CHARACTERIZATION OF SIGNALLING CROSS-TALK BETWEEN THE EP2 AND FP RECEPTORS IN ENDOMETRIAL EPITHELIAL CELLS

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DECLARATION

I herewith declare that I autonomously carried out this work, except where due acknowledgements is made by reference. No portion of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Aron B Abera
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LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylyl cyclase
AKR	aldo-keto reductase
ALP	alkaline phosphatase
AMPS	ammonium persulphate
ANG	angiotensin
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
ANOVA	analysis of variance
ATP	adenosine triphosphate
β-AR	β-adrenergic
BCP	1-bromo-3-chloropropane
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
CaMBD	calmodulin binding domain
CAMK-II	Ca^{2+}/calmodulin-dependent kinase II
cAMP	adenosine 3',5'-cyclic monophosphate
cDNA	complementary DNA
CHO	Chinese hamster ovary
CIS	carcinoma in situ
COX	cyclooxygenase
CRE	cAMP response element
CREB	cAMP regulatory binding protein
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMEM	dulbecco's modified eEagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
EC_{50}	median (half-maximal) effective concentration
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	endothelial growth factor
EGFR	epidermal growth factor receptor
EIC	endometrial intraepithelial carcinoma
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
E_{max}	maximal agonist-stimulated IP production
FAK	focal adhesion kinase

FCS	foetal calf serum
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
G protein	guanine nucleotide-binding protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
GSK-3	glycogen synthase kinase-3
GTP	guanosine triphosphate
HEK-293	human embryonic kidney-293
HEL	human erythroleukemia
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horse-radish peroxidise
Hyg	hygromycin
IBMX	3-isobutyl-1-methyl xanthine
IGF	insulin growth factor
IgG	Immunoglobulin G
IHC	immunohistochemistry
IL	interleukin
IM	intramural
IP3	inositol phosphate
JAK	janus-activated kinase
KDa	kilodalton
LH	luteinizing hormone
mg	milligram
μl	microgram
ml	milliliter
μg	microlitre
MAPK	mitogen-activated protein kinase
MMP	matrixmetalloproteinases
mRNA	messenger ribonucleic acid
NFκB	nuclear factor-kappaB
NP40	"Nonidet" P40
NSAID	non-steroidal anti-inflammatory drug
OSE	ovarian surface epithelial
OH	hydroxyl
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PFA	paraformaldehyde
PG	prostaglandins
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase

$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFS	prostaglandin F synthase
PGG_2	prostaglandin G_2
PGH_2	hydroxy cyclic endoperoxide
PGI_2	prostacyclin
PGT	prostaglandin transporter
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PPAR	peroxisome proliferators-activated receptors
PTEN	*phosphatase and tensin homolog on chromosome ten*
PVDF	polyvinylidene difluoride
RACC	receptor-activated Ca^{2+} channel
Rb	retinoblastoma
rpm	revolutions per minute
RT	reverse transcriptase
RTK	receptor tyrosine kinase
SAT1	spermidine/ N1-acetyltransferase
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
siRNA	small interfering RNA
SM	submucosal
SS	subserosal
STAT	signal transducers and activators of transcription
TAE	tris-acetate EDTA
Taq	*thermus aquaticus*
TBE	tris-boric acid EDTA
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween®20
TGF	transforming growth factor
TM	transmembrane
TNF	tumour necrosis factor
Tris	tris (hydroxymethyl) amino methane
TRP	transient receptor potential
TXA_2	thromboxane A_2
UV	ultraviolet
V	volt
VEGF	vascular endothelial growth factor
W	watt

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PROJECT SYNOPSIS

Uterine fibroids are benign tumors that arise from the smooth-muscle uterine cells (myometrium) and are the most common uterine disorder occurring in as many as 30% of women over 35 years of age. Despite their frequent occurrence, the etiology of uterine fibroids is not well elucidated. Several studies have shown that numerous tumors can be regulated by cyclooxygenase (COX) enzyme products but their role in uterine fibroids is not well established.

The initial aim of the study was to determine the expression level of COX enzymes and prostaglandin receptors in fibroids and autologous myometrium samples from women with fibroids. Real-Time reverse-transcriptase polymerase chain reaction (RT-PCR) revealed that the expression of COX enzymes, EP1, EP2 and EP4 prostanoid receptors and prolactin were not significantly altered while the EP3 subtype receptor was significantly down-regulated in fibroids compared to adjacent myometrium samples. The EP3 receptor has a protective role in tumor development suggesting the role for down-regulation of the receptor in uterine fibroids pathology.

In addition, the expression of COX enzymes, prostaglandin receptors and prostaglandin-mediated genes were assessed in endometrium samples from women with and without uterine fibroids in different stages of the menstrual cycle. COX-2 and interleukin-8 (IL-8) mRNA expressions were significantly higher in both proliferative stage and early-mid secretory, EP2 receptor and IL-11 were elevated in the proliferative stage, vascular endothelial growth factor (VEGF) was highly expressed in the early-mid secretory phase while FP receptor was up-regulated in all stages of the menstrual cycle in endometrium samples from women with fibroids. These data suggest that up-regulation of COX-2 and prostaglandin receptors (EP2 and FP) in endometrium can induce expression of angiogenic and mitogenic factors such as VEGF, IL-8 and IL-11 which might act in a paracrine manner on neighboring myometrial/fibroid tissue to promote angiogenesis and facilitate tumor growth.

Furthermore, since EP2 and FP receptors were up-regulated in the proliferative phase of endometrium from uterine fibroid patients and the receptors are co-expressed in endometrial adenocarcinoma (Ishikawa) cells, this study investigated a possible cross-talk that influences intracellular signalling by using Ishikawa cells stably expressing the EP2 and FP receptors (FPEP2 cells) as a model cell line. Real-Time RT-PCR, Western blot analysis and immunofluorescence microscopy confirmed stable expression of the EP2 and FP receptors in FPEP2 cells localized to the perinuclear and plasma membrane.

Using FPEP2 cells, the integrated effect of Butaprost (EP2 receptor ligand) and PGF (FP receptor ligand) co-administration on inositol phosphate (IP3) and adenosine 3',5'-cyclic monophosphate (cAMP) release was assessed to study a possible heterologous-interaction or cross-talk between the EP2 and FP receptors. The study showed that in FPEP2 cells, PGF alone does not alter cAMP production, but in combination with Butaprost augments EP2 receptor-mediated cAMP release. PGF-mediated potentiation of cAMP release was abolished by antagonism of the FP receptor, inhibition of phospholipase C (PLC) and IP3-receptor whereas inhibition of protein kinase C (PKC) had no effect suggesting the cross-talk is mediated by FP receptor activation of IP3 release. Moreover, inhibition of calcium effectors using calmodulin antagonist (W7) or Ca^{2+}/calmodulin-dependent kinase II (CaMK-II) inhibitor (KN-93) abolished PGF potentiation of Butaprost-mediated cAMP release. Using short interfering RNA (siRNA) molecules targeted against the adenylyl cyclase 3 (AC3) isoform, the study showed the isoform to be responsible for the cross-talk between the FP and EP2 receptors.

In order to determine the integrative effects of the EP2 and FP receptors co-activation on gene expression, a whole genome array profiling in FPEP2 cells in response to Butaprost and/or PGF was performed. The gene array revealed 228 genes that are regulated by co-activation of the EP2 and FP receptors that are involved in cell morphology, proliferation and differentiation.

In addition, co-activation of EP2 and FP receptors with their respective ligands enhanced or repressed a set of EP2 receptor-regulated genes. One of the genes identified, SAT1 (Spermidine/ N1-acetyltransferase), was regulated by the EP2 and FP receptors cross-talk via the calcium sensitive AC3 isoform. SAT1, with known role in regulation of tumorigenesis was also up-regulated in the proliferative stage of endometrium samples from women with uterine fibroids suggesting the EP2 and FP receptor cross-talk characterized *in vitro* can also happen *in vivo*.

In conclusion, this study reports that COX-2, EP2 and FP receptors, VEGF, IL-8, IL-11 and SAT1 are up-regulated in endometrium from women with uterine fibroids. These genes play a major role in development of fibroids by facilitating angiogenesis and cell growth and by inhibiting apoptosis via autocrine/paracrine mechanisms. In addition, this study demonstrates that co-activation of the EP2 and FP receptors results in enhanced release of cAMP via the FP receptor-$G\alpha_q$-Ca^{2+}-calmodulin pathway by activating the calcium-sensitive AC3 isoform and modulates a molecular switch which alters the trans-activation of a subset single-receptor induced genes that have important functions in the pathogenesis of reproductive pathologies.

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Chapter I

General Introduction

1.1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin have been widely used for the treatment of a wide spectrum of pathological conditions over the last century without understanding the mechanism of their action. Only in 1971, was the molecular mechanism of NSAID revealed. Vane (1971) demonstrated that NSAIDs reduce inflammation by inhibiting cyclooxygenase (COX) and won a Nobel Prize in medicine for this discovery. COX is a rate limiting enzyme that catalyzes the first two steps in the biosynthesis of prostaglandins, thromboxanes and leukotrienes (collectively called eicosanoids) from the substrate arachidonic acid (Vane *et al.*, 1998).

1.2 Arachidonic Acid Metabolism

Arachidonic acid, an essential fatty acid derived from elongation and oxidation of linoleic acid is incorporated by ester linkage into membrane phospholipids and triglycerides. In response to stimulation by cytokines, growth factors, circulating hormones or stress, arachidonic acid gets hydrolyzed by secretory or cytoplasmic or both types of phospholipase A_2 ($sPLA_2$, $cPLA_2$) and released from the sn-2 position of the membrane triglycerides (Marnett, 1992; Wang *et al.*, 2007).

Arachidonic acid gets oxidized by the rate limiting enzyme COX to form prostaglandin G_2 (PGG_2). The hydroxy endoperoxide (PGH_2) gets synthesized from PGG_2 reduction and serves as a substrate for terminal prostanoid synthase enzymes to produce specific prostanoids (prostaglandins, prostacyclins and thromboxanes) (Marnett, 1992; Smith *et al.*, 2000) (Fig 1.1). The enzymes are named according to their respective prostanoids, such that prostaglandin E_2 (PGE_2) by prostaglandin E synthase, prostaglandin F_2 (PGF_2) by prostaglandin F synthase, prostaglandin D_2 (PGD_2) by prostaglandin D synthase, prostacyclin (PGI_2) by prostaglandin I synthase (PGIS) and thromboxane (TX) by thromboxane synthase (TXS) (Narumiya *et al.*, 1999; Sales and Jabbour, 2003). The prostanoids formed are released from cytoplasm and transported by prostaglandin transporter (PGT) outside of the cells to act on their cognate receptors (Sales and Jabbour, 2003).

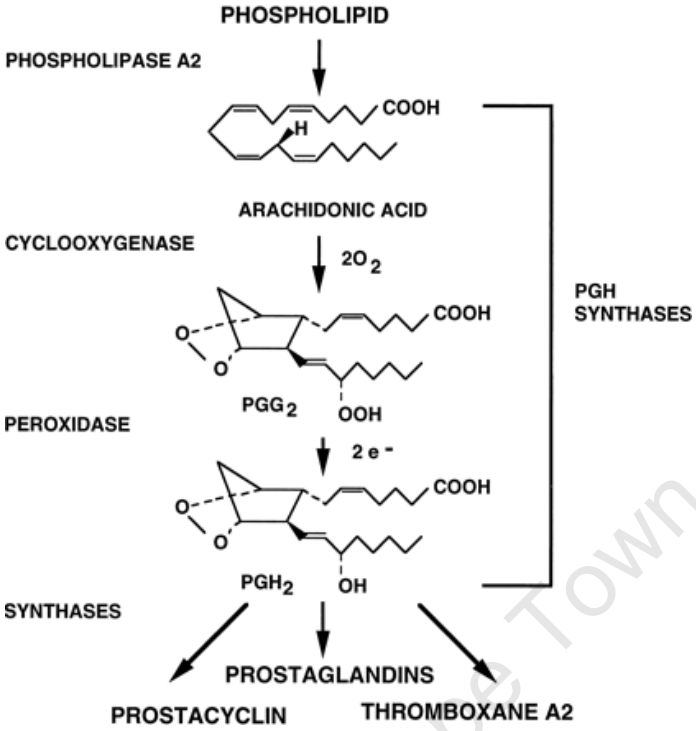

Fig. 1.1: *The arachidonic acid metabolism*[1]

Arachidonic acid gets hydrolyzed by phospholipase A_2 and released from the membrane triglycerides. It then gets oxidized and reduced to PGH_2 by the rate limiting enzyme COX. PGH_2 then serves as a substrate for terminal prostanoid synthase enzymes to produce specific prostanoids (prostaglandins, prostacyclins and thromboxanes).

1.3 Cyclooxygenase Enzyme Isoforms

COX, a membrane-bound heme-containing enzyme, was cloned in 1988 and has three isoforms termed as COX-1, COX-2 and COX-3 according to the order of their discovery. COX-1 and COX-2 are derived from two different genes, while COX-3 is a splice variant of COX-1. COX-1 is believed to be constitutively expressed, while COX-2 is an inducible isoform mostly expressed at sites of inflammation (Fu *et al*., 1990; Vane *et al.*, 1998; Davies *et al.*, 2004). COX-3 was suggested to be a third physiological form of COX enzyme by Willoughby *et al.* (2000) after identifying a

[1] Diagram reproduced from Smith *et al*., 2000

different form of late-induced COX enzyme that appears 48 hours after the onset of inflammation.

COX enzymes catalyze a cyclooxygenase and a peroxidase reaction to synthesize PGH_2, hence the enzymes are also called PGH_2 synthase. The cyclooxygenase reaction occurs in a hydrophobic channel in the core of the enzyme and can be irreversibly inhibited by acetylation of the site with aspirin, while the peroxidase reaction happens at a heme-containing active site that is located near the enzyme surface and other NSAIDs like indomethacin produce both reversible and irreversible inhibition by competing with the substrate (Vane *et al.,* 1998; Smith *et al.,* 2000).

1.3.1 Cyclooxygenase-1 (COX-1)

The human COX-1 gene, approximately 22 kb in length, is located on chromosome 9 and is transcribed as 2.8 kb mRNA. COX-1 cDNA encodes a glycoprotein that is processed from 576 amino acids with molecular mass of 70 kDa. It exists as a homodimer and is preferentially integrated in plasma membrane (Tanabe and Tohnai, 2002; Kang *et al.*, 2007). The promoter region for COX-1 lacks TATA or CAAT box and contains two Sp1 motifs, two AP-2 sites, a GATA and NF-IL6 motif, all characteristics of a "housekeeping" gene. For this reason COX-1 was presumed to be only constitutively active. However, COX-1 has been shown to be up-regulated in some cells by phorbol myristate acetate (PMA), Interleukin-1β (IL-1β), vascular endothelial growth factor (VEGF), bradykinin, estrogen, shear stress and retinoic acid (Kang *et al.,* 2007). For example, Gibson *et al.* (2005) have shown estrogen can up-regulate COX-1 mRNA expression in endothelial cells mediated by Sp1 functional binding site in the promoter of the enzyme.

COX-1 is widely expressed in almost all tissues under basal conditions and it is presumed to be involved in providing prostanoid precursors for homeostatic regulation (DuBois *et al.,* 1998). COX-1, as the only isoform detectable in platelet is important in the blood for a critical role in blood clotting. This was further shown by inhibiting COX-1 using NSAIDs which in turn leads to a failure of complete and successful

aggregation. Induction of COX-1 in platelets has a paracrine effect in adjacent vascular endothelial cells by generating prostaglandin that results in vasodilatation (Schafer, 1995). This physiological phenomenon of COX-1 is also observed in the kidney and stomach by acting in different setting to produce prostaglandins and can maintain the body homeostatic (Vane *et al.,* 1998; DuBois *et al.,* 1998).

COX-1 has been shown to play a major role in gestation and parturition. Trautman *et al.* (1996) showed COX-1 was constitutively expressed in the amnion, chorion and decidua throughout the whole of the third trimester of pregnancy. Another study has shown that treatment of human amnion cells by human chorionic gonadotropin (hCG) can induce COX-1 mRNA and protein expression suggesting that the regulation of COX-1 can help maintain pregnancy (Toth *et al.,* 1996).

1.3.1.1 Cyclooxygenase-1 in Carcinogenesis

Despite being considered a "housekeeping gene", several studies have demonstrated COX-1 over-expression in numerous pathologies. Gupta *et al.* (2003) showed increased expression of COX-1 in ovarian cancer samples compared to normal tissue. COX-1 expression was localized in the epithelial compartment of the tumors with higher expression of VEGF in the same compartment. Daikoku *et al.* (2005) extensively studied the role for COX-1 in mouse ovarian surface epithelial (OSE) cells lacking the functional tumor suppressor gene *p53* with activation of oncogenic pathways. The authors reported that tumor and OSE cells derived from these mice showed an over-expression of COX-1 but not COX-2. Moreover, they showed a decrease in cell proliferation and an increase in apoptosis when OSE cells were treated with a specific COX-1 inhibitor sc-560.

COX-1 has been implicated in colon carcinogenesis. Chulada *et al.* (2000) developed COX-1 knockout *Min*/$^{+}$ mice (mice contain a truncating mutation in the *Apc* gene and are predisposed for developing intestinal adenomas) that showed a significant decrease in intestinal tumorigenesis compared to normal mice. A recent study reported a decrease in cell proliferation and induction of G_0/G_1 arrest in colon cancer cells (HT-29) by treating the cells with specific COX-1 inhibitor sc-560. COX-1 inhibition also

resulted in morphological and biochemical changes in the cells characteristics of macroautophagy which is a cellular process that degrades organelles through lysosomal machinery and may serve as an alternative to the apoptosis pathway (Wu *et al.,* 2009). Narko *et al.* (1997) showed that COX-1 over-expressing immortalized endothelial cell lines (ECV) can enhance tumorigenesis when injected subcutaneously into the athymic nude mice. Histological analysis of tissue samples from these mice revealed that the tumor contained highly malignant ECV cells and vessel-like structures demonstrating COX-1 over-expression can induce tumorigenesis and exacerbate angiogenesis in endothelial cells. Previous work in our laboratory has showed an over-expression of COX-1 enzyme in squamous cell carcinoma and adenocarcinoma of cervical cancer and was associated with expression of VEGF and other growth factors (Sales *et al.,* 2002). COX-1 up-regulation has also been reported in breast cancer, lung tumors and in human prostate cancer (Hwang *et al.,* 1998; Bauer *et al.,* 2000; Kirschenbaum *et al.,* 2000). These data show the major role for COX-1 up-regulation in tumor development by promoting cell proliferation and angiogenesis and by inhibiting apoptosis.

1.3.2 Cyclooxygenase-2 (COX-2)

The human COX-2 gene, approximately 8.3 Kb, is located in on chromosome 1 and demonstrates a remarkable conservation of exon-intron junction with COX-1 gene. Intron 1 of COX-1 is lost in COX-2 and the introns in COX-2 gene are shorter than those in COX-1 gene. The COX-2 gene is transcribed as 4.6, 4.0 and 2.8 kb mRNA splice variants. The cDNA for COX-2 encodes a 604 amino acids polypeptide before cleavage that is 61% identical to COX-1 polypeptide. COX-2 protein has a molecular mass of 70 kDa and localizes to the perinuclear envelope (Tanabe and Tohnai, 2002; Kang *et al.,* 2007). Unlike COX-1, COX-2 has a TATA box and several transcription elements such as NF-IL6 motif, two AP-2 sites, three Sp1 sites, two NF-κB sites, E-box and an adenosine 3',5'-cyclic monophosphate (cAMP) response element (CRE) motif (Kosaka *et al.,* 1994; Tanabe and Tohnai, 2002).

COX-2 is usually expressed in low levels in normal tissues and is inducible by phorbol esters, pro-inflammatory factors, hormones, several growth factors and oncogenes in numerous cell types. The enzyme has been shown to be transcriptionally regulated in bones, monocytes/macrophages, fibroblast cells, endothelial cells, smooth muscle cells and epithelial cells. These transcriptional activations use several binding sites in COX-2 promoter region. For example, lipopolysaccharide (LPS) and PMA act synergistically to activate COX-2 expression in bovine arterial endothelial cells via the NF-κB sites and CAAT enhancer binding protein (C/EBP) elements. Bradykinin has been shown to activate COX-2 expression in smooth muscle cells via the CREB binding elements (Kang *et al.*, 2007).

COX-2 expression has been proven to be indispensible in reproductive physiology, particularly in female reproduction. For example, follicular levels of COX-2 have been reported to increase dramatically before ovulation. This robust increase was shown *in vivo* by administration of hCG in rats. *In vitro* studies have revealed robust COX-2 induction by luteinizing hormone (LH), follicle-stimulating hormone (FSH) or forskolin stimulation of preovulatory granulosa cells. Administration of NSAIDs such as indomethacin blocked ovulation in the granulosa cells (Sirois *et al.*, 2004; Kang *et al.*, 2007). Morham *et al.* (1995) developed the first COX-2 knockout mice and showed that these mice have renal dysplasia and more importantly the females are infertile. This finding is consistent with other reports including those mentioned above about the crucial role for COX-2 for successful ovulation (Scherle *et al.*, 2000). Lim *et al.* (1997) also showed COX-2 null mice were impaired in many events of female reproductive processes such as ovulation, fertilization, implantation and decidualization. These events in early pregnancy are inducible, a fact that mirrors the role of the inducible COX-2 role in these events.

1.3.2.1 Cyclooxygenase-2 in Carcinogenesis

As an inducible gene in response to inflammation, COX-2 has emerged as a key player in cancer-related inflammation and tumor progression. COX-2 up-regulation has been associated with pathogenesis of colon, lung, pancreatic, gastric, breast, head and neck cancer (DuBois *et al.*, 1998; Wang *et al.*, 2007).

The role for COX-2 in colorectal cancer was first shown by the ability of aspirin to reduce colon cancer incidence and mortality (Marnett, 1992). Other study showed COX-2 mRNA expression to be markedly enhanced in 85% of human colorectal carcinomas and 50% of colorectal adenomas compared to accompanying normal mucosa (Eberhart *et al.,* 1994). Studies using COX-2 null mice showed a reduction in intestinal tumorigenesis in *Min/*$^{+}$ mice compared to normal mice (Chulada *et al.,* 2000).

COX-2 over-expression has been reported in human breast cancer. Half *et al.* (2002) reported an induction of COX-2 in 43% of invasive breast cancers and in 63% of adjacent ductal carcinoma in situ (DCIS) samples examined. The higher induction of COX-2 in DCIS samples might suggest that COX-2 up-regulation affects the early stages of mammary carcinogenesis. Furthermore, other studies have shown COX-2 inhibition can lead to a decrease in tumor progression and metastasis in oncogenic mouse models of spontaneous breast cancer. *In vivo* administration of a selective COX-2 inhibitor, celecoxib, caused a significant reduction in mammary gland tumor burden in these mice. This tumor reduction was associated with an increase in tumor cell apoptosis via the expression of pro-apoptotic protein Bax and down-regulation of the anti-apoptotic protein Bcl-2 (Basu *et al.,* 2004).

More importantly COX-2 has been implicated in uterine pathology. Gupta *et al.* (2000) was the first one to report over-expression of COX-2 in prostate adenocarcinoma compared to pair-matched benign tissue samples. Expression of COX-2 in cancer tissue was higher in surrounding stromal cells and in infiltrating inflammatory cells compared to tumor cells. COX-2 up-regulation has been reported in cervical cancer. Kulkarni *et al.* (2001) reported over-expression of COX-2 enzyme in cancer of cervix as well as cervical intraepithelial neoplasia (CIN). Sales *et al.* (2001) also showed an up-regulation of COX-2 mRNA in squamous cell carcinoma and in adenocarcinoma of the human cervix. The authors also showed over-expression of COX-1 enzyme in HeLa cells (cervical adenocarcinoma cells) can induce the expression of COX-2 and PGE synthase (Sales *et al.,* 2002).

These data show COX-2 over-expression can enhance tumor development by up-regulation of angiogenic factors, and pro-apoptotic proteins and by down-regulation of anti-apoptotic proteins.

1.3.3 Cyclooxygenase -3 (COX-3)

COX-3, approximately 5.2 Kb, is a splice variant of COX-1. COX-3 gene has an addition of intron 1 downstream from the start codon that results in-frame insertion of 30 amino acids (Chandrasekharan *et al.,* 2002). COX-3 mRNA has been shown to be abundantly expressed in heart and cerebral cortex but it remains controversial whether COX-3 protein exists in humans. COX-3 shares all the catalytic and important structural features with COX-1 and COX-2. However, it is more sensitive to inhibition by acetaminophen than the other isoforms (Davies *et al*., 2004).

Although COX enzymes are regulators of numerous body functions, it is the expression and regulation of the prostanoids that determine the physiology and pathology of a cell or a tissue.

1.4 Prostanoids and Prostanoid Receptors

Prostanoids were first discovered in 1930s, when semen products were shown to decrease blood pressure *in vivo* (Oates, 1982; Versteeg *et al.,* 1999). Prostanoids, members of the eicosanoid family, consist of either prostaglandins that contain a cyclopentane ring or thromboxanes containing a cyclohexane ring. Prostaglandins include the D, E, F and I series while thromboxanes are divided into TXA and TXB (Versteeg *et al.,* 1999).

These prostanoids exert their biological function by acting on their cognate receptors. There are two classes of prostanoid receptors: G-coupled cytoplasmic receptor class and the nuclear PPAR (peroxisome proliferators-activated receptors) class consisting of PPAR α, γ and δ. The G-coupled cytoplasmic receptor has eight prostanoid receptors (EP1, EP2, EP3, EP4, FP, DP, IP and TP receptors) belonging to it (Breyer *et al.,* 2001).

These prostaglandin receptors have a typical G (guanine nucleotide-binding) protein-coupled receptor (GPCR) characteristic with seven hydrophobic transmembrane domain structures (Hirata *et al.,* 1991). Within the prostanoid family the receptors have an overall homology ranging from 24-44% and have 65 amino acid residues that are conserved. Of these conserved residues, 34 amino acids are identical between the prostanoid receptors (Versteeg *et al.,* 1999; Breyer *et al.,* 2001). Most of the conserved domains lie in the transmembrane regions, although some of the residues exist in the second extracellular loop. The ligand binding domain of most prostanoid receptors is found in the conserved domain of the transmembrane and the extracellular loop (Audoly and Breyer, 1997; Breyer *et al.*, 2001).

A strikingly different feature of prostanoid receptors from other GPCRs is the existence of various splice variants. In the prostanoid family, EP1, EP3, FP and TP receptors have different splice variants, which mostly differ in the intracellular C-terminal region of the receptor where most of the splicing occurs. Alternative splicing does not have any impact in ligand binding but have an effect on G protein coupling specificity, agonist-induced receptor phosphorylation, desensitization/internalization and constitutive activity (Breyer *et al.,* 2001).

1.4.1 Prostaglandin E_2 (PGE_2) and PGE_2 Receptors

PGE_2, a major metabolite of COX-initiated arachidonic acid metabolism, is biosynthesized in cells from PGH_2 by PGES and plays a critical role in regulating renal function and facilitating reproduction. PGES, the rate limiting enzyme for PGE_2 production, has two isoforms termed as cytosolic PGES (cPGES) and membrane bound PGES (mPGES) (Helliwell *et al.,* 2004).

PGE_2 has a well-known role in stimulating myometrial contraction. It also plays a major function in tumor progression by promoting angiogenesis, cell migration, chronic inflammation and inhibition of apoptosis (Wang *et al.,* 2007; Marnett and DuBois, 2002). These roles for PGE_2 are mediated by distinct prostanoid receptors which are encoded by different genes. PGE_2 receptors are designated as EP1,

EP2, EP3 and EP4 receptors and signal through several secondary messengers. EP2 and EP4 receptors signal via increased levels of cAMP, EP1 signals via activation of calcium, while EP3 signals through calcium mobilization and reduction of cAMP (Fig 1.2) (Audoly and Breyer, 1997; Breyer *et al.*, 2001; Wang *et al.*, 2007).

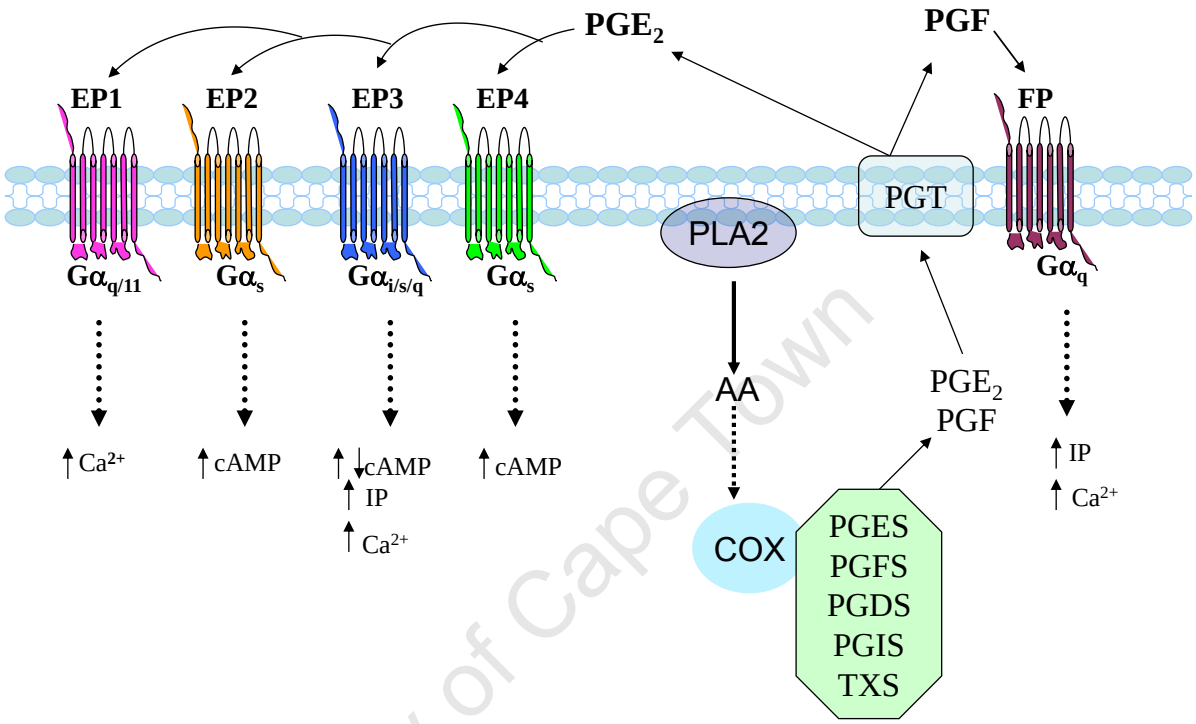

Fig. 1.2: *Schematic representation of COX-prostanoid biosynthetic and signalling pathway. Arachidonic acid (AA) gets released from plasma membrane by phospholipase A_2 (PLA_2) and acts as a substrate for COX enzymes for production of prostanoids. Once prostanoids are formed they get transported out of the cytoplasm by prostaglandin transporter (PGT) and act on their cognate receptors to activate numerous secondary messengers.*[2]

1.4.1.1 EP1 Receptor

The EP1 receptor was cloned and characterized in 1993 from a human erythroleukemia (HEL) cells (Funk *et al.*, 1993). The EP1 receptor cDNA encodes a protein of 402 amino acids and is highly expressed in the kidney, followed by gastric muscularis mucosae and adrenal tissue. The receptor was originally described to act as a smooth muscle constrictor (Breyer *et al.*, 2001).

[2] Diagram taken and modified from Sales and Jabbour, 2003

There are several agonists for the EP1 receptor with this order of affinity: PGE_2 > PGE_1 > $PGF_{2\alpha}$ >> PGD_2. However, these agonists also activate other prostanoid receptors. Selective agonists for the EP1 receptor include SC15089 or SC53122 and their use can facilitate the dissection of the individual receptor signalling characteristics (Narumiya *et al.,* 1999; Breyer *et al.,* 2001).

Activation of the EP1 receptor by PGE_2 leads to release of intracellular calcium in Chinese hamster ovary (CHO) cells. This activation is dependent on extracellular calcium without detectable release of inositol-1,4,5-triphosphate (IP3), suggesting the receptor might regulate a calcium channel gating via an unidentified G protein coupling (Watabe *et al.,* 1993). Another study using the phospholipase C (PLC) inhibitor (U73122) showed suppression of the intracellular calcium release without affecting the sustained phase of Ca^{2+} influx from extracellular space suggesting the presence of receptor-activated Ca^{2+} channel (RACC)-related response following the activation of the EP1 receptor (Katoh *et al.,* 1995). A study done on EP1 receptor stably expressing *Xenopus laevis oocytes* showed Ca^{2+} mobilization was dependent on EP1 receptor binding to $G\alpha_{q/11}$ and transient receptor potential-5 (TRP5), the latter uniquely activating the extracellular calcium influx. These data show EP1 receptor can couple to $G\alpha_{q/11}$ and TRP5 to activate Ca^{2+} mobilization from the intracellular stores via PLC activation and to trigger Ca^{2+} influx from extracellular space, respectively (Tabata *et al.,* 2002).

