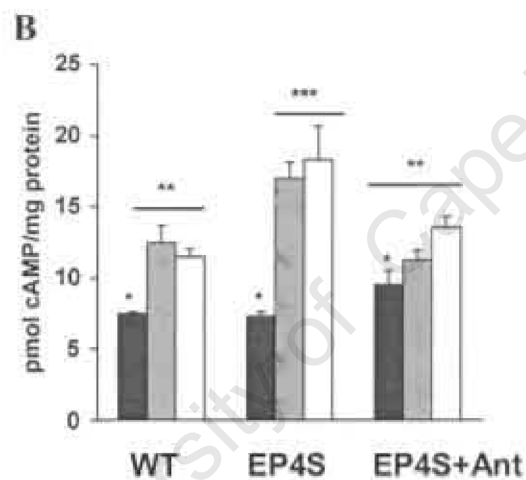

Figure 5.2. B. cAMP accumulation in response to treatment with 300 nM PGE_2 for time periods of 0, 5 and 10 minutes in HeLa wild-type (WT), EP4S and EP4S cells pre-treated for 1 hour with EP4 antagonist (ONO-AE2-227) (EP4S+Ant). Data shown as Mean ± SEM from 3 independent experiments (** is significantly different from * ($p < 0.05$); *** is significantly different from * and ** ($p < 0.05$).

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5.4.2. Investigations into the activation of MAPK or PI3kinase/AKT signalling pathways following treatment with seminal plasma or PGE_2.

5.4.2.1. Phosphorylation of ERK1/2

HeLa WT and EP4S cells were stimulated with seminal plasma (1:500) or 300 nM PGE_2 for 0, 1, 5 and 10 minutes. Ligand receptor activation resulted in a rapid phosphorylation of ERK1/2 in EP4S and WT cells after 1 minute of stimulation with a 1:500 dilution of seminal plasma (7.78 ± 1.11 and 2.87 ± 0.812 fold respectively). This rapid activation of ERK1/2 was elevated and sustained in EP4S cells at 5 and 10 minutes (5.83 ± 0.62 and 4.52 ± 1.11 fold respectively) compared with the activation of ERK observed in WT cells at 5 and 10 minutes (1.81 ± 0.94 and 1.41 ± 0.16 fold respectively). Pre-incubation of HeLa EP4S cells with the specific EP4 receptor antagonist ONO-AE2-227 (EP4S+Ant) abolished the activation of ERK1/2 in EP4S cells at all time points investigated (1.55 ± 0.16; 1.23 ± 0.06 and 1.89 ± 0.70 fold respectively for 1, 5 and 10 minutes) ($p < 0.05$) (Figure 5.3. A).

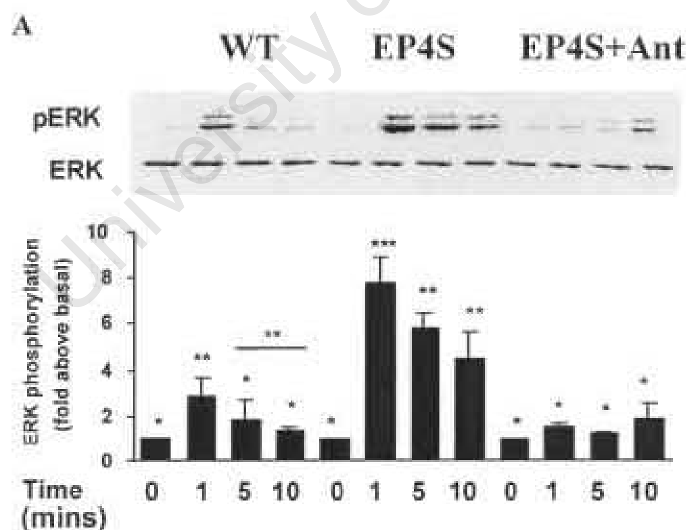

Figure 5.3. A. Representative immunoblot showing phosphorylation of ERK1/2 following treatment with seminal plasma in HeLa wild-type (WT), EP4S and EP4S cells pre-treated with 1 μM ONO-AE2-227 (EP4S+Ant). Cells were stimulated with seminal plasma (1:500) for time periods of 0, 1, 5 and 10 minutes. ERK phosphorylation is shown as fold above basal (Mean ± SEM) from 3 independent experiments (** are statistically significant from * ($p < 0.05$); *** are statistically significant from * and ** ($p < 0.05$).

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Similarly, 300 nM PGE_2 stimulation resulted in significantly elevated ERK1/2 phosphorylation after 1 minute in EP4S (3.79 ± 1.31 fold) cells when compared with HeLa WT (2.60 ± 0.47 fold) ($p < 0.05$), with a sustained increase at 5 minutes in EP4S cells (3.59 ± 0.57 fold) when compared to WT cells at the same time point (2.21 ± 0.84 fold) ($p < 0.05$). ERK1/2 phosphorylation was decreased in both EP4S and WT cells following 10 minutes PGE_2 stimulation (1.36 ± 0.79 and 1.23 ± 0.32 fold respectively). Pre-treatment of EP4S cells with EP4 antagonist (EP4S+Ant) significantly reduced levels of ERK1/2 phosphorylation when at all time points investigated (1.77 ± 0.11; 1.51 ± 0.14 and 1.1 ± 0.17 fold respectively for 1, 5 and 10 minutes) ($p < 0.05$).

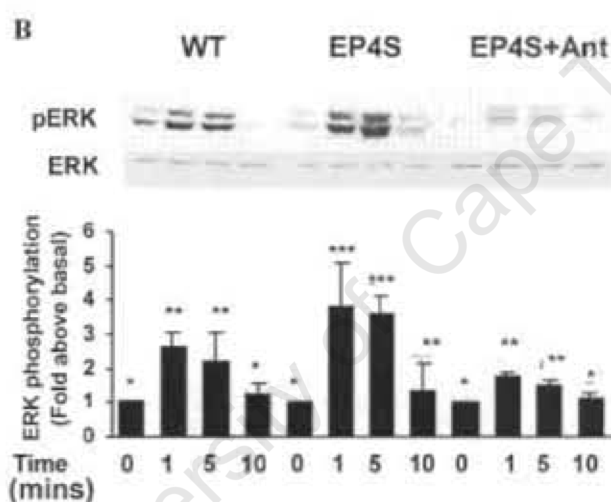

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Figure 5.3. B. Representative immunoblot showing phosphorylation of ERK1/2 following treatment with PGE_2 in HeLa wild-type (WT), EP4S and EP4S cells pre-treated with 1 μM EP 4 antagonist (ONO-AE2-227) (EP4S+Ant). Cells were stimulated with 300 nM PGE_2 for time periods of 0, 1, 5 and 10 minutes. ERK phosphorylation is presented as fold above basal (Mean ± SEM) from 3 independent experiments (** is significantly different from * ($p < 0.05$); *** is significantly different from ** and * ($p < 0.05$)).

These data confirm phosphorylation and activation of ERK1/2 in response to seminal plasma or PGE_2 stimulation of HeLa EP4 overexpressing cells. In order to determine if the mechanism of this PGE_2-EP4 receptor mediated ERK1/2 signalling occurs via the EGF receptor, specific chemical inhibitors were used. These included PD98059 (cell permeable inhibitor that selectively blocks the activation of MEK, thereby inhibiting the phosphorylation and the activation of MAP kinase) (Alessi *et al.* 1995) and AG1478 (selective inhibitor of EGFR tyrosine kinase) (Golubovskaya *et al.* 2002) (Appendix II).

HeLa EP4S cells were pre-treated for 1 hour with 1 μM ONO-AE2-227 (Ant), 50μM PD98059 (PD) or 100nM AG1478 (AG) prior to stimulation with a 1:500 dilution of seminal plasma (+sp) (Figure 5.4. A) or 300 nM PGE_2 (+pge) (Figure 5.4. B) for a time period of 3 minutes. Control EP4S cells were incubated with vehicle and left unstimulated (-sp or –pge).

Seminal plasma (1:500) stimulations of EP4S cells resulted in a 9.60 ± 1.49 fold increase in ERK1/2 phosphorylation above vehicle-treated cells. ERK1/2 phosphorylation was significantly inhibited when cells were pre-treated with PD98059 (1.81 ± 0.91 fold), AG1478 (1.47 ± 0.23 fold) or EP4 antagonist (1.41 ± 0.19 fold) ($p < 0.01$) (Figure 5.4. A).

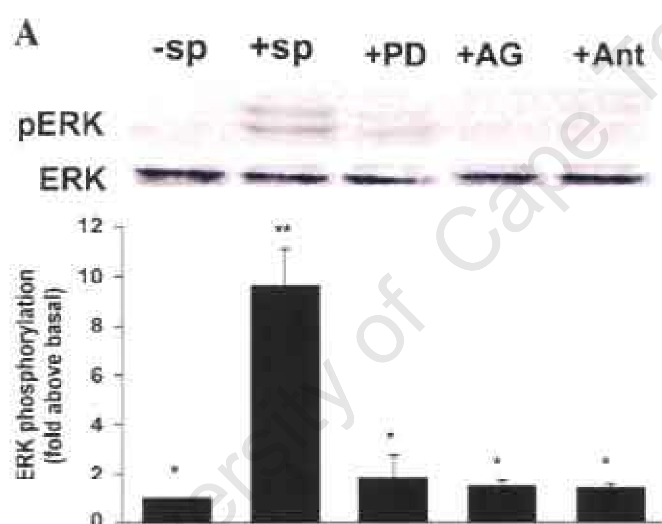

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Figure 5.4. A. Representative immunoblot showing ERK1/2 phosphorylation following seminal plasma (1:500) stimulation of EP4S cells (+sp) and EP4S cells pre-treated with PD98059 (+PD), AG1478 (+AG) or EP4 antagonist (+Ant). Unstimulated control cells are shown as –sp. Results are shown as fold ERK1/2 phosphorylation above basal (Mean ± SEM) from 3 independent experiments (** is statistically significant from *, $p < 0.01$).

Similarly, PGE_2 (300 nM) treatment of EP4S cells resulted in a 6.22 ± 1.27 fold increase in ERK1/2 phosphorylation above vehicle-treated cells. ERK1/2 phosphorylation was significantly inhibited when cells were pre-treated with PD98059 (1.12 ± 0.11 fold), AG1478 (1.01 ± 0.75 fold) or EP4 antagonist (0.98 ± 0.59 fold) ($p < 0.01$) (Figure 5.4. B).

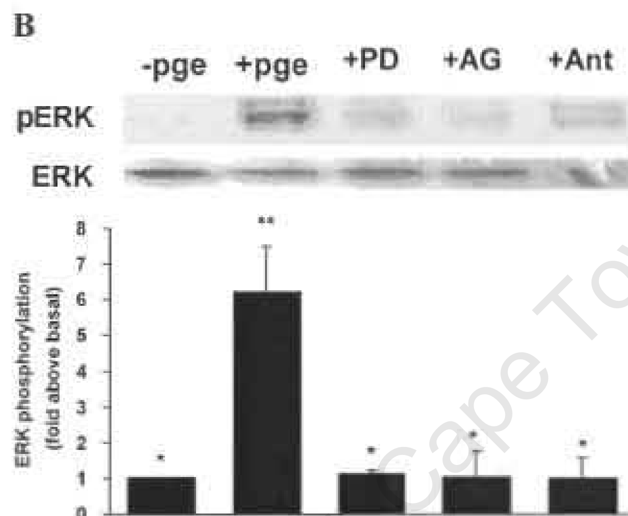

Figure 5.4. B. Representative immunoblot showing ERK1/2 phosphorylation following 3 minutes of 300 nM PGE_2 stimulation of EP4S cells (+pge) and EP4S cells pre-treated with PD98059 (+PD), AG1478 (+AG) or EP4 antagonist (+Ant). Unstimulated control cells are shown as −pge. Results are shown as fold ERK1/2 phosphorylation above basal (Mean ± SEM) from 3 independent experiments (** is statistically significant from *, $p < 0.05$).

To further explore a role for the EGF receptor and small GTPase Ras in activation of ERK1/2, we co-transfected HeLa EP4S cells with cDNA for cMyc-tagged ERK with either the dominant negative (DN) form of MAPK kinase (MEK), EGF receptor, Raf or with an empty vector (pcDNA3.0) as described in Section 2.5. EP4S cells co-transfected with empty vector (pcDNA3.0) were then either pre-treated for 1 hour prior to stimulations with 1 μM ONO-AE2-227 (EP4 Ant) or pre-treated with vehicle and left unstimulated (-sp or -pge) as a control. Co-transfected EP4S cells were then stimulated by seminal plasma (1:500) (Figure 5.5. A) or 300 nM PGE_2 (Figure 5.5 B) for 3 minutes. Following stimulations, cells were lysed and transfected cMyc-ERK was immunoprecipitated from cell lysates and subjected to Western blot analysis.

Seminal plasma (1:500) rapidly phosphorylated cMyc-ERK1/2 in EP4S cells co-transfected with empty vector (pcDNA3) (4.06 ± 0.55 fold above vehicle-treated cells). This elevation in ERK phosphorylation was abolished by co-transfection with the DNEGFR (1.58 ± 0.06 fold), DNRaf (1.79 ± 0.23 fold), or the DNMEK (1.54 ± 0.10 fold) ($p < 0.05$) (Figure 5.5. A). Pre-treatment of EP4S cells for 1 hour with 1 μM ONO-AE2-227 (sp+EP4 Ant) decreased ERK1/2 phosphorylation to 1.59 ± 0.32 fold above vehicle-treated cells (Figure 5.5. A).

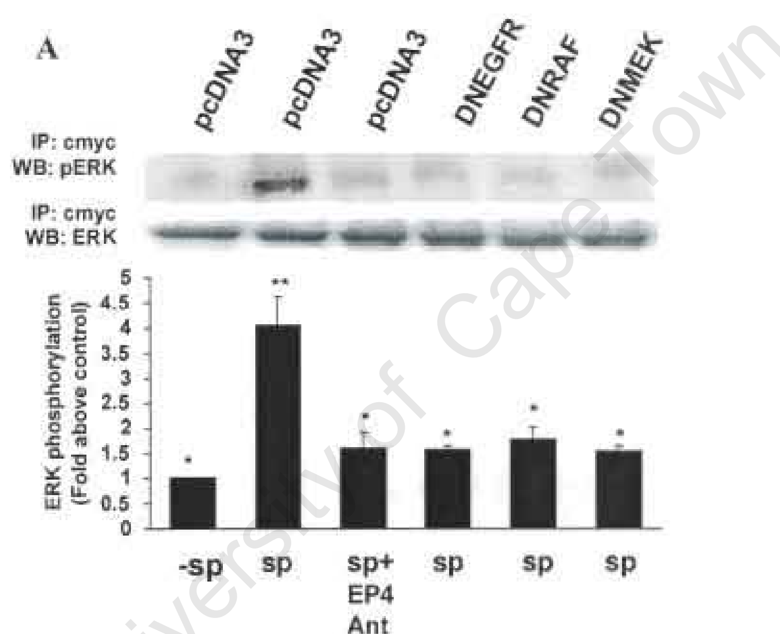

Figure 5.5. A. Immunoblot showing cMyc-ERK1/2 phosphorylation in EP4S cells co-transfected with empty pcDNA3.0 vector, DNEGFR, DNRaf or DNMEK in response to seminal plasma (1:500) stimulation for 3 minutes. EP4S cells co-transfected with empty vector were also pre-treated with 1 μM ONO-AE2-227 (EP4 Ant) prior to stimulations or left unstimulated (-sp). Data are shown as fold ERK1/2 phosphorylation (Mean±SEM) above vehicle-treated cells from 3 independent experiments (** is statistically significant from *, $p < 0.01$).

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Similarly, phosphorylation of cMyc-ERK1/2 was rapidly elevated following 3 minutes of 300 nM PGE_2 stimulation of EP4S cells (2.46 ± 0.30 fold). This elevation in ERK phosphorylation was abolished by co-transfection with the DNEGFR (1.42 ± 1.91 fold), DNRaf (1.34 ± 0.14 fold), or the DNMEK (1.29 ± 0.24 fold) ($p < 0.05$) (Figure 5.5. B). In addition, pre-treatment of EP4S cells for 1 hour with 1 μM ONO-AE2-227 (sp+EP4 Ant) significantly decreased ERK1/2 phosphorylation to 1.34 ± 0.11 fold above vehicle-treated control cells ($p < 0.05$) (Figure 5.5. B).

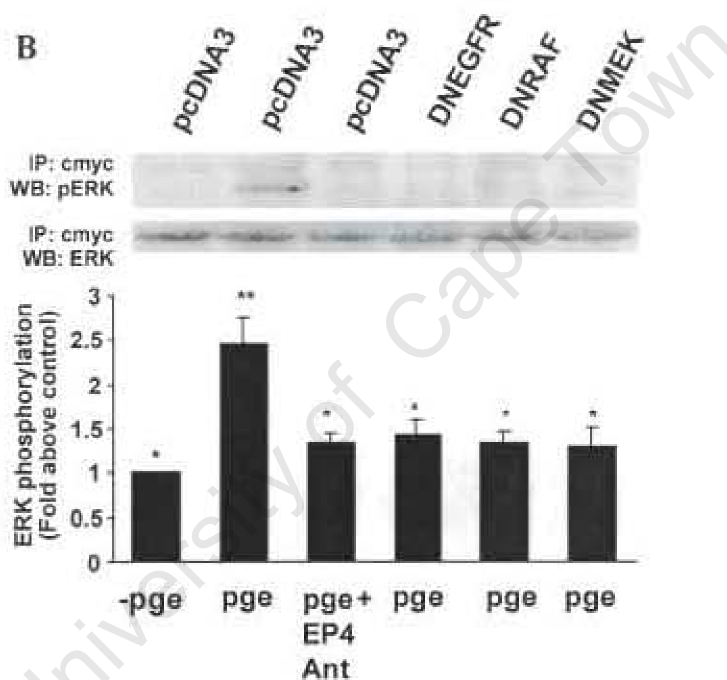

Figure 5.5. B. Immunoblot showing cMyc-ERK1/2 phosphorylation in EP4S cells co-transfected with empty pcDNA3.0 vector, DNEGFR, DNRaf, DNMEK in response to 300 nM PGE_2 stimulation for 3 minutes. EP4S cells co-transfected with empty vector were also pre-treated with 1 μM ONO-AE2-227 (EP4 Ant) prior to stimulations or left unstimulated (-pge). Data are presented as fold ERK1/2 phosphorylation (Mean ± SEM) above control from 3 independent experiments (** is statistically significant from *, $p < 0.05$).

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5.4.2.2. Activation of JNK signalling pathway

In order to investigate whether PGE_2- or seminal plasma-EP4 receptor interaction activated other MAPK signalling pathways, we treated HeLa WT and EP4S cells with seminal plasma (1:500) or PGE_2 (300 nM) for time periods of 0, 1, 3, 5, 10, 15, 20 or 30 minutes. In addition, EP4S cells were pre-treated for 1 hour with 1 μM EP4 antagonist ONO-AE2-227 followed by stimulation with seminal plasma (1:500) or 300 nM PGE_2 for the time periods indicated. No significant differences were observed in JNK phosphorylation for cells treated with seminal plasma (1:500) (Figure 5.6. A, B) or PGE_2 (300 nM) (Figure 5.6. C, D).

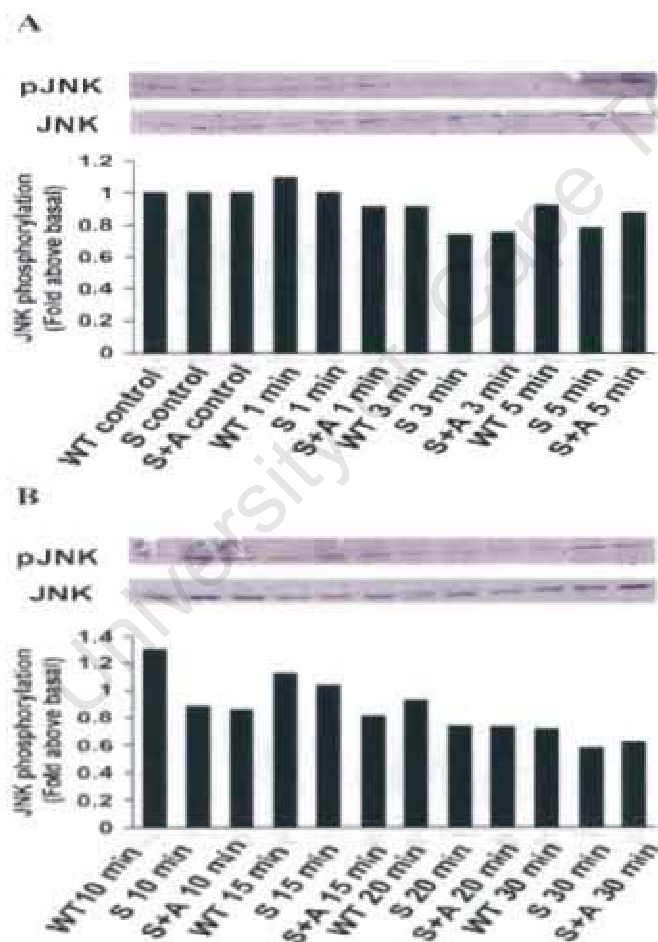

Figure 5.6. A, B. Representative immunoblots showing phosphorylation of JNK following treatment with seminal plasma (1:500) over a time course of 0, 1, 3, 5, 10, 15, 20 and 30 minutes in HeLa WT (WT), EP4S (S) and EP4S cells pre-treated with 1 μM ONO-AE2-227 (S+A).

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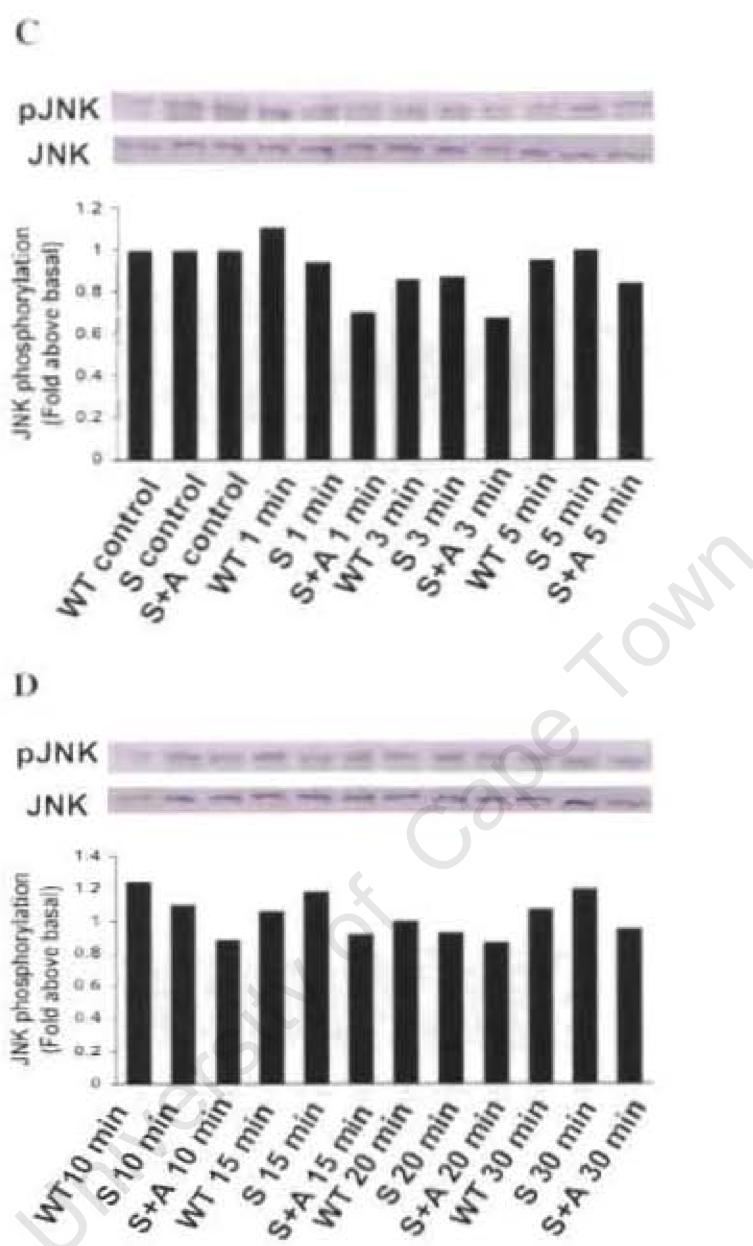

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Figure 5.6. C, D. Representative immunoblots showing phosphorylation of JNK following treatments with 300 nM PGE_2 over a time course of 0, 1, 3, 5, 10, 15, 20 and 30 minutes in HeLa WT (WT), EP4S (S) and EP4S cells pre-treated with 1 μM ONO-AE2-227 (S+A). No significant differences were seen in JNK phosphorylation over the time course investigated.

5.4.2.3. Activation of p38MAPK signalling pathway

Data published by Fiebich *et al.*, 2001, suggest that PGE_2 induces IL-6 via an EP4-like receptor by the activation of p38MAPK (Fiebich *et al.* 2001). Investigations were performed to investigate the possible activation of p38MAPK following PGE-EP4 receptor interaction in our model system. EP4S cells were pre-treated for 1 hour with EP4 antagonist ONO-AE2-227. HeLa WT, EP4S, and pre-treated EP4S cells were then stimulated with seminal plasma (1:500) or PGE_2 (300 nM) for time periods of 0, 1, 3, 5, 10, 15, 20 or 30 minutes. Phosphorylation of p38MAPK is shown as fold above basal. No significant differences were observed for cells treated with seminal plasma (1:500) (Figure 5.7. A, B) or PGE_2 (300 nM) (Figure 5.7. C, D).

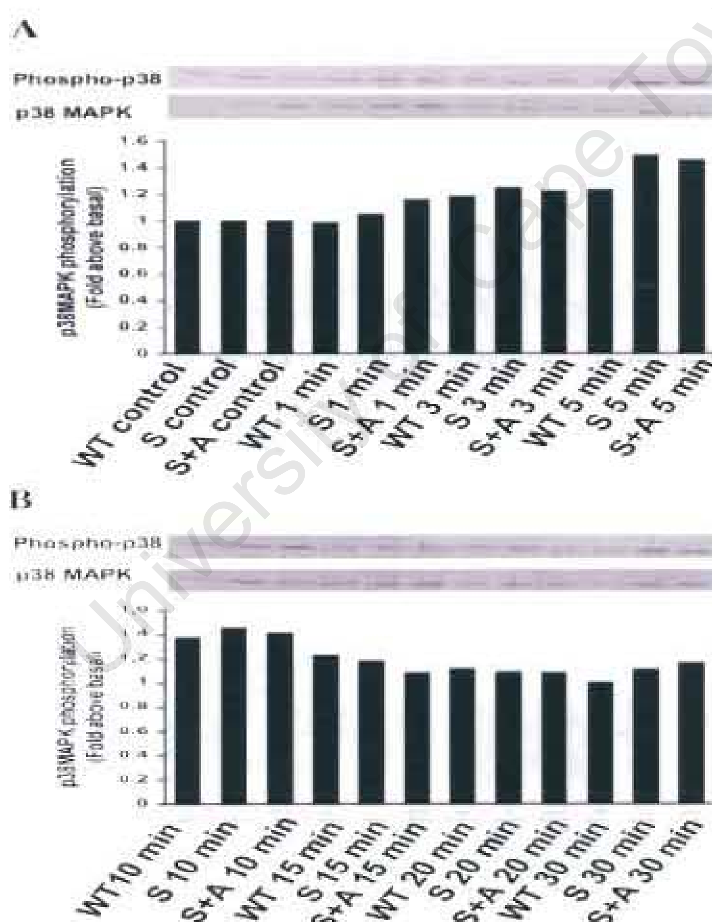

Figure 5.7. A, B. Representative immunoblots showing phosphorylation of p38MAPK following treatment with seminal plasma (1:500) over a time course of 0, 1, 3, 5, 10, 15, 20 or 30 minutes in HeLa WT (WT), EP4S (S) and EP4S cells pre-treated with 1 μM ONO-AE2-227 (S+A).

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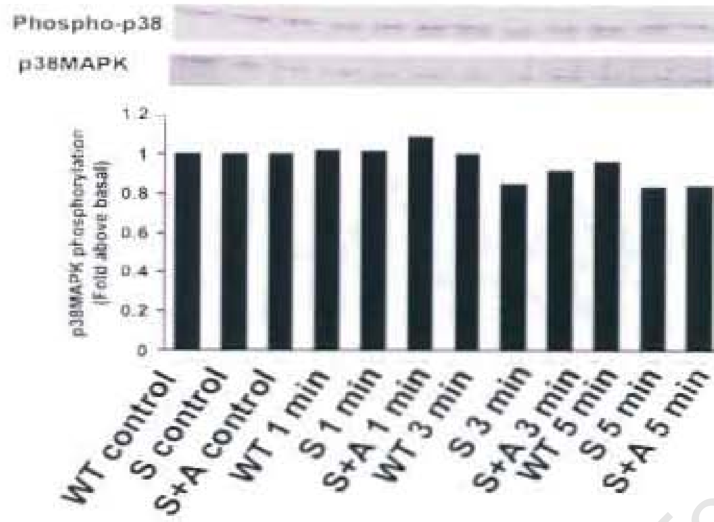
C



D

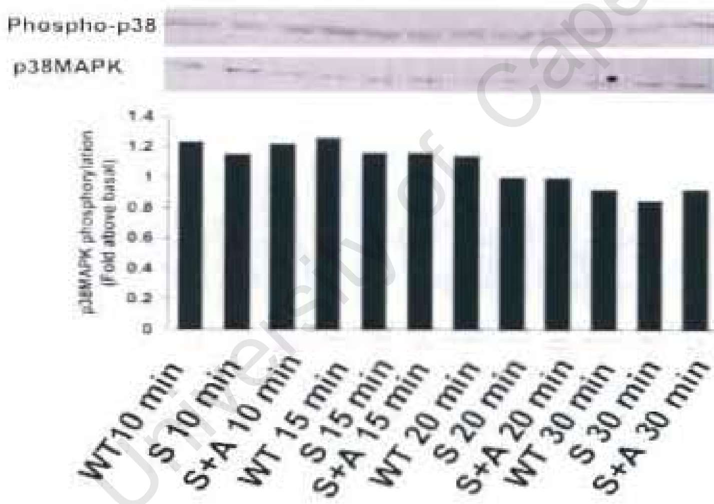


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Figure 5.7. C, D. Representative immunoblots showing phosphorylation of p38MAPK following treatment with 300 nM PGE_2 over a time course of 0, 1, 3, 5, 10, 15, 20 or 30 minutes in HeLa WT (WT), EP4S (S) and EP4S cells pre-treated with 1 µM ONO-AE2-227 (S+A). No significant differences were seen in p38MAPK phosphorylation over the time course investigated.

5.4.2.4. Activation of PI3 Kinase/AKT signalling pathway

Studies performed by Fujino *et al.*, 2003 show activation of the EP4 receptor leads to phosphorylation of ERK1/2 through a PI3kinase-dependent mechanism (Fujino *et al.* 2003). Investigations were performed to investigate whether signalling in our HeLa EP4 over-expressing model system involves the PI3kinase/AKT signalling pathway. EP4S cells were pre-treated for 1 hour with 1 μM ONO-AE2-227. HeLa WT, EP4S and pre-treated EP4S cells were then stimulated with seminal plasma (1:500) or 300 nM PGE_2 for time periods of 0, 1, 3, 5, 10, 15, 20 or 30 minutes. AKT phosphorylation is shown as fold above basal. No significant differences were observed for cells treated with seminal plasma (1:500) (Figure 5.8. A, B) or PGE_2 (300 nM) (Figure 5.8. C, D).

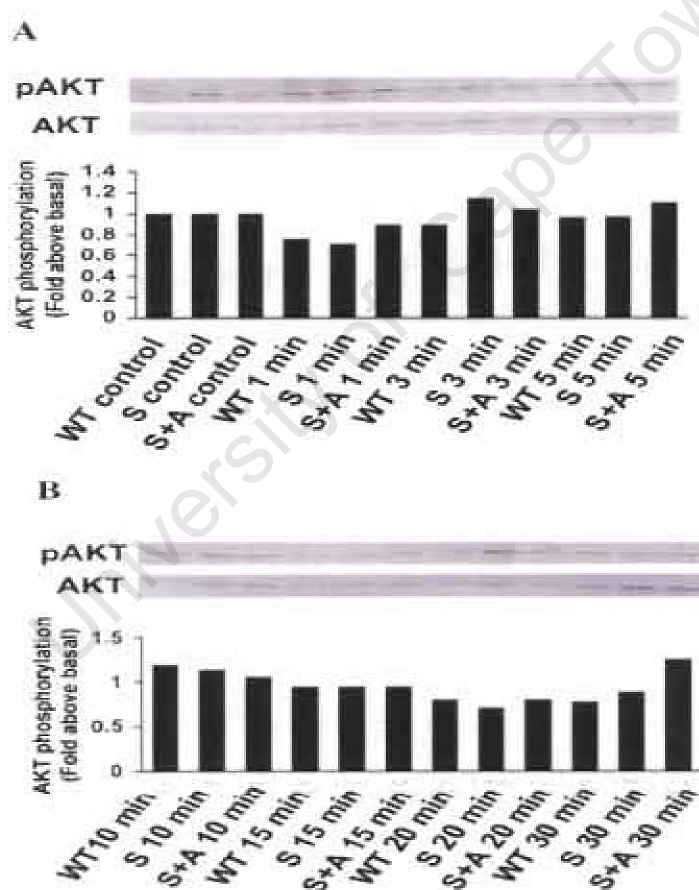

Figure 5.8. A, B. Representative immunoblots showing phosphorylation of AKT following treatment with seminal plasma (1:500) over a time course of 0, 1, 3, 5, 10, 15, 20 or 30 minutes in HeLa WT (WT), EP4S (S) and EP4S cells pre-treated with 1 μM ONO-AE2-227 (S+A).