1.4.1.2 EP2 Receptor

The first EP2 receptor to be cloned by Bastien *et al.* (1994) was later found to be the EP4 receptor by Nishigaki *et al.* (1995). The authentic EP2 receptor was cloned from human placenta library by Regan *et al.* (1994b) and the cDNA encodes a polypeptide of 358 amino acids. The receptor is abundantly expressed in the uterus, lung and spleen but has low expression in the kidney (Breyer *et al.,* 2001).

The EP2 receptor was first characterized in the lung with relaxant/inhibitory effect on smooth muscle cells in cat trachea. The authors showed that a selective EP2 receptor agonist, TR4979 (later called Butaprost), can mediate relaxation of trachea via

the activation of the receptor (Gardiner, 1986). There are several agonists for the EP2 receptor in this order of affinity for the receptor. PGE_2 > 11-deoxy- PGE_2 > Butaprost > AH13205 = 19 R OH PGE_2 >> sulprostone and PGE_1-OH (Kiriyama *et al.,* 1997; Breyer *et al.,* 2001). A recent study on osteopenic rat model has reported a new selective agonist for the EP2 receptor (CP-533536) that promotes bone formation and improves fracture healing in rat models (Cameron *et al.,* 2009). The EP2 receptor is a $G\alpha_s$-coupled receptor and following activation by PGE_2, it increases intracellular cAMP accumulation. cAMP is produced from adenosine triphosphate (ATP) and this reaction is catalysed by the enzymatic activity of members of the adenylyl cyclase family (Regan, 2003).

EP2 receptor has the same pharmacological and functional coupling as the EP4 receptor despite only sharing 38% identity in the transmembrane region. This similarity is not appreciably different from the other EP receptors (Toh *et al.,* 1995). However, several studies have shown that PGE_2 activation of the EP2 receptors generates a higher accumulation of cAMP in comparison to the release by PGE_2-activated EP4 receptors despite the cells having a comparable level of receptor expression. This is probably dependent in the different downstream signalling pathway they use upon PGE_2 activation (Regan, 2003).

Following activation of the EP2 receptor by PGE_2, the downstream signalling is mostly through Protein Kinase A (PKA) activation via the Wnt signalling pathway suggesting it is predominantly cAMP mediated. This involves phosphorylation of glycogen synthase kinase-3 (GSK-3) by Akt to activate β-catenin-mediated transcriptional activation. However, Fujino *et al.* (2003) showed the EP4 receptor pathway primarily uses the phosphatidylinositol 3-kinase (PI3K) and the extracellular signal-regulated kinase (ERK) pathway to activate the β-catenin-mediated transcriptional activation in addition to partial activation of the cAMP pathway. The same group showed EP2 receptor activation does not activate the ERK pathway (Fujino *et al.,* 2002). In contrary, studies in our laboratory using stably expressing EP2 receptor endometrial adenocarcinoma (Ishikawa) cells showed activation of the receptor can lead to the phosphorylation of ERK1/2 via cAMP production and c-Src-

mediated phosphorylation of the epidermal growth factor receptor (EGFR) (Sales *et al.*, 2004).

The other major difference between the EP2 and EP4 receptor is that the latter undergoes rapid agonist-dependent desensitization and internalization, while the EP2 receptor does not (Nishigaki *et al.,* 1996). This was demonstrated in transfected CHO cells, where the EP4 receptor underwent short term desensitization while the EP2 receptor did not. In addition, PGE_2 treatment of transfected human embryonic kidney-293 (HEK-293) cells induced internalization of the EP4 receptor, while the EP2 receptor did not internalize (Desai *et al.*, 2000).

1.4.1.3 EP3 Receptor

The human EP3 receptor was cloned by Yang *et al.* (1994) from a human kidney library and the cDNA encodes a 367 amino acid polypeptide. The EP3 receptor was originally described as smooth muscle constrictor and is highly expressed in kidney, uterus, adrenal gland and stomach tissues (Breyer *et al.,* 2001). This receptor subtype is unique among the prostanoid family as it has multiple splice variant generated with the alternative splicing of the region coding for the C-terminal tail. The human EP3 receptor has at least six isoforms that are identical over the first 359 amino acids and differ in the C-terminus with the variation ranging from 6 to 65 amino acids (Regan *et al.*, 1994a; Pierce and Regan, 1998; Ashby, 1998; Narumiya *et al.,* 1999). All the EP3 splice variants bind PGE_2 but can also be activated by other agonists with this order of affinity to the receptor: Sulprostone = M&B28767 = PGE_2 > GR63799X > 17-phenyl-PGE_2 > misoprostol free acid = enprostil = carbacyclin > misoprostol methyl ester. Although not tested in the above studies, EP3 receptor has a selective agonist, SC-46275 that has been shown to selectively activate the receptor in several animal model cell lines (Savage *et al.*, 1993; Breyer *et al.,* 2001).

EP3 receptor isoforms vary in G protein specificity, receptor phosphorylation and desensitization and intracellular trafficking. These splice variants generally can couple to $G\alpha_i$ protein and inhibit cAMP generation. However, some splice variants can couple to other G proteins including $G\alpha_s$ to generate cAMP or $G\alpha_{q/11}$ to activate PLC-

mediated Ca^{2+} release that is appeared to be differentially regulated by their diverse C-terminal tails (An *et al.,* 1994; Asboth *et al.,* 1996; Breyer *et al.,* 2001).

1.4.1.4 EP4 Receptor

The EP4 receptor was initially cloned from a human cDNA lung library by Bastien *et al.* (1994) and was described as the EP2 receptor. However, subsequent studies showed the receptor does not have similar molecular characteristics compared to the EP2 receptor and was named the EP4 receptor (Nishigaki *et al.,* 1995).

The EP4 receptor protein is composed of 488 amino acids and is longer than the EP2 receptor, most of the difference coming from the carboxyl terminus of the EP4 receptor. This variation between the receptors may have an effect on agonist-dependent desensitization and internalization as the EP2 receptor is insensitive to this regulation as mentioned in section 1.4.1.2 (Nishigaki *et al.,* 1996; Desai *et al.,* 2000). The EP4 receptor is more abundantly and widely expressed than the EP2 receptor with a higher expression in thymus, ileum, lung, spleen, adrenal and kidney tissues (Bastien *et al.,* 1994; Breyer *et al.,* 2001).

The EP4 receptor has the following agonists in order of their affinity to the receptor; PGE_2 = PGE_1 > M&B-28767 > misoprostol-free acid >> iloprost > $PGF_{2\alpha}$ > PGD_2. ONO-AE1-329 agonist has a relative selective specificity for the EP4 receptor (Kiriyama *et al.,* 1997; Breyer *et al.,* 2001). PGE_2 activation of the EP4 receptor leads to coupling of the receptor to $G\alpha_s$ and activates different signalling pathways, the PKA-mediated cAMP pathway being one of them. However, the main signalling pathway is PI3K-dependent and it leads to either activation of the Akt pathway or phosphorylation of ERK to enhance expression of downstream target genes (Fujino *et al.,* 2003).

1.4.2 Prostaglandin $F_{2\alpha}$ (PGF) and PGF Receptor

Biosynthesis of PGF_2 occurs by the action of PGH 9-, 11-endoperoxide reductase, PGE 9-ketoreductase or PGD 11-ketoreductase on PGH_2, PGE_2 or PGD_2, respectively. PGD 11-ketoreductase has two isozymes termed as lung type PGFS (PGFS I) and liver type PGFS (PGFS II) both responsible for the production of $PGF_{2\alpha}$ and 9α-11β-PGF_2. $PGF_{2\alpha}$, together with PGE_2 is called a primary prostaglandin and this review will refer it as PGF (Sakamoto *et al.,* 1995; Watanabe, 2002; Helliwell *et al.,* 2004).

PGF is a group of hormone-like substances that participates in body functions such as smooth muscle contraction and relaxation, dilation and constriction of blood vessels, blood control pressure and regulation of inflammation. PGF has also a well established role in parturition, induction of myometrial contraction and cervical relaxation (Helliwell *et al.,* 2004; Wang *et al*., 2007). A pharmaceutical equivalent of PGF, dinoprost, can be used for the induction of abortion and for treatment of hydatidiform mole. The role of PGF is mediated by a distinct prostanoid receptor named FP receptor (Wang *et al.,* 2007).

1.4.2.1 FP Receptor

The human FP receptor was cloned by Abramovitz *et al*. (1994) from uterus cDNA library and encodes a protein of 359 amino acid residues. The FP receptor endogenous ligand is PGF but it can be activated with the following agonists in order of this potency: PGF = fluprostenol > PGD_2 > PGE_2 > U-46619 > iloprost (Narumiya *et al.,* 1999; Breyer *et al.,* 2001).

Several splice variants have been reported for the FP receptor in different species. Vielhauer *et al.* (2004) reported the first human FP receptor variant (hFP_s) with 71bp exon insertion that created a frame shift mutation resulting in a truncated receptor lacking the seventh transmembrane domain. Expression studies using northern blots showed the mRNA for the hFP_s was present in human heart and skeletal muscle. However, a biochemical function of hFP_s could not be established as the receptor did

not bind with any significant affinity to the endogenous FP receptor ligand PGF (Vielhauer *et al.,* 2004).

Recent studies showed six splice variants of the human FP receptor, each one created by an insertion of a different exon between the existing exon 2 and 3. These splice variants lack the seventh transmembrane domain and their carboxyl terminal was extracellular and the receptors were not functional on their own. The authors showed that one of the splice variants (altFP4) can form a heterodimer with the wild type FP receptor. The heterodimer was different biochemically compared to the altFP4 as it responded to PGF treatment in identical manner with the wild type FP receptor. However, the heterodimer was highly responsive to the prostamide analogue, bimatoprost, compared to the wild type FP receptor. bimatoprost activation of the heterodimer expressing HEK-293 cell line gave a rapid transient Ca^{2+} release followed by secondary Ca^{2+} waves, the latter being Ca^{2+} influx from extracellular space. A specific prostamide antagonist, AGN211335 inhibited the secondary phase of Ca^{2+} signalling. As the prostamide receptor has yet to be cloned and characterized there is a possibility that the heterodimer may represent the putative prostamide receptor (Liang *et al.,* 2008).

FP receptor, a $G\alpha_{q/11}$-coupled receptor, triggers the release of IP3 via the PLC pathway following activation by PGF (Watanabe *et al.,* 1994). Studies in our laboratory using FP receptor stably expressing Ishikawa cells have shown the receptor can also activate the ERK pathway via transactivation of the EGFR (Sales *et al.,* 2005).

Thomas *et al.* (1978) showed co-stimulation of rat luteal cells with LH and PGF inhibited LH-mediated progesterone secretion despite PGF itself being able to stimulate progesterone production in these cells. This inhibition was reversed by addition of dibutyryl cAMP indicating that progesterone secretion inhibition was due to a blockage of cAMP production. These data might suggest PGF can inhibit cAMP production although the mechanism is poorly understood.

1.4.3 Other Prostanoids and Their Cognate Receptors

Prostaglandin D_2 (PGD_2), a PGD synthase (PGDS) product, is a major prostanoid produced in central nervous systems (CNS) in humans. PGD_2 is important for sedation, sleep induction and regulation of body temperature, all mediated by coupling to its cognate DP receptor (Urade and Hayaishi, 1999). The DP receptor was the most recent prostanoid receptor to be cloned and it is expressed in leptomeninges, retina and mucus-secreting cells of the gastrointestinal tract. DP receptor activation by PGD_2 leads to accumulation of intercellular cAMP and mobilization of Ca^{2+} (Boie *et al.*, 1995; Breyer *et al.*, 2001).

Prostacyclin (PGI_2) was discovered in 1976 when it was found that patients with inherited deficiency of PGI_2 production tend to develop thrombophilia and were predisposed to vascular diseases (Wang *et al.*, 2007). PGI_2 synthesis is facilitated by prostacyclin synthase (PGIS) and is involved in numerous body functions that include antithrombosis and vasodilator actions via interaction with its cell surface IP receptor (Helliwell *et al.*, 2004; Breyer *et al.*, 2001). The IP receptor is widely expressed in the dorsal root ganglia suggesting the receptor plays a role in mediation of pain. The receptor is also expressed in aorta, heart, kidney, lung and liver. The IP receptor has a higher affinity to PGE_1 than PGE_2 and is selectively activated by the agonist cicaprost that leads to stimulation of cAMP production (Breyer *et al.*, 2001).

Thromboxane A_2 (TXA_2) synthesis is catalyzed by thromboxane synthase (TXS) and primarily regulates platelet shape change and aggregation and as such has a critical role in blood clotting. In addition it mediates smooth muscle contraction and proliferation. The receptor for TXA_2 was the first eicosanoid family receptor member to be cloned and it has two isoforms designated as TPα and TPβ, differing exclusively in their C-terminus amino acid sequence (Breyer *et al.*, 2001). These TP receptors are abundantly expressed in the thymus followed by lung, kidney and spleen with lower levels in uterus, brain and heart. TPα and TPβ receptors couple to $G\alpha_{q/11}$ and activate the intracellular calcium/diacylglycerol (DAG) molecular pathway (Breyer *et al.*, 2001). Hirata *et al.* (1996) showed TPβ receptors can inhibit adenylyl cyclase by coupling to G_i/G_o, while TPα receptors can activate cAMP by coupling to $G\alpha_s$ protein in CHO cells.

1.4.4 General Physiological Functions of Prostanoids

Prostanoids and their cognate receptors have numerous physiological functions in the body. These physiological roles have been well studied by either pharmacologically inhibiting their action through agents such as NSAIDs or by gene-knockout mice studies (Kobayashi and Narumiya, 2002). The next three sections will discuss the role for prostanoids in allergy, inflammation and pain, vascular hemostasis and reproductive functions.

1.4.4.1 Prostaglandins in Allergy, Inflammation and Pain

Allergic asthma is caused by expression of T_h2 cytokines in lung and is characterized by infiltration of eosinophils and bronchial hyper-reactivity. Mast cells play a role in triggering this disease since they are activated by an immunoglobulin (Ig) E-mediated allergic challenge. PGD_2 is one of the chemical mediators that is released by activated mast cells, and has been proposed to be a marker of mast cell activation (Kobayashi and Narumiya, 2002). The role for PGD_2 in asthma was well characterized by developing DP receptor deficient mice. Using these mice models, the authors proposed that PGD_2 released in response to allergic challenge can act on its cognate receptor DP to recruit lymphocytes to the site of allergy (Matsuoka *et al.*, 2000).

Studies have shown prostanoids can be mediators of vasodilation and inflammatory process by synergizing with other effectors such as histamine and bradykinin to increase vascular permeability and edema. These effects are mostly mediated by PGE_2 and PGI_2 since both prostanoids have been shown to be elevated in sites of inflammation (Kobayashi and Narumiya, 2002). The role for IP receptor in inflammation and pain has been shown using specific knockout mice studies. In IP receptor null mice, acetic acid-induced writhing was reduced to the level observed in indomethacin-treated wild-type mice (Murata *et al.*, 1997; Bley *et al.*, 1998). The same pattern of reduction in writhing was observed in LPS-treated EP3 receptor knockout mice demonstrating the role of this PGE_2 receptor in pain and inflammation (Kobayashi and Narumiya, 2002).

Studies using antagonists for E prostanoid receptors in rodents have shown a role for these receptors in hyperalgesia (response to stimuli that is painful). PGE_2-EP2 receptor signalling has been shown to be involved in generation of inflammatory pain in the zymosan A (a yeast extract that induces inflammatory hyperalgesia) peripheral inflammation model. In this study, zymosan A-treated EP2 receptor null mice recovered much faster from hyperalgesia compared to wild type mice demonstrating the role for EP2 receptor in inflammation and pain (Matsuoka and Narumiya, 2007).

Zymosan-treated peritoneal macrophages derived from IP, EP2 and EP4 receptors null mice were used to examine the effect of PGE_2 and carbacyclin (PGI_2 analogue) on tumor necrosis factor-α (TNF-α) and IL-10 production. Macrophages collected from IP null mice showed a down-regulation of TNF-α production and up-regulation of IL-10 in response to PGE_2 but not to carbacyclin, while macrophages derived from EP2 and EP4 null mice lacked the response to the specific agonists to the EP2 and EP4 receptors, but not to PGE_2 or carbacyclin. These data demonstrate that PGE_2 and PGI_2 regulate pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokine production redundantly by coupling to their cognate EP2, EP4 and IP receptors, respectively via cAMP accumulation (Shinomiya *et al.,* 2001; Kobayashi and Narumiya, 2002).

1.4.4.2 Prostanoids in Vascular Hemostasis

It is well known that most prostanoids elicit contractile and/or relaxant activities on vascular smooth muscle cells. Thromboxanes and prostacyclins are produced abundantly in vascular endothelial cells and platelets and have an opposing action by acting as vasoconstrictor and vasodilator agents, respectively (Kobayashi and Narumiya, 2002; Matsuoka and Narumiya, 2007). IP receptor knockout mice show an enhanced thrombotic tendency when endothelial damage is evoked showing the antithrombotic effect of prostacyclin (Murata *et al*., 1997). In contrast, TP receptor deficient mice show increased bleeding tendencies and are resistant to TP agonist-mediated cardiovascular shock (Thomas *et al.,* 1998; Kobayashi and Narumiya, 2002).

PGE_2 has been implicated in contractile and/or relaxant functions on vascular smooth muscle cells. Kennedy *et al.* (1999) studied the role for EP2 receptor in regulating blood pressure using EP2 receptor null mice. The authors reported that activation of the EP2 receptor by Butaprost induces a hypotensive response in wild type mice while this effect is not observed in EP2 receptor null mice. This result demonstrates PGE_2 elicit arterial dilatation via interaction with its EP2 receptor.

1.4.4.3 Prostanoids in Reproductive Functions

Prostanoids have been shown to play major roles in reproduction functions such as in ovulation, fertilization, luteolysis and parturition. Studies have shown that EP1, EP3, EP4, IP and TP receptors null female mice are fertile demonstrating these prostanoid receptors are dispensable in reproductive physiology. However, functional studies have shown that the EP2 receptor plays an important role in reproductive physiology. Kennedy *et al.* (1999) showed EP2 receptor deficient mice had a reduced litter size and deliver few pups compared to wild type mice. The authors also reported impaired ovulation and a dramatic decrease in fertilization in EP2 receptor deficient mice. Hizaki *et al.* (1999) reported that EP2 null female mice are normal and can give normal birth to their pups in term, but their litter size is significantly smaller. Only very few pups are born alive and grow normally, showing the importance of the EP2 receptor in pregnancy. The authors proposed that the interaction between PGE_2 and EP2 receptors can induce the oophorus maturation that is required for successful fertilization.

Lim and Dey (1997) showed EP2 receptor expression in a temporal manner during the pre-implantation period by the effect of ovarian hormones, progesterone and estrogen. The authors showed expression of the EP2 receptor mRNA in the mouse luminal epithelium on day 3 of pregnancy and a significant increase of the receptor on day 4 where both progesterone and estrogen regulate epithelial cell differentiation for the event of implantation.

In addition, PGF plays a role in ovulation, luteolysis and parturition. Sugimoto *et al.* (1997) showed FP receptor deficient female mice were healthy and were able to get pregnant. However, no parturition was found in these mice due to the lack of induction of labor even when given exogenous oxytocin. A reduction in progesterone by ovariectomy in FP receptor deficient mice resulted in normal delivery of their pups. These data demonstrate that FP receptor plays a key role in parturition by diminishing progesterone level to induce labor (Sugimoto *et al*., 1997).

1.4.5 Prostanoid Receptors in Carcinogenesis

Prostaglandins and their cognate receptors have been implicated in numerous cancers. Several studies have reported NSAIDs can prevent incidence of colorectal cancer. Giardiello *et al.* (1998) evaluated the expression of prostanoids in human colorectal mucosa and showed an increase in PGE_2 and thromboxane production compared to control samples. Treatment of patients with sulindac (NSAID) decreased the concentration of PGF, PGE_2 and PGD_2 compared to healthy control subjects. The decrease in prostaglandins production was correlated with the reduction of colorectal adenomas. A recent study using colonic epithelial cell lines showed an increase in motility in response to PGE_2 and PGF, suggesting a role for prostaglandin in the progression of adenoma by increasing cell motility (Qualtrough *et al.,* 2007). Dysregulation of the EP4 receptor has been implicated in colon cancer. Azoxymethane (a colon carcinogen)-treated EP4 knockout mice showed a decrease in development of putative preneoplastic lesions in the colon compared to wild type mice. The authors confirmed involvement of the EP4 receptor in colon cancer by using an EP4-selective antagonist (ONO-AE2-227) to decrease the number of putative preneoplastic lesions in azoxymethane-treated mice (Mutoh *et al.,* 2002). Others have suggested that EP4 receptors might be involved in the early stages of colon cancer, while EP2 receptors are more important in facilitating the cancer growth (Kawamori *et al.,* 2003; Fujino *et al.,* 2007).

Ovarian cancer has been associated with increased synthesis of prostaglandins and their receptors. Rask *et al.* (2006) showed up-regulation of EP1 and EP2 receptors in malignant ovarian tumors compared to benign tumors. Expression of both receptors

was distributed in ovarian surface epithelial cells suggesting a role for these receptors in malignant transformation of epithelial cells. Spinella *et al.* (2004) used human ovarian carcinoma cells to show that endothelin-1 can induce PGE_2, PGF and PGD_2 production and EP2 and EP4 prostanoid receptor expression via transactivation of EGFR. This activation increases matrix metalloproteinases (MMPs) and VEGF expression leading to ovarian cancer cell invasion and tumor progression.

Prostanoid receptor up-regulation has been implicated in prostate cancer. Miyata *et al.* (2006) showed an up-regulation of EP1, EP2 and EP4 prostanoid receptors in cancer cells compared to non-tumor gland cells. In addition, EP1 and EP2 receptors were associated with increase in cancer cell proliferation suggesting a role for these EP subtype receptors in prostate cancer. *In vitro* studies by Wang and Klein (2007) using PGE_2-stimulated prostate cancer cell line (PC-3) showed a significant induction of VEGF production via the ERK/Akt signalling pathway mediated by the EP2 receptor. Another *in vitro* study looked at the aldo-keto reductase (AKR) family members that are responsible for biosynthesis of PGF from PGD_2. The authors showed that PGD_2 stimulation of AKR expressing PC-3 cells can induce cell proliferation that is mediated by increase in PGF accumulation in the culture system. This cell proliferation was mediated by PI3K/Akt signalling pathway and was significantly decreased by co-stimulation of PGD_2 and the FP antagonist AL8810. These data show the key role of PGF-FP receptor interaction in mediation of cell proliferation in prostate cancer cells (Wang *et al.*, 2008).

Up-regulation of COX enzymes and prostaglandin receptors play a significant role in endometrial pathology (Jabbour *et al.*, 2006). Before discussing the implication of prostanoids in endometrium pathology it is useful to describe the molecular make up and physiological activities of the endometrium.

1.5 Structure and Function of the Endometrium

The human endometrium is a complex tissue that lines the uterine cavity and is composed of both glandular and stromal elements. This dynamic tissue undergoes well-defined cycles of tissue proliferation, differentiation and breakdown. In the absence of pregnancy the endometrium is shed and regenerates itself again. This cyclical nature of tissue breakdown and remodelling is called the menstrual cycle. The endometrium consists of two layers, the upper layer is the one that sheds at menstruation and the tissue regenerates itself from the lower layer of the endometrium (Critchley *et al.,* 2001; Jabbour *et al.,* 2006a). Earlier studies showed that ovarian hormones, estrogen and progesterone are responsible for the changes of endometrial structure and functions throughout the menstrual cycle (Markee, 1940; Corner and Allen, 1929; Jabbour *et al.,* 2006a).

The menstrual cycle has three classic phases: A proliferative phase that is dominated by estrogen, a secretory phase that is dominated by progesterone and a menstrual phase following the withdrawal of progesterone and is associated with the termination of the corpus luteum. The secretory phase is then divided in two parts. Early secretory phase is regulated by both estrogen and progesterone, while the mid secretory phase is only regulated by progesterone (Jabbour *et al.,* 2006a). This has been suggested by observed down-regulation of the estrogen receptor in the mid secretory phase (Snijders *et al.,* 1992)**.** Estrogen plays a major role in promoting the proliferation while progesterone controls the differentiation of the glandular and stromal components (Jabbour *et al.,* 2006a).

For successful menstruation blood vessels are very essential. It has been shown that there are spiral forms of the arterioles in the upper two third of the functional layer that are responsible for leukocyte entry and vasoconstriction. The initiation of menstruation also consists of a higher expression of various MMPs that would help break down the basement membrane comprised of collagen type 4, fibronectin and glycosaminoglycans in response to progesterone (Smith, 1998; Osteen *et al.,* 1994; Jabbour *et al.,* 2006a). Another feature of the menstrual cycle involves migration of the

endothelial cells in the matrix to promote menstruation (Smith, 1998; Rogers *et al.*, 1992).

The endometrial proliferation, differentiation and breakdown require remodelling of endometrial vasculature. VEGF-A plays an essential role in facilitation of new blood vessel formation and triggers proliferation, differentiation and migration of the endometrium**.** Knockout mice studies have shown the binding of VEGF to VEGF-receptor 2 (KDR) can trigger endothelial cell recruitment and proliferation, while interaction with VEGF-receptor 1 (Flt-1) can induce endothelial cells to form tubules with the induction of tight junctions between the endothelial cells (Shalaby *et al.*, 1995; Fong *et al.*, 1995; Smith, 1998). The angiopoietins, Ang-1 and Ang-2, have been shown to facilitate angiogenesis by stabilising and destabilising blood vessels, respectively (Maisonpierre *et al.*, 1997). Other angiogenic growth factors including basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), thrombospondin-1 and endocrine gland VEGF (EG-VEGF) and their cognate receptors are expressed in endometrium, and can stimulate angiogenesis (Smith, 1998; Battersby *et al.*, 2004).

1.5.1 Roles for COX Enzymes and Prostanoid Receptors in Endometrium Physiology

Towards the end of the menstrual cycle in the event of progesterone withdrawal, expression of COX-2 has been reported to be up-regulated in endometrial glandular epithelium cells and lasts through the proliferative phase. Kang *et al.* (2005) showed a higher expression of the human prostaglandin transporter in the luminal, glandular epithelial and stromal cells during the menstrual cycle. This was accompanied by expression of EP2 and EP4 receptors throughout the stages of menstrual cycle, the latter being highly expressed in the late proliferative phase. The expression of the EP2 and EP4 receptors was localized to the peri-vascular and endothelial cells showing the significance of these receptors in vascular function and angiogenesis (Jabbour *et al.*, 2006b; Milne *et al.*, 2001). Milne and Jabbour (2003) have reported expression of the FP receptor throughout the menstrual cycle with higher expression in the mid-late proliferative phase and localizing predominantly to

glandular epithelial cells. The expression of COX-2 and the prostanoid receptors can in turn promote the expression of pro-angiogenic factors (VEGF, bFGF, PDGF, Ang-1 and Ang-2) or inhibit cathepsin-D, which in turn contributes to inhibition of anti-angiogenic factor angiostatin (Perchick and Jabbour, 2003; Jabbour *et al.*, 2006b).

There have been several benign endometrial pathologies reported with clinical symptoms of pain or bleeding disturbances, such as dysmenorrhoea, endometriosis and menorrhagia. COX enzymes and prostaglandins have been implicated in these benign endometrial pathologies. However, the most frequently diagnosed and common malignancy is endometrial carcinoma (Jabbour *et al.*, 2006b).

1.6 Endometrial Carcinoma

Endometrial carcinoma is one of the two most common malignancies of the female genital tract in the western world, also ranking fourth in incidence among invasive tumors in women following breast, lung and colon cancers. Carcinoma of the endometrium is more common in post-menopausal women and approximately 80% of the patients are over 50 years of age. Most women who undergo hysterectomy to treat the non-invasive precursors of endometrial carcinoma will experience morbidity related to endometrial neoplasia. The first symptom of endometrial carcinoma is abnormal vaginal bleeding and overall, patients have an 86% five-year survival rate after diagnosis (Jabbour *et al.*, 2006b; Ellenson and Wu, 2004).

Endometrial carcinoma arises from several types of cells, with endometrial cancer from the glandular epithelium being the most common type accounting for 80-90% of the endometrial cancers. Endometrial carcinoma can be divided in two major types termed as type I and II. Type I is generally associated with clinical findings such as obesity, diabetes and hypertension. The tumors, usually confined to the uterus, are low-grade (well to moderately differentiated) and indolent tumors often associated with endometrial hyperplasia. Histology of type I tumors usually resemble normal proliferative endometrial glands (Fig 1.3). These tumors are usually treated with surgery but if left untreated they can progress to endometrial carcinoma. This group

consists of well and moderately differentiated carcinoma according to histological classification (Kurman *et al.*, 1985; Ellenson and Wu, 2004; Di Cristofano and Ellenson, 2007).

Type II tumors are poorly differentiated tumors that are aggressive in manner and are not associated with unopposed estrogen. The histology of this tumor can be classified into two types of carcinoma termed as serous carcinoma and endometrial intraepithelial carcinoma (EIC). Serous carcinoma consists of highly atypical cells that can grow in papillary, glandular or solid patterns while EIC, precursor of serous carcinoma, is composed of similar cells to its precursor carcinoma but do not invade the underlying stroma (Ellenson and Wu, 2004; Di Cristofano and Ellenson, 2007).

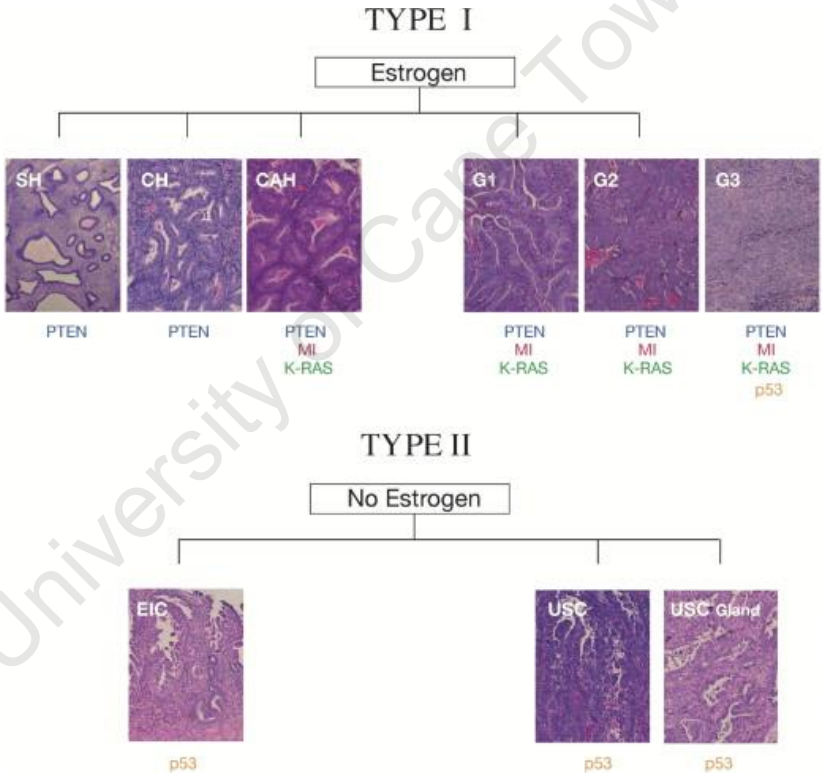

Fig. 1.3: *Histological classification of endometrial cancer: The invasive tumors are shown on the right while the precursors are on the left. The genetic alterations associated with the lesions are shown below each photomicrograph. SH, simple hyperplasia; CH, complex hyperplasia; CAH, complex atypical hyperplasia; G1-G3, Grade 1-3 endometroid carcinoma; EIC, endometrial intraepithelial carcinoma; USC, papillary form of serous carcinoma; PTEN, phosphatase and tensin homolog on chromosome ten. Type I tumors are associated with hyper-estrogenic while type II are not associated with estrogen stimulation (Picture taken from Ellenson and Wu, 2004).*

1.6.1 Etiology of Endometrial Carcinoma

The etiology of endometrial carcinoma is yet to be elucidated. However, over the last decade several theories have been postulated using gene knockout and over-expression systems to provide evidence supporting the involvement of several genes (Ellenson and Wu, 2004). One of the genes that are highly related to endometrial carcinoma is the *phosphatase and tensin homolog on chromosome ten* (*PTEN*) tumor suppressor gene. This gene is the most altered gene in type I endometrial carcinoma and 50% of the tumors contain mutations in this gene (Tashiro *et al.,* 1997). Other studies have shown a correlation between mutations in *PTEN* gene and hyperplastic lesions (20%) suggesting a key role for this gene in early stages of the cancer (Levine *et al.,* 1998; Ellenson and Wu, 2004).

The other gene associated with endometrial cancer is *p53* tumor suppresser gene. Mutations in *p53* gene account for approximately 10-20% of all endometrial carcinoma with higher mutation frequency reported in high-grade tumors (Lax *et al.,* 2000). The third oncogene associated with endometrial cancer, k-ras, has been shown to be mutated in 10-30% of the cases and mutations have been found in all grades of endometrial carcinoma (Ellenson and Wu, 2004).

Once the tumor is formed numerous growth factors play a major role in promoting its growth and the COX-prostanoid biosynthetic pathway play an active role in this process.

1.6.2 Roles for COX Enzymes and Prostanoid Receptors in Endometrial Carcinoma

Several studies have been done to assess the expression of COX enzymes and prostanoid receptors in endometrial carcinoma. COX-1 enzyme has been reported to be unaltered in endometrial carcinoma compared to normal endometrium despite being reported to be involved in other uterine pathologies (Gupta *et al.,* 2003; Tong *et al.,* 2000; Jabbour *et al.,* 2006b). However, COX-2 has been shown to be up-regulated in the glandular epithelium of proliferative endometrium in the cancer cells of human

endometrial carcinoma compared to normal endometrium. It has been shown to be expressed in hyperplastic lesions suggesting the enzyme might be involved in early pathology and progression of endometrial cancer (Ohno *et al.*, 2005; Tong *et al.*, 2000).

Studies in our laboratory showed over-expression of COX-2 and PGES enzyme that co-localized to endothelial cells and neoplastic epithelial cells in endometrial adenocarcinoma suggesting co-regulation of both enzymes. Concomitantly with the elevation of COX-2 and PGES expression, the authors showed up-regulation of the EP2 and EP4 prostaglandin receptors in secretory phase of adenocarcinoma compared with control endometrium samples. These results suggest that COX-2 and PGES enhancement can lead to increased production of PGE_2 to enhance proliferation of epithelial cells in autocrine/paracrine manner via the activation of the EP2 and EP4 receptors (Jabbour *et al.*, 2001). Moreover, other studies in our laboratory have reported dysregulation of the FP receptor in endometrial adenocarcinoma. The authors showed an up-regulation of the FP receptor in human endometrial adenocarcinoma that was localized to the neoplastic glandular epithelial cells (Sales *et al.*, 2004).

Up-regulation of COX-2, prostaglandins and their cognate receptors can influence the growth of endometrial adenocarcinoma by promoting angiogenesis, inhibition of apoptosis, control of cell growth and promoting metastasis in autocrine/paracrine manner (Ohno *et al.*, 2005).

1.6.2.1 Angiogenesis

Angiogenesis is very essential for tumor growth in endometrial carcinoma since the tumor needs oxygen and nutrients to facilitate growth. COX-2 induces tumor angiogenesis through numerous pathways that are regulated by the synthesized prostanoids. The angiogenesis process is initiated by PGE_2- and PGF-mediated production of angiogenic growth factors from tumor, stromal and infiltrating inflammatory cells (Ohno *et al.*, 2005). For example, studies in our laboratory have shown that PGE_2-EP2 receptors and PGF-FP receptors interactions can lead to transphosphorylation of the EGFR to promote expression of VEGF in Ishikawa cells

(Sales *et al.,* 2004; Sales *et al*., 2005). VEGF and other angiogenic growth factors then bind to endothelial cell surface receptors that are expressed on pre-existing venules, activating intracellular signalling pathways. One of these signalling pathways results in release of PGI_2 that regulates VEGF-induced vascular permeability (Murohara *et al.,* 1998). In addition, proteases secreted from proliferating endothelial cells locally degrade the vascular basement membrane, followed by vascular sprouting. The migration of these developing vessels is mediated by integrins at the endothelial-extracellular matrix interface and by release of MMPs at the sprouting vascular front. TXA_2 plays a major role in migration of endothelial cells and PGE_2 has been shown to interact with its cognate receptors to promote MMP-2 and MMP-9 expression. This angiogenic response is sustained by microvascular endothelial cells over-expressing the anti-apoptotic protein Bcl-2 regulated by COX-2 and PGE_2 (Ohno *et al.,* 2005). These data show the COX-2-prostanoid pathway promotes angiogenesis via synthesis of various prostaglandins and their receptors to sustain tumor growth.

1.6.2.2 Inhibition of Apoptosis

Sustainment of tumor growth vastly depends on inhibition of apoptosis. COX-2 has been shown to inhibit the apoptosis pathway in many cell types and this phenomenon can be reversed by the administration of NSAIDs (Ohno *et al.,* 2005). St-Germain *et al*. (2004) used different human endometrial cancer cell lines to show the involvement of COX-2 in inhibition of apoptosis. The authors showed that COX-2 inhibition with NS-398 in endometrial cancer cells resulted in the inhibition of Akt phosphorylation and induction of apoptosis suggesting that the COX-2 pathway plays a key role in control of cell survival. Ohno *et al.* (2007) used 70 endometrial cancer cases to study the role of COX-2 in inhibition of apoptosis. The authors showed that apoptotic index was lower in COX-2 positive cancer cells compared to COX-2 negative cases. Other studies have shown up-regulation of prostaglandin products, which in turn inhibit apoptosis through Bcl-2 mediated pathway and by the nitric oxide pathway (Ohno *et al.,* 2005). Taking these data in account, up-regulation of Cox-2 can facilitate tumor growth in endometrial carcinoma by inhibiting apoptosis.

1.6.2.3 Control of Cell Growth and Metastasis

The prostanoid products of the COX-2 pathway exacerbate tumorigenesis in endometrial adenocarcinoma by enhancing cell proliferation and migration. *In vitro* studies using Ishikawa cells as a model showed over-expression and activation of FP receptor can induce the expression and secretion of fibroblast growth factor-2 (FGF-2) via the ERK pathway. In addition the authors showed that the secreted FGF-2 can enhance proliferation of Ishikawa cells (Sales *et al.,* 2007). Further studies by the same group showed that activation of prostanoid receptors can modulate cell adhesion and invasion. The authors reported FP receptor activation by PGF can lead to adhesion to vitronectin and reorganization of the actin cytoskeleton via the recruitment of focal adhesion kinase (FAK) to focal adhesion sites. These data suggest a role for PGF-FP interaction in regulating cell movement metastasis and adhesion in endometrial carcinoma (Sales *et al.,* 2008).

Recent studies showed that leptin, a secreted hormone with role in obesity has also a role in the enhancement of cell proliferation in six endometrial adenocarcinoma cell lines (Ishikawa, ECC-1, HEC-1A, HEC-1B, RL95-2 and AN3CA cells). The authors showed that leptin can activate Janus-activated kinase-2 (JAK2)/signal transducers and activators of transcription 3 (STAT3) and the mitogen-activated protein kinase (MAPK) pathway to regulate expression of COX-2 and PGE_2 production in different endometrial cancer cell lines. This signalling pathway can in turn activate cell proliferation in the cell lines (Gao *et al.,* 2009).

The above mentioned data suggest that prostaglandins synthesized from the COX-2 pathway play a major role in development of endometrial carcinoma by acting on their cognate receptors to promote angiogenesis, inhibit apoptosis, and facilitate cell adhesion and migration. The endometrium can also be affected by the presence of other tumors arising from underlying or surrounding tissue such as uterine fibroids.

1.7 Uterine Fibroids

Uterine fibroids (leiomyomas) are benign tumors that arise from the smooth-muscle uterine cells (myometrium) and are the most common uterine disorder occurring in as many as 30% of women over 35 years of age. The prevalence of fibroids is two to three times more common in black women than among white women in United States of America (USA) and with a higher incidence with increasing age (Marshall *et al.*, 1997; Flake *et al.*, 2003).

Most cases of fibroids are asymptomatic but some women have significant symptoms warranting therapy. The clinical importance of uterine fibroids is shown by the fact that there are no adequate drugs for therapy and most women undergo hysterectomy for this disorder, accounting for approximately one-third of hysterectomies in USA (Wilcox *et al*., 1994).

The symptoms of uterine fibroids are related to their local mass effect and can be classified in three groups (Stewart, 2001):

- Abnormal uterine bleeding (menorrhagia; heavy and prolonged menses),
- Pelvic pressure and severe pain (dysmenorrhea) and
- Reproductive dysfunction (infertility and repetitive pregnancy loss).

The location of the tumor is an important factor in determining bleeding symptoms. As shown in figure 1.4, the three locations of fibroids are submucosal (SM), intramural (IM) and subserosal (SS) fibroids with most women presenting with a mixed location of fibroids. Submucous fibroids are contained in the uterine cavity and intrude to the endometrial cavity and are associated with menorrhagia, while intramural fibroids are contained within the wall of the uterus with no cavity deformity. Subserosal fibroids extend to the serosal surface and a cause of irregular feel of fibroids (Bajekal and Li, 2000; Stewart, 2001).

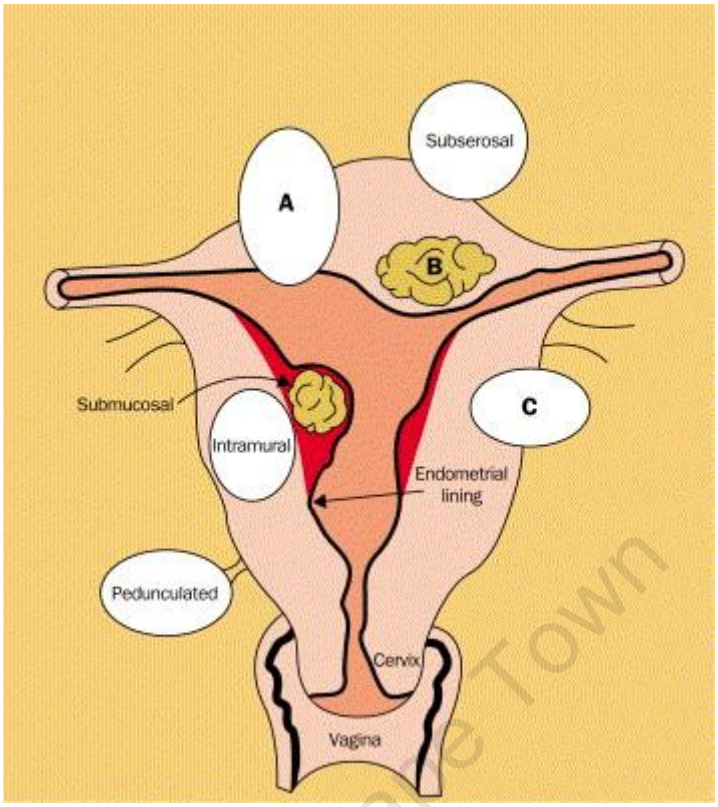

Fig. 1.4: *Different locations of uterine fibroids. A. Submucosal B. Intramural C. Subserosal fibroids*[3].

1.7.1 Risk Factors Associated with Fibroids

Several risk factors have been associated with uterine fibroids, one of them being menarche. Marshall *et al.* (1998) have shown a positive association between an early age menarche and hysterectomy-confirmed fibroid diagnosis. The correlation with early menarche might be due to increased cell divisions of myometrium throughout the reproductive years resulting in increased chance of mutation in genes involved in cell proliferation (Marshall *et al.,* 1998).

One of the risk factors associated with fibroids inversely is parity, reports showing 20-40% reduced risk in uterine fibroids. The possibility for reduced risk with parity could be less time of exposure to unopposed estrogens while reduced fertility might be associated with a long term exposure to estrogens from anovulatory cycles. Other studies have shown reduced risk of fibroids associated with current smoking

[3] Picture taken from Stewart 2001

with 50% of reduction of uterine fibroids requiring surgery. A diminished risk of fibroids requiring surgery has been reported in post-menopausal women showing an inverse correlation with menopause. (Ross *et al*., 1986; Marshal *et al*., 1997; Samadi *et al*., 1996; Parazzini *et al.,* 1996; Flake *et al.*, 2003).

1.7.2 Etiology of Uterine Fibroids

The etiology of uterine fibroids is yet to be elucidated. Several theories have been postulated, one of them being an increased level of ovarian hormones (estrogen and progesterone) resulting in increased mitotic rate of smooth muscle cells that may cause somatic mutations resulting in progressive growth dysregulation (Rein, 2000). This theory is supported by the observation that early onset of menstruation and hormone replacement therapy at menopause increase the risk of symptomatic uterine fibroid while treatment with gonadotropin-releasing hormone (GnRH) analogue causes a remarkable reduction in tumor burden (Shaw, 1998). The hormonal responsiveness of fibroids has been demonstrated in vitro as cells derived from fibroids express estrogen and progesterone receptors and are responsive to steroid hormones (Howe *et al.*, 1995). Another possible theory is that fibroids may develop due to an abnormal myometrium. Studies have shown a significant increase of estrogen receptors in the myometrium of fibroid uteri (Richards & Tiltman, 1996).

Genetic background also plays a role in uterine fibroid incidence on the basis of familial and ethnic predilections. Several studies show an increased incidence of fibroids among the first degree relatives then among the general population, with black women more prone to the disorder than white women. Black women diagnosed with uterine fibroid have multiple lesions, larger fibroids, greater uterine weight and had developed the symptoms at younger age (Schwartz *et al.,* 2000; Marshall *et al.,* 1997; Flake *et al.*, 2003).

Inhibition of apoptosis of myometrial smooth muscle cells have been suggested to be a cause of uterine fibroid formation as suppression of apoptotic pathways may result in development of benign tumors. Some studies have shown that fibroids have a

higher cellular proliferation rate and a lower apoptosis compared to normal myometrium. Vu *et al.* (1998) have shown that fibroid tissue sample from GnRH agonist leuprolide acetate (LA)-treated women had a significant decrease in cellular proliferation index, estrogen and progesterone receptors compared to untreated control, quite likely since treatment decreases the number of cycling cells.

1.7.3 Effectors of Uterine Fibroids

Once a uterine fibroid is formed, estrogen and progesterone play a major role in promoting its growth but their explicit role is yet to be elucidated (Rein, 2000; Flake *et al*., 2003). The effect of ovarian hormones could be partly mediated by the mitogenic effects of growth factors and cytokines expressed locally by the smooth muscle cells. Numerous molecular studies have been done to investigate the expression level of growth factors and cytokines between fibroids and myometrium tissue. Amongst these growth factors epidermal growth factor (EGF), insulin growth factor (IGF), basic fibroblast growth factor (bFGF) and VEGF play major roles in formation of fibroids (Flake *et al*., 2003).

The level of IGF-I isoform has been shown to be higher in fibroids compared to adjacent myometrium sample suggesting it may play a mitogenic role in the development and growth of uterine fibroids (Hoppener *et al*., 1988; Flake *et al.,* 2003). Gao *et al.* (2001) reported IGF promotes growth of fibroids by inhibiting the apoptotic pathway through up-regulation of Bcl-2 expression in fibroid cells. They also showed IGF-I treatment of fibroid cells markedly increased the expression of proliferating nuclear cell antigen (PCNA) that is involved in control of cell proliferation and DNA repair and replication (Mangrulkar *et al*., 1995).

bFGF plays a role in pathology via activating its cognate receptor (FGFR1) to initiate remodelling of extracellular matrix (ECM) and regulate angiogenesis. Support for the role of bFGF in uterine fibroids is its over-expression in ECM of fibroids compared with adjacent myometrium tissue samples (Mangrulkar *et al*., 1995).

EGF has been shown to play a key role in pathologies of uterine fibroids. Higher EGF mRNA expression in the secretory phase of menstrual cycle in fibroid samples compared to normal myometrium suggests a probable role of EGF in fibroid development. In other studies, Maruo *et al.* (2000) have shown progesterone and estrogen up-regulate expression of PCNA and EGF-receptor in cultured fibroid cells.

VEGF is expressed both in myometrium and fibroids, however without significant expression level difference (Harrison-Woolrych *et al*., 1995). Despite this finding, VEGF might still be involved in pathologies of fibroids as it promotes angiogenesis and is very essential for actively growing tumors. In addition, molecular studies have shown that VEGF can exert a biological effect on FGF signalling pathway by prompting the release of bFGF which will in turn stimulate angiogenesis (Jonca *et al*., 1997).

1.7.4 Proposed Roles for Cyclooxygenase and Prostaglandins in Uterine Fibroids

Studies have shown COX-1 to be up-regulated in carcinomas of the uterine cervix (Sales *et al*., 2002), while COX-2 is up-regulated in endometrial (Tong *et al*., 2000; Ferrandina *et al*., 2002) and cervical carcinoma (Ryu *et al*., 2000; Sales *et al*., 2001) but little is known about the role of COX enzymes in uterine fibroids. Cesen-Cummings *et al*. (2003), using the Eker rat as a model for human uterine fibroids showed fibroids and myometrium expressed the same levels of COX-1 mRNA while fibroid cells were deficient in COX-2 expression.

In studies aimed at determining the levels of prostanoids in human myometrium and fibroids, it was found that both produce prostanoids. However, the levels of these prostanoids were reported to be contradictory (Yamaguchi and Mori, 1987; Aitokallio-Tallberg, 1990). Recent *in vitro* studies using cultured fibroid cells have shown that letrozole, a specific aromatase inhibitor can inhibit PGE_2-regulated cell proliferation and induce apoptosis suggesting the key role of prostaglandins in cell proliferation of uterine fibroids (Han *et al.*, 2008).

Tsibris *et al.* (2002) screened up to 12,000 genes in uterine fibroids and control myometrium tissue homogenates from nine samples in the proliferative and secretory stages of the menstrual cycle. Two variants of the EP3 receptors (EP3a1 and EP3-IV receptors) and PGD_2 synthase were down-regulated in fibroids compared to adjacent myometrium tissue samples. Wang *et al.* (2003) showed the EP3-IV receptor and PGD_2 synthase to be down-regulated in fibroid samples compared to adjacent myometrium in gene array analysis from seven patients in the proliferative stage of menstrual cycle. Down-regulation of the EP3 receptor and PGD_2 synthase was also reported in other independent gene array analyses (Quade *et al.,* 2004; Catherino *et al.,* 2004; Arslan *et al.,* 2005).

1.7.5 Endometrial Changes Associated with Uterine Fibroids

It has been reported that the mechanical presence of uterine fibroid can affect the endometrium. Early studies have shown that atrophy of the endometrium, as well as, elongation and distortion of the endometrium glands came as result of mechanical presence of uterine fibroids. This could be a direct effect of the nodular mass of the fibroids on the overlying or adjacent endometrium. The authors also reported ovarian hormonal change in uterine fibroids can cause cystic glandular hyperplasia, edema, polyposis and haemorrhages in endometrium (Deligdish and Loewenthal, 1970).

The only molecular study on the comparison of endometrium from normal and women with fibroids was done by Miura *et al.* (2006). The authors classified fibroids samples according to the location of the tumor and compared pattern of macrophages infiltration in the tissues. Accumulation of macrophages was significantly higher in endometrium of women with submucosal (SM) and intramural (IM) fibroids compared to women with subserosal (SS) fibroids and control tissue. Monocyte chemotactic protein-1 (MCP-1) and PGF were significantly increased in endometrium of women with SM and IM relative to SS or control tissue. The authors proposed an increase in inflammation and PGF synthesis could be a possible mechanism in causing either pregnancy complications or infertility in women having SM or IM uterine fibroids (Miura *et al.,* 2006).

1.8 G Protein-Coupled Receptor (GPCR) Cross-Talk

Prostanoid receptors, as part of GPCR family, are found to be co-expressed in the same cells and co-activation of these receptors might lead to cross-talk between the distinct signalling pathways elicited by each receptor. Receptor cross-talk can also occur among distinct families of receptors such as tyrosine kinase receptors and GPCRs but this review will focus on cross-talk between GPCRs (Ashby, 1998; Selbie and Hill, 1998).

Intracellular signalling in a cell expressing multiple GPCRs comprises the integration of input from each G proteins coupling (G_s, $G_{i/o}$ and $G_{q/11}$) following ligand binding. These signalling outputs may act synergistically or antagonistically to amplify or attenuate cell signalling. This leads to a broad spectrum of signalling pathways which can alter physiological or pathological make up of a cell or a tissue. These heterologous interactions or cross-talks between GPCRs can result in a loss of function (desensitization), and also a gain or a potentiation of function (Werry *et al.*, 2003; Selbie and Hill, 1998). Since dysregulation of the EP2 and FP receptors in endometrial adenocarcinoma is reported and these receptors are co-expressed in endometrial adenocarcinoma cells, the next sections will focus on cross-talk between $G\alpha_s$- and $G\alpha_q$-coupled receptors.

1.8.1 Intracellular Calcium in GPCR Cross-Talks

Alteration of intracellular calcium is a key regulator of many biological processes. The release of intracellular calcium can be regulated by either activation of a single GPCR or co-activation of multiple GPCRs in the same cell (Werry *et al.*, 2003). Stimulation of $G\alpha_q$-coupled receptor can lead to activation of PLC and generation of IP3 and a subsequent release of intracellular calcium from cellular stores (Watanabe *et al.*, 1994). However, several studies have shown PLC-mediated Ca^{2+} release can be influenced by co-activation of $G\alpha_s$-coupled receptors regulating a heterologous interaction between $G\alpha_s$ and $G\alpha_q$-coupled receptors (Werry *et al.*, 2003).

For example, Jimenez *et al.* (1999) reported a GPCR cross-talk that involves enhancement of intracellular calcium by co-activation of $G\alpha_s$ and $G\alpha_q$ proteins in type I cerebellar astrocytes. The authors showed that stimulation of the cells with ATP can activate release of intracellular calcium via the P2Y receptors coupled to $G\alpha_q$ protein and PLC activation. Furthermore, they showed co-activation of the P2 and P1 purinoceptors by ATP and adenosine, respectively augmented P2Y receptor-mediated intracellular calcium release. This increase was mediated by the activation of A_{2B} adenosine receptors that couple to $G\alpha_s$, suggesting a cross-talk between the P2Y and P1 purinoceptors to potentiate PLC-mediated intracellular calcium. Werry *et al.* (2002) showed a cross-talk can happen upon co-activation of the $G\alpha_s$-coupled β-adrenoceptors and $G\alpha_q$-coupled P2Y receptors to potentiate $G\alpha_q$-mediated intracellular calcium release in HEK-293 cells.

The mechanism by which activation of $G\alpha_s$ can regulate intracellular calcium is not well known. A previous study showed that vasopressin-stimulated IP3 production was augmented by co-stimulation with a cAMP analogue in cultured rat hepatocytes. The authors suggested this could be a direct effect of cAMP on either phosphorylation of the vasopressin receptor or the PLC enzyme itself (Pittner and Fain, 1989). Another study showed that the increase in $G\alpha_q$-mediated Ca^{2+} release by $G\alpha_s$ activation could be by a direct action of PKA on IP3 receptor/channel to facilitate Ca^{2+} release from endoplasmic reticulum. Another indirect effect of PKA on intracellular Ca^{2+} stores could be by stimulation of ATP-dependent Ca^{2+} pump that in turn modifies the functional distribution between the IP3-sensitive and IP3-insensitive Ca^{2+} pools (Hajnoczky *et al.,* 1993).

1.8.2 Adenylyl Cyclases (ACs) in GPCR Cross-Talks

Activation of $G\alpha_s$-coupled receptor leads to accumulation of intracellular cAMP. The AC family are responsible for this enzymatic reaction by catalyzing the synthesis of cAMP from ATP. ACs have been shown to play a major role in mediating a $G\alpha_s$-$G\alpha_q$ cross-talk by forming a critical link between Ca^{2+} and cAMP signalling (Wang and Storm, 2003; Willoughby and Cooper, 2007).

The first AC was cloned in 1989. The enzyme consists of two hydrophobic transmembrane domains (TM1 and TM2 each containing six transmembrane domains), two long cytoplasmic domain (C1 and C2) and a short N-terminal region as shown in figure 1.5 (Krupinski *et al*., 1989). The enzyme has two highly conserved ATP-binding domains on the cytoplasmic domain that are designated as C1a and C2a. These domains are highly homologous and complementary and need to dimerize for complete AC activity. C1b and C2b refer to the less conserved domains in AC (Hanoune *et al*., 1997; Tesmer *et al.,* 1997; Willoughby and Cooper, 2007).

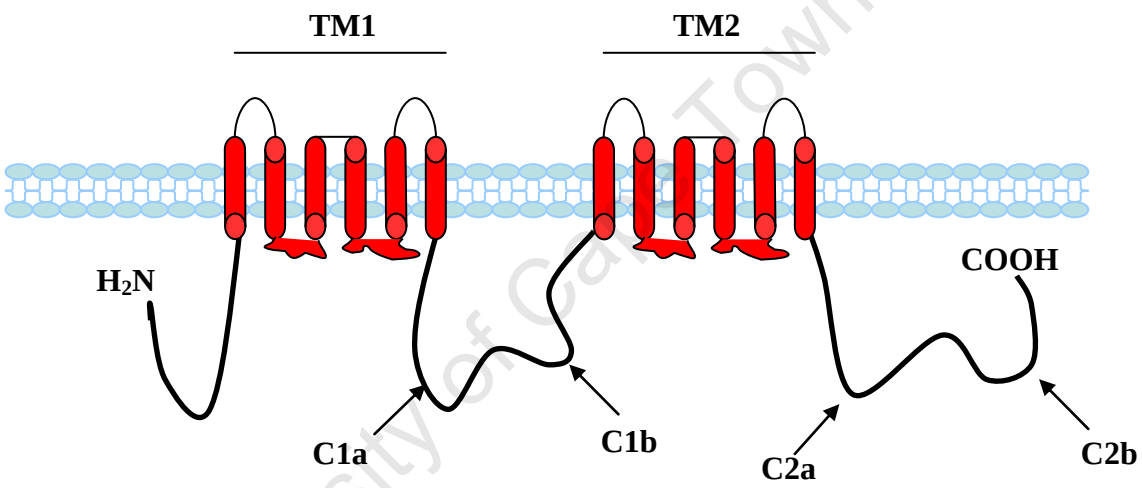

Fig. 1.5: *Adenylyl cyclase structure. ACs can be divided in five major domains: The hydroxyl terminus, the first six transmembrane group (TM1), the first cytoplasmic loop (C1a and C1b), the second group of six transmembrane (TM2) and the second cytoplasmic loop (C2a and C2b) (Figure taken and modified from Hanoune et al., 1997; Willoughby and Cooper, 2007).*

There are multiple AC isoforms (9 membrane-bound and 1 soluble) each with a molecular weight about 120-150 kDa, except the soluble isoform which is half the size of the others and is found in spermatozoa. This observed high mass is contributed by glycosylation of the enzymes that is found in the second transmembrane cluster. The AC isoforms differ in their amino acid sequence, tissue distribution and regulation and are named in the order of their publication (Krupinski *et al.*, 1989; Bakalyar and Reed, 1990; Gao and Gilman, 1991; Katsushika *et al.*, 1992; Watson *et al.*, 1994; Defer *et al.*, 1994; Premont *et al.*, 1996; Buck *et al.*, 1999).

Despite their difference in amino acid sequence they share a large sequence homology in the primary structure and the same predicted three-dimensional structure. All the AC isoforms can be stimulated by $G\alpha_s$ and forskolin. However, ACs can also be regulated by several regulatory factors such as calcium, G proteins ($G\alpha_i$ and $G\beta_\gamma$), protein kinases (PKA, PKC, calmodulin and calmodulin-kinase) and phosphatases (calcineurin). Based on their responsiveness to the above regulatory elements, ACs have been classified into four groups. Group 1 consists of enzymes that are stimulated by calcium or calmodulin (AC1, AC3 and AC8) while group 2 contains AC enzymes that are activated by PKC and $\beta\gamma$ subunit (AC2, AC4 and AC7). Group 3 are enzymes inhibited by low concentration of calcium (AC5 and AC6) and Group 4 (AC9) is insensitive to both calcium and $\beta\gamma$ subunit (Hanoune *et al*., 1997, Willoughby and Cooper, 2007). The major source of AC isoforms expression and their response to several regulatory factors are shown in table 1.1.

Table 1.1: Regulation and distribution of adenylyl cyclases (adapted and modified from Willoughby and Cooper, 2007; Hanoune and Defer, 2001)

AC isoform	Major source of expression	Response to cAMP signalling pathway component				
		Gαs	Gαi	Gβγ	Calcium	Protein kinases
AC1	Brain	↑	↓	↓	↑ (via CaM)	↓ CaMK IV ↑ RTK
AC2	Lung, brain	↑	→	↑	→	↑ PKC
AC3	Olfactory epithelium, pancreas	↑	↓	→	↑ ?(via CaM) ↓ ?(CaMKII)	↓ CaMK II
AC4	Widespread	↑	→	↑	→	↑ PKC
AC5	Heart, striatum	↑	↓	→	↓	↑ PKC ↓ PKA ↑ RTK
AC6	Heart, kidney, widespread	↑	↓	→	↓	↓or ↑ PKC ↓ PKA ↑ RTK
AC7	Widespread	↑	→	→	→	↑ PKC
AC8	Brain, pancreas	↑	↓	↓	↑ (via CaM)	→ PKC
AC9	Testis, widespread	↑	↓	→	↓ (via Calcineurin)	↓ PKC
Soluble AC (SAC)	Sperm, kidney	→	→			

1.8.2.1 Calcium-Regulated Adenylyl Cyclase Isoforms

All mammalian ACs are inhibited by high Ca^{2+} concentrations (supramicromolar). This effect could be due to the competition of Ca^{2+} on binding to metal binding sites on the catalytic domain. Out of the nine membrane-bound AC isoforms three of them (AC1, AC3 and AC8) are stimulated by calcium while AC5 and AC6 are normally inhibited by physiological calcium changes *in vivo* (Tang *et al.,* 1991; Choi *et al.,* 1992; Cali *et al.,* 1994). In the next sections this review will discuss the regulation of calcium stimulated AC isoforms in detail.

1.8.2.2 Adenylyl Cyclase 1 (AC1)

AC1 was the first AC isoform to be cloned and characterized by Krupinski *et al.* (1989). The characterization of AC1 using Sf9 cells showed accumulation of intracellular cAMP regulated by calcium and calmodulin directly without the presence of $G\alpha_s$ activator (Tang *et al.,* 1990). Choi *et al.* (1992) has shown that AC1 can be stimulated by extracellular calcium by stably expressing the isoform in HEK-293 cells. The authors showed that in the presence of the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methyl xanthine), extracellular calcium and Ca^{2+} ionophore (A23187) can increase the intracellular accumulation of cAMP by 16-fold. They also showed forskolin-activated cAMP can be enhanced by the addition of A23187 by about 9-fold even in the absence of IBMX (Choi *et al.,* 1992).

Wu *et al.* (1993) dissected the calmodulin binding domain by mutating specific sites that are reported to contribute to calmodulin sensitivity of the enzymes (aromatic amino acids in C1b region of the molecule). A specific F503R mutation completely abolished calcium stimulated cAMP. This mutation was specific to the regulatory sites as neither the basal cAMP level nor the forskolin-stimulated cAMP was altered by the mutation showing the mutation had no effect on the catalytic activity of the enzyme (Wu *et al.,* 1993).

1.8.2.3 Adenylyl Cyclase 3 (AC3)

AC3 was cloned by Bakalyar and Reed (1990) from olfactory neurons and was presumed to be specific to the sensory system. But other studies have shown the enzyme to be expressed in pancreas, heart, vascular smooth muscle, brain and lung tissues (Wang and Storm, 2003).

The regulation of AC3 by calcium is not totally clear. Although AC3 is generally regarded as calcium stimulable isoform, studies in HEK-293 cells have shown direct inhibition of the isoform by physiologically relevant concentrations of intracellular calcium. This inhibition was blocked by chemical inhibitor for calmodulin kinases showing the mechanism of AC3 inhibition (Wayman *et al.,* 1995). Other

studies have shown that increase in intracellular calcium can directly activate CaMK-II to phosphorylate AC3 in HEK-293 cells. The authors identified the site of phosphorylation on AC3 using a S1076A mutation that completely inhibited intracellular calcium inhibition of AC3 (Wei *et al.,* 1996; Wei *et al.*, 1998). However, another study has revealed when it is concomitantly activated with $G\alpha_s$, AC3 can be stimulated by intracellular calcium to potentiate cAMP production. The authors employing HEK-293 cells stably expressing the AC3 isoform showed an increase in intracellular accumulation of cAMP when activated with calcium in the presence of forskolin or GppNHp (a nonhydrolyzable guanosine triphosphate (GTP) analogue that persistently activate $G\alpha_s$ proteins) while calcium by itself did not have any effect on cAMP (Choi *et al*., 1992).

1.8.2.4 Adenylyl Cyclase 8 (AC8)

AC8 was the last calcium-regulated isoform to be isolated and it was cloned from human newborn brain-stem tissue in 1994 (Defer *et al.,* 1994). The enzymatic property of this isoform is very similar to AC1 despite sharing only 28% amino acid similarity (Tang *et al.,* 1991). This well studied isoform is activated by calcium and calmodulin in HEK-293 cells at almost the same concentration as AC1 (Cali *et al.*, 1994).