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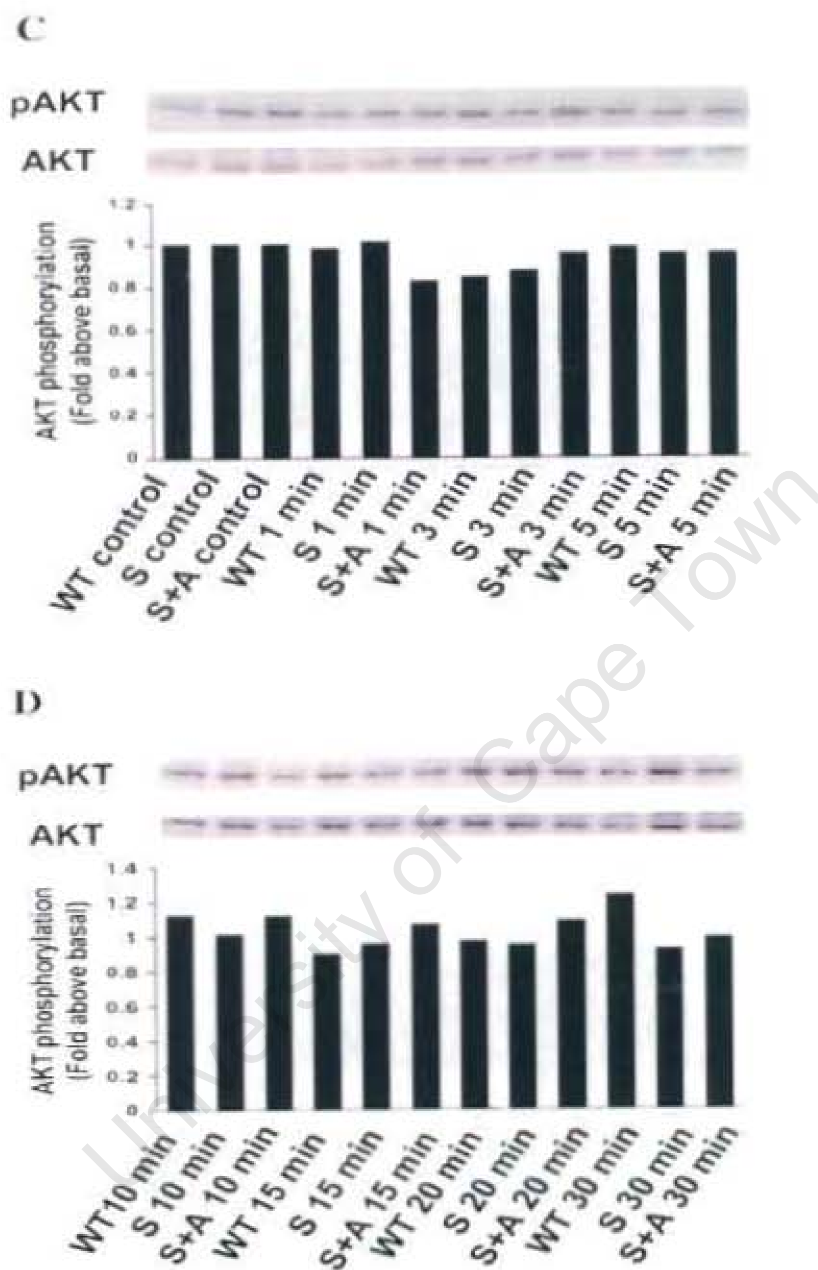

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Figure 5.8. C, D. Representative immunoblots showing phosphorylation of AKT following treatment with 300 nM PGE_2 over a time course of 0, 1, 3, 5, 10, 15, 20 or 30 minutes in HeLa WT (WT), EP4S (S) and EP4S cells pre-treated with 1 μM ONO-AE2-227 (S+A). No significant differences were seen in AKT phosphorylation over the time course investigated.

5.5. Discussion

In the classical model of GPCR signalling, stimulation of seven transmembrane spanning GPCR leads to the activation of heterotrimeric G proteins, which dissociate into α- and βγ-subunits (Hur *et al.* 2002; Yang *et al.* 2003). On the basis of G protein-coupling preference, GPCRs can be broadly classified in to G_s-, $G_{i/o}$- and $G_{q/11}$-coupled receptors (Yang *et al.* 2003). Dissociation of the heterotrimeric subunits activate a host of effector molecules, which include second messenger generating systems, adapter molecules and receptor tyrosine kinases (RTK), giving rise to various cellular, physiological, and biological responses. Many cells express multiple types of GPCRs that signal through various effector molecules resulting in cross regulation and activation of multiple signalling pathways to modulate the net physio or patho-physiological response (Hur *et al.* 2002).

The EP4 receptor is a GPCR which is known to couple to $G\alpha_s$, leading to an increase in adenylyl cyclase. Adenylyl cyclases integrate positive and negative signals triggered by GPCRs to regulate the levels of cAMP. Nine isoforms of adenylyl cyclase have been cloned to date, and all of them are regulated by $G\alpha_s$ (Hur *et al.* 2002). However their expression patterns and regulatory properties vary widely. Studies have also revealed variable sensitivity of adenylyl cyclases to regulators such as protein kinases and Ca^{2+} (Hur *et al.* 2002). The effects of various kinds of regulators on adenylyl cyclase isozymes suggests that crosstalk can occur between adenylyl cyclase-coupled receptors and other signals that regulate Ca^{2+}, or protein kinases, such as growth factor receptors or even other GPCRs (Hur *et al.* 2002). The vast majority of the cellular effects of cAMP are mediated by cAMP-dependent protein kinase A (PKA), which is involved in activating numerous signalling cascades, including the MAPK pathway.

As discussed previously, both EP2 and EP4 receptors have been identified on HeLa cells, and both have been shown to activate $G\alpha_s$. Data presented by Fujino *et al.*, 2002, demonstrate an interesting observation that despite nearly identical receptor expression, maximal levels of PGE_2-stimulated cAMP formation in EP4-expressing HEK293 cells

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was only 20 % of the level obtained in EP2-expressing HEK293 cells. However under the same conditions, the ability of PGE_2 to stimulate Tcf signalling was about 50 % greater in EP4-expressing cells as compared with EP2-expressing cells (Fujino *et al.* 2002). This suggest that the lower amounts of PGE_2-stimulated cAMP formation in EP4-expressing HEK293 cells is because of less efficient coupling to this pathway and not because of an overall impairment in the signalling potential of these receptors (Fujino *et al.* 2002). These data are interesting in view of results depicted in Figure 5.2, which demonstrate intracellular accumulation of cAMP in the EP4-overexpressing HeLa cells following PGE-EP4 receptor interaction. This may be indicative of EP4 cell-type specific signalling, where EP4 may play a different physiological role in the cervix and couple to alternate signalling pathways.

The more efficient coupling of the EP2 receptor to intracellular cAMP formation however, may be significant with respect to findings made by Dhillon AS *et al.*, 2002 and Sidovar MF *et al.*, 2000. These data reported that the phosphorylation of Raf kinase by PKA inhibits the activity of Raf kinase, and subsequently decreases Raf-mediated MAPK signalling in their cell model system. In EP2-overexpressing COS cells, therefore, a robust activation of PKA may inhibit RAF kinase and block the phosphorylation and activation of ERKs (Sidovar *et al.* 2000; Dhillon *et al.* 2002). In our HeLa model system, we have demonstrated that PGE-EP4 receptor activation leads to an accumulation of cAMP, however inefficient coupling to the PKA pathway by EP4 may allow for the activation of Raf kinase, and subsequent phosphorylation and activation of ERKs. It is clear from these findings that the EP4 receptor could work in concert with the EP2 receptor to initiate physiological cell responses. In addition, the involvement of other GPCRs may also be crucial to various cell signalling responses.

Cross-talk between cell surface receptors is recognised as a mechanism capable of expanding the cellular communication signalling network. Receptor cross-talk can, in fact, also occur among distinct families of receptors, such as tyrosine kinase receptor and GPCR (Gschwind *et al.* 2002; Schafer *et al.* 2004). In this context, studies performed by Spinella *et al.* (2004) demonstrated a role for another GPCR, termed endothelin receptor

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(ET_AR) in the progression of ovarian carcinoma. In this study, they show that in ovarian carcinoma cells, endothelin-1 (ET-1), through the binding with ET_AR, induces PGE_2 production, and increases the expression of PGE_2 receptor EP2 and EP4 (Spinella *et al.* 2004). The use of pharmacological EP agonists and antagonists indicated that ET-1 and PGE_2 stimulate VEGF production principally through these EP receptors. They also demonstrated that this induction of PGE_2 and VEGF by ET-1 involves *Src*-mediated EGFR transactivation. These results implicate EP2 and EP4 receptors in the induction of VEGF expression and cell invasiveness by ET-1 and provide a mechanism by which ETAR/ET-1 can promote and interact with PGE_2-dependent machinery to amplify its pro-angiogenic and invasive phenotype in ovarian carcinoma cells (Spinella *et al.* 2004).

Recent studies have also revealed that several distinct signalling mechanisms contribute to the activation of the MAPK pathway by GPCRs, as suggested in the case of the EGFR (Maudsley *et al.* 2000; Pai *et al.* 2002; Yang *et al.* 2003). EGFR belongs to a family of receptor tyrosine kinases (RTK), which undergo auto-phosphorylation upon agonist stimulation and activates the Ras/MAPK pathway (Vlahovic *et al.* 2003). It has recently been reported that ERK1/2 activation in endometrial carcinoma is dependent on transactivation of the EGFR by prostaglandins, including PGE_2 (Sales *et al.* 2004). This study confirms the potential mode of ERK1/2 activation in EP4S cells by seminal plasma and PGE_2 occurs via the EGFR. Although the mechanism of transactivation of the EGFR has not been investigated in this study, other studies have proposed possible mechanisms for transactivation of EGF receptor by GPCR's (Maudsley *et al.* 2000; Pierce *et al.* 2001; Pai *et al.* 2002). These mechanisms involve the activation of transmembrane matrix metalloproteinases (MMP) and extracellular release of heparin-binding EGF (HB-EGF) from its latent membrane-spanning precursor in the plasma membrane. Once cleaved the HB-EGF ligand can associate with and activate the EGF receptor and induce ERK1/2 MAPK signalling (Pai *et al.* 2002). Alternatively, studies have shown that activation of the c-Src family of non-receptor tyrosine kinases is involved in GPCR-mediated transactivation of the EGFR. It is feasible to consider that in the HeLa EP4 overexpressing cell model system, either method may be responsible for transducing signalling following EP4 receptor activation.

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Data from this study have demonstrated that seminal plasma (1:500) or PGE_2 (300 nM) stimulation of EP4-overexpressing cervical adenocarcinoma cells activates signal transduction cascades, which culminate in the phosphorylation of ERK1/2 MAPK (Figure 5.9). This phosphorylatyion of ERK1/2 does not appear to be related to cAMP levels, as cAMP accumulation in the EP4 overexpressing cell line was increased to a similar extent in both seminal plasma-treated and PGE_2-treated cells. Studies performed by Faour *et al.*, 2001, implicate the EP4 receptor and downstream kinases p38 MAPK and, perhaps cAMP-dependent protein kinase in release of PGE_2 by rhIL-1 beta in human synovial fibroblasts. In addition, studies by Martineau et al., 2004, indicate that key components of the eicosanoid pathway are upregulated by mechanically stimulated p38 MAPK via the EP4 receptor in podocytes. These studies suggest the possible involvement of alternate signalling pathways that may be activated via the EP4 receptor, however in our study, at the time points investigated, no other MAPK or PI3 kinase pathways were activated at the time points investigated. Investigations into the mode of ERK1/2 activation demonstrated that the EGFR was essential for activation of downstream ERK1/2, since co-treatment of the cells with AG1478 (specific EGF receptor kinase inhibitor) (Figure 5.4), and co-transfection of cells with DNEGFR (Figure 5.5) significantly reduced the phosphorylation of ERK1/2. This suggests that PGE-EP4 signalling to ERK1/2 may involve the EGF receptor (Figure 5.9). These findings are thus consistent with a growing body of evidence suggesting that transactivation of the EGFR by GPCRs is a recurrent theme in cell signalling to promote target gene transcription (Gschwind *et al.* 2002; Schafer *et al.* 2004; Spinella *et al.* 2004).

To our knowledge, there have been no studies performed on the effects of seminal plasma on the cervix in humans, and very little is known regarding any possible consequences that contact between seminal plasma and pre-neoplastic epithelial cells might yield. Prostaglandin levels in seminal plasma are very high, up to 10 000 times those found at a site of inflammation. It is also noteworthy to consider that seminal plasma is a heterogeneous fluid, and that any differences in kinetics between seminal plasma and PGE_2 in regulating EP4 receptor signalling observed in this study could suggest that other factors and effectors of signal transduction pathways may be functioning in synergy with

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PGE_2 to modulate neoplastic cervical epithelial cell function. These factors may include other prostanoid derivatives of the E series (PGE and 19-hydroxy-PGE), which may be acting in combination to shift the kinetics of signal transduction in cervical carcinoma cells.

Taken together, these data indicate that endogenous PGE_2 (which may synthesised de novo by COX enzymes), as well as prostaglandins available in seminal plasma can activate EP receptor signalling in cervical adenocarcinoma cells. Figure 5.9 is a cartoon, which depicts schematically the activation of EP4 receptor in the HeLa cell model system to potentially modulate the transcription of target genes. In this figure, PGE, either produced intracellularly via the COX-enzyme biosynthetic pathway or present in seminal plasma, activates EP4 receptor initiating production of the second messenger cAMP (Figure 5.9). Activation of these second messenger systems may then initiate kinase signalling by the ERK1/2 MAPK pathway and target gene transcription (Figure 5.9.). Data suggests that target gene transcription can occur via EP4-receptor-mediated transactivation of EGFR, either via the release of a heparin-bound EGF-like molecule to activate RTK directly or by intracellular mechanisms involving c-Src. In turn, the activation of target genes by EP4 receptor signalling may promote tumourigenesis or angiogenesis (by modulating vascular function and vascular tone).

Although this study has provided a model system in which to investigate EP4 receptor signal transduction following PGE_2 or seminal plasma stimulation, further studies are needed to map out the integrative network of signalling pathways activated in HeLa EP4s cells following ligand receptor activation.

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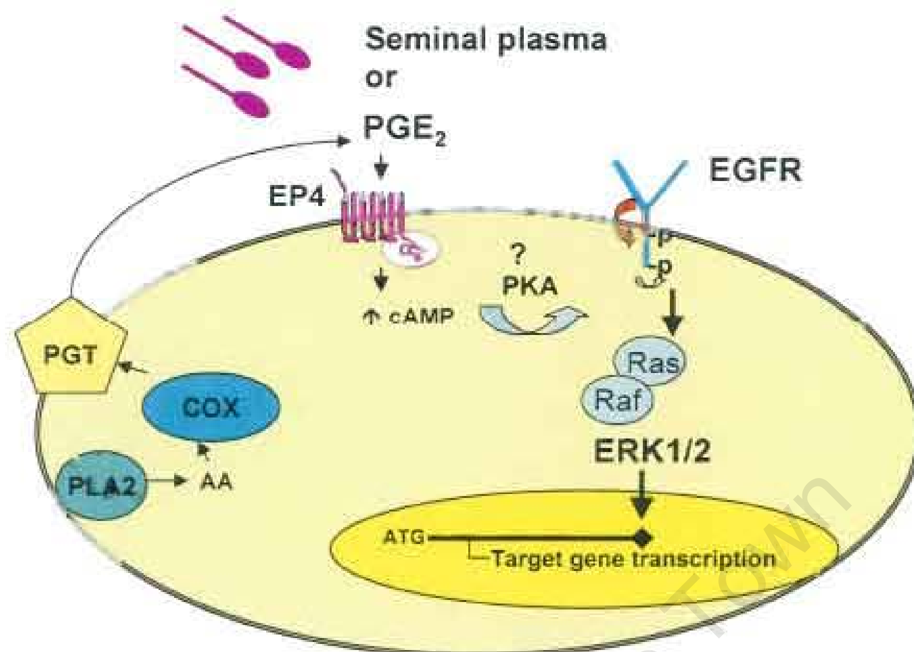

Figure 5.9. Signal transduction pathways activated by the EP4 receptor in response to stimulations by PGE_2 and seminal plasma. Prostaglandin, either produced intracellularly via the COX biosynthetic pathway or present in seminal plasma, activates the EP4 receptor initiating the production of cAMP. Activation of second messenger systems can then initiate kinase signalling by ERK1/2 and target gene transcription. Target gene transcription may occur via transactivation of the EGFR. In turn, the activation of target genes by EP4 receptor signalling can promote angiogenesis, cellular proliferation, and cellular adhesion, migration or metastasis.

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After these investigations into the role that MAPK signalling proteins may play in cervical tumourigenesis following PGE-EP receptor activation, the next chapter will go on to investigate how these signals are integrated to give a gene-specific transcriptional response. As mentioned previously, this is an important consideration in sexually active women with pre-neoplastic lesions, as exposure to high levels of prostaglandins may upregulate transcription of various genes involved in tumourigenesis, such as COX-2, or angiogenesis, such as VEGF. We therefore decided to investigate possible target genes, which might be upregulated in response to seminal plasma or PGE_2 stimulation of EP4 overexpressing adenocarcinoma cells.

CHAPTER 6 - GENE TRANSCRIPTION IN RESPONSE TO EP4 RECEPTOR ACTIVATION BY SEMINAL PLASMA OR PGE_2

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6.1. Introduction

As discussed in the previous section, seminal plasma or PGE_2-EP4 interaction leads to activation of various signalling pathways, and involves the phosphorylation of ERK1/2 and transactivation of the EGF receptor. Activation of these pathways may ultimately result in transcription of target genes to promote inflammation, growth and angiogenesis.

Some of the target genes that have been shown to be regulated by the prostanoid signalling pathways include COX-2, PGE_2 synthase and vascular endothelial growth factor (VEGF). Studies by Fujino and Regan (2003) have proposed a mechanism that could explain how the EP4 receptor could induce the expression of COX-2 and PGE_2 synthase, which are both known to be upregulated in cancer and inflammation. PGE_2 stimulation of the EP4 receptor can activate a PI3K/ERK signalling pathway resulting in the induction of functional EGR-1 expression (Fujino *et al.* 2003). Likewise, EP4 receptor stimulation can activate a PI3K dependent pathway resulting in Tcf transcriptional activation (Fujino *et al.* 2002) and COX-2 expression has been shown to be upregulated by nuclear β-catenin accumulation and Tcf-mediated transcriptional activation (Araki *et al.* 2003). Thus, stimulation of the EP4 receptor has the potential to induce expression of both PGES and COX-2, thereby setting up a positive feedback loop in which the increased synthesis of PGE_2 may increase tumourigenic potential of cells (Regan 2003).