Gu and Cooper (1999) showed two calmodulin binding sites in AC8, located at C- and the N-terminus of the enzyme. The complex interaction on how calmodulin activates AC8 was studied by Simpson *et al.* (2006). The authors showed the N-terminus plays a key role in the activation of the enzyme by calcium as it has an amphipathic helix calmodulin binding domain (CaMBD). At resting calcium, AC8 is auto-inhibited by the interaction of calmodulin with CaMBD in N terminus. In the event of calcium entry, Ca^{2+} binds to the calmodulin and eventually interacts with the main regulation site in the COOH terminus then eventually triggers relief autoinhibition allowing ATP to bind to the catalytic domain of the enzyme (Simpson *et al.*, 2006).

1.8.3 Intracellular cAMP Augmentation via GPCR Cross-Talks

Intracellular calcium and the adenylyl cyclase pathway have been shown to play a major part in $G\alpha_s$-$G\alpha_q$ cross talk by activating cAMP. Meszaros *et al.* (2000) showed a potentiation of isoproterenol (β-adrenergic (β-AR) receptor agonist)-induced cAMP production by angiotensin II (ANG-II) activation of AT_1 receptors coupled to $G\alpha_q$ protein pathway indication of a signalling cross talk between AT_1 and β-AR receptors in cardiac fibroblasts cells.

The mechanism in which the cross-talk occurs was further studied by Ostrom *et al.* (2003). The authors showed the $G\alpha_s$-$G\alpha_q$ cross-talk was mediated by the activation of PLC and release of intracellular calcium. They further showed Ca^{2+} in turn activate the calmodulin-CaMK-II to trigger the cross talk between the receptors probably by the activation of calcium stimulable AC3 isoform, since it is the only calcium regulated isoform expressed in their model cells.

Zhang *et al.* (1997) also reported $G\alpha_s$-$G\alpha_q$ cross talk in cultures vascular smooth muscle cells (VSMC). In these cells, Isoproterenol-stimulated cAMP accumulation was increased to 2-2.5 fold when it is co-stimulated by arginine vasopressin (AVP). Further studies of the molecular pathway underlying this augmentation revealed the cross-talk is mediated by the intracellular calcium pathway and is independent of PKC. Here too the cross talk between the receptors is probably by the activation of calcium-stimulable AC3 isoform, since AC3 is the only calcium regulated isoform expressed in these cells (Zhang *et al.,* 1997).

The other $G\alpha_s$-$G\alpha_q$ cross-talk involves the calcium inhibitable AC6 isoform. Studies using AC6 over-expressing HEK-293 cells showed augmentation of Isoproterenol and forskolin-stimulated cAMP by carbachol activation of the muscarinic receptor. In this study the authors showed AC6 can be regulated in two ways as it was inhibited by capacitative calcium entry (CCE) while carbachol activated release of intracellular calcium augmented forskolin-stimulated cAMP. The authors dissected the pathway involved in this cross-talk and found it to be mediated by the calcium-calmodulin pathway (Beazely and Watts, 2005). Another study using AC9

over-expressing HEK-293 cell lines also showed a bi-directional regulation of the AC9 isoform by calcium (Cumbay and Watts, 2005).

1.8.4 Cross-Talks in Eicosanoid Receptors Family

There have been several reports for cross-talk in the eicosanoid family. The most notable is the cross-talks reported between the thromboxane and prostacyclin receptors. These two eicosanoid receptors have opposing roles in physiology of vascular hemostasis which are mediated via different signalling pathways. As mentioned in section 1.4.3, TXA_2 binds to TPα and TPβ receptors to activate intracellular IP3 release while prostacyclin binds to IP receptor to activate release of cAMP from intracellular stores (Breyer *et al.*, 2001).

Walsh *et al.* (2000) reported a cross-talk between TPα and IP receptors in HEK-293 cells stably expressing the TPα receptor. The authors reported stimulation of the cells using selective IP agonist (cicaprost) completely desensitized U46619-TP mediated PLC activation and Ca^{2+} mobilization. The desensitization of TPα receptors by cicaprost was mediated by PKA pathway and inhibition of PKC did not have any effect on this heterologous desensitization. However, signalling of the TPβ receptors was not affected by stimulation of IP receptors (Walsh *et al.*, 2000).

The other GPCR cross-talk reported in eicosanoid family is between the PGD_2 receptors and TXA_2 receptors. The action of PGD_2 mimics that of prostacyclin by inhibition of platelet aggregation in vascular hemostasis. Activation of the DP receptor by BW235C desensitized TPα receptor by inhibiting PLC activation and was mediated by the PKA pathway. This effect was limited to the TPα receptor as TPβ was unaffected by stimulation of DP receptors in HEK-293 cells over-expressing the TPβ receptors (Foley *et al.*, 2001).

Walsh and Kinsella (2000) showed a cross-talk between the TXA_2 receptors (TPα and TPβ) and the EP1 receptors in HEK-293 cells. The authors showed that subjecting TPα and TPβ receptors to 17 phenyl trinor PGE_2 (EP1 receptor agonist) causes desensitization and inhibition of signalling of the TP receptors in a PKC-

dependent manner. The authors further indentified the activation of the FP receptor by PGF can also mediate desensitization of the TPα and TPβ receptors via the PKC pathway in HEK-293 cells (Kelley-Hickie and Kinsella 2004).

In another study, Wilson *et al.* (2004) showed co-expression of the IP and TPα receptors in HEK-293 cells can augment TPα-receptor mediated cAMP without activation of the IP receptor. The cAMP augmentation was further increased when both receptors were co-activated by their respective ligands. Further studies with the receptors showed that heterodimerization can occur between IP and TPα receptors that probably leads to augmentation of cAMP production by IP receptors.

1.9 General Aims and Objectives of the Project

As mentioned in section 1.7, uterine fibroids are an important clinical problem in women in the reproductive years. Despite being a major cause of pregnancy related complications and gynecological morbidity, the etiology of uterine fibroid are still not well elucidated (Marshall *et al.,* 1997; Flake *et al.,* 2003). There is a strong evidence to support a role for COX enzymes and prostaglandin receptors dysregulation in reproductive pathologies (Lumsden *et al.,* 1983; Karck *et al.,* 1996; Smith *et al.,* 1981; Sales *et al*., 2002; Ryu *et al*., 2000; Jabbour *et al.,* 2006b). However, there have not been sufficient studies to assess the effect of COX and prostaglandin receptors in uterine fibroids.

The objectives of this study were two fold. The first aim was to assess the expression of COX enzymes and prostaglandin receptors in fibroids, autologous myometrium and endometrium samples from women with fibroids. The second aim comes from co-expression of prostaglandin receptors in several cell types and tissues (Ashby, 1998). For example, EP2 and FP receptors are co-expressed in Ishikawa cells. Studies in our laboratory have shown that up-regulation of the EP2 and FP receptors in endometrial adenocarcinomas. In addition, the authors have shown elevated expression and activation of the EP2 and FP receptors can promote the expression of angiogenic genes via the ERK1/2 pathway in Ishikawa cells (Sales *et al*., 2004; Sales *et al*., 2005).

This observation suggests that co-activation of prostanoid receptors in the same cell could change the physiological/pathophysiological gene expression profile. The second aim of this study was to address the integrative signalling effects of the EP2 and FP receptors on signal transduction and gene expression. The specific aims of the research were to:

1. generate a model cell line co-expressing the EP2 and FP receptors in Ishikawa cells (FPEP2 cells)
2. identify a possible intracellular signalling cross-talk mediated by co-activation of the EP2 and FP receptors and to further characterize the molecular mechanism underlying the potential cross-talk in FPEP2 cells
3. identify which genes or cluster of genes are differentially regulated by co-activation of the EP2 and FP receptors relative to activation of EP2 or FP receptor separately in FPEP2 cells

Chapter II

General Materials and Methods

2.1 Chemicals and Reagents

All chemicals used were molecular biology grade, and were obtained from Sigma (Dorset, United Kingdom (UK) or South Africa (SA) or IBI (Cambridge, UK). Cell culture media was purchased from Gibco (Gibco, Paisely, UK). Foetal Calf Serum (FCS) and Penicillin-Streptomycin were purchased from PAA Laboratories (PAA Laboratories, Middlesex, UK). MycoAlert® Mycoplsma detection kit was purchased from LONZA (LONZA, Rockland, USA), while phosphate buffer saline (PBS) and trypsin-EDTA were purchased from BioWhittaker (BioWhittaker, Berkshire, UK).

Hygromycin (100mg/ml stock in HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer) was purchased from Invivogen (Invivogen, Autogen Bioclear UK). G418 (100mg/ml stock in PBS), indomethcin (3mg/ml stock in ethanol), Butaprost (5mM stock in ethanol), PGE_2 (PGE, 100µM stock in ethanol), $PGF_{2\alpha}$ (PGF, 100µM stock in ethanol) and IBMX (20mM stock in 50% ethanol) were purchased from Sigma (Sigma chemical Co., Nottingham, UK). FP receptor antagonist (AL8810, 50mM stock in ethanol), IP3-receptor blocker (2-APB, 40mM stock in dimethyl sulfoxide (DMSO)), PLC inhibitor (U73122, 10mM stock in DMSO), Calmodulin antagonist (W7, 25mM stock in DMSO), Ca^{2+}/calmodulin-dependent kinase II (CaMK-II) inhibitor (KN-93, 50mM stock in DMSO) and PKC inhibitor (Ro-31-822, 1mM stock in DMSO) were all purchased from Calbiochem (Calbiochem, Nottingham, UK). Inhibitor of $G\alpha_q$ (YM254890, 1mM stock in DMSO) was a kind gift from M. Taniguchi, (A stellas Pharmaceuticals Inc., Tokyo, Japan).

Pre-cast 4% to 12% Tris-Glycine gels and SeeBlue® Plus2 Pre-stained marker were purchased from Novex (Novex, UK). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Millipore, Watford, UK). Whatman no. 3 paper was obtained from Whatman (Whatman, UK). Odyssey blocking buffer was obtained from Li-Cor Bioscience (Li-Cor Bioscience, Cambridge, UK). The EP2 and FP receptor primary antibodies were purchased from Cayman Chemical Company (Axxora, Nottingham, UK) and primary antibody for β-actin was purchased from Santa Cruz Biotechnology (Santa

Cruz, Wiltshire, UK). Fluorescent secondary antibodies were purchased from Li-Cor Biosciences (Li-Cor Biosciences, Cambridge, UK). Stealth short interfering RNA (siRNA) duplex oligoribonucleotides for AC1 and AC3 were purchased from Invitrogen (Invitrogen, Paisley, UK).

2.2 Cellular Investigations

2.2.1 Cell Culture

The complete medium used to culture human endometrial adenocarcinoma (Ishikawa) cells (Nishida *et al.,* 1985; European Collection of Cell Culture, Centre for Applied Microbiology, Wiltshire, UK) was Dulbecco's Modified Eagle Medium supplemented with nutrient F-12, glutamax-1 and pyridoxine (DMEM-F12), 10% FCS and 1% antibiotics (stock solution of 500IU/ml penicillin and 500µg/ml streptomycin). The medium was stored at 4°C, and was warmed at 37°C before use. Monolayer cultures of Ishikawa cells were grown in 125 cm^2 tissue culture (TC) flasks and kept in a humidified incubator (37°C , 95% relative humidity and 5% CO_2). The cells were checked for growth every other day using an inverted microscope. Confluent cells were passaged by washing twice with PBS and dissociating with 5ml trypsin-EDTA, for 10 min at 37°C. The cell suspension was centrifuged in a fresh tube at 1000 rpm for 5 min and the pellet was re-suspended in fresh medium. Cells were seeded into new flasks after 1:10 dilution with a new passage number being recorded on each flask. Early passage cells were frozen down for later use by re-suspending the trypsinized cells (~1×10^6 cells/ml) in FCS containing 10% DMSO and transferred into small sterile cryogenic vials. The vials containing cell aliquots (1ml each) were placed upright in a -80°C freezer and later transferred to liquid nitrogen for long term storage. Thawing of frozen vials was done quickly at 37°C and cells were suspended in 10ml complete medium to neutralize the DMSO. The mix was centrifuged at 1000 rpm for 5 min and cell pellet was re-suspended in 10 ml of complete medium and transferred into a 75 cm^2 flask. The cells were frequently checked for Mycoplasma contamination using MycoAlert® Mycoplasma detection kit (LONZA, Rockland, USA) according to the manufacturer's protocol.

2.2.2 Cell Transfection

Stable cell lines expressing both EP2 and FP receptors (FPEP2 cells) were prepared from Ishikawa cells stably expressing the FP receptor (FPS32 cell lines) (Sales *et al.,* 2005). These cells were maintained in a selective medium containing 200µg/ml of G418. EP2 receptor cDNA construct was prepared as described in section 2.3.1. The EP2 receptor cDNA was transfected into the FPS32 cells using SuperFect® transfection reagent (QIAGEN, UK) according to manufacturer's recommendations.

Briefly, 1×10^6 FPS32 Ishikawa cells were plated into 100 mm cell culture dishes and were allowed to adhere overnight in a humidified incubator. The following day EP2 receptor cDNA (10µg) in Tris-EDTA buffer (10mM Tris and 1mM EDTA pH 7) was added to cell growth medium lacking serum and antibiotics. Subsequently, 60µl of SuperFect® transfection reagent was added to the DNA mix and left for 15 min at room temperature to allow transfection-complex formation. Cells were then washed with PBS and 3 ml of complete growth medium was added to the transfection mix and immediately added to the cells. Afters five hrs of incubation under a normal growth condition, the medium was aspirated and cells were washed with PBS and incubated in a normal growth medium overnight. The following day the cells were passaged (1:5, 1:10, 1:20 and 1:50) and were grown in the presence of a selective medium containing 800µg/ml of hygromycin. For control, untransfected cells were grown under the same selective media in parallel. Once untransfected cells had died, 50 hygromycin-resistant clones were picked using sterile cloning cylinders (Sigma) by trypsinizing each colony and were transferred into 24-well plates. Single clones were then allowed to grow in the presence of selective medium and transferred into 25 cm^2 flasks. Clones were then screened for the expression of the EP2 receptor by Real-time RT-PCR and for the accumulation of intracellular cAMP in the presence of the EP2 receptor ligands, PGE_2 and Butaprost. All clones were maintained in hygromycin and G418 (200µg/ml each) selective for the expression of the EP2 and FP receptors, respectively.

2.2.3 Ligand Stimulation and cAMP Assay

Ligand-induced cAMP accumulation was determined by seeding 2×10^5 cells in 6-well plates. The following day, the cells were serum-starved with serum free medium in the presence of 3µg/ml of indomethacin (a dual COX enzyme inhibitor used to inhibit production of endogenous prostaglandins). After 18 hrs, the cells were pre-treated in serum free medium containing the phosphodiesterase inhibitor IBMX (0.2mM) for 30 min in humidified incubator. Subsequently, the cells were treated for 5 min with vehicle (0.1% ethanol and/or DMSO), PGE_2 (100nM), Butaprost (5µM) or PGF (100nM) in the presence/absence of different chemical inhibitors as indicated. After the incubation, cells were washed twice with cold PBS to stop the reaction and lysed in 200µl of cell lysis buffer (R&D Systems, Oxford, UK). Insoluble material was removed from lysates by centrifuging the samples at 14000 rpm for 15 min at 4°C. Intracellular accumulation of cAMP was determined by an Enzyme-Linked ImmunoSorbent Assay (ELISA) cAMP Kit (R&D Systems, Oxford, UK) according to the manufacturer's protocol.

In short, the ELISA was performed using goat anti-mouse polyclonal antibody-coated 96-well polystyrene microplates. The cAMP present in the samples was measured by competition with a fixed amount of horse-radish peroxidise (HRP)-labelled cAMP for sites on a mouse monoclonal antibody. 100µl of cAMP standards (200pmol/ml to 1.56pmol/ml) and diluted samples were added to the plate in duplicate. The wells were then coated with 50µl/well of mouse monoclonal antibody to cAMP in buffer with blue dye™ and cAMP conjugated to HRP with red dye™ and incubated at room temperature for 3 hrs on a horizontal orbital microplate shaker (0.12" orbit) set at 550 rpm. Thereafter the wells were aspirated and washed four times with 400µl R&D wash buffer™ to remove excess conjugate and unbound sample. Subsequently, a substrate solution (a 1:1 ratio of Colour Reagent A™ (stabilized hydrogen peroxide) and Colour Reagent B™ (stabilized chromogen (tetramethylbenzidine)) was added to determine the bound enzyme activity. The colour development was stopped using Stop solution™ (Sulfuric acid) and the absorbance was read at 450 nm using Labsystems Multiskan EX plate reader (Thermo Labsystems, UK). The concentration of cAMP was calculated by a standard curve using

Assay Zap computer programe (Biosoft, Cambridge, UK). Data were normalized by the respective protein concentration of each sample using the protein assay described in section 2.2.8.

2.2.4 Ligand Stimulation and Total Inositol Phosphate (IP3) Assay

Ligand-induced accumulation of IP3 was determined by plating 50,000 cells in 24-well plates containing 1ml/well of complete medium. Cells were allowed to attach overnight and on the following day, the cells were washed with PBS and then labeled with 0.5µCi/well myo-[3H]-inositol (Amersham Bioscineces, RSA) in an inositol-free DMEM 199 medium supplemented with 2% dialyzed FCS overnight. The next day, the medium was removed and cells were washed with Buffer I (140mM NaCl, 20mM HEPES, 4mM KCL, 8mM Glucose, 1mM $MgCl_2$, 1mM $CaCl_2$, 1mg/ml bovine serum and 10mM LiCl) and preincubated for 30 min in Buffer I. Subsequently, cells were stimulated with PGF and/or Butaprost at the indicated concentrations in Buffer I for 60 min at 37°C in an incubator, while non-stimulated samples served as control for each experiment. After aspirating the buffer, the cells were lysed by the addition of 1 ml ice cold 10mM formic acid and the plates were placed on ice for 30 min.

Total [3H]-inositol phosphates were separated from cell extracts on AG 1-X8 resin by anion exchange chromatography. Briefly, the columns were washed with 3ml of 3mM of amonium formate/0.1M formic acid and then with 10ml of distilled water. Subsequently, 1ml of sample was added to the columns and columns were rinsed once with 10ml of distilled water and 5ml of 0.1M formic acid/ 5mM myo-inositol. The labeled inositol phosphates were eluted with 3ml of 1M ammonium formate/0.1M formic acid solution and mixed with 16ml of Quick Safe A scintillation fluid (Zinser analytic, Frankfurt, Germany). The associated radioactivity was determined by counting each vial for a minute in Liquid Scintillation Analyzer (Packard GmbH, Frankfurt, Germany).

2.2.5 Wound Healing Assay (Scratch Assay)

Ishikawa cells were seeded at a density of 2×10^5 cells/well into 6-well plates in complete media. On the following day cells were serum starved overnight in media containing indomethacin (3µg/ml). Thereafter, the cell monolayer was scraped using a p1000 tip to create a straight line. The debris of the smooth edge were removed by washing with PBS and cells were treated with vehicle, Butaprost (5µM) and/or PGF (100nM). The cells were incubated for 24 hrs in a tissue culture incubator and an image was acquired every four hrs. The distance of each scratch closure was measured and expressed as fold increase above vehicle treated.

2.2.6 Proliferation Assay

Ishikawa cells were seeded at a density of 5000 cells/well in a 96-well plate and were left to adhere overnight in complete media. The next day the cells were serum starved in 1% FCS/DMEM in the presence of 3µg/ml of Indomethacin. Cells were then treated with vehicle, Butaprost (5µM) and/or PGF (100nM) for 24, 48 and 72 hrs. After each time point, proliferation was assessed by incubating the cells with CellTitre 96AQueous One Solution reagent (Promega Corp., Madison, WI) for two hrs in a humidified incubator. After the incubation the amount of soluble formazan produced by cellular reduction of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was determined by reading the absorbance at 492 nm using Labsystems Multiskan EX plate reader (Thermo Labsystems, UK).

2.2.7 Protein Extraction from Cells

Ishikawa cells were seeded at a density of 2.5×10^5cells in a 6-well plate and cultured in complete media overnight. The cells were lysed by an addition of lysis buffer (150mM NaCl, 10% Glycerol, 0.6% (v/v) NP-40, 50mM Tris/HCL pH 7.4 and 10mM EDTA containing mini complete tablet cocktail consisting of protease inhibitors (chymotrypsin, thermolysin, papain, pronase, pancreatic extract and trypsin inhibitors) (Roche)). The insoluble material was pelleted by centrifugation at 14000 rpm for 15 min

at 4°C and the clarified lysate was transferred to a new tube for further protein quantification.

2.2.8 Protein Concentration Determination

Protein concentrations were determined using the standard BIO-RAD D_C assay (BIO-RAD, UK) as directed by the manufacturer's instructions. Bovine serum albumin (BSA) was used as a protein standard and diluted from 400 to 0μg in 100μl of distilled water in tubes. 20μl of standards or samples were loaded into a 96-well plate (in duplicate) followed by the addition of 25μl of working reagent (20μl of Reagent S™ in 1ml of Reagent A™ (alkaline copper tartrate solution)). Thereafter, 100μl Reagent B™ (Folin Reagent) was added to the well and after 15 min incubation the absorbance at 690 nm was measured using Labsystems Multiskan EX plate reader (Thermo Labsystems, UK). A standard curve was plotted using the program Assay Zap (Biosoft, Cambridge, UK) and the unknown protein concentrations were determined from the standard curve.

2.2.9 SDS-PAGE and Western Blotting

A total of 20-50μg of protein was resuspended in 1 × Laemmli buffer (125mM Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercapthoethanol and 0.05% bromophenol blue) and boiled for 5 min at 95°C. Samples and 4μl of SeeBlue® (Novex, Invitrogen) pre-stained protein marker were then fractionated by electrophoresis (50mA, 2 hrs) on a pre-cast 4% to 12% Bis-Tris gels (NOVEX, Invitrogen). The gel and three Whatman paper (Whatman, UK) were placed to a protein free tray and equilibrated with transfer buffer (25mM Tris/HCL, 0.192M Glycine and 20% Methanol). Millipore Immobilon™-FL PVDF membrane (Millipore, Watford, UK) cut to the dimension of the gel was equilibrated in transfer buffer after immersing in methanol for 1 min. The blot was assembled by overlying three pieces of Whatman paper with a membrane followed by the gel and the three layers of Whatman paper. The gel was transferred to the Immobilon™-FL PVDF membrane at constant voltage of 14V using semi-dry blotter (BIO-RAD) for 90 min. After incubating the membrane in 10ml of Odyssey blocking buffer (Li-Cor Bioscience, Cambridge, UK) for an hour the membrane was incubated with relevant

primary antibody at 4°C overnight with gentle shaking. The membrane was then washed four times in 10 ml of PBS-Tween (PBS with 0.1% Tween 20) and was incubated with the secondary fluorescence antibody for 1 hour. After washing the membrane thrice with PBS-Tween, proteins were then viewed using Odyssey Infrared imaging system (Li-Cor Bioscience, Cambridge, UK).

2.2.10 Immunofluorescence Microscopy of Cells

The site of EP2 and FP receptors expression in FPS32 and FPEP2 cell lines were localized using an immunofluorescence microscopy. Ishikawa cells (10×10^5 cells/well) were plated out in 2-well cell chamber slides and left to adhere overnight. The next day the cells were fixed with 4% paraformaldehyde (PFA) for 20 min and washed with PBS, before being blocked in 5% BSA diluted in normal goat serum for 2 hrs. Thereafter, the cells were incubated with polyclonal rabbit anti-EP2 and FP receptor antibody diluted (1:100) in normal goat serum overnight at 4°C. To check the specificity of the signals, the primary antibodies were preadsorbed (10 fold in weight) with a specific blocking peptide for each receptor and used as control. The next day cells were washed three times with PBS for 10 min and incubated with Alexa flour conjugated secondary goat anti-rabbit antibody (Molecular probes) for 2 hrs. After three 10 min of PBS washes the nuclear was stained using DAPI (1:1000 in PBS; Sigma) for 10 min. Thereafter slides were washed and mounted in Permafluor (Immunotech-Coulter, Buckinghamshire, UK) and coverslipped for microscopic analysis.

2.3 Molecular Studies

2.3.1 Construction of EP2 Receptor cDNA Expression Vector

Total RNA was extracted from proliferative phase of human endometrial tissue and reverse transcribed into cDNA using a method described 2.3.3 and 2.3.5, respectively. EP2 receptor cDNA was synthesised using forward 5'-CTCTTTTCCAGGCACCCCAC-3' and reverse 5'-TTTTAAACTGACCTCAAAGGTCAGC-3' primers. Subsequently, the EP2 receptor cDNA was ligated into the pcDNA3.1 (Invitrogen) expression vector.

2.3.2 Total RNA Extraction from Cells

Throughout total RNA extraction, a ribonuclease (RNase) free environment was maintained by wiping all the surfaces with RNase-Away™ (Gibco-BRL) and by using double distilled deionized water that was pretreated with 0.01% diethylpyrocarbonate (DEPC; Sigma). Total RNA was extracted from Ishikawa cells using the RNA extraction reagent Trizol™ (Invitrogen, Paisley, UK) according to the manufacturer's protocol. The cells were lysed in 1 ml Trizol reagent by pipetting and incubating at room temperature for 5 min to ensure complete disassociation of nucleoproteins and were then transferred to Phase lock gel heavy tubes (5 PRIME). BCP (1-bromo-3-chloropropane (Sigma), 200µl per 1ml of Trizol) was added and the sample was shaken vigorously for 15 sec. After 10 min incubation at room temperature, the tube was centrifuged at 14000 rpm for 15 min at 4°C for phase separation. The colourless upper aqueous phase that contains RNA was transferred to a clean tube and precipitated by the addition of isopropanol (Sigma, 500µl per 1ml of Trizol). The tubes were incubated at room temperature for 10 min and centrifuged at 14000 rpm for 15 min at 4°C. After discarding the supernatant, the RNA precipitate was washed with 1ml of 75% ethanol and centrifuged at 14000 rpm for 5 min at 4°C. The RNA pellet was air dried for 5 min and then resuspended in approximately 40-60µl of DEPC- ddH_2O depending on the size of the pellet. The sample was then aliquoted to avoid repeated freeze/thaw cycles and stored at -70°C for later use. RNA concentration was determined by measuring the absorbance at 260nm and 280nm (OD_{260} and OD_{280}) using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

2.3.3 Tissue Collection and RNA Isolation

Fibroids, myometrial and endometrial tissues were collected from women undergoing hysterectomy for uterine fibroids. Collected tissues were quickly transferred to 2ml microfuge tubes with RNAlater and were snap frozen on dry ice after collection. The tissue was then disrupted and homogenized twice in RLT™ buffer using a Tissuelyser II (QIAGEN, Crawley, UK) for 3 min at 25Hz frequency according to manufacturer's protocol. The mix was centrifuged for 3 min at 10000 rpm and supernatant was transferred

to a fresh tube and mixed with 70% ethanol. The lysate was transferred into an RNeasy spin column (QIAGEN) placed in a 2ml collection tube and was centrifuged for 15 sec at 10000 rpm at room temperature. The column was then washed with RW1™ buffer (QIAGEN) by spinning at 10000 rpm for 15 sec. To eliminate any genomic DNA contamination the column was directly treated with DNase I (10µl of DNase I stock in 70µl of buffer RDD™ (QIAGEN)) for 15 min at room temperature. The column was then washed with RW1™ and RPE™ buffer (QIAGEN) by centrifuging for 15 sec at 10000 rpm at room temperature. Thereafter, the RNeasy spin column was placed on a fresh 1.5ml collection tube and 30-50µl of RNase-free water was added to the column directly. The column was spun at 1000 rpm for 1 min at room temperature to elute the RNA. The concentration and purity of the RNA was determined as described in 2.3.2.

2.3.4 Primer Design

All primer pairs and probes used in this study were designed using PRIMER express program (Perkin Elmer, PE biosystems, Warrington UK) unless otherwise stated. At least one primer of each pair spanned an exon-exon junction to prevent amplification of genomic DNA during the PCR reaction. The specificity of the designed primers was checked by blasting the sequence using a BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3.5 cDNA Synthesis

The cDNA template was synthesized from total RNA by reverse transcription (RT). For each 20µl RT reaction, a mix of RNA template (400ng) and primers (Random hexamers and/or oligo dT) in a sterile nuclease-free tube was added (the components used in the PCR reaction mix are mentioned in table 2.1). Synthesis of cDNA was performed using a TaqMan® Reverse Transcription Reagents (PE Applied Biosystems, Warrington, UK) in the following conditions; 1 hr 42°C, 5 min 99°C and 5 min at 5°C. A mix with no reverse transcriptase was included to control for any genomic DNA contamination. For non-quantitative RT-PCR, cDNA synthesis was performed with some modification. The

cDNA template for RT-PCR was synthesized from 1µg total mRNA and both random hexamers (1.25µM) and oligo dT (1.25µM) were used for the reaction.

Table 2.1: Reverse transcription reaction mix; components and concentrations.

Component	**Stock concentration**	**Final concentration**
RT-PCR Reaction Buffer	10×	1×
deoxynucleoside triphosphate (dNTP Mix)	10mM	2mM
$MgCl_2$	25mM	5.5mM
Random hexamers	50µM	2.5µM
RNase Inhibitor	20U/µl	0.4U/ul
MultiScribe™ Reverse Transcriptase	50U/µl	1.25U/µl

2.3.6 RT-PCR and Agarose Gel Analysis

Specific adenylyl cyclase (AC) primers (0.4pmol/µl each; Sequences of primers mentioned in table 2.2) were used to amplify individual isoforms of AC in a reaction mix containing AmpliTaq Gold® PCR Master Mix (5mM $MgCl_2$ and 400µM each dNTPs, 0.05 U/µL of AmpliTaq Gold DNA polymerase) and 2µl of cDNA in 20µl final volume. PCR reaction was performed for 35 cycles (94, 56, and 72°C for 30, 45 and 60 sec, respectively) after 5 min at 95°C unless otherwise stated. After the reaction, 4µl of gel tracking dye (Appendix B) was added to increase the density of the PCR product. The bromophenol blue in the dye acts as a tracking dye to follow the progress of the electrophoresis. The mix was then loaded onto a 2% agarose gel that was melted in tris-acetate/EDTA (TAE) buffer (Appendix B) with 2µl of ethidium bromide (10mg/dl). To check the PCR products, electrophoresis was performed at 100 volts/20cm for 30 min and the gel was visualized using ultraviolet light.

Table 2.2: Primer sequences for all adenylyl cyclase isoforms and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene*

Target gene		Sequence (5'-3')
AC1	Forward primer	CCACGTCCTACATCCTCGTT
	Reverse primer	AAGTGGTAGGGGCACCTTCT
AC2	Forward primer	GCTCATGTGGCTTTTGAA
	Reverse primer	GGAAGTGCTCAGCCACGT
AC3	Forward primer	TACCCAGCTGTCTTCTGCCT
	Reverse primer	GAGATGGCCTCCACCATG
AC4	Forward primer	ATGCACAACAGGATGCTGAAC
	Reverse primer	GCCCCAATAACTCCAGCTAC
AC5	Forward primer	CGGGCGAGTACACTGCGG
	Reverse primer	GGTTCTCTGGCGGTTCAT
AC6	Forward primer	TCGGGCAACACTGCAGTACCTG
	Reverse primer	CCCGAGTCCGCTGCAGCTTG
AC7	Forward primer	CTCTCTGTGCTGATGTACGTCGAG
	Reverse primer	TCTTGACGTAGAGGCTGTGGAAGT
AC8	Forward primer	TTAGGAACCCCTCCTCCG
	Reverse primer	TGCTAGGGGCACAGTCAAG
AC9	Forward primer	GTGGAGCATCTATTTTGCGGTC
	Reverse primer	CGAACACCAGCAGGGTGAG
Soluble AC	Forward primer	AAAACCTGTCACCTCCAACG
	Reverse primer	TCTAAAGCGTTGAGCCGAAT
GAPDH	Forward primer	GAGTCAACGGATTTGGTCGTATTG
	Reverse primer	GCTGTAGCCAAATTCGTTGTC

* Primers taken from Bernatchez *et al.*, 2003

2.3.7 Real-Time RT-PCR

The synthesized cDNA was amplified using the specific primers, probes and PCR components that are mentioned in table 2.3. Primers and probes for 18S were also added in the reaction mix for control. After mixing, 20µl of the reaction mix from each samples including a no reverse transcriptase control and a calibrator (positive control) were added to Fast optical 96-well MicroAMP PCR plates (PE Applied Biosystems, Warrington, UK) in duplicates. The plate was sealed with nuclease free MicroAMP optical adhesion film (PE Applied Biosystems, Warrington, UK) and the reaction was run on ABI Prism 7900 Quantitative PCR machine. Data were analyzed using sequence detector version 1.6.3 and expression of target genes were normalized for each samples using their respective 18S rRNA and were expressed as fold increase above vehicle treated samples (Sequences of primer and probes are mentioned in table 2.4).

Table 2.3: Real time RT-PCR reaction mix; components and concentrations

Component	**Stock concentration**	**Final concentration or volume**
Taqman buffer (5.5mM $MgCl_2$ and 200µm of each dNTPs, 0.025U/µl of AmpliTaq Gold DNA polymerase)	2×	1×
Ribosomal 18S primers (forward, reverse and probe)	25µM	50nM each
Target gene forward and reverse primers	25µM	300nM each
Target gene probe	5µM	100nM each
cDNA	-	2µl/50µl

Table 2.4: Primers and probes for Real Time RT-PCR

Target gene		Sequence (5'-3')
COX-1	Forward primer	TGTTCGGTGTCCAGTTCCAATA
	Reverse primer	ACCTTGAAGGAGTCAGGCATGAG
	Probe (FAM)	CGCAACCGCATTGCCATGGAG T
COX-2	Forward primer	CCTTCCTCCTGTGCCTGATG
	Reverse primer	ACAATCTCATTTGAATCAGGAAGCT
	Probe (FAM)	TGCCCGACTCCCTTGGGTGTCA
EP1 Receptor	Forward primer	AGATGGTGGGCCAGCTTGT
	Reverse primer	GCCACCAACACCAGCATTG
	Probe (FAM)	CAGCAGATGCACGACACCACCATG
EP2 Receptor	Forward primer	GACCGCTTACCTGCAGCTGTA C
	Reverse primer	TGAAGTTGCAGGCGAGCA
	Probe (FAM)	CCACCCTGCTGCTGCTTCTCATTG TCT
EP3 Receptor	Forward primer	GACGGCCATTCAGCTTATGG
	Reverse primer	TTGAAGATCATTTTCAACATCATTATCA
	Probe (FAM)	CTGTCGGTCTGCTGGTCTCCGCTC
EP4 Receptor	Forward primer	ACGCCGCCTACTCCTACATG
	Reverse primer	AGAGGACGGTGGCGAGAAT
	Probe (FAM)	ACGCGGGCTTCAGCTCCTTCCT
FP Receptor	Forward primer	GCAGCTGCGCTTCTTTCAA
	Reverse primer	CACTGTCATGAAGATTACTGAAAA AAATAC
	Probe (FAM)	CAC AAC CTG CCA GAC GGA AAA CCG
VEGF	Forward primer	TACCTCCACCATGCCAAGTG
	Reverse primer	TAGCTGCGCTGATAGACATCCA
	Probe (FAM)	ACTTCGTGATGATTCTGCCCTCCTCCT T
IL-8	Forward primer	CTGGCCGTGGCTCTCTT
	Reverse	TTAGCACTCCTTGGCAAAACTG
	Probe (FAM)	CCTTCCTGATTTCTGCAGCTCTGTGTGAA
IL-11	Forward primer	CCCAGTTACCCAAGCATCCA
	Reverse primer	AGACAGAGAACAGGGAATTAAATGTGT
	Probe (FAM)	CCCCAGCTCTCAGACAAATCGCCC
SAT1	Forward primer	CGGGCCGACTGGTGTTTA
	Reverse primer	AGTCAGGCTGGCACCATGAC
	Probe (FAM)	CCGTCACTCGCCGAGGTTCCTTG
AC1	Forward primer	TCTTCGGCAAGTTCGATGAA
	Reverse primer	GCAGTCCCCGAGAATCTTGA
	Probe (FAM)	TAGCCACGGAGAACCACTGTCGCC
AC3	Forward primer	CTGATGTCACTGTAGCCAACAAGA
	Reverse primer	CCACATCAAACTCCCCTTTCA
	Probe (FAM)	CATCCCTGGGCGCGTGCAC
Prolactin	Forward primer	CGGAAGTACGTGGTATGCAAGA
	Reverse primer	TCAGGATGAACCTGGCTGACT
	Probe (FAM)	CCCCGGAGGCTATCCTATCCAAAGCT
18S	Forward primer	CGGCTACCACATCCAAGGAA
	Reverse primer	GCTGGAATTACCGCGGCT
	Probe (VIC)	TGCTGGCACCAGACTTGCCCTC

2.4 Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significant differences were determined by one-way analysis of variance or unpaired student's *t*-test using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) (*, $p<0.05$; **, $p<0.001$; ***, $p<0.0001$).

Chapter III

Expression of Cyclooxygenase Enzymes and Prostaglandin Receptors in Fibroids, Myometrium and Endometrium Samples from Women with Uterine Fibroids

3.1 Introduction

Uterine fibroids, myometrium-derived benign tumors, are the most common uterine disorder occurring in as many as 30% of women over 35 years of age (Flake *et al.,* 2003). The etiology of uterine fibroids is unknown but several studies have shown estrogen and progesterone play a major role in promoting its growth (Rein, 2000; Flake *et al*., 2003).

Numerous growth factors have been implicated with tumor development of fibroids since they are essential elements in controlling the proliferation rate of cells. Amongst these growth factors, the role for EGF, IGF and bFGF in uterine fibroids are well documented (Hoppener *et al*., 1988; Flake *et al.,* 2003).

The COX-prostanoid pathway has been implicated in numerous cancers including reproductive pathologies (Jabbour *et al.,* 2006b). However, studies on the expression of COX enzymes and prostaglandin receptors in uterine fibroids and surrounding tissues are limited.

3.2 Aim of the Study

Previous molecular studies have shown role for genes that are regulated by COX enzymes and prostaglandins in reproductive pathology. The aims of this study were to compare the expression of

(a) COX enzymes and prostaglandin receptors between fibroid and adjacent myometrium tissue samples.

(b) COX enzymes, prostaglandin receptors and genes activated by prostaglandins between endometrium from women with and without fibroids.

3.3 Materials and Methods

3.3.1 Tissue Collection and RNA Extraction

Biopsy specimens from fibroids and adjacent myometrium were collected from women undergoing hysterectomy for the disorder (n=14 each). Endometrial tissues were collected from women undergoing hysterectomy for uterine fibroids (n=8 proliferative, n=11 early-mid secretory and n=2 from late secretory phase). Normal endometrium samples from different cycle stages were collected from women undergoing hysterectomy for benign gynecological disorders (n=10 proliferative, n=17 from early-mid secretory and n=3 from late secretory phase). The cycle stage dates were confirmed by histological staging by a pathologist and by blood estrogen and progesterone levels. Samples were quickly transferred to microfuge tubes with RNAlater®, snap frozen on dry ice and stored at -70°C until processing. Total RNA was extracted from cell lysates as described in section 2.3.3. The concentration and purity of the RNA were determined by measuring the absorbance at 260nm and 280nm (OD_{260} and OD_{280}) using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

3.3.2 cDNA Synthesis and Real-Time RT-PCR

Real-Time RT-PCR was performed to investigate the levels of COX-1, COX-2, prostaglandin receptors (EP1, EP2, EP3, EP4 and FP receptors), prolactin, VEGF, IL-8 and IL-11 mRNA expression in tissue homogenates derived from women with and without fibroids. cDNA was synthesized from total mRNA (400ng) by reverse transcription in the presence of PCR reaction buffer (1×), dNTPs (0.5mM each), $MgCl_2$ (5.5mM), random hexamers (2.5µM), RNase inhibitor (0.4U/µl), and MultiScribe™ reverse transcriptase (1.25U/µl) (PE Applied Biosystems, Warrington, UK). Thereafter, Real-Time RT-PCR was performed using specific primers and probes (300nM and 100nM, respectively; sequences of the primers used are mentioned in table 2.4) in a reaction mix containing Taqman buffer (5.5mM $MgCl_2$ and 200µm of each dNTPs, 0.025U/µl of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Warrington,

UK)), ribosomal 18S forward and reverse primers and probe (all at 50nM), and 2µl of the cDNA.

3.3.3 Statistical Analysis

All data are presented as mean ± SEM. Statistical significant differences were determined by unpaired student's *t*-test using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) (*, $p < 0.05$; **, $p < 0.001$; ***, $p<0.0001$).

3.4 Results

3.4.1 Expression of COX-1 and COX-2 in Fibroids and Adjacent Myometrium Tissue Samples

The levels of COX-1 and COX-2 expressions were determined in fibroids and adjacent myometrium tissue homogenates obtained from women with fibroids using Real-Time RT-PCR. As shown in figure 3.1A, level of COX-1 expression was not statistically different in fibroids samples (0.18 ± 0.04) compared to adjacent myometrium samples (0.33 ± 0.10). The level of COX-2 mRNA expression also showed no significant difference between the samples with the following values: Myometrium (0.39 ± 0.14) and Fibroids (0.16 ± 0.03) (Fig.3.1B).

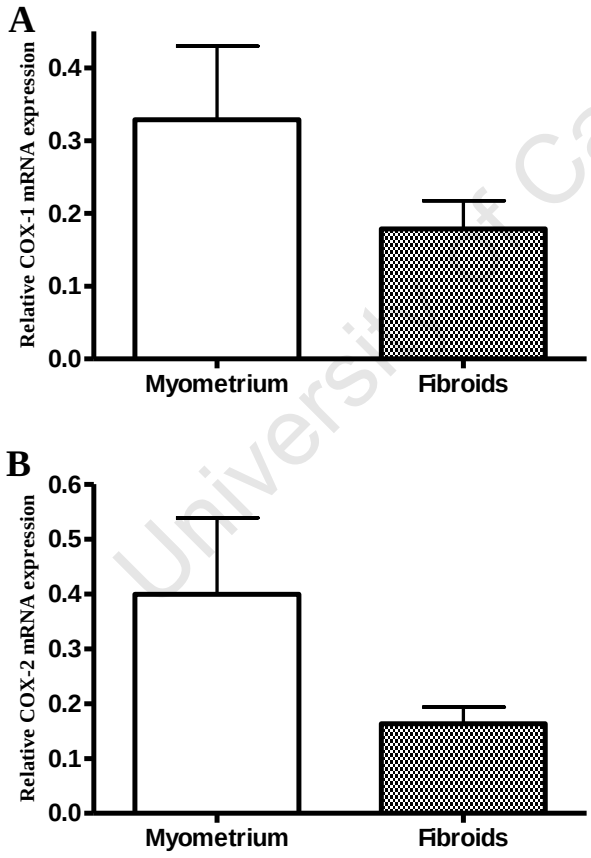

Fig. 3.1: *Relative mRNA expression of* ***(A)*** *COX-1 and* ***(B)*** *COX-2 in fibroids (n=7) and adjacent myometrium (n=7) tissue homogenates from women with fibroids as determined by Real-Time RT-PCR. Data are presented as mean ± SEM from three independent experiments.*

3.4.2 Expression of Prostanoid Receptors and Prolactin in Fibroids and Adjacent Myometrium Tissue Samples

Real-Time RT-PCR was used to assess mRNA expression levels of prostanoid receptors (EP1, EP2, EP3, EP4 and FP receptors) and prolactin between fibroids and adjacent myometrium tissue homogenates from women with fibroids. As shown in figure 3.2A and 3.2B, the level of EP1 and EP2 receptors in myometrium were slightly different compared to fibroids samples however, the difference was not statistically significant (EP1 receptor; 0.31 ± 0.09 vs. 0.58 ± 0.29 and EP2 receptor; 9.94 ± 2.66 vs. 6.09 ± 2.6 respectively). EP3 receptor mRNA expression was significantly lower ($p<0.001$) in fibroid tissue samples (19.45 ± 4.98) compared to the adjacent myometrium samples (51.43 ± 7.21) (Fig 3.2C). The values of EP4 and FP receptor mRNA expression were almost similar in myometrium and in fibroid samples (EP4 receptor; 1.65 ± 0.39 vs. 1.66 ± 0.53 and FP receptor; 399.0 ± 99.29 vs. 316.8 ± 103.2 respectively) (Fig 3.2D and 3.2E). Prolactin mRNA expression was not statistically different in fibroid tissue samples (3.06 ± 0.98) compared to myometrium samples (2.08 ± 0.36) (Fig 3.2F).

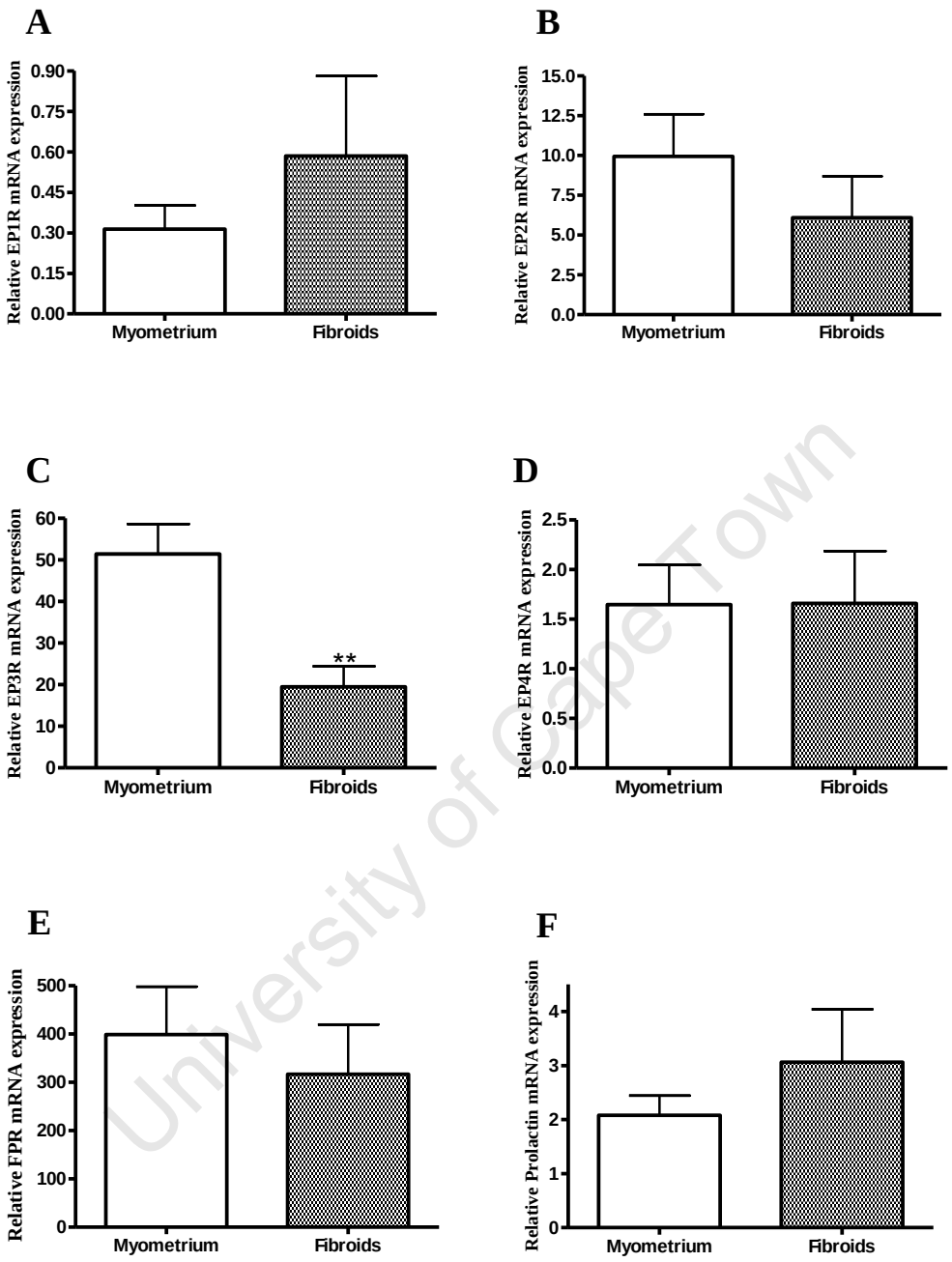

Fig. 3.2: *Relative mRNA expression of prostanoid receptors. (**A**) EP1 (**B**) EP2 (**C**) EP3 (**D**) EP4 (**E**) FP receptors (**F**) prolactin in fibroids (n=14) and adjacent myometrium tissue (n=14) homogenates from women with fibroids as determined by Real-Time RT-PCR. Data are presented as mean ± SEM from three independent experiments (**, $p<0.001$).*

3.4.3 Expression of COX-1 and COX-2 in Endometrium from Women with and without Fibroids

The expression of COX-1 and COX-2 was assessed throughout the menstrual cycle (proliferative, early-mid secretory and late secretory phase) using cDNA samples from endometrium tissue of women with and without fibroids. As shown in figure 3.3A, COX-1 mRNA expression did not show any significant difference between endometrium from women with fibroid and control samples in all the menstrual cycle stages. The level of COX-2 mRNA expression was significantly higher ($p<0.0001$) in endometrium from women with fibroids compared to control samples in both proliferative (4.34 ± 1.09 vs. 0.58 ± 0.11) and early-mid secretory phase (10.15 ± 6.33 vs. 3.73 ± 1.1) (Fig 3.3B).

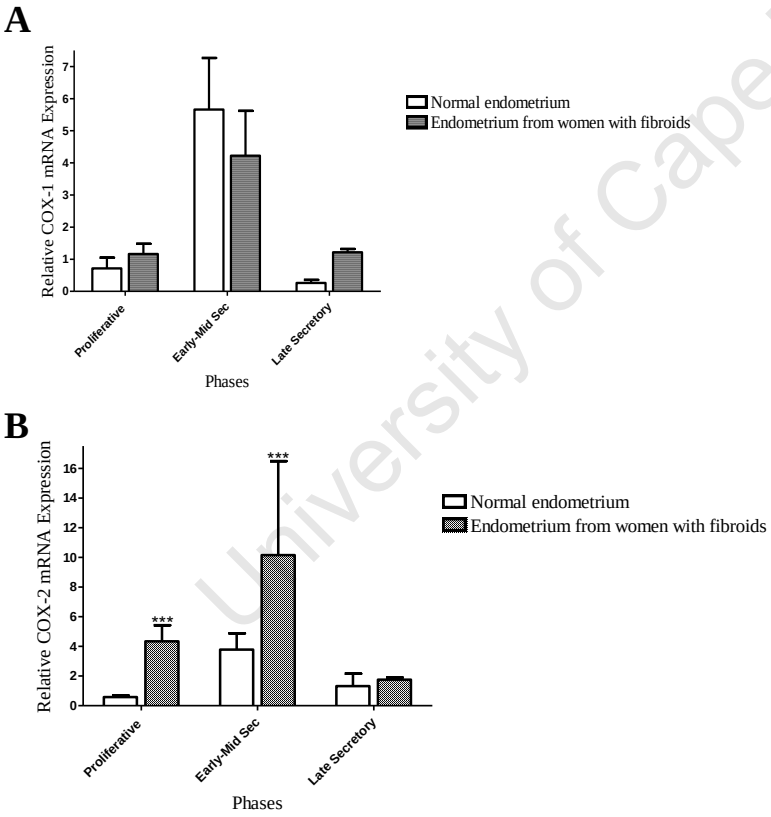

Fig. 3.3: *Relative mRNA expression of* ***(A)*** *COX-1* ***(B)*** *COX-2 in endometrium tissue of women with (n=8 proliferative, n=11 early-mid secretory and n=2 from late secretory phase) or without fibroids (n=10 proliferative, n=17 from early-mid secretory and n=3 from late secretory phase) as determined by Real-Time RT-PCR across different stages of menstrual cycle. Data are presented as mean ± SEM from three independent experiments (****, $p<0.0001$).

3.4.4 Expression of Prostanoid Receptors in Endometrium from Women with and without Fibroids

Expression of EP2, EP4 and FP receptors was assessed in endometrium tissue from normal and of women with fibroids using Real-Time RT-PCR. As shown in figure 3.4A, the level of EP2 receptor showed a significant increase in endometrium tissue derived from women with fibroids in proliferative stage (9.79 ± 3.35 vs. 3.82 ± 0.80; $p<0.001$), while it was lower in the early-mid secretory phase compared to normal endometrium (8.67 ± 2.74 vs. 20.34 ± 4.41; $p<0.05$). FP receptor level was significantly higher in all the stages of the menstrual cycle in endometrium from women with fibroids compared to normal endometrium with the following values; proliferative (10.11 ± 3.57 vs. 3.01 ± 0.7; $p<0.0001$), early-mid secretory (6.74 ± 1.59 vs. 1.91 ± 0.63; $p<0.05$) and late secretory phase (5.61 ± 3.35 vs. 0.73 ± 0.23; $p<0.05$) (Fig 3.4B). The level of EP4 receptor expression was not significantly different between endometrium of women with or without fibroids at any stage of the menstrual cycle (Fig 3.4C).

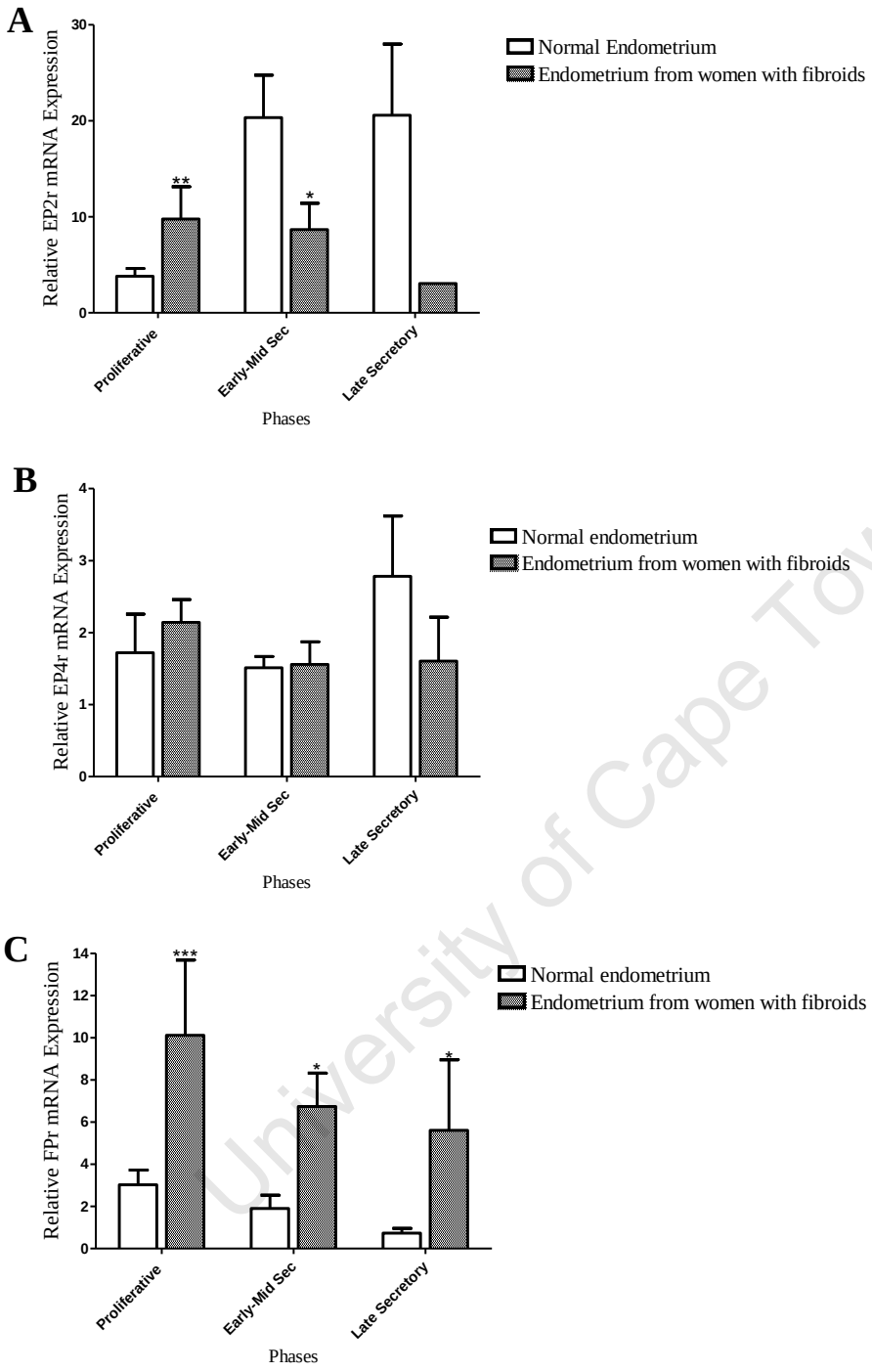

Fig. 3.4: *Relative mRNA expression of* ***(A)*** *EP2* ***(B)*** *EP4* ***(C)*** *FP receptors in endometrium tissue of women with (n=8 proliferative, n=11 early-mid secretory and n=2 from late secretory phase) or without fibroids (n=10 proliferative, n=17 from early-mid secretory and n=3 from late secretory phase) as determined by Real-Time RT-PCR across different stages of menstrual cycle. Data are presented as mean ± SEM from three independent experiments (*, $p < 0.05$; **, $p < 0.001$; ***, $p<0.0001$).*

3.4.5 Expression VEGF, IL-8 and IL-11 in Endometrium from Women with and without Fibroids

Real-Time RT-PCR was used to determine the level of VEGF, IL-8 and IL-11 mRNA expression in endometrium from women with and without fibroids. As shown in figure 3.5A, level of VEGF mRNA expression in early-mid secretory was shown to be significantly higher ($p<0.001$) in endometrium from women with fibroids compared to control samples (20.12 ± 3.84 vs. 12.31 ± 1.3) while there was no significant difference in the other phases. The Level of IL-8 expression was significantly higher in endometrium from women with fibroids in the proliferative phase (99.03 ± 26.63 vs. 3.09 ± 0.86; $p<0.0001$) and in early-mid secretory phase (56.58 ± 16.26 vs. 3.34 ± 0.77; $p<0.0001$) in comparison to normal endometrium samples (Fig 3.5B). Expression of IL-11 was significantly higher in endometrium from women with fibroids in the proliferative stage (26.33 ± 10.84 vs. 4.48 ± 2.21; $p<0.0001$), while it was lower in the early-mid secretory phase compared to normal samples (10.56 ± 2.24 vs. 23.12 ± 5.66; $p<0.0001$) (Fig 3.5C). Both IL-8 and IL-11 mRNA expression were not altered in the late secretory phase.

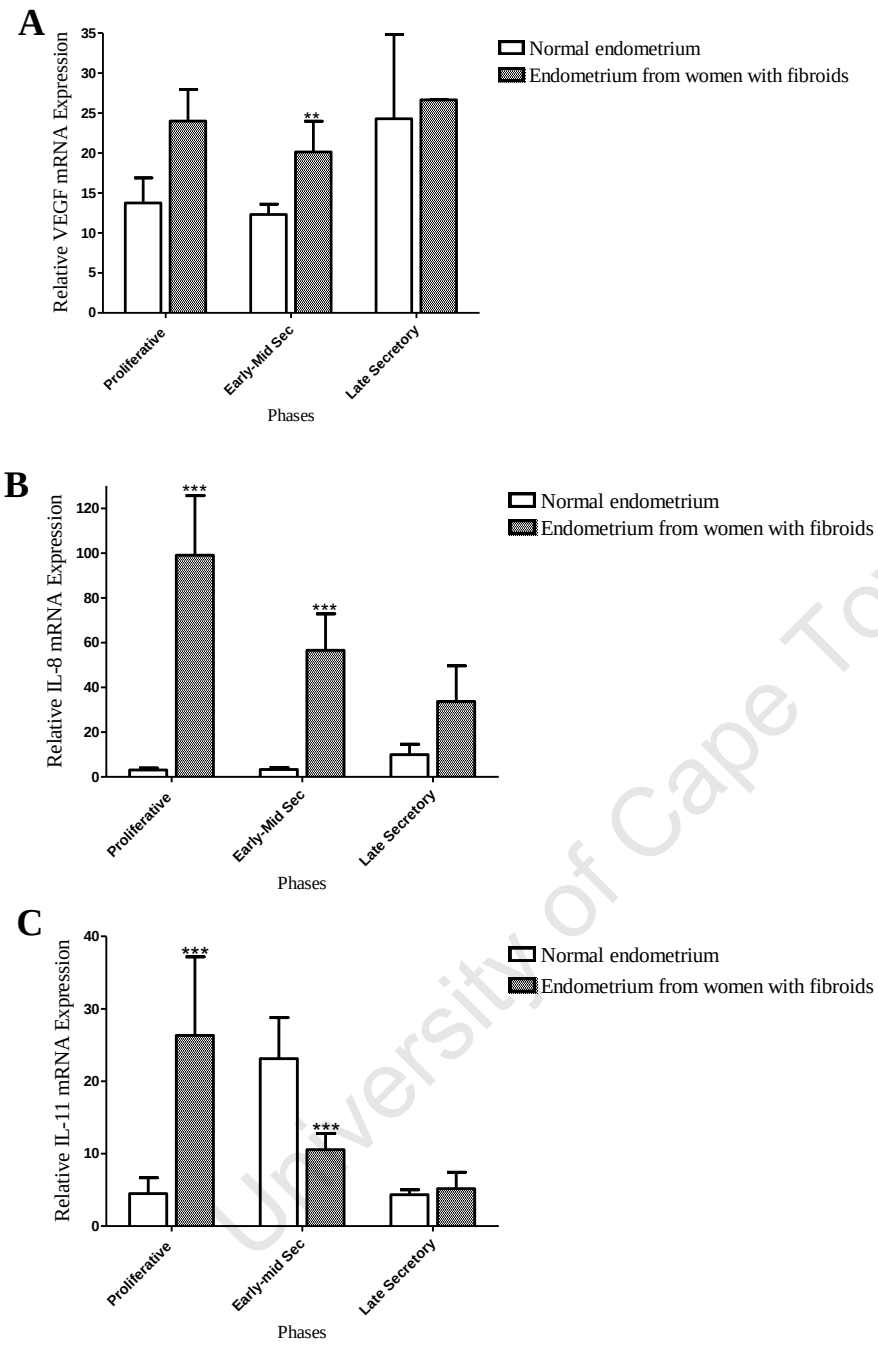

Fig. 3.5: *Relative mRNA expression of* ***(A)*** *VEGF* ***(B)*** *IL-8* ***(C)*** *IL-11 in endometrium tissue of women with (n=8 proliferative, n=11 early-mid secretory and n=2 from late secretory phase) or without fibroids (n=10 proliferative, n=17 from early-mid secretory and n=3 from late secretory phase) as determined by Real-Time RT-PCR across different stages of menstrual cycle. Data are presented as mean ± SEM from three independent experiments (**, p < 0.001; ***, p<0.0001).*

3.5 Discussion

Uterine fibroids are a fairly frequent occurrence in women in the reproductive age group. Despite being a major cause of gynecological morbidity, the etiology of uterine fibroid is still not yet elucidated (Marshall *et al.,* 1997). Several studies have shown ovarian hormones estrogen and progesterone to play a major role in the development of uterine fibroids (Flake *et al*., 2003). Furthermore, there have been studies showing the effectors of uterine fibroids once they are formed. Since uterine fibroids are mostly characterized by changes in cell proliferation and differentiation, there has been more focus on studying the effect of growth factors in uterine fibroids. As mentioned in the introduction numerous growth factors have been implicated with the pathology of uterine fibroids (Hoppener *et al*., 1988; Flake *et al.,* 2003; Gao *et al.,* 2001; Mangrulkar *et al*., 1995; Maruo *et al*., 2000).

There is a compelling evidence to support a role for COX enzymes and prostaglandins in reproductive pathologies such as dysmenorrhea, endometriosis, menorrhagia, cervical cancer and endometrial adenocarcinoma (Lumsden *et al.*, 1983; Karck *et al.,* 1996; Smith *et al.,* 1981; Sales *et al*., 2002; Ryu *et al*., 2000; Jabbour *et al.*, 2006b). However, there haven't been sufficient studies to assess the effects of prostaglandins on the development of uterine fibroids. The objective of this study was to assess the expression level of COX enzymes and prostaglandin receptors in fibroids, autologous myometrium and endometrium samples from women with fibroids.

The growth of uterine fibroid arises from the myometrium and was practical to first compare the expression of target genes between fibroids and adjacent myometrium samples. In the first part of this study, fibroids and patient-matched myometrial samples from 14 women with uterine fibroids were assessed for mRNA expression of COX enzymes (COX-1 and COX-2), prostaglandin receptors (EP1, EP2, EP3, EP4 and FP receptors) and prolactin. The expressions of COX-1 and COX-2 were not statistically different in fibroids compared to adjacent myometrium samples. Prostanoid receptors (EP1, EP2, EP4 and FP receptors) and prolactin also did not show any significance difference between fibroid and patient-matched myometrium samples.

However, expression of the EP3 prostanoid receptor showed a significant decrease in fibroids compared to myometrium samples. Four independent gene array analyses have reported down-regulation of the EP3 subtype receptor in fibroids compared to adjacent myometrium samples (Tsibris *et al*., 2002; Wang *et al*., 2003; Quade *et al*., 2003; Catherino *et al.*, 2004). Down-regulation of the EP3 receptor has been shown to play a role in colorectal cancer. Shoji *et al.* (2004) showed that the EP3 receptor decreased markedly in colon cancer tissue compared to normal colon mucosa samples. The authors also showed azoxymethane-induced colon cancer was more enhanced in EP3 receptor knockout mice compared to wild type mice. Cell lines studies by the group also revealed EP3 receptor agonist can markedly decrease the number of viable cells in the HCA-7 human colon cancer cell lines expressing the EP3 receptor, supporting its role in decreasing cell proliferation (Shoji *et al.,* 2004). Based on these observations, down-regulation of the EP3 receptor might not influence the early stages of uterine fibroids but could still play a role in development of the tumor at a later stage once the fibroid is formed.