As mentioned in the previous chapter, studies by Spinella *et al.* (2004) implicate EP2 and EP4 receptors in the induction of VEGF expression and cell invasiveness by endothelin-1 (ET-1). They demonstrated that ET-1 through the endothelin-A receptor (ET_AR) induces COX-enzyme expression and that both enzymes contribute to PGE_2 and VEGF production in ovarian carcinoma cells through EGFR transactivation (Spinella *et al.* 2004). The reduction of ET-1-induced VEGF production and cell invasion by using a specific EP4 antagonist (AH23848) suggests that the production of PGE_2 by ET_AR-mediated EGFR transactivation could activate EP4 receptor-mediated signalling, and in turn stimulate angiogenic and migratory action of ovarian carcinoma cells.

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Several studies have shown a correlation between PGE_2-EP4 receptor interaction and tumour phenotype in various tumour types and in other model systems (Sheng *et al.* 2001; Dohadwala *et al.* 2002; Sales *et al.* 2002; Seno *et al.* 2002; Fujino *et al.* 2003; Sales *et al.* 2004) in which EP4 receptors promote tumour progression, increasing pro-angiogenic factors, and tumour cell invasion. Moreover, previous reports demonstrate that EP2 and EP4 receptor transcripts are inducible by cytokine (Narko *et al.* 2001) and that PGE_2 may regulate EP4 receptor expression in non-small cell lung carcinoma cells (Dohadwala *et al.* 2002) and EP2/4 receptors in COX-1 transfected cervical carcinoma cells (Sales *et al.* 2002). In endometrial cancer cells, elevated EP2 receptor expression may facilitate PGE_2-induced release of pro-angiogenic factors (Sales *et al.* 2004). Thus, an increase in PGE-EP receptor interaction may promote a positive feedback mechanism in carcinoma cells by activating specific signalling pathways to modulate the expression of EP receptors and synthesis of PGE_2 to enhance and sustain tumourigenesis.

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6.2. Aim

The aim of this section is to investigate possible target genes regulated by seminal plasma or PGE_2-EP4 interaction. The genes to be investigated include EP1, EP2, EP3, EP4 receptor and genes involved in tumourigenesis, such as COX-2, or angiogenesis such as bFGF and VEGF.

6.3. Materials and Methods

In this study we investigated the effect of seminal plasma (1:500) or 300 nM PGE_2 on the expression of various genes. HeLa wild-type (WT) and EP4S cells were pre-treated for 1 hour prior to stimulations with 1 μM EP4 antagonist (ONO-AE2-227), or in some experiments with specific inhibitors 50 μM PD98059 (Inhibitor of MEK) or 100 nM AG1478 (Inhibitor of EGFR kinase) for 1 hour prior to stimulations. Thereafter, HeLa WT, EP4S, pre-treated WT and pre-treated EP4S cells were stimulated for various time periods (as indicated in figure legends) with either seminal plasma (1:500) or 300 nM PGE_2. Thereafter, RNA was isolated and samples were subjected to quantitative real-time RT-PCR using primers specific for various genes. Data are presented as Mean ± SEM from 3 independent experiments.

6.3.1. RNA Isolation and Quantitative real-time PCR analysis

RNA isolation was performed on cells as described in Section 2.7.1. Following spectophotometry to determine RNA concentration and quality (Section 2.7.2), real time quantitative RT-PCR was performed for genes encoding EP receptors, COX-2, VEGF and bFGF using specific primers and probes (Table 5) (Section 2.7.3 and Section 2.7.4). Fold induction was calculated by dividing the relative mRNA expression of cells treated with PGE_2 (300nm) or seminal plasma (1:500) by the relative mRNA expression in vehicle-treated cells at the same time point.

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6.3.2. Western blot analysis of protein

In order to investigate protein expression following EP4 receptor activation, cells were pre-treated and/or stimulated with seminal plasma (1:500) or 300 nM PGE_2 as described in Section 6.3. Protein was extracted from stimulated cells and subjected to Western blot analysis using β-actin and COX-2 specific antibodies as described in Section 2.8. Protein expression was normalised to β-actin to correct for any differences in protein loading.

6.3.3. VEGF ELISA analysis

Secretion of VEGF protein into culture medium was investigated using VEGF ELISA assays as described in Section 2.10. Quantification is achieved by the construction of a standard curve using known concentrations serial dilutions of human VEGF standards (15.6 pg/ml up to 1000 pg/ml). By comparing the absorbance obtained from a sample containing an unknown amount of human VEGF protein with that obtained from the standards, the concentration of human VEGF protein in the test samples were determined. Results are presented as secreted VEGF protein (pg/ml) (Mean ± SEM) present in the sample culture medium from 3 independent experiments.

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6.4. Results

6.4.1. QPCR analysis of EP-receptor mRNA expression

HeLa EP4S (EP4S+Ant) and WT (WT+Ant) cells were pre-treated with 1 μM specific EP4 receptor antagonist (ONO-AE2-227) for 1 hour prior to stimulation with 300 nM PGE_2. Thereafter HeLa EP4S, WT, pre-treated EP4S (EP4S+Ant) and pre-treated WT (WT+Ant) cells were stimulated for time periods of 0, 2, 4, 8, 16 and 24 hours (h) with 300 nM PGE_2.

Real-time quantitative RT-PCR analysis showed no significant changes in the levels of EP1 receptor (Figure 6.1. A), EP2 receptor (Figure 6.1. B) or EP3 receptor (Figure 6.1. C) mRNA expression at each of the time points investigated. This indicates that signalling via PGE-EP4 interaction does not result in an upregulation of EP1, EP2 or EP3 receptor mRNA at the time points investigated.

However, EP4 receptor mRNA (Figure 6.1. D) in EP4S cells was significantly increased at the 2 h (1.66 ± 0.15 fold), 4 h (1.83 ± 0.19 fold), and 8 h (3.47 ± 0.65 fold) time points when compared to EP4S cells treated with EP4 antagonist and 300 nM PGE_2 (EP4S+Ant) (0.71 ± 0.20; 0.60 ± 0.24; 1.44 ± 0.28; respectively) ($p < 0.01$) (Figure 6.1. D). In addition, WT cells treated with PGE_2 also respond in a receptor-specific manner with an increase in EP4 mRNA observed following 8 hours of 300 nM PGE_2 stimulation (Figure 6.1 D). These data indicate the possibility of a positive feedback loop in which increased PGE_2-EP4 receptor interaction may promote up-regulation of expression of the EP4 receptor.

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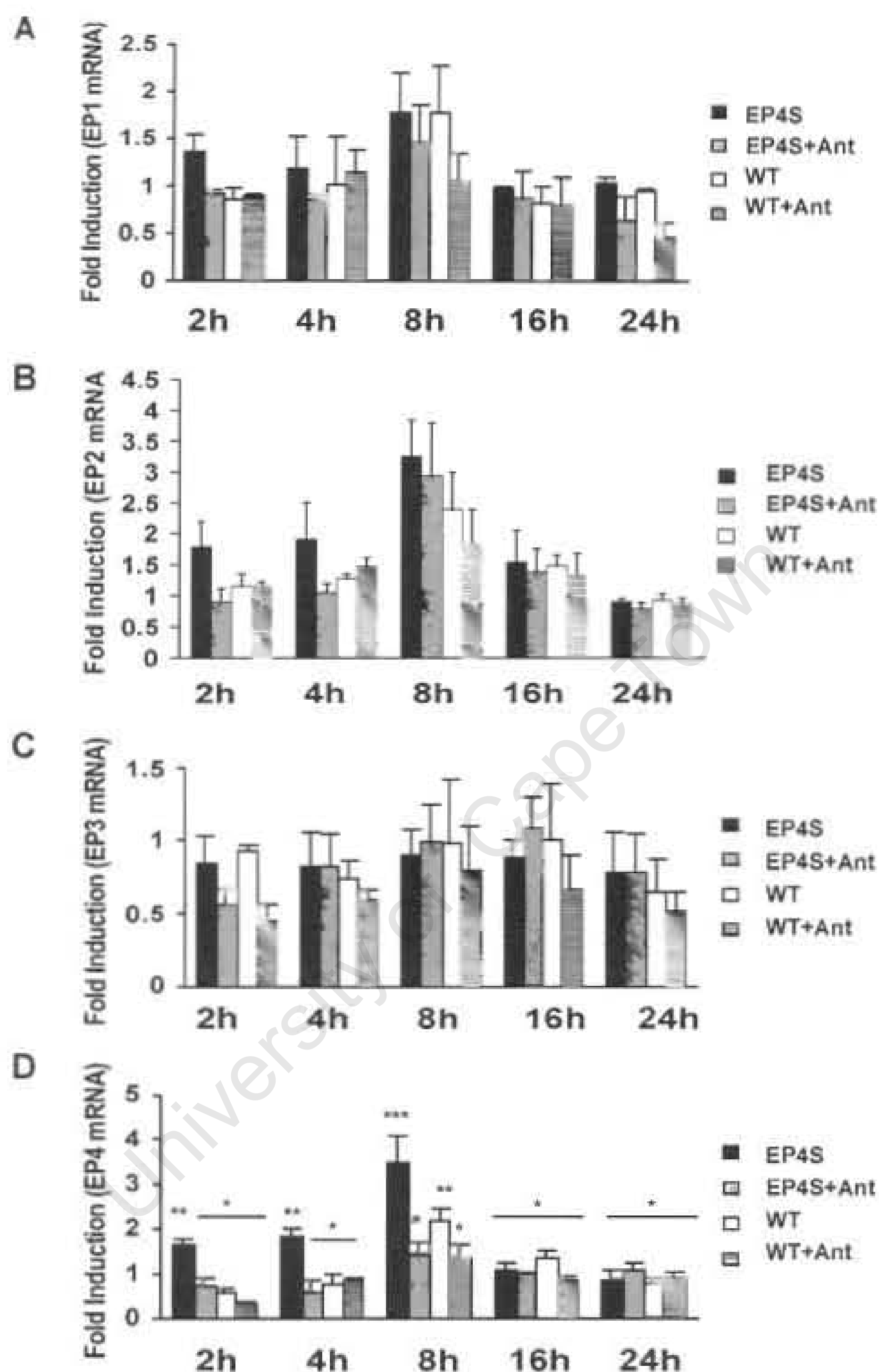

Figure 6.1. EP1 (Figure A), EP2 (Figure B), EP3 (Figure C) and EP4 (Figure D) receptor mRNA expression in response to PGE_2. HeLa EP4S cells, WT cells, were stimulated with 300 nM PGE_2 for 2, 4, 8, 16 and 24 hours either alone or following pre-treatment with 1 μM ONO-AE2-227 (+Ant). Data are shown as fold increase (Mean ± SEM) of EP receptor mRNA above control from 3 independent experiments (* is statistically significant from ** ($p < 0.05$), *** is statistically significant from both * and ** ($p < 0.05$).

6.4.2.1. QPCR analysis of COX-2 mRNA expression

The effect of EP4 receptor activation on the upregulation of COX-2 mRNA in response to seminal plasma (Figure 6.2. A) or PGE_2 (Figure 6.2. B) was investigated. HeLa EP4S and WT cells were stimulated with either seminal plasma (1:500) (Figure 6.2. A) or 300 nM PGE_2 (Figure 6.2. B) for 0, 2, 4, 8, 16 and 24 hours (h) either alone or following pre-treatment for 1 hour with 1 μM specific EP4 receptor antagonist (ONO-AE2-227).

Treatment of WT cells with seminal plasma had no significant effect on COX-2 mRNA levels. However, treatment of EP4S cells with seminal plasma (1:500) resulted in a significant increase in COX-2 mRNA at 8 h (5.03 ± 0.59 fold), when compared to HeLa WT (2.27 ± 0.18) ($p < 0.01$) cells. Pre-treatment of EP4S cells with 1μM ONO-AE2-227 (EP4S+Ant) abolished the seminal plasma induced up-regulation of COX-2 expression (1.40 ± 0.25 fold) ($p < 0.01$) (Figure 6.2. A).

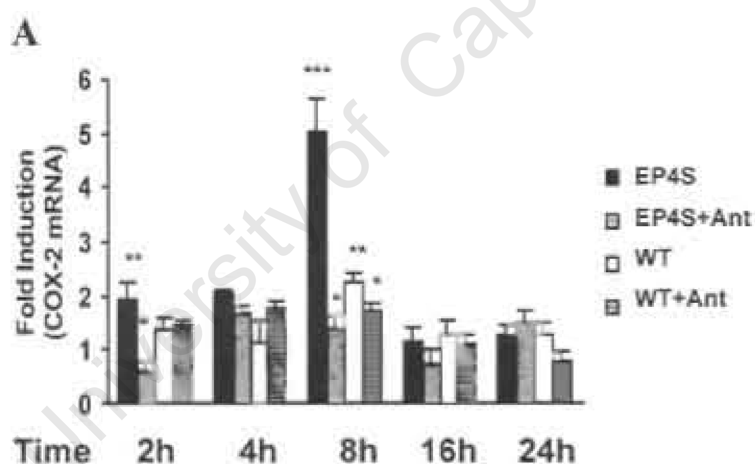

Figure 6.2. A. COX-2 mRNA expression following seminal plasma (1:500) stimulation of HeLa cells. EP4S (EP4S+Ant) and WT (WT+Ant) cells were pre-treated with 1 μM ONO-AE2-227. Subsequently, EP4S, WT, pre-treated EP4S (EP4S+Ant) and pre-treated WT (WT+Ant) cells were stimulated with seminal plasma (1:500) for 2, 4, 8, 16 and 24 hours. Data are presented as fold increase (Mean ± SEM) of COX-2 mRNA above control from 3 independent experiments (** is statistically significant from * ($p < 0.05$), *** is statistically significant from ** and * ($p < 0.05$).

Stimulation of EP4S cells with 300 nM PGE_2 resulted in a significant increase in COX-2 mRNA at the 8 and 16 hour time points (1.61 ± 0.13 and 1.25 ± 0.01 fold respectively), when compared to EP4S cells pre-treated with ONO-AE2-227 (EP4S+Ant) (0.617 ± 0.07 and 0.85 ± 0.09 fold respectively at 8 and 16 hours) ($p < 0.01$) (Figure 6.2. B). Similarly, stimulation of WT cells with 300 nM PGE_2 resulted in a significant increase in COX-2 mRNA at 8 hours (1.19 ± 0.06 fold) when compared to pre-treated WT cells (WT+Ant) at the same time point (0.64 ± 0.05 fold) ($p < 0.05$).

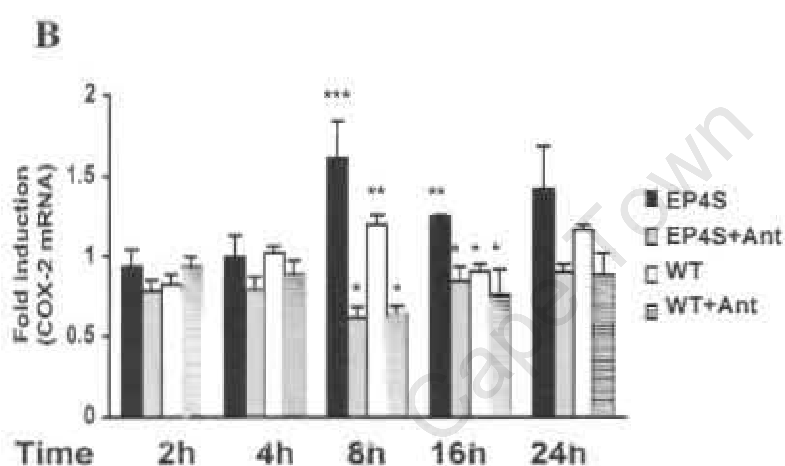

Figure 6.2. B. COX-2 mRNA expression following 300 nM PGE_2 stimulation of HeLa cells. HeLa EP4S and WT cells were stimulated for 2, 4, 8, 16 and 24 hours (h) with 300 nM PGE_2 either alone or following pre-treatment with 1 µM ONO-AE2-227 (+Ant). Data are presented as fold increase (Mean ± SEM) of COX-2 mRNA above control from 3 independent experiments (** is statistically significant from * ($p < 0.05$), *** is statistically significant from ** and * ($p < 0.05$).

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In order to investigate the signal transduction pathways by which COX-2 gene transcription may be upregulated, HeLa WT and EP4S cells were stimulated with either seminal plasma (1:500) (Figure 6.3. A) or 300 nM PGE_2 (Figure 6.3. B) alone or following pre-treatment for 1 hour with specific chemical inhibitors. These inhibitors included PD98059 (MAPK kinase inhibitor) (50 µM), AG1478 (EGFR kinase inhibitor) (100 nM) or ONO-AE2-227 (EP4 antagonist) (1 µM). Control cells were pre-treated with vehicle and left unstimulated.

Seminal plasma (1:500) (sp) stimulation of EP4S cells resulted in a 5.17 ± 0.85 fold increase in COX-2 mRNA, which was abolished by pre-incubation with PD98059 (sp+PD) (1.40 ± 0.14 fold) ($p < 0.01$), AG1478 (sp+AG) (0.73 ± 0.16 fold) ($p < 0.01$) or ONO-AE2-227 (sp+Ant) (1.05 ± 0.11 fold) ($p < 0.01$) (Figure 6.3. A). PGE_2 (300 nM) (pge) stimulation of EP4S (S) cells resulted in a 1.57 ± 0.03 fold increase in COX-2 mRNA, which was abolished by pre-treatment with PD98059 (pge+PD) (0.74 ± 0.05 fold) ($p < 0.01$), AG1478 (pge+AG) (1.21 ± 0.03 fold) ($p < 0.01$) and ONO-AE2-227 (pge+Ant) (1.11 ± 0.07 fold) ($p < 0.01$) (Figure 6.3. B).