Despite several reports showing up-regulation of prostanoid receptors (mainly EP2 and FP receptors) in uterine pathology (Jabbour *et al*., 2001; Sales *et al*., 2004; Sales *et al.*, 2005; Jabbour *et al.*, 2006b), in this current study there was no significant change observed between fibroids and patient-matched myometrium samples. These findings led me to the second part the study. As mentioned in the introduction, the growth of fibroids can affect autologous endometrium possibly leading to a dysregulated endometrium and pregnancy complications. Molecular studies have shown macrophage infiltration, concentration of MCP-1 and PGF expression to be higher in endometrium from women with fibroids (submucosal and intramural) compared to control endometrium samples (Deligdish and Loewenthal, 1970; Miura *et al.*, 2006).

In light of these studies, I decided to look at the expression level of COX enzymes prostaglandin receptors, and prostanoids-mediated genes in endometrium from women with and without fibroids. These samples were matched according to their respective

menstrual cycle stage and women treated with GnRH analog were excluded from the study. First, I assessed the expression level of COX-1 and COX-2 since both isoforms of COX enzymes have been shown to be involved in reproductive pathology (Sales *et al*., 2002; Tong *et al*., 2000; Ferrandina *et al*., 2002; Ryu *et al*., 2000; Sales *et al*., 2001; Sales *et al.,* 2002). The level of COX-1 expression was not altered between the samples but COX-2 mRNA expression was significantly higher in both proliferative and early-mid secretory phase in endometrium from women with fibroids compared to control samples. The level of EP2 receptor was also elevated in the proliferative stage while FP receptor was up-regulated in all stages of menstrual cycles in endometrium samples from women with fibroids. COX-2 has been shown to play a role in uterine pathologies by promoting angiogenesis, inhibiting apoptosis, controlling cell growth and increasing cell migration and cell adhesion. This is mainly facilitated by production of prostaglandins all products of COX-2 or COX-1 pathway (Ohno *et al.,* 2005; Jabbour *et al.,* 2006b). In the human endometrium, production of PGF and PGE_2 by separated epithelial cells is higher in proliferative phase of menstrual cycle to regulate epithelial cell proliferation (Smith and Kelly, 1988; Milne and Jabbour, 2003). PGF and PGE_2 can interact with EP2 and FP receptors to activate expression of angiogenic and mitogenic genes such as VEGF and IL-8 to act in a paracrine manner on neighboring myometrial/fibroid tissue.

Angiogenesis is essential for actively growing tumors as the tumor needs oxygen and nutrients to facilitate growth. COX-2, PGE_2 and PGF and their cognate receptors have been shown to promote expression of angiogenic factors such as VEGF (Jabbour *et al.,* 2006b; Sales *et al.,* 2004; Sales *et al.,* 2005). VEGF is a potent angiogenic and mitogenic growth factor that plays a great role in the development of reproductive tumors by promoting angiogenesis (Leung *et al.,* 1989). Previous studies have shown expression of VEGF was not altered between fibroids and matched myometrial samples, despite studies showing over-expression of VEGF in other reproductive pathologies (Harrison-Woolrych *et al*., 1995; Guidi *et al.,* 1996). In this current study VEGF was highly expressed in the early-mid secretory phase of the menstrual cycle in endometrium from women with fibroids compared to control samples suggesting its possible paracrine role in promoting angiogenesis to sustain tumor development in fibroids.

IL-8, a chemoattractant chemokine, was the other gene that was assessed and it is involved in angiogenesis and mitogenesis. IL-8 has shown to be over-expressed in cancer cell lines including cancer of breast, ovary and prostate (Green *et al*, 1997; Ivarsson *et al.*, 1998; Ferrer *et al.*, 1998). Arici *et al.* (1998) have shown that IL-8 can play a role in the growth and proliferation of endometrium by directly stimulating endometrial stromal cell proliferation. Recent study also showed lysophosphatidic acid (LPA)-mediated expression of IL-8 can trigger multi-step process of angiogenesis including migration and proliferation of endothelial cells (Chen *et al.*, 2008). Comparative studies done on uterine fibroids and adjacent myometrium samples showed high level of IL-8 and its receptor in myometrium tissue immediately surrounding fibroids. Furthermore, the authors showed blocking of IL-8 by neutralizing antibody can inhibit cell proliferation showing its role in the growth of fibroids (Senturk *et al.*, 2001). In this study IL-8 mRNA expression was significantly higher in the proliferative and early-mid secretory phase in endometrium samples from women with fibroids compared to control samples. These data suggest that up-regulation of VEGF and IL-8 in endometrium collectively can have a paracrine effect on fibroids to promote angiogenesis and sustain tumor development.

This study also showed up-regulation of IL-11 in the proliferative stage of endometrium from women with fibroids. IL-11 is a hematopoietic growth factor and can affect cell growth, differentiation and proliferation (Du and Williams, 1994). Studies done using IL-11 over-expressing transgenic mice showed subepithelial and adventitial tissue fibrosis in lung tissue compared to wild type mice. Immunohistochemistry analysis of tissue samples from IL-11 over-expressing mice also revealed an enhancement in deposition of type I and type III collagen, suggesting a role for IL-11 in the progression of fibrotic disorders (Zhu *et al.*, 2001). Luo *et al.* (2005) showed transforming growth factor-β (TGF-β) treated fibroid cells expressed significantly higher IL-11 mRNA expression compared to normal myometrial smooth muscle cells using microarray analysis. Our findings show an up-regulation of COX-2 and prostanoid receptors (EP2 and FP) in endometrium of women with fibroids. These prostaglandin receptors can in turn enhance expression of genes that are involved in angiogenesis and mitogenesis such

as VEGF, IL-8 and IL-11 to sustain development of uterine fibroids via paracrine manner.

Up-regulation of the EP2 and FP receptors in the proliferative phase of endometrium from women with fibroids was also observed in this study. For many years it has been known that prostanoid receptors are co-expressed in many cell types (Ashby, 1998). For example, our laboratory has shown that EP2 and FP receptors are co-expressed in endometrial adenocarcinoma cells (Sales *et al*., 2004; Sales *et al*., 2005). However, to my knowledge there have been no studies addressing the integrative signalling effects of the two prostanoid receptors on signal transduction and gene expression in endometrium. Taking these in account, I decided to investigate a possible cross-talk between the EP2 and FP receptors and the molecular mechanism underlying the intracellular signalling pathway in response to co-activation of both receptors.

Chapter IV

Generation and Characterization of Ishikawa Cells Stably Expressing the EP2 and FP Receptors

4.1 Introduction

The arachidonic acid cascade produces a family of bioactive lipids including prostaglandins, thromboxanes and leukotrienes (collectively called eicosanoids) that modulates different physiological and pathophysiological responses in the reproductive tract (Marnett, 1992). Arachidonic acid gets hydrolyzed by PLA_2 and is released from sn-2 position of the membrane. Once it is released from cellular stores, arachidonic acid gets oxidized by the rate limiting enzyme COX of which there are three isoforms (COX-1, COX-2 and COX-3) to form prostaglandin G_2 (PGG_2). PGG_2 then gets reduced and serves as the substrate for terminal prostanoid synthase enzymes to produce specific prostaglandins (Marnett, 1992; Narumiya *et al.,* 1999).

Prostaglandins have a relatively short half-life and are believed to exert paracrine and autocrine effects on target cells by coupling to their specific GPCRs to activate intracellular signaling. PGE_2 and PGF are the most abundantly biosynthesized prostaglandins and are major metabolites of COX enzymes in the human endometrium (Narumiya *et al.,* 1999; Jabbour *et al.,* 2006b). COX enzyme expression, prostaglandin synthesis and their cognate receptor expression (mainly EP2 and FP) are dysregulated in numerous reproductive pathologies including endometrial adenocarcinoma (Lumsden *et al*., 1983; Karck *et al*., 1996; Smith *et al.,* 1981; Jabbour *et al.,* 2006b).

Researchers have studied the molecular pathway of prostaglandin receptors by over-expressing receptors in model cell lines. Studies done in our laboratory have shown elevated expression and activation of EP2 and FP receptors, which promote the expression of angiogenic genes via the ERK1/2 pathway in Ishikawa cells (Sales *et al*., 2004; Sales *et al*., 2005). In addition others have shown activation of the EP2 receptors can trigger the Wnt signalling pathway that involves phosphorylation of GSK-3 by Akt to activate β-catenin-mediated transcriptional activation in HEK-293 cells (Fujino *et al.*, 2002).

EP2 and FP receptors are co-expressed in Ishikawa cells and use different intracellular signalling pathways (Sales *et al.*, 2004; Sales *et al.*, 2005). EP2 receptors couple to $G\alpha_{s,}$ resulting in increased formation of cAMP, while FP receptors couple to $G\alpha_{q}$ leading to the accumulation of IP3 and DAG (Watanabe *et al.*, 1994; Jabbour *et al.*, 2001; Milne and Jabbour, 2003).

4.2 Aim of the Study

The EP2 and FP receptors are often co-expressed in numerous types of cells including Ishikawa cells. The aim of this study was to generate a model cell line co-expressing the EP2 and FP receptors in Ishikawa cells in order to study a possible cross-talk between both signalling pathways.

4.3 Materials and Methods

4.3.1 Generation of EP2 and FP Receptors Stable Cell Lines and Selection Procedure

To generate cells expressing the EP2 and FP receptors (FPEP2 cells), cells stably expressing the FP receptor (FPS32) were used as a parental cell line. FPS32 cell lines were generated by Sales *et al.* (2005), which were used for individual FP receptor signalling pathway studies. EP2 receptor cDNA was transfected into FPS32 cell lines as described in section 2.2.2. Briefly, EP2 receptor cDNA expression construct was ligated into the pcDNA3.1 expression vector (Invitrogen) and transfected into the FPS32 cell lines using SuperFect® transfection reagent. Transfected cells were selected in a medium containing 800µg/ml of hygromycin. Fifty single hygromycin-resistant clones were picked and allowed to grow under the selective medium. Clones were then screened for expression of the EP2 receptor by Real-Time RT-PCR and by determining intracellular accumulation of cAMP in the presence of the EP2 receptor ligands, PGE_2 and Butaprost.

4.3.2 cDNA Synthesis and Real-Time RT-PCR

Real-Time RT-PCR was performed to investigate the levels of E prostanoid receptors (EP1, EP2, EP3 and EP4) and FP receptor mRNA expression in parental FPS32 cell line and selected FPEP2 clones. Total mRNA was extracted from the clones as described in section 2.3.2 and cDNA was synthesized from total mRNA (400ng) by reverse transcription in the presence of RT reaction buffer (1×), dNTPs (0.5mM each), $MgCl_2$ (5.5mM), random hexamers (2.5µM), RNase inhibitor (0.4U/µl), and MultiScribe™ reverse transcriptase (1.25U/µl). Thereafter, Real-Time RT-PCR was performed using specific EPs and FP receptors primers (300nM) and probes (100nM) in a reaction mix containing Taqman buffer (5.5mM $MgCl_2$ and 200µm of each dNTPs, 0.025U/µl of AmpliTaq Gold DNA polymerase), ribosomal 18S forward and reverse primers and probe (all at 50nM), and 2µl of the cDNA (Sequences of the primers used are mentioned in Table 2.4).

4.3.3 Ligand Stimulation and cAMP Assay

In order to determine the functionality of the EP2 receptor, cAMP accumulation was determined in response to PGE_2 and Butaprost induction. Cells (2×10^5 cells/well) were seeded in 6-well plates and serum starved the following day in the presence of 3µg/ml of indomethacin. A day later, the cells were pre-treated with 0.2mM of IBMX for 30 min, before being treated with vehicle (0.1% ethanol), PGE_2 (100nM) or Butaprost (5µM) for 5 and 10 min in serum free medium. cAMP accumulation was determined by ELISA using cAMP Kit (R&D Systems) according to method mentioned in section 2.2.3. The concentration of cAMP was calculated using a standard curve by Assay Zap computer programe (Biosoft, Cambridge, UK) and was normalized according to the respective protein concentration of each sample. Data are presented as mean ± SEM.

4.3.4 Ligand Stimulation and Total IP3 Assay

PGF induced accumulation of IP3 in FPS32 and FPEP2 cells was determined by plating 50,000 cells in 24-well plate containing 1ml/well of complete medium. The following day, cells were labeled with 0.5µCi/well myo-[3H]-inositol in an inositol-free DMEM 199 medium (2% FCS) overnight. A day later cells were washed and stimulated with increasing doses of PGF (10^{-10} to 10^{-6}M) in Buffer I (Appendix B) for 1 hr at 37°C. After aspirating the buffer, cells were lysed by the addition of 1 ml ice cold 10mM formic acid and by placing the plates on ice for 30 min. Total [3H]-inositol phosphates was then separated and analyzed by the method described in section 2.2.4. Data were expressed as fold increase above non-stimulated samples and presented as mean ± SEM.

4.3.5 SDS-PAGE and Western Blot Analysis

To assess protein expression level of FP and EP2 receptors, total protein was extracted from FPS32 and FPEP2 and quantified as mentioned in section 2.2.7 and 2.2.8, respectively. A total of 40µg of protein was resuspended in 1× Laemmli buffer (Appendix B) and boiled for 5 min at 95°C. Samples and SeeBlue® pre-stained protein marker

(Novex, Invitrogen) were then fractionated by electrophoresis (50mA, 2 hrs) on a pre-cast 4% to 12% Bis-Tris gels (NOVEX, Invitrogen). The gel was then transferred onto Immobilon™-FL PVDF membrane (Millipore, Watford, UK) in a transfer buffer (Appendix B) at constant voltage of 14V using semi-dry blotter for 90 min. After incubating the membrane in 10ml of Odyssey blocking buffer for 1 hr the membrane was either incubated with the rabbit primary antibody raised against the EP2 or the FP receptor in combination with goat anti-actin primary antibody (all in 1:1000) at 4°C overnight with gentle shaking. The membrane was then washed four times in 10 ml of PBS-Tween and was incubated with two fluorescent secondary antibodies, donkey anti-rabbit IgG conjugated to Alexa Fluor 680 (Invitrogen) and donkey anti-goat IgG conjugated to IRDYE 800 (Tebu-bio, Peterborough, UK) for 1 hr in the dark at room temperature. After washing the membrane three times with PBS-Tween, proteins were then viewed using Odyssey Infrared imaging system (LI-COR biosciences, Cambridge, UK).

4.3.6 Immunofluorescence Microscopy of Ishikawa Cells

To localize the expression of the EP2 and FP receptors, FPEP2 and FPS32 Ishikawa cells (1×10^5 cells/well) were plated out in 2-well chamber slides, fixed with 4% PFA for 20 min and blocked in 5% BSA diluted in normal goat serum for 2 hrs. Thereafter, the cells were incubated with rabbit anti-EP2 or FP receptor antibody diluted in normal goat serum containing 5% BSA (1:100) overnight at 4°C and washed three times in PBS for 10 min, before being incubated with Alexa flour conjugated secondary goat anti-rabbit antibody (Molecular probes) for 2 hrs. After three 10 min PBS washes, the nuclear was stained using DAPI (1:1000 in PBS; Sigma) for 10 min. Thereafter, slides were washed and mounted in Permafluor for microscope analysis. Control cells were incubated with preadsorbed primary antibodies with a specific blocking peptide.

4.3.7 Statistical Analysis

All data are presented as mean ± SEM. Statistical significant differences were determined by one-way analysis of variance using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) (*, $p< 0.05$; **, $p< 0.001$; ***, $p<0.0001$).

4.4 Results

4.4.1 Analysis of EP2 and FP Receptors mRNA Expression of the Ishikawa Stably Transfected Clones

FPS32 cells were transfected with the EP2 receptor cDNA and grown under selective media containing hygromycin parallel to untransfected cells as controls. After control cells have died, 50-hygromycin resistant clones were picked and allowed to grow in a selective media. Real-Time RT-PCR was performed on three selected clones (FPEP2 clone 4, 8 and 10) to check the level of EP2 receptor expression in comparison with the parental FPS32 cells. As shown in figure 4.1A, FPEP2 clone 4 and 8 showed higher levels of EP2 receptor mRNA expression with a significant fold increase (78.30 ± 5.99 and 83.93 ± 6.70 vs. 1, respectively; $p<0.0001$), while clone 10 had about 26 fold increase (26.43 ± 6.98 vs. 1; $p<0.05$) above the parental FPS32 cells. The expression of EP1, EP3, EP4 and the FP receptor was not significantly altered as shown in figure 4.1B.

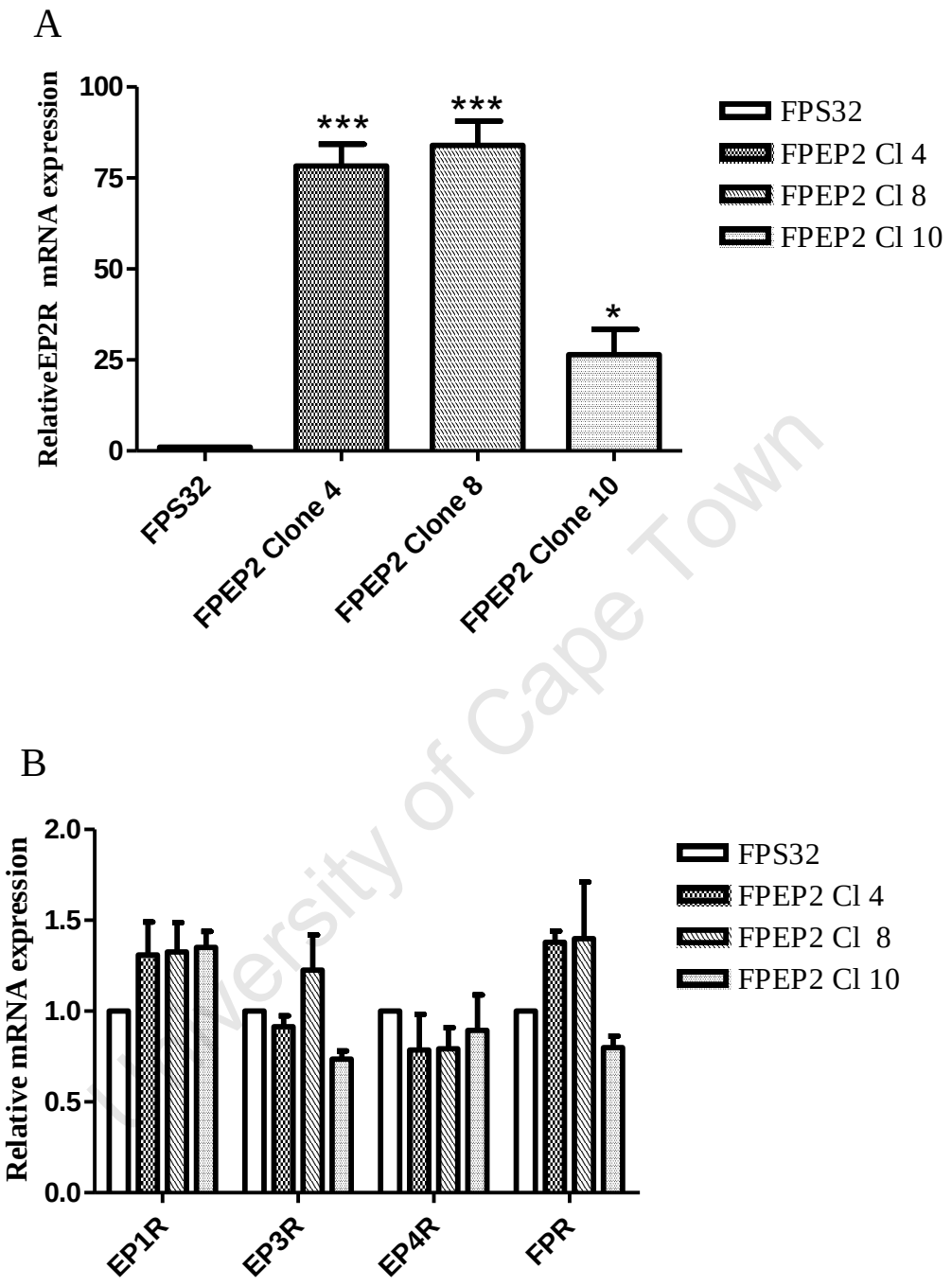

Fig. 4.1: *Relative expression of E and F prostanoid receptors in FPS32, FPEP2 clone 4, 8 and 10.* ***A.*** *Expression of the EP2 receptor and* ***B.*** *Expression of EP1, EP3, EP4 and FP receptors as determined by Real-Time RT-PCR analysis. Expression levels in FPEP2 clones are expressed as fold increase above FPS32 cells. Data are presented as mean ± SEM from four independent experiments (*, $p < 0.05$; ***, $p<0.0001$).*

4.4.2 Production of cAMP by the FPS32 and FPEP2 Clones

Activation of the EP2 receptor by PGE_2 or Butaprost leads to intracellular accumulation of cAMP (Jabbour *et al.*, 2001; Regan, 2003). FPEP2 Clone 4 and 8 were chosen for cAMP assay as they both express relatively higher EP2 receptor than clone 10. In order to assess the functionality of the EP2 receptor in FPEP2 clone 4 and 8, the ability to generate cAMP was determined by treating the cells with vehicle (0.1% ethanol) or Butaprost (5μM) or PGE_2 (100nM) for 5 and 10 min. As shown in figure 4.2, treatment of FPEP2 clone 4 cells with Butaprost or PGE_2 for 5 min increased intracellular cAMP accumulation significantly compared to vehicle treated (348 ± 52.1 and 3539.5 ± 475.2 vs. 10.7 ± 1.8 pmol cAMP/mg protein, respectively) while clone 8 had a much higher cAMP release (1658 ± 326.2 and 5424.2 ± 619.9 vs. 4.6 ± 1.4 pmol cAMP/mg protein, respectively). The relative accumulation of cAMP in response to 10 min Butaprost or PGE_2 induction was 1798.5 ± 392.5; 5468.2 ± 968.8; 3604.2 ± 278.9 and 7956.6 ±1 482.9 pmol cAMP/mg protein for clone 4 and 8 respectively. PGE_2 stimulation of the FPEP2 cells exhibited a higher cAMP release compared to Butaprost-treated cells. Butaprost induction (10min) of the FPS32 cells had no effect while PGE_2 stimulation (10min) showed a slight increase in intracellular cAMP accumulation compared to treated vehicle (9.7 ± 1.8 and 19.8 ± 2.7 vs. 9.6 ± 1.8 pmol cAMP/mg protein, respectively). Since the FPEP2 clone 8 directed a higher accumulation of intracellular cAMP in response to EP2 receptor specific ligands it was chosen for subsequent studies.

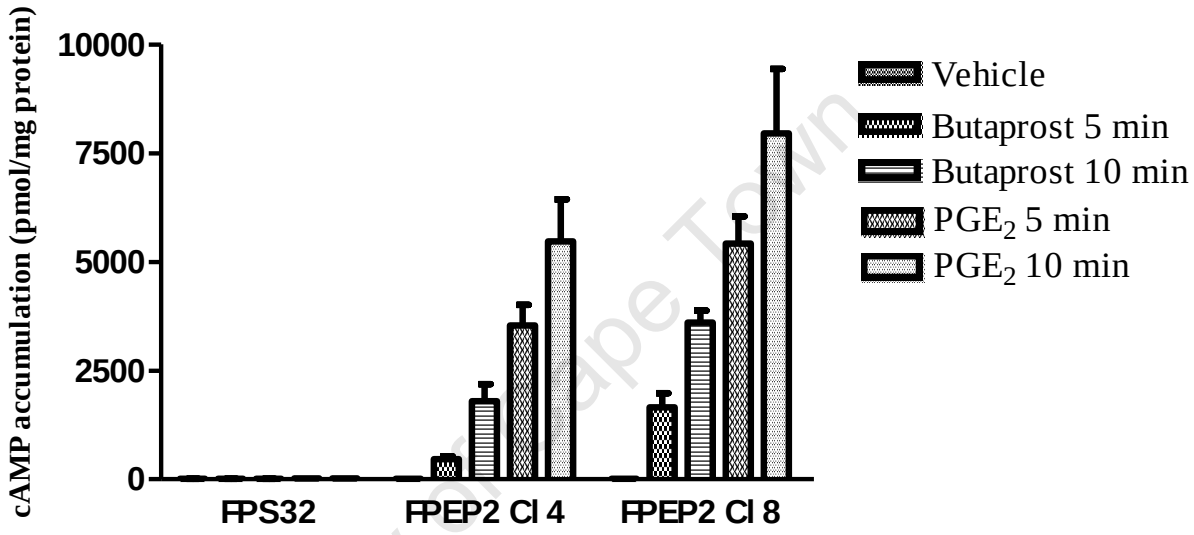

Fig. 4.2: *The effect of 5 and 10 min treatment with vehicle, Butaprost (5μM) or PGE_2 (100nM) on cAMP release in FPS32, FPEP2 clone 4 and 8 cell lines as determined by cAMP ELISA analysis. Data are presented as mean ± SEM from four independent experiments.*

4.4.3 Production of IP3 by the FPS32 and FPEP2 Clones

The FP receptor is a $G\alpha_q$-coupled receptor, which upon PGF activation leads to an accumulation of intracellular IP3 (Watanabe *et al.*, 1994). In order to compare the functionality of the FP receptors in the parental FPS32 and FPEP2 cell lines, the cells were subjected to increasing doses of PGF stimulation (10^{-10} to 10^{-6}M) while control samples were left untreated for 1 hr. Both the parental FPS32 and FPEP2 cell line resulted in an increase in IP3 release reaching an E_{max} of 15.7 ± 0.6 and 16.6 ± 2.1, while the EC_{50} values for the FPS32 and FPEP2 were 2.3nM ± 0.3 and 1.95nM ± 0.8, respectively. There was no significance difference in IP3 release between the parental and FPEP2 cell lines validating the Real-Time RT-PCR data on the level of FP receptor expression in section 4.4.1.

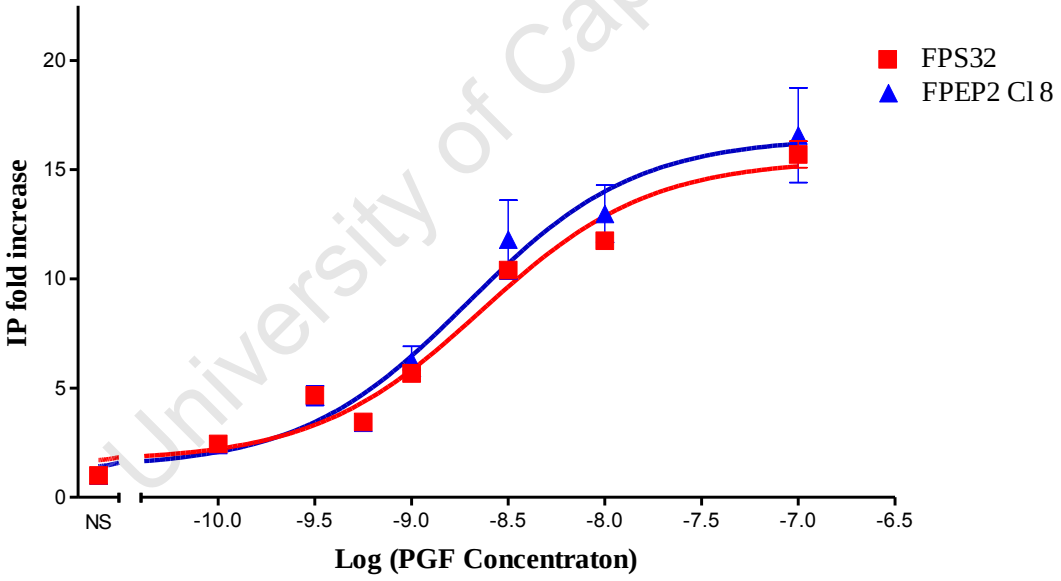

Fig. 4.3: *The effect of 1 hr PGF stimulation (10^{-10} to 10^{-6}M) on IP3 response in FPS32 and FPEP2 clone 8 cells as determined by an IP assay. Data are expressed as fold increase above non-stimulated samples. Data are presented as mean ± SEM from four independent experiments.*

4.4.4 Protein Expression of the FP and EP2 Receptors in FPS32 and FPEP2 Cells

Western blot analysis was done on the FPS32 and FPEP2 cells to compare the expression level of EP2 and FP receptor proteins and to validate the Real-Time RT-PCR data shown in figure 4.1. Protein samples (40µg) from both cell lines were subjected to Western blot analysis using specific EP2 and FP receptor antibodies. Protein expression was normalized using β-actin as a loading control on the same blots. As shown in figure 4.4A, the level of FP receptor expression was not different between the parental FPS32 and FPEP2 cell lines, while figure 4.4B shows the level of EP2 receptor to be higher (4.5 fold) in the FPEP2 cells relative to the parental clone FPS32, validating the Real-Time RT-PCR data.

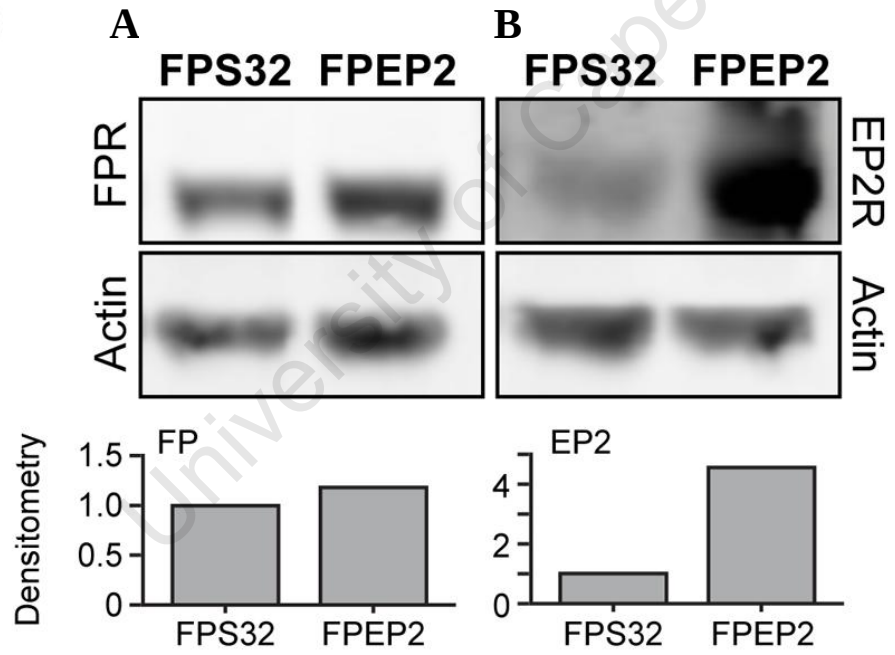

Fig. 4.4: *Representative Western blot analysis in FPS32 and FPEP2 protein samples (40µg).*
A. *Protein expression of FP receptor* ***B.*** *Protein expression of EP2 receptor as determined by Western blot analysis. EP2 and FP receptor protein expression levels were normalized for loading using ß-actin expression and expressed as fold increase above FPS32 cells.*

4.4.5 Localization of the FP and EP2 Receptors in FPS32 and FPEP2 Cells

EP2 and FP receptor expression was visualized in the FPS32 and FPEP2 cells using Immunofluorescence microscopy. This was done to investigate if the receptors are expressed on the plasma membrane compartment after synthesis. A specific blocking peptide to which the antibody was raised against was used as a negative control. As shown in figure 4.5 Panel A, FPEP2 cells showed the same level of immunoreactivity to the FP receptor antibody in comparison to the FPS32 cells and revealed a strong and clear plasma membrane and perinuclear labeling. No EP2 receptor staining is seen in FPS32 cells but the FPEP2 cells showed a marked expression with the same distribution pattern shown for the FP receptor. Both EP2 and FP receptor signals were almost abolished when the antibody for the receptors was preadsorbed with a specific blocking peptide as shown in figure 4.5.

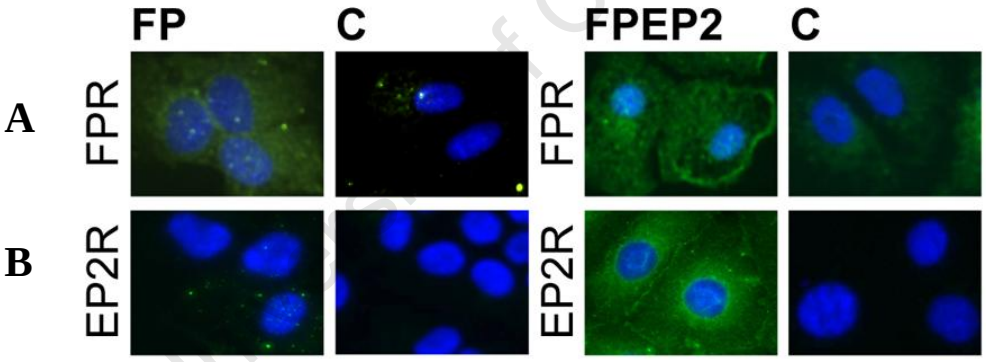

Fig. 4.5: *Localization of the FP and EP2 receptor in FPS32 and FPEP2 cells.*

A. *localization of the FP receptor* ***B.*** *Localization of the EP2 receptor as determined by immunofluorescence microscopy. Control cells (C) were incubated with preadsorbed primary antibody using specific blocking peptides.*

4.4 Discussion

It has been known that prostanoid receptors are co-expressed in many cell types and tissues (Ashby, 1998). This observation suggests that co-activation of prostanoid receptors in the same cell could alter the signalling cascades which are normally activated by each receptors. The altered signalling cascades in turn could change the gene expression profile and the physiology of these cells that might be an outcome of receptor heterologous interaction or cross-talk.

GPCR cross-talk in the eicosanoid family has been shown in several studies by stably expressing the receptors in HEK-293 cell lines. Studies using TPα expressing HEK-293 cells showed an IP receptor agonist-dependent desensitization of the TPα receptor (Walsh *et al.,* 2000). The same isoform of TP receptor has also been shown to be inhibited by activation of PGD_2 receptor via the PKA pathway in HEK-293 cells (Foley *et al.,* 2001). Walsh and Kinsella (2000) showed TPα and TPβ receptors are subjected to 17 phenyl trinor PGE_2 (EP1 receptor agonist) mediated desensitization. The authors further indentified that the activation of the FP receptor by PGF can also mediate desensitization of the TPα and TPβ receptors in HEK-293 cells (Kelley-Hickie and Kinsella, 2004). In another study, Wilson *et al.* (2004) showed that co-expression of IP and TP receptors in HEK-293 cells can augment TP-receptor mediated cAMP without activation of the IP receptor. The cAMP augmentation was further potentiated when both receptors were co-activated by their respective ligands.

EP2 and FP receptors are co-expressed in Ishikawa cells (Sales *et al.,* 2004; Sales *et al*., 2005). However, there have been no studies addressing the integrative signalling effects of the EP2 and FP receptors. In order to investigate this, EP2 receptor was stably transfected into Ishikawa cells expressing the FP receptor stably (FPS32 cells). Out of 50-hygromycin-resistant clones, three clones (FPEP2 clone 4, 8 and 10) were selected for further expression analysis. Expression level of the EP2 receptor in FPEP2 clones was compared to the parental FPS32 cells using Real-Time RT-PCR. I found that all the FPEP2 clones showed higher level of EP2 receptor mRNA expression than the parental

FPS32 cells with clone 10 giving a lower fold increase. Introduction of the EP2 receptor had no effect on the expression profile of the other E-series receptors and of FP receptor which could be activated by prostanoid ligands in our study.

The EP2 receptor is a $G\alpha_s$-coupled receptor, which upon PGE_2 or Butaprost activation leads to an accumulation of intracellular accumulation of cAMP (Jabbour *et al*., 2001; Regan, 2003). Due to their higher mRNA expression of the EP2 receptor, FPEP2 clone 4 and 8 were chosen for functional studies. This was done by assessing the accumulation of intracellular cAMP after activation of the EP2 receptor by either PGE_2 or Butaprost. Both clones gave a significant increase in cAMP accumulation compared to the parental cell line FPS32. However, FPEP2 clone 8 showed a robust increase in cAMP accumulation that was significantly higher than the FPEP2 clone 4 and was chosen for subsequent studies. PGE_2 induction of the cells exhibited more intracellular cAMP accumulation than Butaprost. Studies have shown the inhibitory constant (*Ki*) values of EP2 receptor and its agonists (PGE_2 and Butaprost) to be 10nM and 110nM, respectively in CHO cells. This shows PGE_2 has about 10 fold increased affinity to bind to the EP2 receptor than Butaprost, explaining the higher release in cAMP exhibited by PGE_2 activation of the FPEP2 clones (Kiriyama *et al.,* 1997).

Activation of cells by PGF leads to FP receptor-$G\alpha_q$ coupling to trigger IP3 production (Watanabe *et al.,* 1994). To confirm the similar level of FP receptor mRNA expression between FPS32 and FPEP2 clone 8 cells functionally, cells were treated with PGF and assessed for IP3 accumulation. There was no significance difference in the E_{max} and EC_{50} values between PGF treated FPS32 and FPEP2 clone 8 cells, suggesting the levels of FP receptor were similar between the two cell lines.

Protein expression level of the EP2 receptor in FPEP2 clone 8 cells was compared to the parental FPS32 cells using Western blot analysis. EP2 receptor protein expression was higher in FPEP2 cells while FP receptor protein levels were not altered compared to FPS32 cells. Immunofluorescence microscopy analysis also confirmed that the EP2

receptor is stably expressed in the FPEP2 clone 8 cells without altering the expression of the FP receptor. Both EP2 and FP receptors were localized in the perinuclear and cytoplasmic membrane regions, a lower expression observed in cytoplasmic regions. Prostaglandin receptors have a short half-life and rapidly cycle between the cell surface and the intracellular pool explaining the small fraction of the receptors in the plasma membrane (Naor *et al.,* 2007).

In conclusion, FPEP2 clone 8 stably over-expresses the EP2 and FP receptors and they are both localized in the perinuclear and cytoplasmic membrane compartments. Furthermore, the functionality of EP2 and FP receptors was also confirmed by the release of their respective secondary messengers, cAMP and IP3 in the presence of their respective ligands. Subsequent studies investigated the intricate signalling system between the EP2 and FP receptors using FPEP2 clone 8 cells as a model system.

Chapter V

EP2 Receptor-Mediated cAMP Release is Augmented Following Activation of the Calcium-Calmodulin Pathway by PGF-FP Receptor Interaction via Adenylyl Cyclase 3 Isoform

5.1 Introduction

Activation of the EP2 receptor by Butaprost leads to intracellular accumulation of cAMP (Jabbour *et al*., 2001; Regan, 2003). cAMP is a prototypical secondary messenger that impacts in every aspect of a cellular life, from differentiation and development through to cell death. It is generated in cells by the enzymatic activity of the adenylyl cyclase (AC) family which catalyses the synthesis of cAMP from ATP. The AC enzyme is made of 12 transmembrane spanning domains, extensive amino and carboxyl termini and contains two catalytic ATP-binding domains that are found in the cytoplasmic region of the enzyme (Krupinski *et al*., 1989; Willoughby and Cooper, 2007).

There are nine membrane-bound AC isoforms with different amino acid sequence, tissue distribution and regulation and are named from AC1-AC9 in the order of their identification (Hanoune *et al*., 1997). All the isoforms of ACs can be stimulated by $G\alpha_s$ and forskolin. However, ACs can be subclassified in terms of their responsiveness or not to other regulatory factors. Group 1 comprises enzymes that are stimulated by physiological concentration of calcium or calmodulin (AC1, AC3 and AC8) while group 2 contains AC enzymes (AC2, AC4 and AC7) that are activated by PKC and $\beta\gamma$ subunit. Group 3 are enzymes inhibited by low concentration of calcium and Group 4 (AC9) is insensitive to both calcium and $\beta\gamma$ subunit (Hanoune *et al*., 1997, Willoughby and Cooper, 2007).

PGF-stimulated FP receptor-$G\alpha_q$ coupling leads to activation of PLC that can either lead to PKC activation by the release of DAG or trigger the production of IP3, which then acts on specific IP3 receptors (IP3R) found at the ER promoting the release of intracellular calcium into the cell cytosol (Watanabe *et al.,* 1994). The concentration of intracellular calcium in cytoplasm is maintained regularly by different factors. One of these regulatory factors is an activation of a calcium buffering protein called calmodulin. Activated calmodulin can in turn act on other calcium effectors like CaMK-II to activate calcium-sensitive isoforms of AC to alter cAMP production in cells (Persechini and Stemmer, 2002).

5.2 Aim of the Study

As described in the previous chapter, I developed an Ishikawa cell line over-expressing the EP2 and FP receptors (FPEP2 cell line). The aim of this study is to identify a possible intracellular signalling cross-talk following co-activation of both receptors and to further characterize the molecular mechanism underlying the potential cross-talk.

5.3 Materials and Methods

5.3.1 Ligand Stimulation and cAMP Assay

In order to determine ligand-stimulated cAMP accumulation, FPEP2 cells (2×10^5 cells/well) were plated out in 6-well dishes. The cells were serum starved the following day in the presence of 3µg/ml of Indomethacin. A day later, the cells were pre-treated in serum free medium containing 0.2mM of IBMX and incubated for 5 min with vehicle (0.1% ethanol and/or 0.1% DMSO), Butaprost (5µM) and/or PGF (100nM) in the presence/absence of different chemical inhibitors. cAMP release was determined by ELISA using cAMP Kit (R&D Systems) according to method mentioned in section 2.2.3. The concentration of cAMP released was calculated using a standard curve by Assay Zap computer programe (Biosoft) and was normalized according to the protein concentration of each sample.

5.3.2 Ligand Stimulation and IP3 assay

In order to assess ligand-stimulated IP3 release, FPEP2 cells (50,000 cells/well) were seeded in 24-well plates overnight. After serum starving the cells overnight, the cells were labeled with 0.5µCi/well myo-[3H]-inositol in an inositol-free DMEM 199 medium (2% FCS) overnight. A day later, cells were washed and stimulated with PGF or Butaprost (10^{-10} to 10^{-6}M) alone or PGF (10^{-10} to 10^{-6}M) and Butaprost (5µM) together in Buffer I for 60 min at 37°C. Total [3H]-inositol phosphates were separated from cell extracts on AG 1-X8 resin by anion exchange chromatography and were analyzed as described in section 2.2.4.

5.3.3 Knockdown of AC1 and AC3 in FPEP2 Cells with siRNA Transfection

Three different Stealth siRNA duplex oligoribonucleotides were used to abolish the expression and function of AC1 and AC3. FPEP2 Ishikawa cells were seeded in complete media at a density of 7.5×10^4 cells/well in 12-well plates to achieve 60-80% confluence on the day of transfection. The next day the cells were transfected with 60nM of AC isoform specific siRNA (20nM from each Stealth siRNA) or scrambled sequence siRNA in the presence of SuperFect (QIAGEN). After 48 hrs, the cells were subjected to RNA extraction for AC1 and AC3 mRNA expression analysis or serum-starved overnight in medium containing indomethacin (3µg/ml) for cAMP assay. For cAMP assay, cells were then exposed to vehicle (0.1% ethanol), Butaprost (5µM) and/or PGF (100nM) for 5 min and lysed with lysis buffer and subjected to cAMP analysis as described in section 2.2.3.

5.3.4 RT-PCR

Total mRNA was extracted from Ishikawa cells as described in section 2.3.2. The cDNA template for RT-PCR was synthesized from total mRNA (1µg) by reverse transcription in the presence of RT-PCR reaction buffer (1×), dNTPs (0.5mM each), $MgCl_2$ (5.5mM), random hexamers (1.25µM), oligo dT (1.25µM), RNase inhibitor (0.4U/µl), and MultiScribe™ reverse transcriptase (1.25U/µl). Thereafter, for PCR amplification specific AC primers (0.4pmol/µl each; Sequences of the primers are mentioned in table 2.2) were used to amplify individual isoforms of AC in a reaction mix containing AmpliTaq Gold® PCR Master Mix (5mM $MgCl_2$ and 400µM each dNTPs, 0.05 U/µL of AmpliTaq Gold DNA polymerase) and 2µl of cDNA in 20µl final volume. Isoforms of AC proved to be difficult to amplify under normal PCR conditions. The following optimizing alterations were introduced to enable successful amplification.

I. AmpliTaq Gold® DNA Polymerase: This Applied biosystems product has been developed for "hot-start" PCR reaction, which provides greater specificity of amplification in general and is particularly suited to amplification of difficult templates, such as GC rich regions. This enzyme is inactive and has no polymerase activity at ambient temperatures and is activated by a 5 min, 95°C incubation step, which can easily be incorporated into existing thermal cycling programs. This enzyme was used for the amplification of all the AC isoforms (AmpliTaq Gold® PCR handbook).

II. Touchdown PCR: This involves decreasing the annealing temperature (0.5°C every second cycle in this case) to a 'touchdown' annealing temperature, which is then used for the remaining cycles. This allows for higher specificity of amplification during the initial cycles and preferential amplification of the early templates at the lower annealing temperature. Touchdown PCR was performed for AC1 and the soluble AC isoforms as shown in table 5.1. For the other AC isoforms, PCR reaction was performed for 35 cycles (94, 56, and 72°C for 30, 45 and 60 sec, respectively) after 5 min at 95°C.

Table 5.1: Touchdown PCR reaction conditions for AC1 and Soluble AC

Cycles	**Denaturation Temp (°C) /time**	**Annealing Temp (°C)/time**	**Extension Temp (°C)/ time**
First hold	95/5 min	-	-
First 10 cycles	95/30 sec	60-55/ 1 min (decreases 0.5 °C per cycle)	72/ 45 sec
Second 25 cycles	92/30 sec	55/1 min	72/45 sec
Final hold	-	-	72/7 min

5.3.5 Real-Time RT-PCR

Total mRNA was extracted and cDNA was synthesized from FPEP2 cells after 48 hrs of siRNA transfection. To assay knockdown efficiency, Real-Time RT-PCR was performed with specific AC primers and probes (300nM and 100nM respectively) in a reaction mix containing Taqman buffer (5.5mM $MgCl_2$ and 200µm of each dNTPs, 0.025U/µl of AmpliTaq Gold DNA polymerase), ribosomal 18S forward and reverse primers and probe (all at 50nM), and 2µl of the cDNA (Sequences of the primers are mentioned in table 2.4). The reaction was run on ABI Prism 7900 Quantitative PCR machine. Data were analyzed using sequence detector version 1.6.3 and expression of AC isoforms were normalized for each treatment using their respective 18S rRNA and were expressed as fold decrease below control siRNA treated samples.

5.3.6 Statistical Analysis

All data are presented as mean ± SEM. Statistical significant differences were determined by one-way analysis of variance using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) (*, $p < 0.05$; **, $p < 0.001$; ***, $p<0.0001$).

5.4 Results

5.4.1 Butaprost Does not Affect PGF-Stimulated IP3 Release in FPEP2 Cells

To study the cross-effects of the two different intracellular signalling pathways involved in EP2 and FP receptors activation, FPEP2 cells were treated with Butaprost and/or PGF. First, the effect of Butaprost on PGF-mediated IP3 release was assessed by treating FPEP2 cells with PGF or Butaprost alone (10^{-10} to 10^{-6}M) or PGF (10^{-10} to 10^{-6}M) in the presence of Butaprost (5μM). As shown in figure 5.1, Butaprost (10^{-10} to 10^{-6}M) treatment of the FPEP2 cells alone had no effect on IP3 release, while treatment with PGF (10^{-10} to 10^{-6}M) had a significant increase in IP3 release (E_{max} 15.5 ± 0.6). Co-treatment of FPEP2 cells with PGF (10^{-10} to 10^{-6}M) and Butaprost (5μM) resulted in an E_{max} of 16.6 ± 2.2 which is similar to the E_{max} achieved with treating the cells with PGF alone (E_{max} 15.5 ± 0.6). The EC_{50} value for the co-stimulation with PGF and Butaprost was slightly smaller compared to PGF treated, but was not statistically significant (EC_{50} 0.7nM ± 0.5 and 1.95nM ± 1.04, respectively). These data demonstrate that PGF-mediated IP3 release is not affected by Butaprost treatment**.**

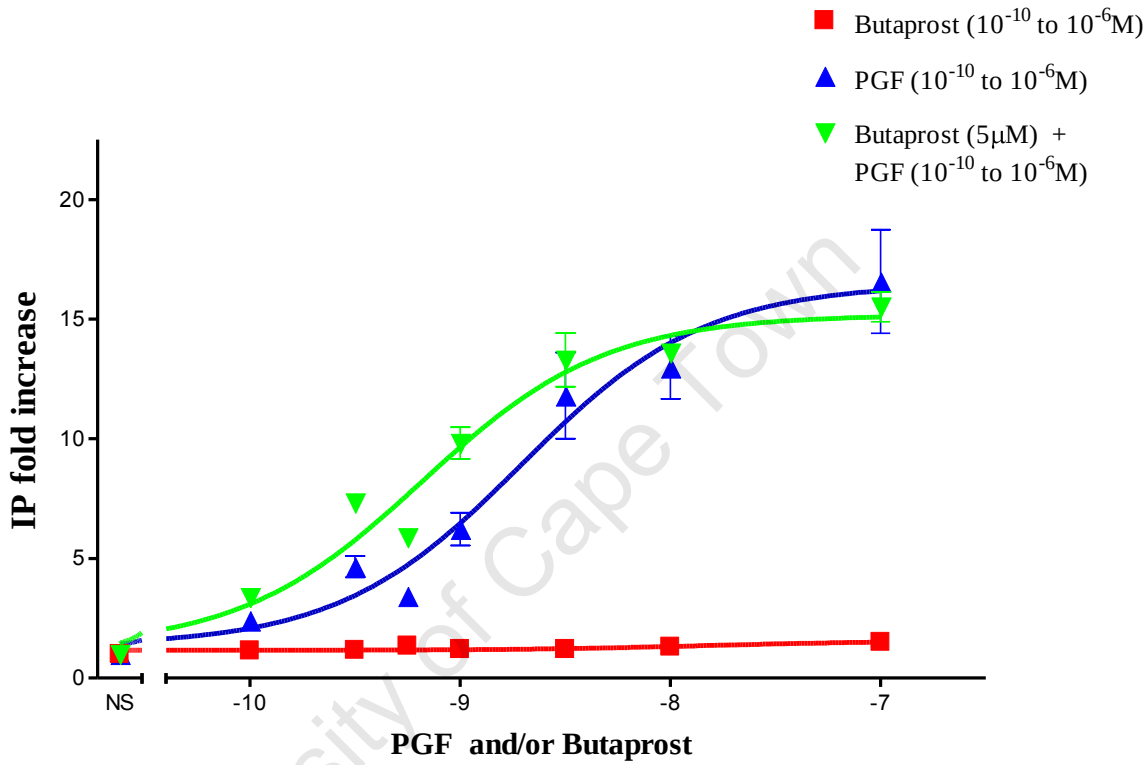

Fig. 5.1: *IP3 release in FPEP2 cells after treatment with PGF or Butaprost (10^{-10} to 10^{-6}M) alone or PGF (10^{-10} to 10^{-6}M) and Butaprost (5μM) together for 60 min as determined by an IP3 assay. Data are expressed as fold increase above non-stimulated samples (NS). Data are presented as mean ± SEM from four independent experiments.*

5.4.2 PGF Potentiates Butaprost-Stimulated cAMP Production in FPEP2 Cells

To assess the effect of FP receptor induction by PGF on Butaprost-stimulated cAMP accumulation, FPEP2 cells were treated with vehicle, Butaprost (5μM) and/or PGF (100nM) for 5 min. As shown in figure 5.2, treatment of the cells with Butaprost resulted in a robust increase in intracellular cAMP accumulation compared with vehicle treated (2172 ± 314 vs. 18.6 ± 1.98 pmol cAMP/mg protein; $p<0.001$), while treatment with PGF alone had minimal effect (73.5 ± 4.7 pmol cAMP/mg protein). However, co-treatment of the cells with Butaprost and PGF significantly increased (~2 fold) the cAMP response compared to treatment with Butaprost alone (4141 ± 511 vs. 2172 ± 314 pmol cAMP/mg protein; $p<0.001$; Fig 5.2). These results demonstrate that co-activation of the EP2 and FP receptors results in enhanced release of Butaprost-stimulated cAMP by PGF.

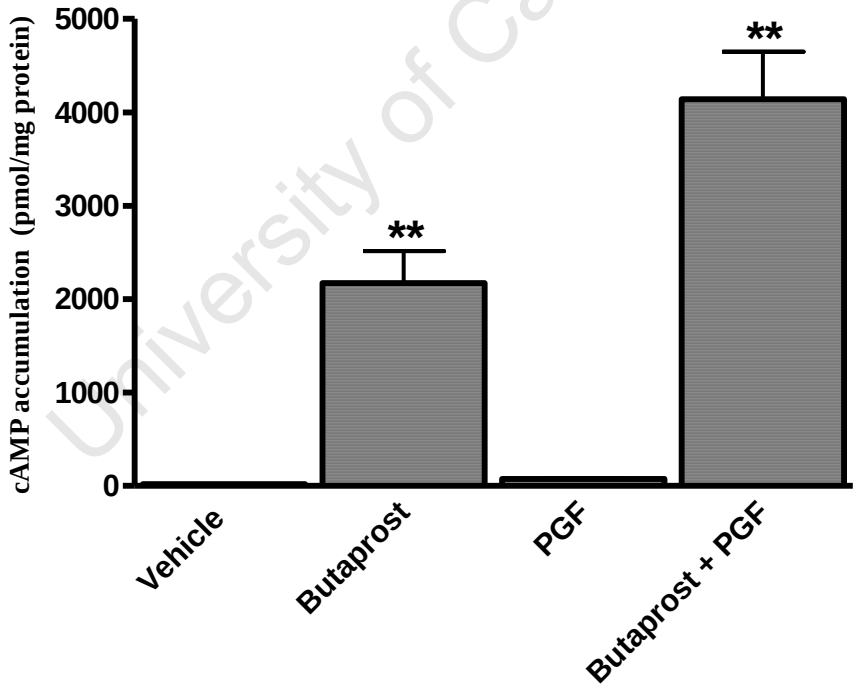

Fig. 5.2: *cAMP accumulation (pmol/mg protein) in FPEP2 cells after treatment with vehicle, Butaprost (5μM) and/or PGF (100nM) for 5 min. Data are presented as mean ± SEM from four independent experiments (**, $p<0.001$).*

5.4.3 PGF-Induced cAMP Potentiation is Mediated by FP Receptor-Gα_q Coupling in FPEP2 Cells

To investigate whether the augmentation of cAMP release by PGF is mediated by either EP2 or FP receptor, the cells were co-treated with specific antagonist for EP2 receptor (10µM of AH6809 with 3min pretreatment) or FP receptor (50µM of AL8810 with 3min pretreatment). As shown in figure 5.3, antagonism of the EP2 receptor significantly decreased ($p<0.001$) the Butaprost-induced cAMP in both groups of treatment (Butaprost only and Butaprost and PGF) but had no effect on the enhancement of cAMP accumulation by PGF (727.1 ± 66.4 and 1397 ± 105.9 pmol cAMP/mg protein, respectively). Whereas, the use of FP receptor antagonist completely abolished the potentiation of cAMP by PGF without altering Butaprost-stimulated cAMP (4141 ± 510.6 vs. 2305 ± 116 pmol cAMP/mg protein; $p<0.001$). To assess if the PGF potentiation of Butaprost-stimulated cAMP is mediated by FP receptor-Gα_q coupling, FPEP2 cells were co-treated with Gα_q inhibitor (1µM of YM254890 with 30 min pretreatment). As shown in figure 5.3, the use of Gα_q inhibitor reduced the level of cAMP release significantly to the Butaprost stimulated level (4141 ± 510.6 vs. 1588 ± 137 pmol cAMP/mg protein respectively; $p<0.001$). These data indicate that, PGF potentiation of Butaprost-stimulated cAMP is mediated by PGF via the FP receptor-activation of Gα_q.

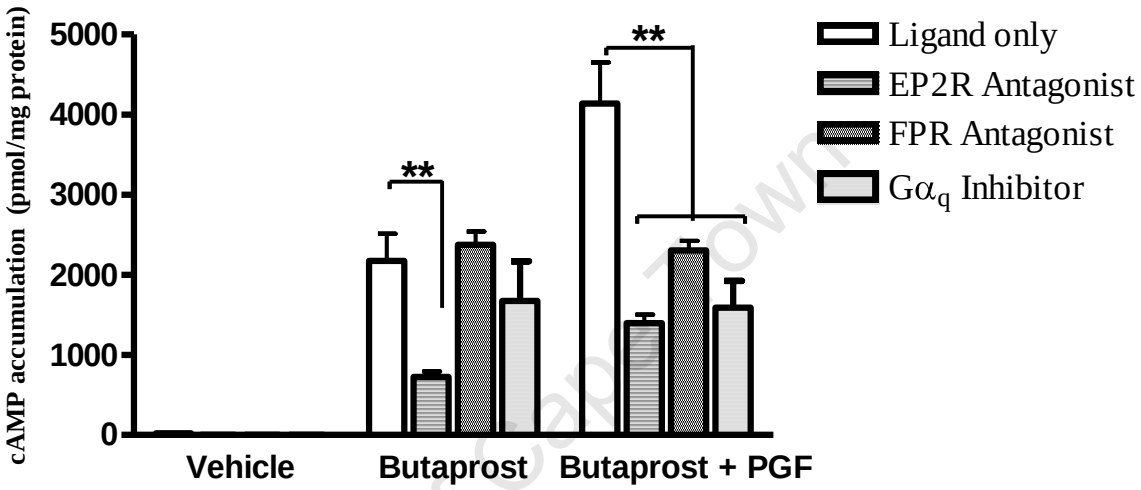

Fig. 5.3: *cAMP accumulation (pmol/mg protein) in FPEP2 cells after 5 min treatment with Butaprost (5μM) and/or PGF (100nM) in the presence/absence of the EP2 receptor antagonist (AH6809; 10μm), FP receptor antagonist (AL8810; 50μm) or $G\alpha_q$ inhibitor (YM-254890; 1μm). Data are presented as mean ± SEM from four independent experiments (**, p<0.001).*

5.4.4 The $G\alpha_q$-$G\alpha_s$ Cross-Talk is Dependent of PLC-Mediated Calcium Release but not on PKC Activation

PGF activated FP receptor-$G\alpha_q$ coupling leads to activation of PLC that results in both IP3 release that increases accumulation of intracellular calcium and DAG release that activates PKC (Watanabe *et al*., 1994). Inhibition of PLC using U73122 (10µM with 30 min pretreatment) abolished the augmentation of cAMP directed by PGF significantly (4141 ± 510.6 vs. 2261 ± 236 pmol cAMP/mg protein; $p<0.001$) demonstrating that the $G\alpha_s$-$G\alpha_q$ cross-talk is mediated by PLC activation. However, in order to determine whether intracellular calcium or PKC activation mediate the observed cross-talk, the cells were incubated with specific IP3-Receptor blocker (40µm of 2-APB with 15 min pretreatment) or PKC inhibitor (1µm of Ro-31-822 with 30 min of pretreatment) in the absence/presence of Butaprost (5µM) and/or PGF (100nM). Co-treatment of the cells with 2-APB inhibited the PGF-mediated potentiation in cAMP release in response to Butaprost but had no effect on Butaprost treatment alone (4141 ± 510.6 vs. 2252 ± 327 pmol cAMP/mg protein; $p<0.001$). Whereas inhibition of PKC failed to attenuate the potentiation of Butaprost-stimulated cAMP release by PGF (4141 ± 510.6 vs. 3655 ± 416 pmol cAMP/mg protein) demonstrating that PGF-mediated increase in Butaprost-stimulated cAMP is via the accumulation of intracellular calcium and not through DAG/PKC activation.

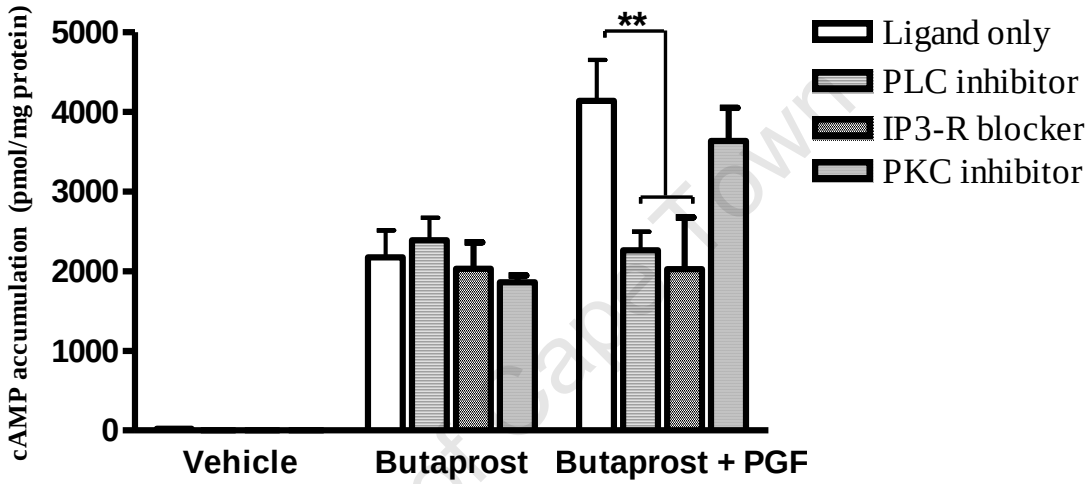

Fig. 5.4: *cAMP accumulation (pmol/mg protein) in FPEP2 cells after 5 min treatment with Butaprost (5μM) and/or PGF (100nM) in the presence/absence of the PLC inhibitor (10μM U73122), IP3-R blocker (40μm 2-APB) or PKC inhibitor (1μm Ro-31-822). Data are presented as mean ± SEM from four independent experiments (**, p<0.001).*

5.4.5 PGF-Induced cAMP Potentiation is Mediated by Intracellular Ca^{2+} Transients

Intracellular calcium can modulate calcium sensitive isoforms of AC to enhance cAMP via activation of calmodulin-CaMK-II pathway (Wang and Storm, 2003; Hanoune and Defer, 2001). To determine whether the PGF-mediated enhancement of Butaprost-stimulated cAMP is mediated by the calcium-calmodulin pathway, chemical inhibitors against two calcium effectors were used. Inhibition of calcium signalling, using calmodulin antagonist (25μm W7) or CaMK-II inhibitor (50μm KN-93) in the presence of Butaprost and PGF reduced the level of cAMP release significantly to the Butaprost-stimulated level (2656 ± 181.7 and 2611 ± 179.9 vs. 4130 ± 187.9 pmol cAMP/mg protein, respectively; $p<0.001$) (Fig 5.5). These results indicate that PGF potentiation of Butaprost-stimulated cAMP is mediated by the release of Ca^{2+} from intracellular stores leading to activation of the calmodulin-CaMK-II pathway.

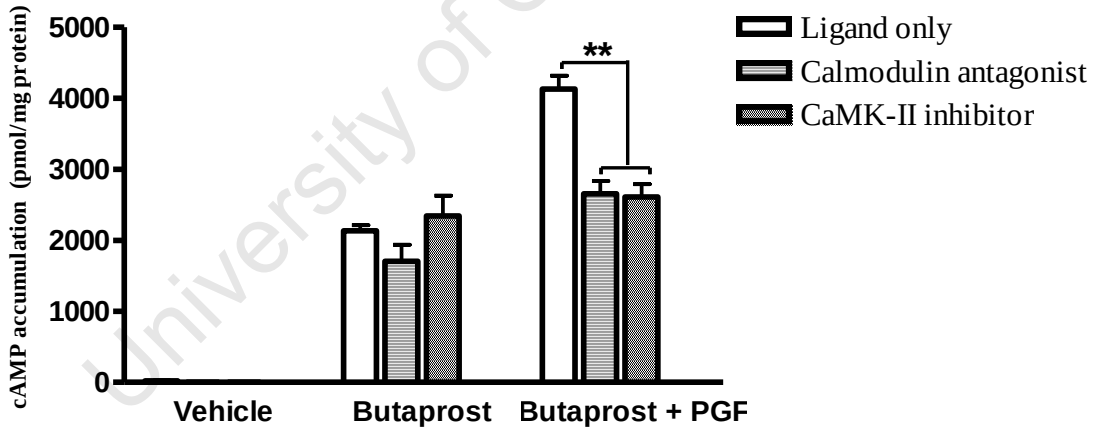

Fig. 5.5: *cAMP accumulation (pmol/mg protein) in FPEP2 cells after 5 min treatment with Butaprost (5μM) and/or PGF (100nM) in the presence/absence of the inhibitors for calmodulin (25μm of W7) or CaMK-II (50μm of KN-93). Data are presented as mean ± SEM from four independent experiments (**, p<0.001).*

5.4.6 Adenylyl Cyclase Isoforms Expression in FPEP2 Ishikawa cells

There are ten isoforms of AC, of which three of them are calcium stimulated (Willoughby and Cooper, 2007). Since I have shown intracellular calcium has a role in the $G\alpha_s$-$G\alpha_q$ cross-talk system mentioned above, I decided to check the expression of multiple AC isoforms in Ishikawa cells. To investigate which isoforms are present in FPEP2 cells, total RNA was extracted from Ishikawa cells and RT-PCR was performed using AC isoform-specific primers (sequences of the primers used are mentioned in table 2.4). As shown in the representative agarose gel in figure 5.6, Ishikawa cells express mRNA for AC1, AC3, AC4, AC5, AC6, AC7, AC9 and the soluble AC (SAC). However, AC2 and AC8 primers did not yield a PCR product at the expected size indicating they are not expressed. Therefore, out of the three calcium regulated isoforms (AC1, AC3 and AC8), Ishikawa cells express two of them (AC1 and AC3) and either both or one of them may be mediating the cAMP potentiation mentioned above.