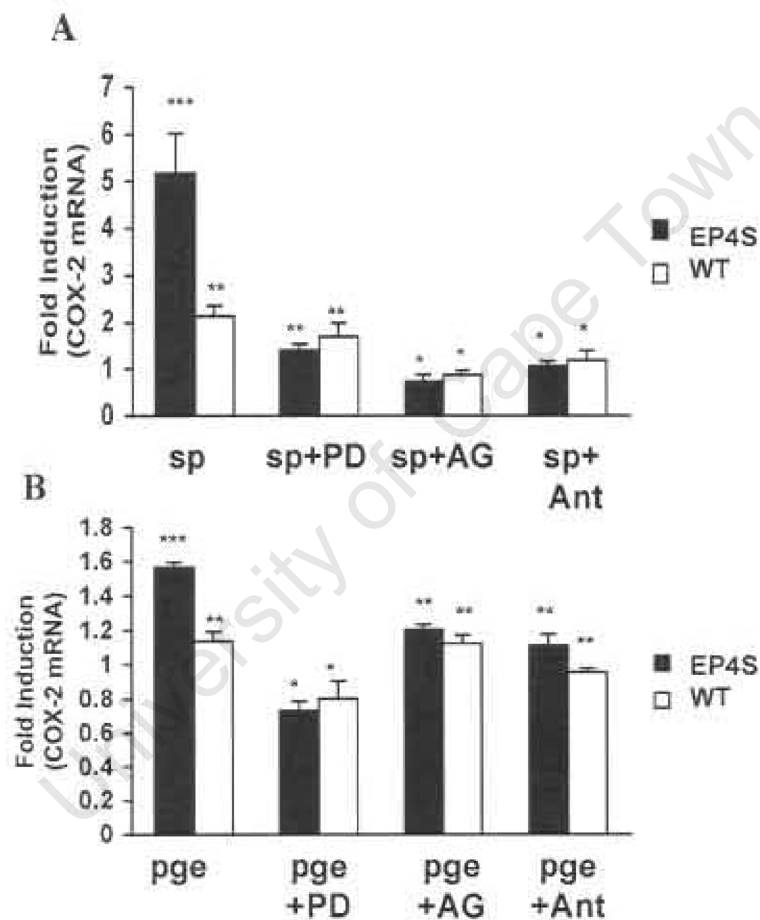

Figure 6.3. Fold induction of COX-2 mRNA expression. EP4S or WT cells were pre-treated with PD98059 (+PD), AG1478 (+AG) or ONO-AE2-227 (+Ant) for 1 hour prior to stimulations. Subsequently, EP4S (S), WT cells (WT), pre-treated EP4S and pre-treated WT cells were stimulated for 8 hours with seminal plasma (sp) (1:500) (Figure 6.3. A) or 300 nM PGE_2 (pge) (Figure 6.3. B). Data are presented as fold increase (Mean ± SEM) of COX-2 mRNA above control from 3 independent experiments (** is significantly different from * ($p < 0.05$), *** is significantly different from ** and * ($p < 0.05$).

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6.4.2.2. COX-2 protein expression in HeLa WT and EP4S cells

In order to investigate whether up-regulation of COX-2 mRNA initiates gene transcription and resultant translation of COX-2 protein, Western blot analysis (as outlined in Section 2.8) was used to determine COX-2 protein expression in seminal plasma or PGE_2 stimulated HeLa cells in the absence or presence of various chemical inhibitors. These chemical inhibitors included PD98059, AG1478 or ONO-AE2-227.

HeLa WT and EP4S cells were stimulated with either seminal plasma (1:500) (Figure 6.4. A) or 300 nM PGE_2 (Figure 6.4. B) for 8 hours either alone or following pre-treatment for 1 hour with PD98059 (5 μM), AG1478 (100 nM), or ONO-AE2-227 (1 μM). Control HeLa EP4S and WT cells were pre-treated for 1 hour with vehicle and left unstimulated.

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Stimulation of HeLa EP4S cells with seminal plasma (1:500) for 8 hours resulted in a 4.40 ± 0.56 fold increase in COX-2 protein ($p < 0.05$) (Figure 6.4. A), which corresponds with the increase in COX-2 mRNA observed at the same time point (Figure 6.3. A). This increase in COX-2 protein expression was abolished by pre-incubation with AG1478 (EP4S+sp+AG) (1.07 ± 0.38 fold; $p < 0.01$), PD98059 (EP4S+sp+PD) (0.97 ± 0.24 fold) ($p < 0.01$) or ONO-AE2-227 (EP4S+sp+Ant) (1.02 ± 0.08 fold) ($p < 0.01$) (Figure 6.4. A). Similarly, stimulation of HeLa WT cells (WT) with seminal plasma (1:500) for 8 hours resulted in a 2.34 ± 0.35 fold increase in COX-2 protein ($p < 0.05$) (Figure 6.4. A), which corresponds with the increase observed in COX-2 mRNA at the same time point (Figure 6.3. A). This increase in COX-2 protein expression was abolished by pre-incubation with AG1478 (WT+sp+AG) (1.07 ± 0.31 fold) ($p < 0.01$), PD98059 (WT+sp+PD) (1.13 ± 0.91 fold) ($p < 0.01$) or ONO-AE2-227 (WT+sp+Ant) (1.03 ± 0.15 fold) ($p < 0.01$) (Figure 6.4. A).

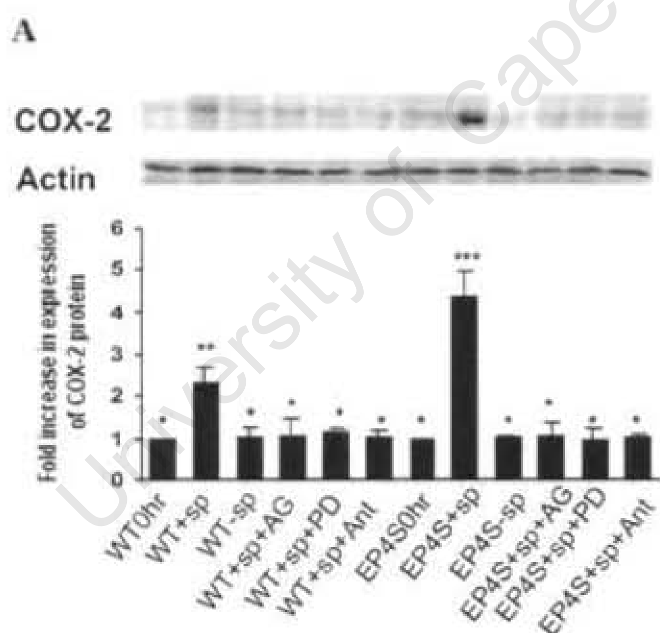

Figure 6.4. A. Immunoblot showing COX-2 protein. HeLa WT or EP4S cells were stimulated with seminal plasma (1:500) (+sp) for 8 hours either alone or following pre-treatment with AG1478 (+AG), PD98059 (+PD) or ONO-AE2-227 (+Ant). Control cells were pre-incubated with vehicle and left unstimulated (-sp). Results were normalised to β-actin to correct for any differences in protein loading. Data are presented as fold increase (Mean ± SEM) in COX-2 protein expression above control from 3 independent experiments (** is statistically significant from * ($p < 0.05$), *** is statistically significant from ** and * ($p < 0.05$).

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Stimulation of HeLa EP4S cells with 300 nM PGE_2 for 8 hours resulted in a 4.28 ± 0.74 fold increase in COX-2 protein ($p < 0.05$) (Figure 6.4. B), which corresponds with the increase in COX-2 mRNA observed at the same time point (Figure 6.3. B). This increase in COX-2 protein expression was abolished by pre-incubation with AG1478 (EP4S+pge+AG) (1.13 ± 0.2 fold) ($p < 0.01$), PD98059 (EP4S+pge+PD) (1.10 ± 0.08 fold) ($p < 0.01$) or ONO-AE2-227 (EP4S+pge+Ant) (1.25 ± 0.08 fold) ($p < 0.01$) (Figure 6.4. B). Similarly, stimulation of HeLa WT cells (WT) with 300 nM PGE_2 for 8 hours resulted in a 2.25 ± 0.05 fold increase in COX-2 protein ($p < 0.05$) (Figure 6.4. B), which corresponds with the increase observed in COX-2 mRNA at the same time point (Figure 6.3. B). This increase in COX-2 protein expression was abolished by pre-incubation with AG1478 (WT+pge+AG) (0.89 ± 0.03 fold) ($p < 0.01$), PD98059 (WT+pge+PD) (0.83 ± 0.10 fold) ($p < 0.01$) or ONO-AE2-227 (WT+pge+Ant) (0.86 ± 0.09 fold) ($p < 0.01$) (Figure 6.4. B).

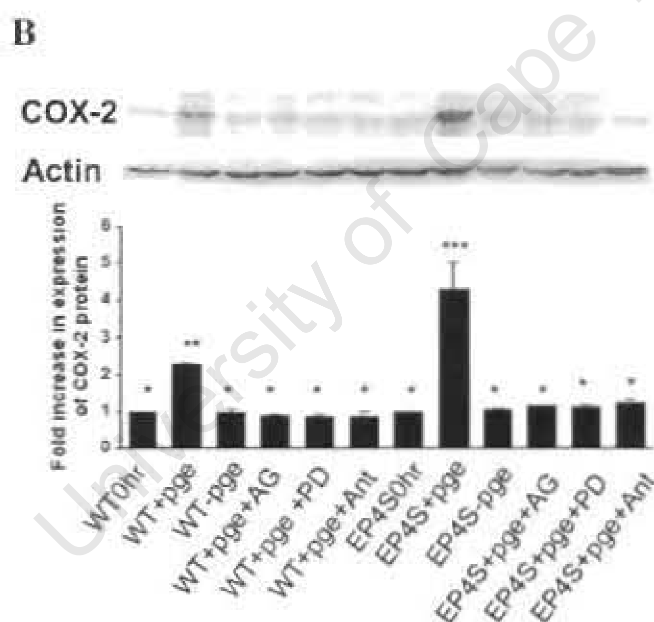

Figure 6.4. B. Immunoblot showing COX-2 protein. HeLa WT or EP4S cells were stimulated for 8 hours with either PGE_2 (300 nM) (+pge) alone or following pre-treatment with AG1478 (+AG), PD98059 (+PD) or ONO-AE2-227 (+Ant). Control cells were pre-incubated with vehicle and left unstimulated (-pge). Results are shown as fold increase (Mean ± SEM) in COX-2 protein expression above control (** is significantly different from * ($p < 0.05$), *** is significantly different from ** and * ($p < 0.05$).

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6.4.3.1. QPCR analysis of VEGF mRNA expression

The effect of EP4 receptor activation on the upregulation of VEGF mRNA in response to seminal plasma (Figure 6.5. A) or PGE_2 (Figure 6.5. B) was investigated. HeLa EP4S and WT cells were stimulated with either seminal plasma (1:500) (Figure 6.5. A) or 300 nM PGE_2 (Figure 6.5. B) for time periods of 0, 2, 4, 8, 16 and 24 hours (h) alone or following pre-treatment for 1 hour with 1 μM ONO-AE2-227.

Stimulation of EP4S cells with seminal plasma resulted in a significant increase of VEGF mRNA at 8 hours (3.73 ± 0.59 fold), which was abolished in EP4S cells pre-treatment with ONO-AE2-227 (EP4S+Ant) (0.80 ± 0.06 fold) ($p < 0.01$) at the same time point (Figure 6.5. A). Similarly, stimulation of HeLa WT cells resulted in a significant increase in VEGF mRNA at 8 hours (3.13 ± 0.34 fold), which was abolished in WT cells pre-treated with ONO-AE2-227 (WT+Ant) (1.31 ± 0.11 fold) ($p < 0.05$) (Figure 6.5. A). No significant differences were observed at the other time points of 2h, 4h, 16h or 24h.

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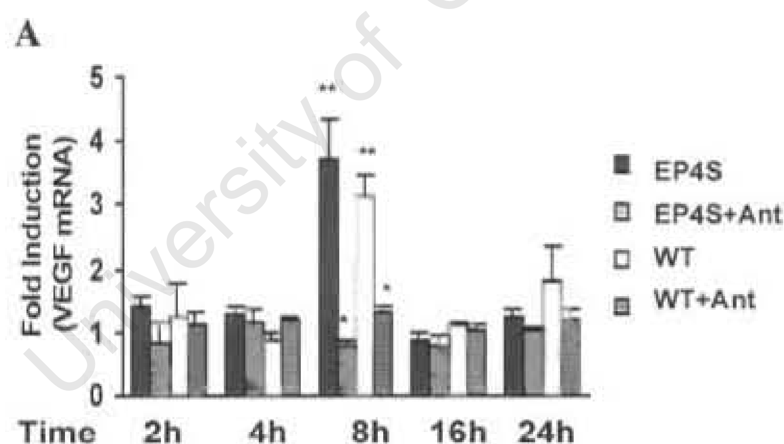

Figure 6.5. A. VEGF mRNA induction following seminal plasma stimulation of HeLa cells. EP4S and WT cells were stimulated with seminal plasma (1:500) for 2, 4, 8, 16 and 24 hours either alone or following pre-treatment with 1 μM ONO-AE2-227 (+Ant). Data are presented as fold induction (Mean ± SEM) of VEGF mRNA above control from 3 independent experiments (** is statistically significant from *, ($p < 0.05$).

Stimulation of EP4S cells with 300 nM PGE_2 resulted in a significant increase of VEGF mRNA at 8 hours (2.64 ± 0.32 fold), which was abolished in EP4S cells pre-treated with ONO-AE2-227 (EP4S+Ant) (0.78 ± 0.12 fold) ($p < 0.01$) at the same time point (Figure 6.5. B). Similarly, stimulation of HeLa WT cells resulted in a significant increase in VEGF mRNA at 8 hours (1.50 ± 0.37 fold), which was abolished in WT cells pre-treated with ONO-AE2-227 (WT+Ant) (0.95 ± 0.16 fold) ($p < 0.05$) (Figure 6.5. B). No significant differences were observed at the other time points of 2h, 4h, 16h or 24h.

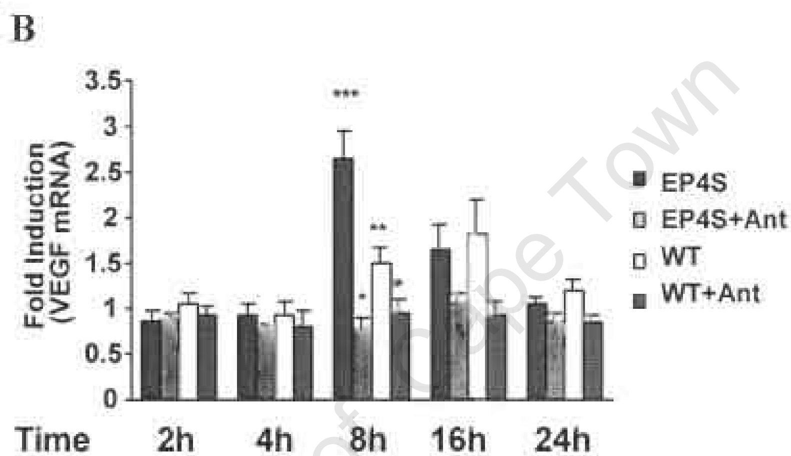

Figure 6.5. B. VEGF mRNA induction following PGE_2 stimulation of HeLa cells. EP4S and WT cells were stimulated with 300 nM PGE_2 for time periods of 2, 4, 8, 16 and 24 hours either alone or following pre-treatment with 1 μM ONO-AE2-227 (+Ant). Data are presented as fold induction (Mean ± SEM) of VEGF mRNA above control from 3 independent experiments (** is statistically significant from * ($p < 0.05$), *** is statistically significant from ** and * ($p < 0.05$).

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In order to investigate the signal transduction pathways by which VEGF gene transcription may be upregulated, HeLa WT and EP4S cells were stimulated for 8 hours with either seminal plasma (1:500) (Figure 6.6. A) or 300 nM PGE_2 (Figure 6.6. B) alone or following pre-treatment for 1 hour with specific chemical inhibitors. These inhibitors included PD98059 (MAPK kinase inhibitor) (50 μM), AG1478 (EGFR kinase inhibitor) (100 nM), or ONO-AE2-227 (1 μM). Control cells were treated with vehicle for 1 hour and then left unstimulated.

Seminal plasma (1:500) (sp) stimulation of EP4S cells resulted in a 3.22 ± 0.16 fold increase in VEGF mRNA, which was abolished by co-treatment with PD98059 (sp+PD) (1.29 ± 0.128 fold) ($p < 0.01$), AG1478 (sp+AG) (0.79 ± 0.03 fold) ($p < 0.01$) or ONO-AE2-227 (sp+Ant) (0.90 ± 0.08 fold) ($p < 0.01$) (Figure 6.6. A). 300 nM PGE_2 (pge) stimulation of EP4S cells resulted in a 2.15 ± 0.16 fold increase in VEGF mRNA, which was abolished by co-treatment with PD98059 (pge+PD) (0.89 ± 0.74 fold) ($p < 0.01$), AG1478 (pge+AG) (1.08 ± 0.09 fold) ($p < 0.01$) or ONO-AE2-227 (pge+Ant) (1.02 ± 0.12 fold) ($p < 0.01$) (Figure 6.6. B).

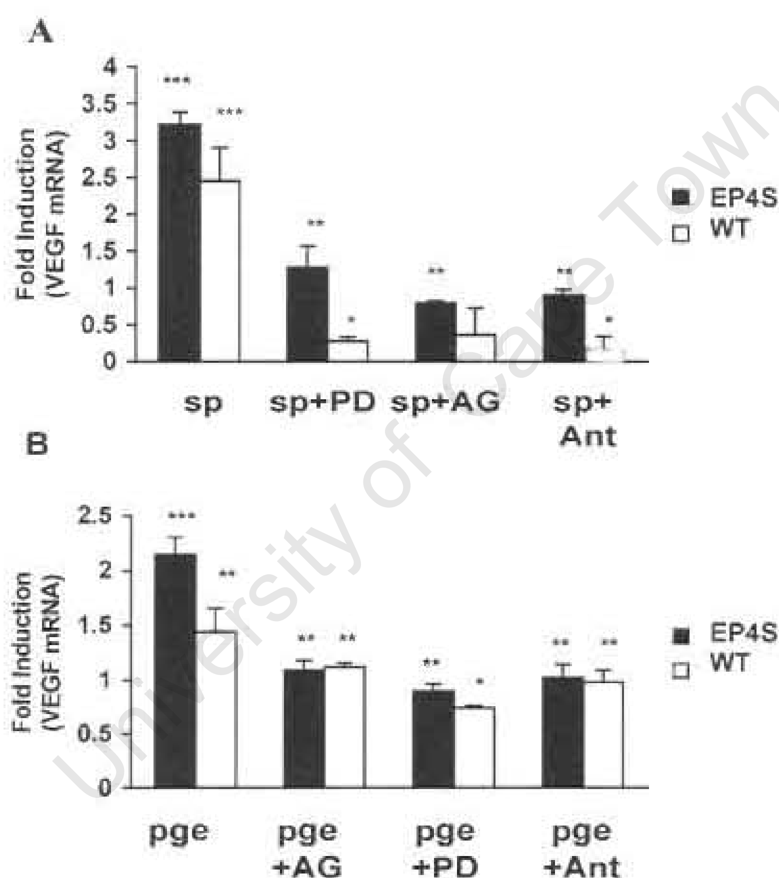

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Figure 6.6. Fold induction of VEGF mRNA expression. HeLa EP4S or WT cells were stimulated with seminal plasma (sp) (1:500) (Figure 6.6. A) or 300 nM PGE_2 (pge) (Figure 6.6. B) for 8 hours either alone or following pre-treatment with AG1478 (+AG), PD98059 (+PD) or ONO-AE2-227 (+Ant). Data are presented as fold induction (Mean ± SEM) of VEGF mRNA above control from 3 independent experiments (** is statistically significant from * ($p < 0.05$), *** is statistically significant from ** and * ($p < 0.05$).

6.4.3.2. VEGF protein expression in HeLa WT and EP4S cells

Secretion of VEGF protein into the culture medium following treatment of cells with seminal plasma (1:500) (Figure 6.7. A) or 300 nM PGE_2 (Figure 6.7. B) was investigated using a VEGF ELISA kit. HeLa WT and HeLa EP4S cells were stimulated with either seminal plasma (1:500) or 300 nM PGE_2 for 8 hours alone or following pre-treatment for 1 hour with specific chemical inhibitors. These inhibitors included PD98059 (5 μM), AG1478 (100 nM) or ONO-AE2-227 (1μM). Control cells were treated with vehicle and left unstimulated.

Treatment of HeLa EP4S cells (black bars) with seminal plasma (sp) resulted in VEGF accumulation of 8540.17 ± 615.36 pg/ml, when compared to control (-sp) (299.03 ± 56.56 pg/ml) ($p < 0.05$) (Figure 6.7. A). VEGF accumulation was significantly reduced in EP4S cells following pre-treatment with PD98059 (+PD) (5085.23 ± 568.75 pg/ml) ($p < 0.05$), AG1478 (+AG) (4579.87 ± 390.06 pg/ml) ($p < 0.01$) or ONO-AE2-227 (+Ant) (5109.33 ± 550.52 pg/ml) ($p < 0.01$) (Figure 6.7. A). Similarly, treatment of HeLa WT cells (open bars) with seminal plasma (sp) resulted in VEGF accumulation of 6621.27 ± 818.79 pg/ml, when compared to vehicle-treated cells (-sp) (417.77 ± 46.79 pg/ml) ($p < 0.05$) (Figure 6.7. A). VEGF accumulation was significantly reduced in WT cells following pre-treatment with PD98059 (sp+PD) (3952.97 ± 451.34 pg/ml) ($p < 0.01$), AG1478 (sp+AG) (5060.43 ± 682.04 pg/ml) ($p < 0.01$) or ONO-AE2-227 (sp+Ant) (4825.23 ± 614.25 pg/ml) ($p < 0.01$) (Figure 6.7. A). In addition, a significant increase was observed in seminal plasma-treated EP4S cells (8540.17 ± 615.36 pg/ml) when compared to seminal plasma-treated WT cells (6621.27 ± 818.79 pg/ml) ($p < 0.05$) (Figure 6.7. A).

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Treatment of HeLa EP4S cells (black bars) with 300 nM PGE_2 (pge) resulted in VEGF accumulation of 906.97 ± 67.06 pg/ml, when compared to control (-pge) (516.87 ± 48.33 pg/ml) ($p < 0.05$) (Figure 6.7. B). VEGF accumulation was significantly reduced in EP4S cells following pre-treatment with PD98059 (+PD) (535.93 ± 125.19 pg/ml) ($p < 0.05$), AG1478 (+AG) (428.43 ± 65.66 pg/ml) ($p < 0.01$) or ONO-AE2-227 (+Ant) (449.90 ±

86.60 pg/ml) ($p < 0.01$) (Figure 6.7. B). Similarly, treatment of HeLa WT cells (open bars) with 300 nM PGE_2 (pge) resulted in VEGF accumulation of 552.83 ± 48.13 pg/ml, when compared to control (-pge) (333.40 ± 18.36 pg/ml) ($p < 0.05$) (Figure 6.7. B). VEGF accumulation was significantly reduced in WT cells following pre-treatment with PD98059 (sp+PD) (339.80 ± 45.39 pg/ml) ($p < 0.05$), AG1478 (sp+AG) (363.90 ± 21.34 pg/ml) ($p < 0.05$) or ONO-AE2-227 (sp+Ant) (402.03 ± 36.12 pg/ml) ($p < 0.05$) (Figure 6.7. B). In addition, a significant increase was observed in PGE_2-treated EP4S cells (906.97 ± 67.06 pg/ml) when compared to seminal plasma-treated WT cells (552.83 ± 48.13 pg/ml) ($p < 0.01$) (Figure 6.7. B).

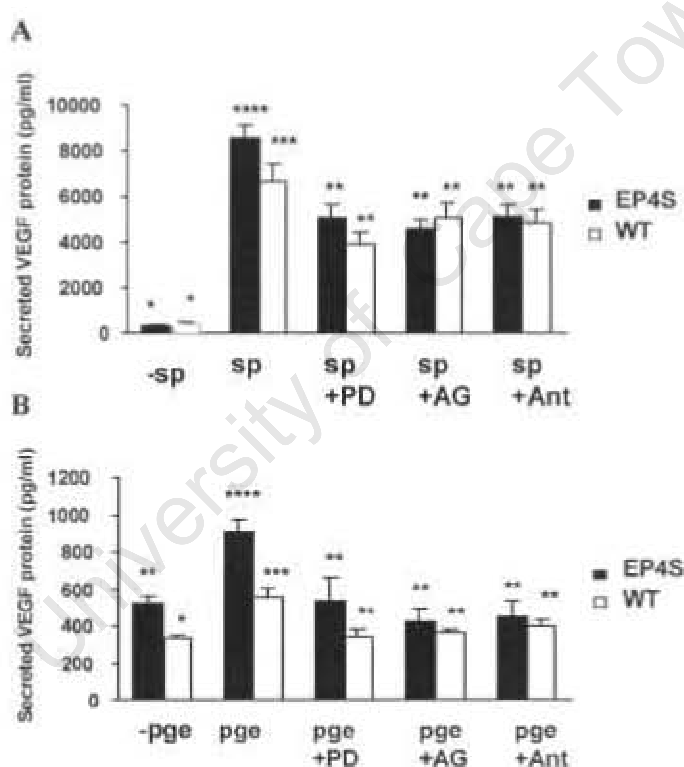

Figure 6.7. VEGF accumulation (pg/ml) in culture medium. HeLa WT and EP4S cells were stimulated with seminal plasma (sp) (Figure A) or 300 nM PGE_2 (pge) (Figure B) for 8 hours either alone or following pre-treatment with PD98059 (sp+PD), AG1478 (sp+AG) or ONO-AE2-227 (sp+Ant). Vehicle-treated (v) cells were included as a control. Data are presented as Mean ± SEM from 3 independent experiments (** is statistically significant from * ($p < 0.05$), *** is statistically significant from ** and * ($p < 0.05$), **** is statistically significant from ***, ** and * ($p < 0.05$).

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6.4.4. Expression of bFGF mRNA following PGE-EP4 receptor interaction

The effect of EP4 receptor activation on the upregulation of bFGF mRNA in response to seminal plasma (1:500) (Figure 6.8. A) or 300 nM PGE_2 (Figure 6.8. B) was investigated using Real-time quantitative RT-PCR analysis. HeLa EP4S and WT cells were stimulated with either seminal plasma (1:500) or 300 nM PGE_2 for 0, 2, 4, 8, 16 and 24 hours (h) alone or following pre-treatment for 1 hour with 1 μM EP4 antagonist (ONO-AE2-227).

Treatment of EP4S cells with seminal plasma (1:500) resulted in a significant increase in bFGF mRNA at 2 h and 8 h (1.70 ± 0.06 and 2.57 ± 0.09 fold, respectively), when compared to EP4S cells treated with ONO-AE2-227 and seminal plasma (EP4S+Ant) at the same time points (1.03 ± 0.07 and 1.57 ± 0.17 fold) ($p < 0.05$) (Figure 6.8. A). Similarly, treatment of WT cells with seminal plasma resulted in a significant increase in bFGF mRNA at 8 hours (2.57 ± 0.38 fold) ($p < 0.05$), when compared to WT cells treated with 1μM ONO-AE2-227 and seminal plasma (WT+Ant) (1.03 ± 0.09 fold) (Figure 6.8. A).

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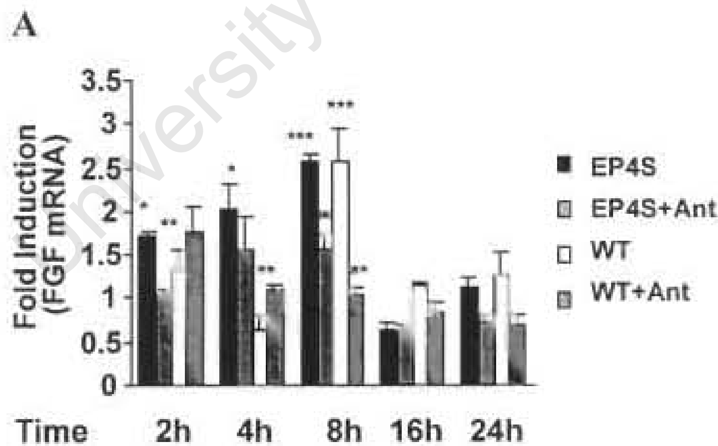

Figure 6.8. A. bFGF mRNA expression following seminal plasma stimulation of HeLa cells. HeLa EP4S and WT cells were stimulated for 2, 8, 16, and 24 hours (h) with seminal plasma (1:500) alone or following pre-treatment with 1 μM ONO-AE2-227 (+Ant). Data are presented as fold increase of bFGF mRNA (Mean ± SEM) above basal levels from 3 independent experiments (** is significantly different from * ($p < 0.05$), *** is significantly different from ** and * ($p < 0.05$).

Stimulation of HeLa EP4S cells with 300 nM PGE_2 resulted in a significant increase in bFGF mRNA at the 2 hour and 8 hour time points (1.19 ± 0.03 and 1.72 ± 0.27 fold, respectively), which was reduced in EP4S cells pre-treated with ONO-AE2-227 (EP4S+Ant) (0.89 ± 0.06 and 0.27 ± 0.10 fold, respectively) ($p < 0.01$) (Figure 6.8. B). Similarly, stimulation of WT cells with 300 nM PGE_2 resulted in a significant increase in bFGF mRNA at 8 hours. EP4 antagonist (WT+Ant) significantly decreased bFGF mRNA expression at 8 hours (1.74 ± 0.28 fold), which was reduced in WT cells pre-treated with ONO-AE2-227 (WT+Ant) (1.04 ± 0.16 fold) ($p < 0.01$) (Figure 6.8. B). No significant differences were observed at the other time points.

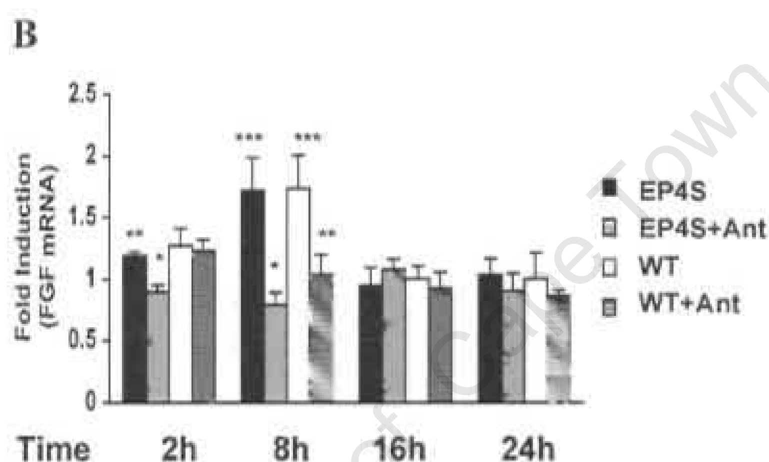

Figure 6.8. B. bFGF mRNA expression following PGE_2 stimulation of HeLa cells. HeLa EP4S and WT cells were stimulated for 2, 8, 16, and 24 hours (h) with 300 nM PGE_2 alone or following pre-treatment with 1 μM ONO-AE2-227 (+Ant). Data are presented as fold increase of bFGF mRNA (Mean ± SEM) above basal levels from 3 independent experiments (** is statistically significant from * ($p < 0.05$), *** is statistically significant from ** and * ($p < 0.05$).

In order to investigate the signal transduction pathways by which bFGF mRNA may be upregulated, HeLa WT and EP4S cells were stimulated for 8 hours with either seminal plasma (1:500) (Figure 6.9. A) or 300 nM PGE_2 (Figure 6.9. B) alone or following pre-treatment for 1 hour with specific chemical inhibitors. These included PD98059 (MAPK kinase inhibitor) (50 μM), AG1478 (EGFR kinase inhibitor) (100 nM), or ONO-AE2-227 (1 μM). Control cells were pre-treated with vehicle and left unstimulated.

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Treatment of EP4S cells with seminal plasma (1:500) (sp) resulted in a 2.49 ± 0.19 fold increase in bFGF mRNA, which was significantly decreased by co-treatment with PD98059 (sp+PD) (1.86 ± 0.18 fold) ($p < 0.05$), AG1478 (sp+AG) (1.11 ± 0.14 fold) ($p < 0.01$) or ONO-AE2-227 (sp+Ant) (1.30 ± 0.24 fold) ($p < 0.01$) (Figure 6.9. A). PGE_2 (300 nM) (pge) stimulation of EP4S cells resulted in a 1.43 ± 0.09 fold increase in bFGF mRNA, which was significantly decreased by pre-incubation with PD98059 (pge+PD) (0.81 ± 0.04 fold) ($p < 0.01$), AG1478 (pge+AG) (0.93 ± 0.05 fold) ($p < 0.01$) or ONO-AE2-227 (pge+Ant) (1.07 ± 0.08 fold) ($p < 0.01$) (Figure 6.9. B).

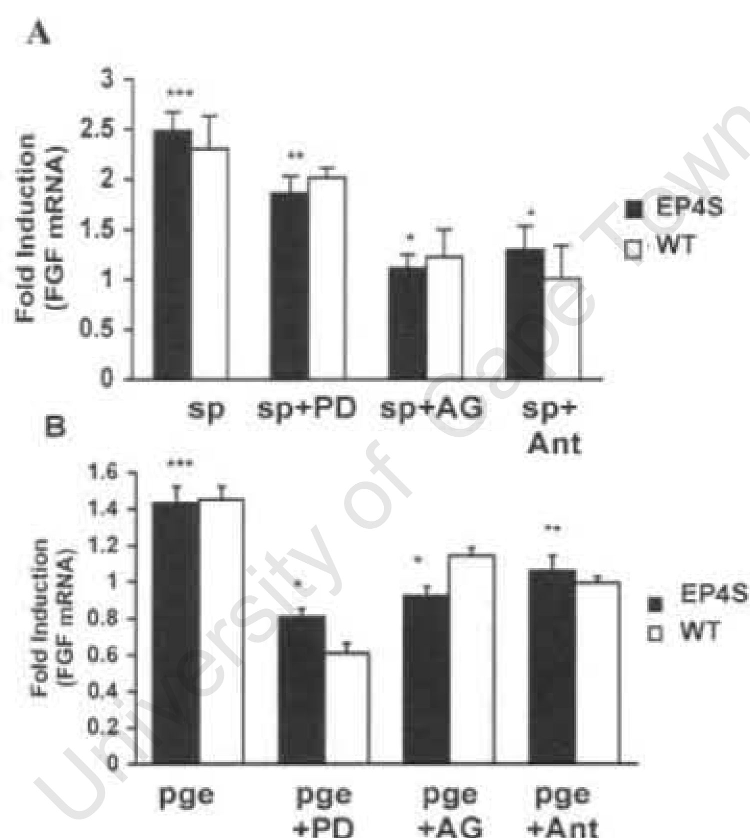

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Figure 6.9. Fold induction of bFGF mRNA. HeLa EP4S and WT cells were stimulated for 8 hours with either seminal plasma (sp) (1:500) (Figure 6.9. A) or 300 nM PGE_2 (pge) (Figure 6.9. B) alone or following pre-treatment with PD98059 (+PD), AG1478 (+AG) or ONO-AE2-227 (+Ant). Data is shown as fold increase (Mean ± SEM) of bFGF mRNA above basal from 3 independent experiments (** is statistically significant from * ($p < 0.05$), *** is statistically significant from * and * ($p < 0.05$).

6.5. Discussion

As discussed previously, the action of PGE_2 is mediated through a family of G-protein coupled receptors (GPCR's) termed EP receptors. Recent studies have suggested a role for the EP receptors in tumourigenesis. Sheng *et al.* (2001) attributed a direct role for PGE_2 and EP receptors in colorectal carcinomas. In this model, enhanced proliferative and tumourigenic effects were mediated by PGE_2 following interaction with the EP4 receptor (Sheng *et al.* 2001). Similarly, other data has demonstrated a role for PGE_2 and EP2 receptor in accelerating intestinal polyp formation in $APC^{\Delta 716}$ knock-out mice models (Sonoshita *et al.* 2001). In addition, other studies have shown that COX-enzyme products may autoregulate the expression of their own receptors (Spinella *et al.* 2004). This lead to investigations into whether up-regulated EP4 receptor expression, and resulting enhanced synthesis of PGE_2 may form a feedback loop to further upregulate expression of EP receptors. Our data indicates that PGE-EP4 interaction does not alter expression of EP1 or EP3 mRNA in EP4S cells (Figure 6.1. A, C). Although preliminary data indicated that there might be an increase in EP2 mRNA at the 2 and 4 hour time points, subsequent statistical analysis confirmed that EP2 mRNA expression levels were not significantly increased at these time points in PGE_2 stimulated EP4S cells (Figure 6.1. B). However, a significant increase in EP4 mRNA expression was observed in the EP4S cells following 2, 4, and 8 hour stimulations with 300 nM PGE_2 (Figure 6.1. B). This increase in EP4 mRNA might be indicative of autoregulation, where increased levels of PGE_2 may lead to upregulation of its own receptors. In this case, PGE_2 stimulation of HeLa EP4-overexpressing cells may lead to an increase in various factors, which might act synergistically to autoregulate expression of EP4 receptor. This will lead to elevated receptor signalling in HeLa cells, and enhance tumourigenic gene transcription.

Investigations were then performed into the possible upregulation of target genes associated with tumourigenesis, such as COX-2. The COX-2 gene is an immediate early-response gene (Chandrasekharan *et al.* 2004). Post-translational processing of the COX-2 transcript results in the expression of protein homodimers of 72 kDa or heterodimers of 72 kDa and 74 kDa. The COX isoforms exists as dimers both structurally and

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functionally (Kniss 1999). COX-2 is present on the luminal surfaces of the endoplasmic reticulum and inner and outer membranes of the nuclear envelope. This nuclear localisation of COX-2 raises the possibility that it may be involved in gene transcription or regulation at the nuclear level (Fritsche *et al.* 2001; Hawk *et al.* 2002). COX-2 has been shown to be upregulated in numerous cancers, including colon, pancreas, oesophagus, lung, prostate, bladder and cervical (Tsujii *et al.* 1997; Jabbour *et al.* 2001; Sales *et al.* 2001; Hawk *et al.* 2002; Kundu *et al.* 2002; van Rees *et al.* 2002; Zweifel *et al.* 2002; Mann *et al.* 2004; Wang *et al.* 2004; Zha *et al.* 2004). COX-2 is rapidly induced after stimulation of quiescent cells by growth factors, oncogenes, carcinogens, and tumour-promoting phorbol esters (Sales *et al.* 2001). COX-2 can also be upregulated by dibutyryl cAMP (Maldve *et al.* 2000). Although the exact role for COX enzymes and their respective products in cervical pathologies remain to be elucidated, studies using *in vitro* model systems have shown that enhanced synthesis of PGE_2 resulting from upregulated COX-2 enzyme expression plays a role in promoting angiogenesis (Tsujii *et al.* 1998), inhibiting apoptosis and enhancing proliferation (Tsujii *et al.* 1995) and metastatic potential of epithelial cells (Tsujii *et al.* 1997; Sales *et al.* 2003).

Moreover, COX-2 and PGE_2 may promote cancer development and invasiveness by mediating the transcription of angiogenic factors, such as vascular endothelial growth factor (VEGF), that promote both the migration of endothelial cells and their arrangement into tubular structures. Several studies have shown a relation between angiogenesis and COX-2 (Tsujii *et al.* 1998; Leahy *et al.* 2002). By use of two *in vitro* models using co-culture of endothelial cells, Tsujii *et al.*, 1998, showed that COX-2 modulates the production of angiogenic factors by tumour cells, whereas COX-1 regulates angiogenesis of endothelial cells and normal tissues. In endothelial cells *in vitro*, basic fibroblast growth factor (bFGF) and VEGF increased by three to five times the synthesis of another prostaglandin, thromboxane A_2 and increased the migration activity of the cells as well (Daniel OT *et al.* 1999). Studies by Dormond O *et al.*, 2001, showed that PGE_2 and PGI_2 are involved in activation of the small GTPases CDC 42 and Rac (as described in Chapter 5), as a result of engagement of integrin-$\alpha_v\beta_3$-dependent with its substrate (Dormond *et al.* 2001). Inhibition of COX-2 suppresses integrin-$\alpha_v\beta_3$-dependent activation of small

GTPases and as a result, inhibits endothelial cell spreading and migration *in vitro*, as well as FGF-induced angiogenesis *in vivo* (Dormond *et al.* 2001).

Since COX-2 derived PGE_2 is the most abundant PG found in solid malignancies that express elevated enzyme levels, and studies have shown that COX enzyme products can autoregulate the expression of their own receptors (Spinella *et al.* 2004), we decide to investigate whether PGE-EP4 interaction would lead to enhanced signalling and possible upregulation of genes involved in tumourigenesis, such as COX-2 or angiogenesis, such as VEGF and bFGF. Studies performed in Section 6.4.2. investigated the upregulation of COX-2 mRNA expression in HeLa EP4S and WT cells following stimulations with both seminal plasma (1:500) (Figure 6.2. A) or PGE_2 (300 nM) (Figure 6.2. B). Levels of COX-2 mRNA expression were found to be significantly increased in the EP4S cells following 8 hours of stimulation with either seminal plasma (1:500) or PGE_2 (300 nM) (Figure 6.2.). This indicated that prostaglandins (PGs) present in seminal plasma may be acting via the EP4 receptors to upregulate tumourigenesis via the upregulation of tumourigenic factors, such as COX-2. Although levels of COX-2 mRNA induction after PGE_2 stimulation (Figure 6.2. B) do not reach those achieved following 8 hours of stimulation with seminal plasma (Figure 6.2. A). Seminal plasma contains 19-hydroxyPGE_2 and various other prostaglandins. These are present in seminal plasma at very high levels, and although the levels of PGE_2 we measured in seminal plasma are comparable with those used for the PGE_2 stimulations, levels of other prostaglandins at such high concentrations in seminal plasma may exert effects or cellular responses not observed when cells are stimulated with PGE_2 alone.

Studies performed by Therland *et al.* (2004) on the kidney, demonstrated co-localisation of COX-2 with EP4 receptors in the vasculature (Therland *et al.* 2004). This implies a role for the EP4 receptor in transducing PG signalling as a result COX-2 expression in the vasculature of the kidney and demonstrates a close association between the EP4 receptor and COX-2. As mentioned previously, upregulation of COX-2 is an important factor in regulating angiogenesis and maintenance of neoplastic tissue. Therefore, elevated PGE_2 levels in seminal plasma may act in an aurocrine/paracrine manner via

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cAMP-linked PGE_2 receptors to mediate an effect on target genes, such as COX-2. Thus, it would use the cAMP-dependent protein kinase pathway to activate adenylate cyclase and increase intracellular cAMP. This in turn may elevate expression of COX-2 via the cAMP responsive element (CRE) on the COX-2 promotor. This positive feedback loop between COX-2 and PGE_2 may potentiate the progression of the disease, which may be further enhanced in sexually active women.

As discussed previously, cellular responses, such as proliferation or differentiation involves the regulation of transcriptional events through intracellular signalling cascades, including pathways that activate kinases of the MAPK family. The ERK sub-family become activated in response to growth factors either through receptor tyrosine kinases (RTK)- or through GPCR-triggered signals. Recently, the epidermal growth factor receptor (EGFR) was identified as an essential link in the GPCR-mediated MAPK activation pathway in rat-1 fibroblasts. These studies suggest that transactivation of distinct receptor tyrosine kinases (RTKs) might contribute in a cell-type specific manner to GPCR-mediated mitogenic signalling (Daub *et al.* 1997). In addition, studies performed by Pai *et al.* (2002) demonstrate that PGE_2 transactivated the EGFR, resulting in gastric and intestinal hypertrophy as well as growth of colonic polyps and cancers (Pai *et al.* 2002).

In order to investigate possible signalling pathways involved in this upregulation of COX-2 expression following stimulation by seminal plasma and PGE_2, specific chemical inhibitors were used (Section 6.4.2.1). Treatment of EP4S cells with PD98059 (inhibits ERK activation), AG 1478 (inhibits EGFR kinase), and the EP4 antagonist (ONO-AE2-227), results in a significant decrease in COX-2 mRNA expression. These data suggest that stimulation of HeLa EP4S cells by both seminal plasma and PGE_2 results in the upregulation COX-2 mRNA via the EP4 receptor and may require phosphorylation of ERK1/2 and transactivation of the EGF receptor. These data correspond well with results published by other groups, which also implicate the involvement of EGFR and COX-2 in other cancers (Araki *et al.* 2003; Kim *et al.* 2004). Kim *et al.*, 2004, demonstrated that synchronous expression of EGFR and COX-2 in carcinomas of the uterine cervix, were

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an indication of poor survival rate in these patients. Data published by Araki K *et al.*, 2004, also implied an important role for EGFR signalling in regulating COX-2 expression in human bronchial adenocarcinomas (Araki *et al.* 2003). Detection of EGFR mRNA in peripheral blood of cervical cancer patients was also suggested as a useful marker of tumourigenesis (Cho *et al.* 2003). It has been postulated by several authors that autocrine activation of the EGFR may provide a growth advantage to EGFR-expressing tumours (Laskin *et al.* 2004). In addition, activation of the EGFR may contribute to several other essential tumourigenic mechanisms, including tumour survival, invasion, metastatic spread, and angiogenesis (Eccles *et al.* 1994; Salomon *et al.* 1995; Woodburn 1999; Wells 2000; Yarden 2001). It is therefore not surprising that our data indicates an important role for the EGFR in signalling via the EP4 receptor in our cervical adenocarcinoma model system.

There is a distinct relationship between the prostaglandin-cyclooxygenase system and VEGF (Buchanan *et al.* 2004). Prostaglandins influence renal blood flow, are important in inflammation and are also pro-angiogenic (Wilkinson-Berka 2004). Recent evidence has suggested that COX-2 modulates angiogenesis by interacting with the VEGF system. Like prostaglandins, nitric oxide is a vasodilator and is implicated in VEGF-mediated vascular permeability and angiogenesis. Emerging evidence also suggests that COX-2 interacts with nitric oxide, and that these two systems have reciprocal effects on each other (Wilkinson-Berka 2004). Studies performed by Miura S *et al.*, 2004, suggest that COX-2 plays a key role in VEGF production in gastric fibroblasts, and that angiogenesis induced by the COX-2-VEGF pathway might be involved in gastric ulcer healing (Miura *et al.* 2004). COX-2 has also been shown to be a key enzyme for IL-1-induced angiogenesis via the upregulation of VEGF (Kuwano *et al.* 2004).

As described previously, the onset of angiogenesis is believed to be an early event in tumourigenesis, which facilitates tumour progression and metastasis (Folkman *et al.*, 1997). Both VEGF and bFGF are growth factors with angiogenic potential. VEGF expression has been demonstrated in a number of tumours, and it has been linked to promotion of tumour growth and metastasis (Folkman 1997; Jain 2003). Studies were

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performed to investigate the possible upregulation of VEGF in our model system following stimulation of HeLa EP4S and WT cells with seminal plasma or PGE_2. Data from Section 6.4.3. demonstrate stimulation of HeLa EP4S cells by both seminal plasma (1:500) (Figure 6.5. A) and 300 nM PGE_2 (Figure 6.5. B) result in the upregulation VEGF mRNA via the EP4 receptor and requires the phosphorylation of ERK1/2 and transactivation of the EGF receptor (Figure 6.6. A and Figure 6.6. B). In our EP4-overexpressing cervical adenocarcinoma cell model system, intracellular signalling upregulates VEGF in response to PGE_2, which could act on endothelial cells and lead to recruitment of new blood vessels to enhance tumour mass. Recent studies by Spinella F *et al.*, 2004, have also implicated the EP2/EP4 receptors in the induction of VEGF expression and invasiveness by endothelin-1 (Spinella *et al.* 2004). These studies therefore suggest that pharmacological blocking of the EP4 receptor may represent an additional strategy to control PGE_2 signalling, which has been associated with cancer progression.

The vast differences observed in the accumulation of VEGF between cells stimulated with seminal plasma (Figure 6.7. A) or PGE_2 (Figure 6.7. B) may be attributed to the fact that seminal plasma contains very high levels of VEGF. This is supported by the observation that in PGE_2-treated cells, co-treatment with EP4 antagonist reduced VEGF levels to those observed in vehicle-treated cells. Whereas VEGF levels in cells treated with seminal plasma and EP4 antagonist remain significantly higher than levels observed in vehicle-treated cells (Figure 6.7). The ability of PD 98059, AG1478 and ONO-AE2-227 to reduce VEGF accumulation in culture medium (Figure 6.7.), demonstrate that this upregulation may occur via phosphorylation of ERK1/2 MAPK and transactivation of the EGF receptor. These data therefore confirm previous results obtained in Chapter 5, which demonstrated that PGE_2 or seminal plasma rapidly augments the activation of the ERK1/2 signalling pathway in an EGFR mediated manner. Activation of these molecular signal transduction pathways resulted in an increase in VEGF mRNA expression (Figure 6.6. A and Figure 6.6. B) and secretion of VEGF (Figure 6.7. A and Figure 6.7. B), indicating that the EGFR and ERK1/2 pathways are necessary for transducing signalling via the EP4 receptor.

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Data obtained from Section 6.4.4 investigate the expression of bFGF mRNA following stimulation of cells with seminal plasma or PGE_2. These data demonstrate that stimulation of HeLa EP4S cells by both seminal plasma and PGE_2 results in the upregulation of bFGF mRNA via the EP4 receptor and may require phosphorylation of ERK1/2 and transactivation of the EGF receptor (Figure 6.9). Although, pre-treatment of cells with the MEK kinase inhibitor and EGFR kinase inhibitor did not reduce bFGF gene expression to basal levels, which indicates that stimulation of cells with seminal plasma may activate alternate signal transduction pathways, which are contributing to the upregulation of bFGF mRNA expression. Therefore, in this model system, activation of these molecular signal transduction pathways via the EP4 receptor resulted in an increase in bFGF mRNA expression (Figure 6.9. A, B), possibly involving EGFR and ERK1/2 pathways, however other signalling pathways may also be playing a role. The resultant production of bFGF could act on endothelial cells and lead to recruitment of new blood vessels to enhance tumour mass.

In summary, data from this Chapter demonstrate that PGE_2-EP4 receptor interaction results in an increase in EP4 receptor mRNA expression, which may involve a positive feedback loop, in which exposure to PGE_2 may lead to upregulation of its EP4 receptor. In addition, both seminal plasma and PGE_2 stimulation of EP4 overexpressing cervical adenocarcinoma cells resulted in a significant increase in both COX-2 and VEGF gene expression. This upregulation of COX-2 and VEGF seems to be transduced via the EP4 receptor by ERK1/2 and EGFR. bFGF mRNA was also upregulated in this model system following EP4 receptor activation, however the mechanism via which this target gene is upregulated appears to be partially transduced via the EP4 receptor through ERK1/2 and EGFR. This upregulation of angiogenic factors by seminal plasma has important implications for sexually active women with pre-neoplastic cervical epithelial cells. These cells may be in contact with seminal plasma containing very high levels of prostaglandins, which may contribute to their neoplastic transformation. In women with cervical neoplastic cells, the upregulation of angiogenic factors such as VEGF and bFGF would allow for the endothelial cells to migrate, divide and form new vessels which may further contribute to enhanced tumour mass and invasion into surrounding tissue. These

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studies, therefore, raise the possibility of using receptor-specific antagonists together with NSAIDs in order to reduce the tumourigenic potential of pre-neoplastic cervical epithelial cells in sexually active women. The use of these two therapeutic agents in combination may provide a more effective treatment than simply using NSAIDs to block COX-enzyme activation, as enhanced signalling and gene transcription resulting from EP4 receptor activation would also be inhibited.

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CHAPTER 7 – DISCUSSION AND CONCLUSIONS

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Discussion and Conclusions

As mentioned in Section 1.1, a role for cyclooxygenase (COX) enzymes, prostanoids and prostanoid receptors has now been ascertained for promoting inflammation and growth of numerous tumours. Recent data have demonstrated elevated expression of COX enzymes and prostanoid EP2 and EP4 receptors and synthesis and signalling of prostaglandin E_2 (PGE_2) in cervical carcinomas. Gynaecological malignancies of this nature have an enormous impact on the socio-economic status of developing countries, including South Africa, and place a huge burden on the national health care system. An understanding of the molecular mechanisms whereby prostaglandins (PGs) may mediate their role in cervical epithelial cells which could exacerbate cervical tumourigenesis may lead to the development of improved therapies for women presenting with cervical carcinoma. An important consideration in the aetiology of cervical cancer is the potential role of seminal plasma PGs in mediating the activation of bio-molecular signalling pathways, which in sexually active women, may further influence the progression of cervical cancer. The aim of this research was to investigate the potential role of seminal plasma PGs in mediating cell signalling and targeting gene transcription to facilitate cervical tumourigenesis via the EP4 receptor.

Chapter 3 reported on the localisation of EP4 receptor in cervical sections, the role of elevated EP4 receptor in cervical carcinomas and the interaction between seminal plasma and EP4 receptor in cervical neoplastic epithelial cells.

Initially, studies were designed to localise the site of expression of the EP4 receptor in cervical sections. Previous data generated in this laboratory had confirmed upregulated expression of EP4 receptor in all cervical adenocarcinoma and squamous cell carcinoma cases investigated. The site of expression of the EP4 receptor was localised by immunohistochemistry using paraffin wax-embedded cervical carcinoma and normal cervical tissue. EP4 receptor expression was localised to the neoplastic epithelial cells of the cervical carcinoma tissue.

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In order to ascertain a role for EP4 receptor in cervical carcinomas, we constructed an EP4-overexpressing epithelial adenocarcinoma cell model system in HeLa (cervical carcinoma) cells (Chapter 4). EP4 receptor cDNA was introduced into the cells and made stable by antibiotic selection. Over-expression of EP4 receptor in this cell line was confirmed by Western blot analysis and real-time quantitative RT-PCR, which showed significantly elevated levels of EP4 receptor mRNA in HeLa EP4 overexpressing cells when compared to wild-type HeLa cells. Since EP4 receptor activation is coupled to cAMP accumulation, cAMP assays were performed to confirm increased accumulation of cAMP in the EP4-overexpressing HeLa cells (EP4S) following PGE_2 stimulations.

Next, to investigate the interaction between seminal plasma and EP4 receptor in neoplastic cervical epithelial cells, investigations were performed into possible signal transduction pathways and gene expression regulated by PGE_2-EP4 receptor activation (Chapter 5 and 6). Treatment of EP4S cells with PGE_2 or seminal plasma resulted in a rapid accumulation of cAMP and phosphorylation of ERK1/2, which was significantly greater compared with WT cells. The phosphorylation of ERK1/2 was inhibited by co-treatment of cells with seminal plasma and MEK inhibitor (PD98059), EP4-selective receptor antagonist (ONO-AE2-227) or an inhibitor of epidermal growth factor (EGF) receptor tyrosine kinase (AG1478). Our data demonstrate that PGE_2 or seminal plasma prostaglandins can activate signalling via EP4 receptors, which may involve transactivation of the EGF receptor and ERK1/2 in the downstream signalling cascade (Figure 8.1). These data are supported by several studies providing evidence that prostanoid GPCRs activate receptor tyrosine kinases (RTKs) (Pai *et al.* 2002; Buchanan *et al.* 2003; Sales *et al.* 2004; Sales *et al.* 2004). This cross-communication results in increased auto-phosphorylation and dimerization of RTKs (such as the EGFR and platelet-derived growth factor receptor) culminating in the activation of MAPK or PI3kinase signalling (Pai *et al.* 2002; Buchanan *et al.* 2003; Sales *et al.* 2004; Sales *et al.* 2004). The diversity of RTK activation by prostanoid receptors, the exact intracellular mechanisms of the activation, and the physiological or pathological significance of this cross-communication are not, however, fully elucidated.

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Investigations were also performed to investigate possible signalling via the p38 MAPK, JNK or the PI3 kinase/AKT signalling pathways. These pathways do not appear to be activated by seminal plasma or PGE_2 stimulation of HeLa EP4S or WT cells over the time course investigated, which suggest that they may not be involved in signalling via the EP4 receptor in our model system, or alternatively that they may be involved at later time points.

PGE_2 or seminal plasma stimulation of EP4S cells culminate in elevated expression of COX-2 mRNA and protein, which has been implicated in tumourigenesis, and elevated expression of two genes associated with angiogenesis, namely vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Expression of COX-2 and VEGF mRNA was inhibited by co-treatment of cells with seminal plasma and the MEK inhibitor, the EP4-selective receptor antagonist or the EGF receptor tyrosine kinase inhibitor. This implies that EP4 receptor signalling can activate expression of inflammatory and angiogenic genes via downstream signalling pathways involving productive cross-communication with the EGFR. Furthermore, co-treatment of seminal plasma-treated HeLa EP4S or WT cells with Indomethacin (COX-enzyme inhibitor) or the EP4-selective antagonist resulted in a significant decrease in proliferation of both EP4S and WT cells.

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The autocrine/paracrine regulation of EP4 prostanoid receptor signalling and the downstream effects on biological function are illustrated in Figure 8.1. PGs, either produced intracellularly via the COX-enzyme biosynthetic pathway or present in seminal plasma, activate the EP4 receptor, initiating the production of the second messenger cAMP. Activation of cAMP can then initiate kinase signalling by the MAPK pathway and target gene transcription. Data suggest that target gene transcription can occur via prostanoid-receptor-mediated transactivation of RTKs, such as the EGFR. In turn, the activation of target genes (COX-2, VEGF and bFGF) by prostanoid receptor signalling can promote angiogenesis (by modulating vascular function and vascular tone), cellular proliferation, and cellular adhesion, migration or metastasis.

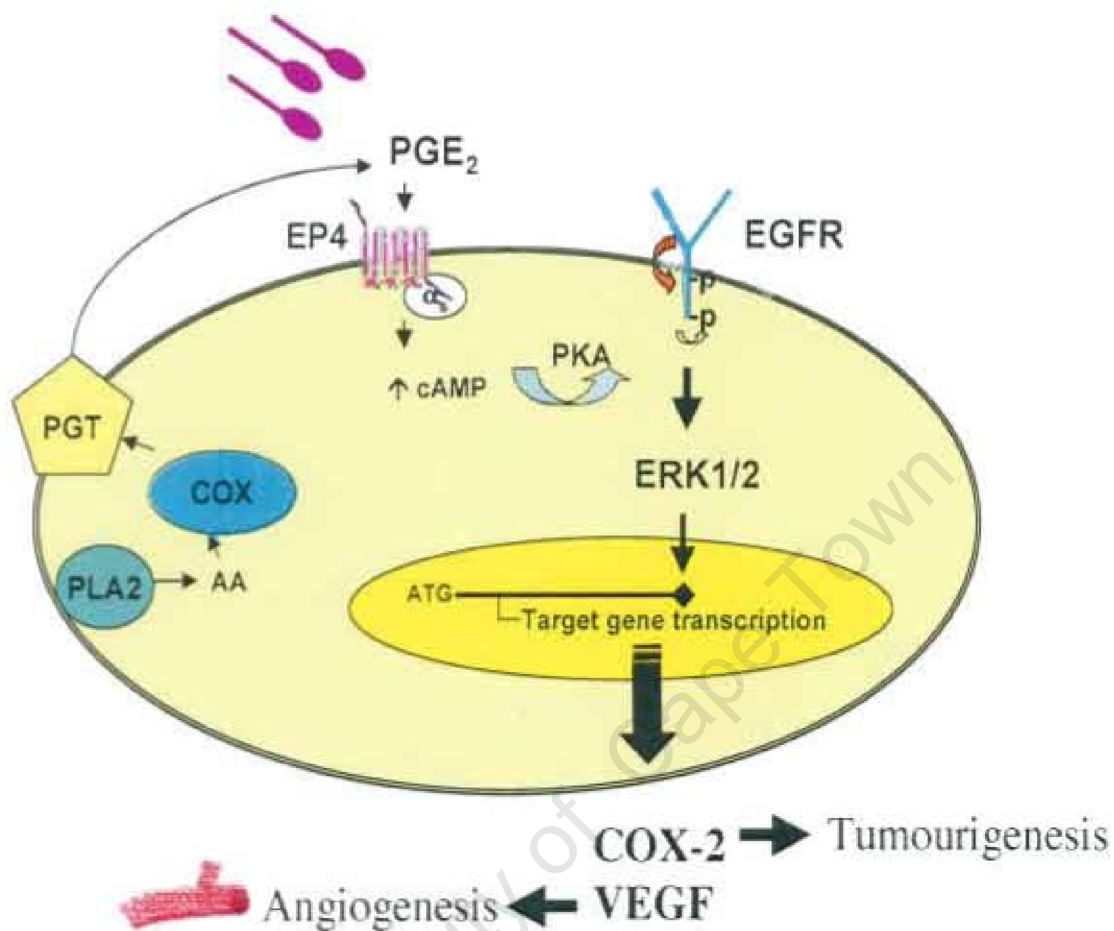

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Figure 8.1. Autocrine or paracrine regulation of prostanoid receptor signalling and the downstream effects on biological function. Prostaglandins, either produced intracellularly via the cyclooxygenase (COX) enzyme biosynthetic pathway or present in seminal plasma, activate the EP4 receptor, initiating the production of the second messenger cyclic AMP (cAMP). Activation of cAMP can then initiate kinase signalling by the mitogen-activated protein kinase (MAPK) ERK1/2 pathway and target gene transcription. Data suggest that target gene transcription can occur via prostanoid-receptor-mediated transactivation of receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR). In turn, the activation of target genes (such as COX-2, VEGF and bFGF) by prostanoid receptor signalling can promote angiogenesis (by modulating vascular function and vascular tone), cellular proliferation, and cellular adhesion, migration or metastasis.

Non-steroidal anti-inflammatory drugs (NSAIDs) and specific inhibitors of COX-2 are therapeutic groups widely used for the treatment of pain, inflammation and fever. However, there is growing experimental and clinical evidence indicating that NSAIDs and COX-2 inhibitors also have anti-cancer activity. Epidemiological studies have shown that regular use of aspirin and other NSAIDs reduces the risk of developing cancer. Studies have demonstrated that COX-2 overexpression promotes tumourigenesis, and that NSAIDs and COX-2 inhibitors suppress tumourigenesis and tumour progression. Recent advances in the understanding of the cellular and molecular mechanisms of the anti-cancer effects of NSAIDs and COX-enzyme inhibitors have demonstrated that these drugs target both tumour cells and the tumour vasculature. New vessel formation permits tumour growth, survival and metastasis. We have identified an upregulation in both bFGF and VEGF, which are both able to promote formation of tumour-induced angiogenic blood vessels. In pre-clinical models, numerous approaches to inhibit VEGF activity lead to decreased tumour growth and angiogenesis. However, although inhibiting either COX-2 or VEGF may be valid therapeutic strategies to treat cervical carcinoma, this will only lead to suppression of endogenous prostaglandins. In sexually active women, any increase in prostanoid receptor activation can be further enhanced by seminal plasma prostaglandins. In addition, seminal plasma contains high levels of VEGF, which could act enhance angiogenesis. From the observations reported in this thesis, it is envisaged that an understanding of the role of specific prostanoid receptors, their signalling pathways and their phenotypic effects in the female reproductive tract might result ultimately in the development and implementation of more efficacious interventions in the clinic. This focus has opened a new vista into the understanding of the complexity of signalling networks and cross-communication that exist between prostaglandin receptors, and between these receptors and RTKs. Unravelling these networks might lead to a better understanding of the role of the prostanoid receptors in cervical carcinoma and might outline further novel therapeutic directions in the clinic.

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In conclusion, these data suggest a role for the EP4 receptor in regulating tumour cell growth and proliferation through target gene transcription. They also imply that cervical carcinoma in sexually active women may be exacerbated following exposure to seminal plasma and activation of EP4-receptor signal transduction pathways. Once again, this opens up the possibility for the use of selective receptor antagonists and modulators of signal transduction in combination with NSAIDs, to block signal transduction and resultant gene transcription, as well as COX-enzyme actions. This may be of particular therapeutic benefit to sexually active women receiving treatment for cervical carcinoma, as they may not only be exposed to endogenous prostaglandins, but also very high levels of exogenous prostaglandins available in seminal fluid.

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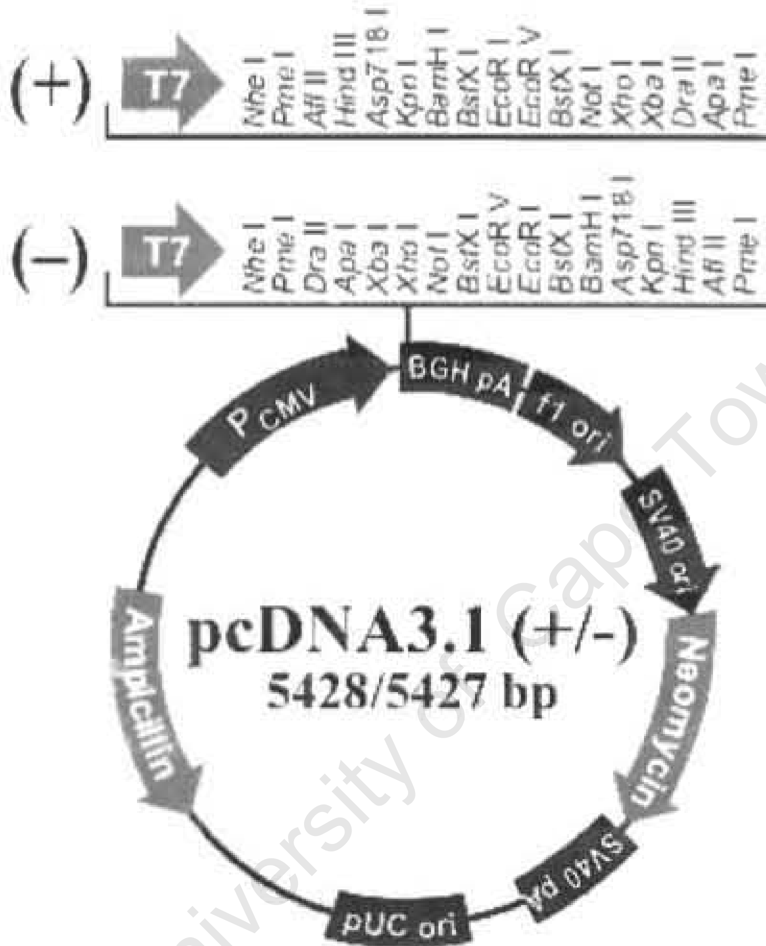
APPENDIX I

Plasmid pcDNA3.1 (Invitrogen)



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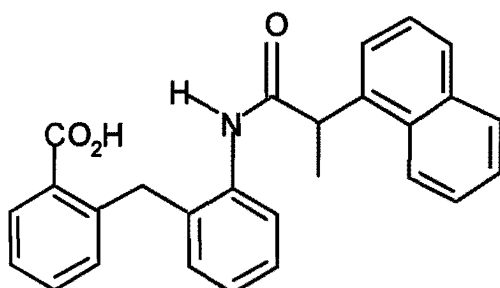
The cDNA (1.5 kb) for human prostaniod receptor EP4 was ligated into the *EcoRV* site of the mammalian expression vectort, pcDNA3.1 (Invitrogen). The size of the EP4 pcDNA3 construct is 6.9 kb. The cDNA can be cut out using *HindIII* and *XhoI*.
Adapted from Invitrogen website (www.invitrogen.com).

APPENDIX II

Chemical structure of compounds



ONO-AE2-227

(Charnwood Molecular Ltd., UK)

2-[2-[2-(1-naphthyl)propanoylamino]phenylmethyl]benzoic acid

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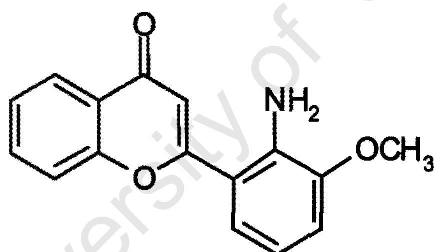
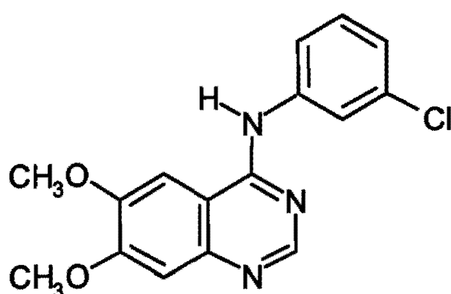


PD 98059

(Calbiochem®, UK)

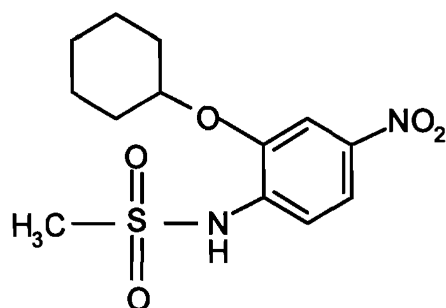
2'-Amino-3'methoxyflavone



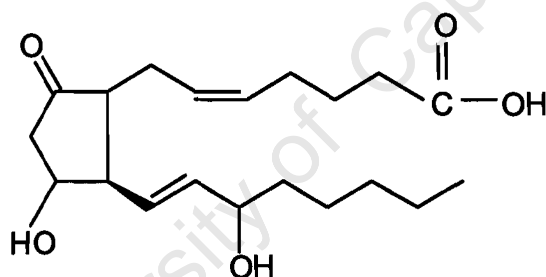
AG 1478

(Calbiochem®, UK)

4-(3-Chloroanilio)6,7-dimethoxyquinazoline



NS-398
(Calbiochem®, UK)
N-(2-Cyclohexyloxy-4-nitrophenyl)methanesulfonamide



Prostaglandin E_2
(Sigma-Aldrich, UK)
(5Z,11α,13E,15S)-11,15-Dihydroxy-9-oxoprosta-5,13-dienoic acid

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