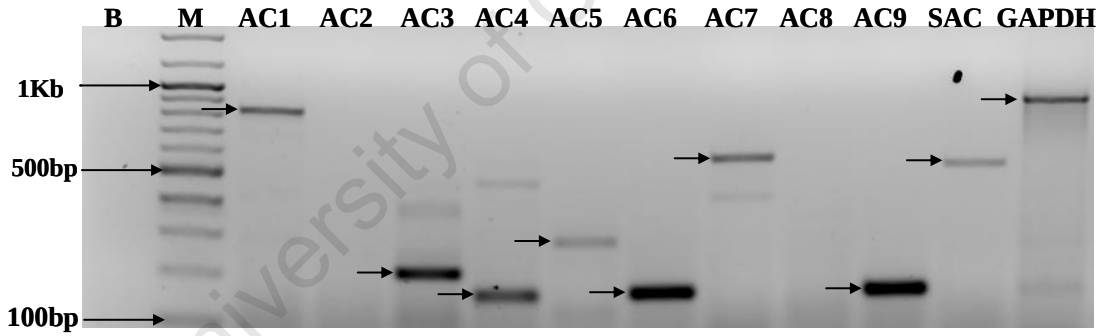

Fig. 5.6: *RT-PCR analysis of AC isoforms mRNA expression in FPEP2 Ishikawa cells.*
Ishikawa cells express mRNA for eight of the ten AC isoforms including the soluble form of AC (SAC) among those two of them are calcium stimulated ones (AC1 & AC3) as determined by RT-PCR and agarose gel (2%) analysis. Arrows indicate the size of the expected PCR product from each primer pair and no reverse transcriptase control is designated as B (blank). PCR product size: AC1= 826bp, AC2= 625bp, AC3= 213bp, AC4= 166bp, AC5= 316bp, AC6= 170bp, AC7= 587bp, AC8= 476bp, AC9= 166bp, SAC= 556bp and GAPDH=956bp.

5.4.7 siRNA Knockdown of AC1 and AC3 Transcripts in FPEP2 Ishikawa Cells

Since AC1 and AC3 are the only calcium regulated isoforms that are expressed in Ishikawa cells, siRNA designed to these specific isoforms were used to knockdown endogenous mRNA expression. After 48 hrs of specific siRNA transfection, Real-Time RT-PCR showed that AC1 and AC3 mRNA were reduced by 76% and 70%, respectively compared with scrambled sequence siRNA. The specificity of the siRNA was also proven as both AC1 and AC3 siRNA inhibited only the expression of their cognate targets without altering the other (Fig 5.7).

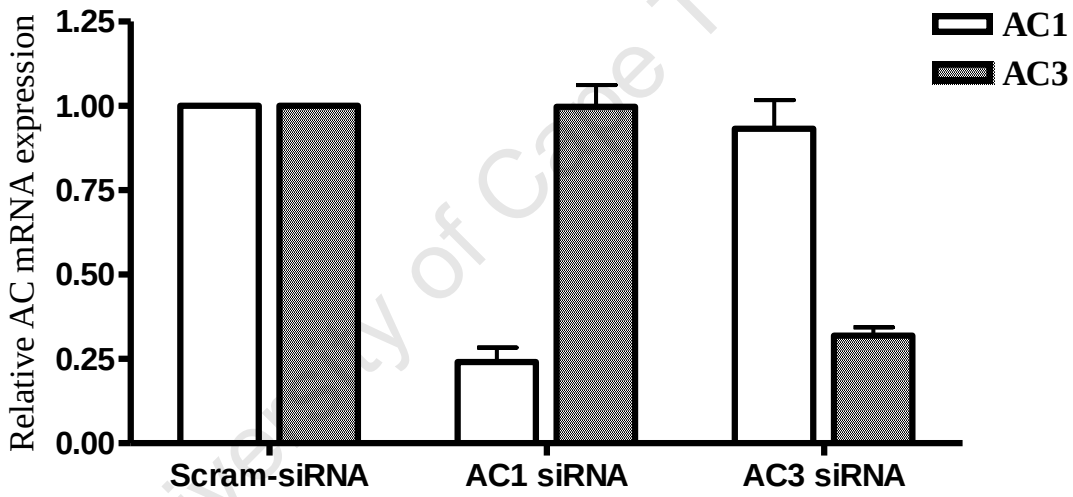

Fig. 5.7: *Real-Time RT-PCR analysis of AC1 and AC3 in FPEP2 cells transfected with isoform specific AC siRNA (60nM) compared to the control siRNA (scrambled sequence). Data are presented as mean ± SEM from four independent experiments.*

5.4.8 siRNA Knockdown of AC3 Abolishes PGF-Potentiation of Butaprost-Stimulated cAMP in FPEP2 Cells

To determine which calcium sensitive AC isoform is involved in $G\alpha_s$-$G\alpha_q$ cross-talk mentioned above, AC1 and AC3 siRNA transfected cells were exposed to either vehicle, Butaprost (5µM) and/or PGF (100nM) for 5min. Thereafter, cells were lysed and subjected to cAMP analysis as described in section 2.2.3 to assess the functional effect of the knockdown. As shown in figure 5.8, transfection of FPEP2 cells with AC3 siRNA completely abolished the potentiation of Butaprost-stimulated cAMP by PGF (3718 ± 114.0 vs. 1882 ± 83.66 pmol cAMP/mg protein; $p<0.001$) while AC1 siRNA transfection had no effect on cAMP accumulation compared to Butaprost and PGF treatment (3718 ± 114.0 vs. 3724 ± 187.0 pmol cAMP/mg protein). Both AC1 and AC3 siRNA did not have any significant effect on Butaprost-stimulated cAMP. This result suggests that PGF potentiates Butaprost-stimulated cAMP through Ca^{2+}-calmodulin pathway by activating the calcium sensitive AC3 isoform in FPEP2 cells.

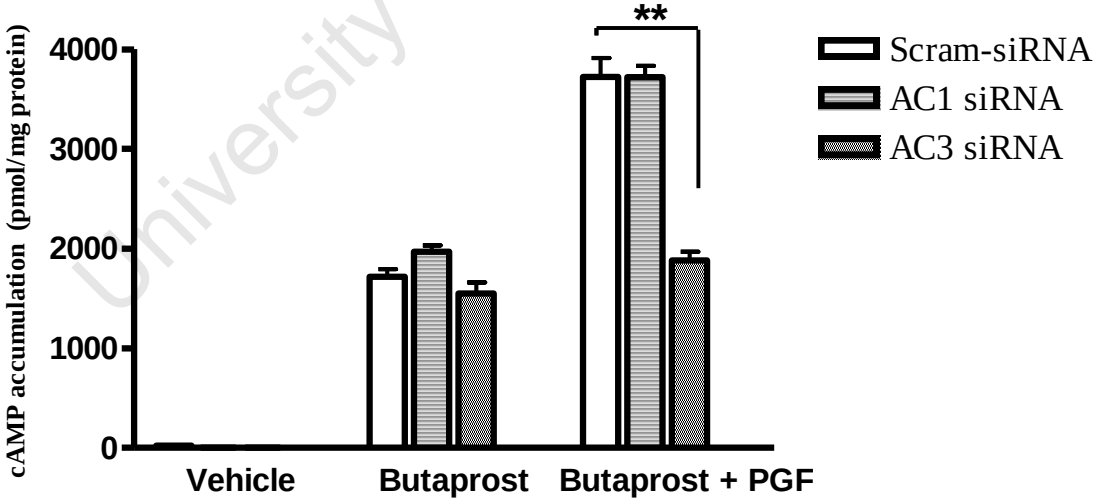

Fig. 5.8: *cAMP accumulation (pmol/mg protein) in AC1 or AC3 knockdown FPEP2 cells after 5 min treatment with vehicle, Butaprost (5µM) and/or PGF (100nM) as determined by cAMP analysis. Data are presented as mean ± SEM from four independent experiments (**, $p<0.001$).*

5.5 Discussion

Regulation of intracellular calcium plays a major role in heterologous-interaction between $G\alpha_s$- and $G\alpha_q$–coupled receptors. The intracellular calcium pathway can activate calcium-stimulated AC isoforms to augment cAMP production or PKA can regulate either the IP3 receptors or the intracellular calcium pump to potentiate production of intracellular calcium in cells (Wang and Storm, 2003; Hajonoczky *et al.*, 1993).

For example, Ostrom *et al.* (2003) showed a potentiation of β-adrenergic (β-AR) receptor induced cAMP production by activation of angiotensin II (ANG-II) receptor coupled to $G\alpha_q$ protein through calcium/calmodulin pathway indication of a signalling cross-talk between ANG -II and β-AR receptors in cardiac fibroblasts cells.

In other study, Jimenez *et al.* (1999) have shown a cross-talk between the P1 and P2 purinoceptors that augments P2 receptor-mediated Ca^{2+} release. This cross-talk was mediated by adenosine activation of the $G\alpha_s$-coupled P1 receptors demonstrating a $G\alpha_s$-$G\alpha_q$ cross-talk that potentiates intracellular calcium in cerebellar cells. Werry *et al.* (2002) have also shown β-adrenoceptors activation can augment P2Y receptor-mediated intracellular Ca^{2+} release in HEK-293 cells.

EP2 and FP receptors are co-expressed in Ishikawa cells and a cross-talk between these prostanoid receptors has not yet been studied. To identify a possible signalling interaction between both receptors, Ishikawa cells that stably express the EP2 and FP receptors (FPEP2 cells) were used as a model system. The aim of this study was to assess the integrated effect of Butaprost and PGF co-administration on IP3 and cAMP release. Using the FPEP2 cells, we showed PGF-stimulated IP3 production was not altered by co-stimulation with Butaprost suggesting heterologous interaction between the two receptors does not have a synergetic effect on IP3 release. However, co-stimulation of FPEP2 cells with Butaprost and PGF augmented Butaprost-stimulated cAMP release despite the fact that PGF-mediated FP receptor activation on its own had no effect on cAMP accumulation. This phenomenon was also observed by Zhang *et al.* (1997) and Meszaros

et al. (2000) in other receptors with similar fold increase (2 to 2.5 fold) to the one reported in this current study.

Using specific receptor antagonists and small molecule chemical inhibitors of cell signaling, the molecular pathways mediating the PGF-induced augmentation of cAMP in cells treated with the combination of ligands was dissected. The PGF-mediated enhancement of cAMP observed in FPEP2 cells treated with Butaprost and PGF was mediated via the FP receptor- $G\alpha_q$-mediated activation of IP3 via PLC and was independent of PKC activation. Following its release IP3 could activate its receptors (IP3R) present on the endoplasmic reticulum (ER) membrane to promote intracellular calcium release and activation of calmodulin and CaMK-II.

The composite of the abovementioned data show PGF potentiation of Butaprost-stimulated cAMP could be via the adenylyl cyclase pathway since several studies have shown that the calmodulin pathway can activate calcium sensitive AC isoforms to regulate intracellular cAMP accumulation (Wang and Storm, 2003; Hanoune and Defer, 2001). Clearly, identifying the expression of multiple AC isoforms in FPEP2 Ishikawa cells, in particular the calcium-stimulated isoforms provides an answer for the intricate cross-talk system between the EP2 and FP receptors. With the use of RT-PCR, data were obtained that show Ishikawa cells express mRNA for eight of the ten AC isoform (AC1, AC3, AC4, AC5, AC6, AC7, AC9 and the soluble AC), AC1 and AC3 being the calcium regulated ones. Even if AC3 is generally regarded as a calcium-stimulable isoform, studies have shown direct inhibition of the isoform by the phosphorylation of CaMK-II in HEK-293 cells (Wei *et al.,* 1996; Wei *el al.,* 1998). However, other study has revealed when it is concomitantly activated with $G\alpha_s$, AC3 can be stimulated by intracellular calcium to potentiate cAMP (Choi *et al*., 1992). Zhang *et al.* (1997) and Ostrom *et al.* (2003) reported AC3 to be the only calcium-regulated isoform that is expressed in their model cells, showing the likely involvement of the isoform in the $G\alpha_s$-$G\alpha_q$ cross-talk reported in their studies. Transfection studies using siRNA to abolish expression of AC1 or AC3 in FPEP2 cells revealed that the calcium sensitive AC3 isoform is responsible for PGF-mediated

potentiation of Butaprost-stimulated cAMP. These data suggest that the $G\alpha_s$-$G\alpha_q$ cross-talk reported here is via the calcium sensitive isoform AC3.

Other studies done on brown adipose tissue showed that neural stimulation can increase norephrine-stimulated AC activity. This is the only study to determine the $G\alpha_s$-$G\alpha_q$ cross-talk between the receptors was followed by a rapid increase in mRNA expression level of the AC3 isoform (Granneman, 1995). Using Real-Time RT-PCR analysis, regulation of AC3 mRNA by co-activation of the EP2 and FP receptors was determined. At all time points (1, 2, 4, 6 and 8 hrs), AC3 mRNA expression was not affected by co-activation of the receptors suggesting the cross-talk does not have an effect on auto-regulation of the isoform itself (Data not shown).

Interestingly, both AC1 and AC3 siRNA inhibition did not have any significant effect on Butaprost-stimulated cAMP suggesting these two isoforms are not regulated by Butaprost. In contrast, Wong *et al.* (2001) showed that AC3 is the principal isoform that is activated by PGE_2 despite the expression of other AC isoforms in human arterial smooth muscle cells (SMC). Another study showed that PGE_2 can interact with its cognate receptors to activate solely the AC6 isoform when it is over-expressed in rat SMCs while it did not couple efficiently to the AC3 isoform. The authors suggested these could be due to localization of the AC isoforms in the cells. They showed that AC3 and AC6 are expressed in caveolin-rich fractions while the PGE_2 receptors EP2 and EP4 were detected primarily in the non-caveolin fraction of the membrane. When AC6 isoform is over-expressed it localizes to non-caveolin fraction explaining why PGE_2 solely activates the AC6 isoform in RSMCs. Differences in these data might be related to cell-specific or different localization of ACs and the components that can activate ACs (Ostrom *et al.*, 2002). In Ishikawa cells Butaprost stimulation of cAMP accumulation can be mediated by other AC isoforms but not by AC1 and AC3 isoforms.

In conclusion, this study demonstrates that co-activation of the EP2 and FP receptors results in enhanced release of cAMP via FP receptor-$G\alpha_q$-Ca^{2+}-calmodulin pathway by activating the calcium-sensitive AC3 isoform in FPEP2 cells. The effect of this cross-talk on gene expression profiling is studied in the next chapter.

Chapter VI

Gene Array Analysis:

Target Genes for EP2 and FP Receptors Co-Activation

6.1 Introduction

In chapter three expression of COX enzymes, prostaglandin receptors and prostaglandin-regulated genes were compared in endometrium samples from women with and without fibroids. I reported enhancement of COX-2, EP2 receptor, FP receptor, VEGF, IL-8 and IL-11 mRNA expression in endometrium from women with fibroids compared to control samples.

Interestingly, endometrial samples derived from women with fibroids had higher EP2 and FP receptors mRNA expression in the proliferative stage of the menstrual cycle compared to normal samples. In order to investigate the integrated signalling and the possible cross-talk between the two receptors, I established Ishikawa cells over-expressing EP2 and FP receptors cell line as a model. Using this cell line, I reported a novel and integrative cross-talk observed following co-activation of the EP2 and FP receptors. An important question is to determine the integrative effects of these receptors co-activation on gene expression profiling.

Gene array analysis has been used in a large number of studies to assess gene expression in cell lines and tissue samples with and without exposure to different external stimuli. One of the great advantages of this analysis is the ability to use a single RNA sample to study the global transcription profile. Another advantage of gene array analysis is the reduction of human technical error since the processes are mostly automated.

There have been several global gene array studies using Ishikawa cells as models to study the genetic signatures underlying endometrial pathologies. Paulssen *et al.* (2008) treated Ishikawa cells with progesterone for 4 hours and compared expression of genes to untreated samples using gene array analysis. This analysis revealed 247 differentially expressed genes of which 126 were up-regulated. Of the genes that were up-regulated some of them were shown to be involved in biological processes like apoptosis (7.2%), developmental process (13.9%), immune response and defence (11%) and intracellular protein trafficking (10.5%). However, genes implicated in cell cycle (12.6%), cell proliferation and differentiation (8.4%), cell

structure and motility (15.4%) and developmental process (14%) and transport (7%) were down-regulated by progesterone treatment.

Davis *et al*. (2006) also assessed genes that are temporally regulated by progesterone and glucocorticoids in Ishikawa cells using gene array analysis. The authors identified unique lists of genes that are hormonally regulated and might be involved in the early process of carcinogenesis. Out of these genes, legumain, a gene associated with metastasis was down-regulated by progesterone while upstream c-fos related transcription factor-2 (USF-2), an anti-proliferative factor was shown to be induced by both progesterone and glucocorticoids (Davies *et al.*, 2006).

Another Global gene array analysis studied the effect of estrogen in Ishikawa cells. This study reported differentially regulated genes using estrogen receptor proficient and deficient Ishikawa cells treated with well-known estrogen active compounds. The authors reported up-regulation of novel genes by estrogen involved in cell growth, cell migration and tissue remodelling. Most down-regulated genes in response to estrogen were related to cell growth inhibition, apoptosis induction and estrogen metabolism (Boehme *et al.*, 2009).

Since EP2 and FP receptors are co-expressed, I set out to examine target genes that are regulated by co-activation of both receptors in endometrial epithelial cells. This was carried out by employing whole genome array profiling in FPEP2 cells in response to Butaprost, PGF or the combination of Butaprost and PGF.

6.2 Aim of the Study

Previously, I have shown that there is cross-talk between the signalling cascades elicited by Butaprost and PGF in FPEP2 Ishikawa cells. This cross-talk can culminate in regulation of different target genes. This current study was designed to identify which genes or cluster of genes are differentially regulated by co-activation of EP2 and FP receptors relative to activation of EP2 or FP receptors separately.

6.3 Materials and Methods

6.3.1 Gene Array

6.3.1.1 Treatment of Cells and RNA Extraction

For gene array analysis, Ishikawa cells were seeded at a density of 5×10^5cells per 6-cm dishes. The following day the cells were serum-starved for 18 hrs in the presence of 3µg/ml of indomethacin. Cells were then treated in serum free media for 8 hrs by incubation with vehicle (0.1% ethanol), Butaprost (5µM) and/or PGF (100nM). Experiments were done four times in triplicates (N=12) for each treatment. After treatment, the cells were washed with cold PBS and incubated with Trizol for 5 min at room temperature. Cells were scraped from the dish surface and lysed through 0.22 gauge needle a number of times. The lysate was centrifuged for 3 min at 10000 rpm and supernatant was removed to a fresh tube and was then mixed with 70% ethanol. The mix was then transferred to RNeasy spin column and total RNA was extracted as mentioned on section 2.3.3. The concentration and purity of the RNA were determined by measuring the absorbance at 260nm and 280nm (OD_{260} and OD_{280}) using NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). The criterion for sample inclusion in the gene array was A260/A280> 1.9. The integrity of the RNA was further assessed by Agilent 2100 bioanalyzer (Agilent Technologies UK Ltd). This automated RNA analyzer determined the quality of RNA by calculation of RNA integrity number (RIN) using microcapillary electrophoresis and fluorescence detection. The RIN values range from 10 (fully intact RNA) to 1 (totally degraded RNA) (Schroeder *et al.*, 2006). Triplicates for each experiment with good RNA quality were pooled and sent for Gene array analysis. Hybridization of RNA samples to the gene array was carried out by GeneService by Applied biosystems human genome survey analysis and described in the section below.

6.3.1.2 Hybridization of RNA Samples and Data Analysis

The hybridization of RNA samples was done by GeneService (Cambridge, UK) employing the method described below. The cDNA template was made by reverse transcriptase using in vitro transcription (IVT) reaction. The RT enzyme was used to incorporate deoxynucleotides to prepare single stranded DNA while the use of T7 poly dT primers enabled addition of the T7 polymerase promoter to the 5' end. To synthesise 2nd strand DNA, RNase H was used to expose the priming sites of DNA polymerase by degrading the RNA in DNA-RNA duplexes. Thereafter, the double stranded cDNA was washed to clear excess buffer and salts and then eluted in suitable solution for IVT labelling. During the IVT labelling the T7 polymerase incorporated ribonucleotides and digoxigenin (DIG)-UTP to make up to 1000-fold cRNA from double stranded cDNA. The cRNA was eventually washed to get rid of excess buffer and eluted to a solution suitable for hybridisation to the microarray. Prior to hybridisation, the cRNA was fragmented using divalent cations by catalysing the 2' hydroxyl cleavage to reduce the secondary structure that interferes with hybridisation kinetics. After blocking the microarrays to prevent non-specific binding, the DIG labelled cRNA was then hybridised to the microarray chip. The microarray chip was then washed to reduce nonspecific hybridisation of the labelled cRNA. The chemiluminescent reaction was performed by subjecting the DIG in the cRNA targets bound to the microarray to anti-DIG alkaline phosphatase (ALP) conjugated antibody. Non-specific antibody binding was then removed by washing prior to adding an enhancement solution in the microarray to increase the quantum yield of the chemiluminescent reaction. The chemiluminescent substrate was added which reacts with the ALP to emit a light signal. The signal was then read by the applied biosystems 1700 chemiluminescent microarray analyzer. This software excludes the signal intensity emitted from the blank features (non-specific signals) and calculates the correct amount of chemiluminescent signals on the microarray. In order to exclude technical variations and non-specific binding, several controls were used for each step. The RT controls, three synthetic mRNAs with bacterial gene sequences, were used to assess how well the RT reaction worked. The IVT controls were three synthetic double-stranded cDNA with a T7 promoter and bacterial control gene sequences. They were used to check the efficiency of labelling of the cRNA. Hybridization controls,

three DIG-labelled 60-mer oligo control targets, were used to determine the effectiveness hybridization to the microarray. To normalize the chemiluminescent signal, a fluorescent doubled 24-mer oligo was used as internal control target to bind with internal control probe on the microarray.

Data acquired using ABI technology was preprocessed according to the manufacturers' recommendations (Applied biosystems 1700 chemiluminescent microarray analyzer chemistry guide). The data was normalized using variance stabilized normalization (Huber *et al.,* 2002). Linear Models for Microarray Data (LIMMA) software package was used to analyze the differential expression of genes which provides fold change, standard error, t-statistics and *P* values for the level of expression (Smyth, 2004). The *P* values were adjusted for multiple testing using Benjamini and Hochberg method (Benjamini and Hochberg, 1995). The resulting gene list included only the genes that had a fold change value of 2.0 or higher and a *P* of < 0.05. Bioinformatics was performed using the gene set analysis tool kit (Zhang *et al.,* 2005).

6.3.2 Gene Ontology Classification

Gene Ontologys (GO) were assigned to classify Butaprost and/or PGF regulated genes for biological processes and molecular functions using a web tool provided by the gene ontology database (www.geneontology.org).

6.3.3 Verification of the Gene Array Data

6.3.3.1 Treatment of Cells

In order to asses the temporal expression of Butaprost and/or PGF regulated genes, FPEP2 cells were treated with vehicle (0.1% ethanol), Butaprost (5µM) and/or PGF (100nM) for 4, 6 and 8 hrs. To assess specific molecular pathway FPEP2 cells were treated with either FP antagonist (50µM of AL8810 with 3min pretreatment) or transfected with AC3 siRNA as mentioned in section 5.3.3 in the presence/absence of Butaprost (5µM) and/or PGF (100nM) for 6 hrs. Cells were washed with cold PBS and were incubated with Trizol for 5 min at room temperature.

6.3.3.2 RNA Extraction and cDNA Synthesis

Total mRNA was then extracted from the treated samples as described in section 2.3.2. cDNA was synthesized from 400ng of mRNA by reverse transcription in the presence of RT-PCR reaction buffer (1×), dNTPs (0.5mM each), $MgCl_2$ (5.5mM), random hexamers (2.5µM), RNase inhibitor (0.4U/µl), and MultiScribe™ reverse transcriptase (1.25U/µl).

6.3.3.3 Real-Time RT-PCR Analysis

Real-Time RT-PCR was performed on the cDNA samples (2µl per reaction) from treated FPEP2 cells using specific primers (300nM) and probes (100nM) respectively) to detect COX-2, VEGF, IL-8 and SAT1 genes in a reaction mix containing Taqman buffer (5.5mM $MgCl_2$ and 200µm of each dNTPs, 0.025U/µl of AmpliTaq Gold DNA polymerase). Primers and probes for 18S (all at 50nM) were also added in the reaction mix as a loading control for the amount of cDNA added in each sample (Sequences of the primers is mentioned Table 2.4). The data was analysed using the comparative C_T method for relative quantification.

6.3.4 Statistical Analysis

All data are presented as mean ± SEM. Statistical significant differences were determined by one-way analysis of variance using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) (*, $p < 0.05$; **, $p < 0.001$; ***, $p<0.0001$).

6.4 Results

6.4.1 Real-Time RT-PCR Validation of COX-2, VEGF and IL-8 Gene Expression in Response to Butaprost and PGF in FPEP2 Ishikawa Cells

Prior to Gene Array analysis, cDNA made from treated RNA samples were validated by measuring the expression of genes that have already been shown to be up-regulated by prostaglandins. Studies in our laboratory have shown a significant increase of COX-2, VEGF and IL-8 mRNA expression level by the induction of Ishikawa cells with either PGE_2 or PGF (Sales *et al.,* 2004; Sales *et al*., 2005). To assess whether these genes are transcriptionally regulated in FPEP2 cells, cDNA samples from FPEP2 cells treated with vehicle, Butaprost and/or PGF were subjected to Real-Time RT-PCR analysis. As shown in figure 6.1A, 8 hrs treatment of the cells with PGF or Butaprost alone gave a significant increase in COX-2 mRNA expression compared to vehicle treated (5.46 ± 0.44 and 5.25 ± 0.69 vs. 1.0, respectively; $p<0.0001$), while treatment with both ligands had an even higher effect (11.35 ± 1.14; $p<0.0001$). Treatment with both ligands (PGF and Butaprost) or Butaprost alone elevated VEGF mRNA expression level significantly (5.55 ± 0.90 and 4.45 ± 0.36 vs. 1.0, respectively; $p<0.0001$), while PGF induction of VEGF mRNA was slightly lower than the former two treatments (3.73 ± 0.38; $p<0.001$) (Fig 6.1B). As shown in figure 6.1C, the relative expression of IL-8 mRNA with PGF or both treatments (PGF and Butaprost) was 455.20 ± 41.51 and 683.1 ± 56.70 respectively ($p<0.0001$). Butaprost alone gave 154.90 ± 19.63 fold increase ($p<0.05$) compared to vehicle treated (1.0). These results validated the cell treatment experiment and samples were sent for gene array analysis.

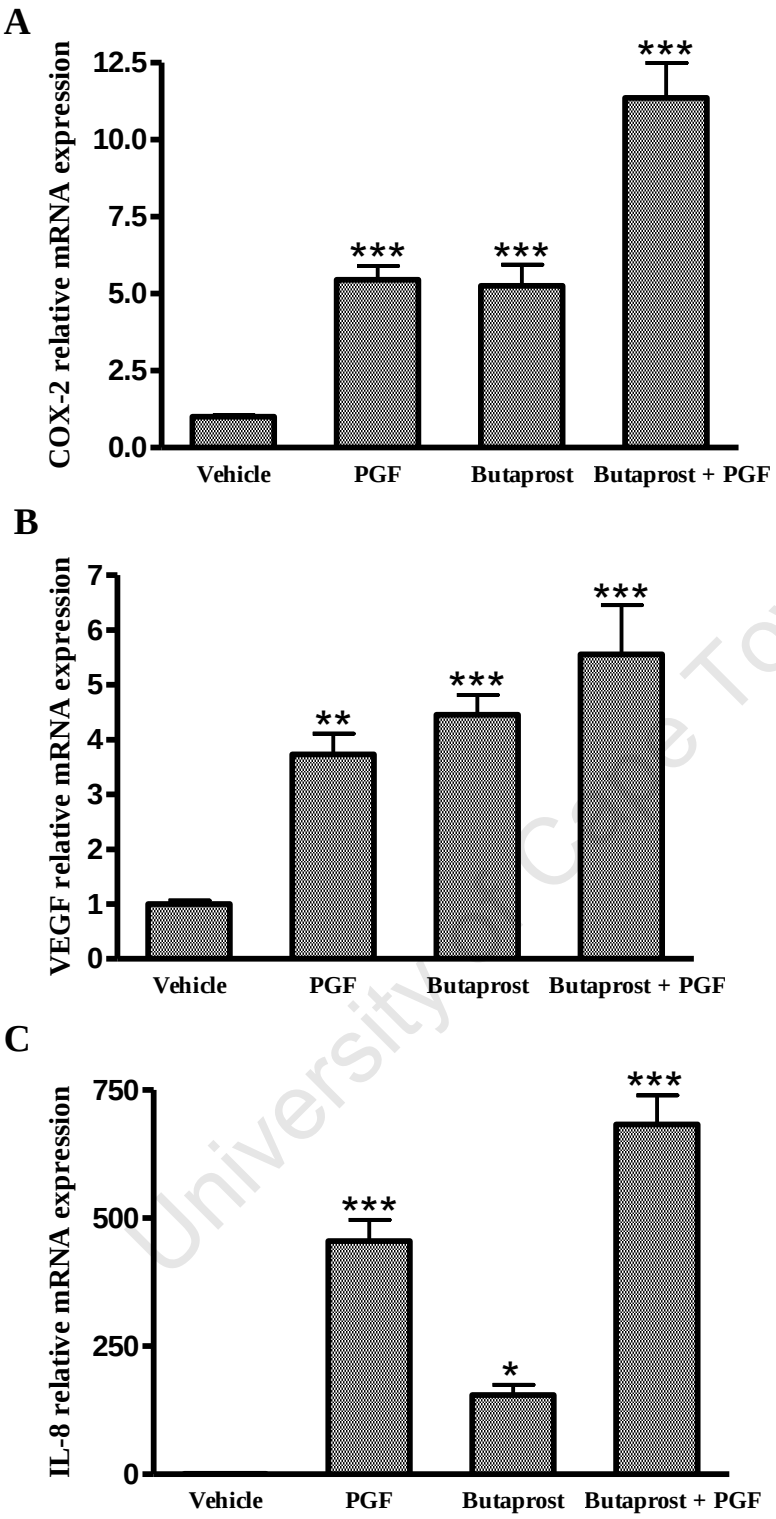

Fig. 6.1: *Relative mRNA expression of (A) COX-2, (B) VEGF and (C) IL-8 in FPEP2 cells after 8 hrs treatment with vehicle, Butaprost (5μM) and/or PGF (100nM) as determined by Real-Time RT-PCR. Data are presented as mean ± SEM from four independent experiments (*, $p < 0.05$; **, $p<0.001$; ***, $p<0.0001$).*

6.4.2 Genes Regulated by PGF and/or Butaprost Treatment

The data from the gene array was analysed by GeneService. Genes were considered to be regulated by PGF and/or Butaprost if they showed an increase or decrease of 1.5 fold relative to vehicle treated. The analysis revealed 1045 genes that are regulated by PGF and/or Butaprost treatment (The complete list of the genes and the fold increases are mentioned in Appendix A). Out of these 1045 genes, 704 genes were regulated by PGF treatment only, of which 312 genes were transcriptionally up-regulated and almost half of the genes (309) were only regulated by PGF and not by the other treatments. Treatment with Butaprost modulated the transcription of 363 genes, of which 202 genes were up-regulated and 161 genes were down-regulated. However, only 71 were uniquely regulated by Butaprost. Co-treatment with the two ligands regulated 631 genes, of which 358 genes were up-regulated and 273 genes down-regulated. Co-activation of the EP2 and FP receptor also revealed regulation of 228 unique genes. Of these, 94 genes were up-regulated while 134 genes were down-regulated. The list of the genes and the fold increase/decrease are mentioned in table 6.1 and 6.2 and selected seven genes that are important in endometrial cancer development are indicated in bold text. Fold induction of their gene expression by PGF and Butaprost co-stimulation was as follows: Basonuclin-1 (3.72 fold more), parathyroid hormone like hormone (PTHLH), (3.32 fold more), interleukin-1 receptor accessory protein (IL-1RAP), (3.29 fold more), 11β-hydroxysteroid dehydrogenases-2 (11βHSD-2) (3.27 fold more), CD2 associated protein (CD2AP), (3.08 fold more), thrombospondin type I, domain containing 1 gene (THSD1), (3.04 fold less) and tumor protein p73 (3.02 fold less). Interestingly, 48 genes were found to be regulated by Butaprost and not by PGF and were either enhanced or repressed by co-stimulation with PGF (Table 6.3). These genes are quite likely regulated by the PGF-potentiation of the Butaprost induced cAMP accumulation and one of these genes was further investigated.

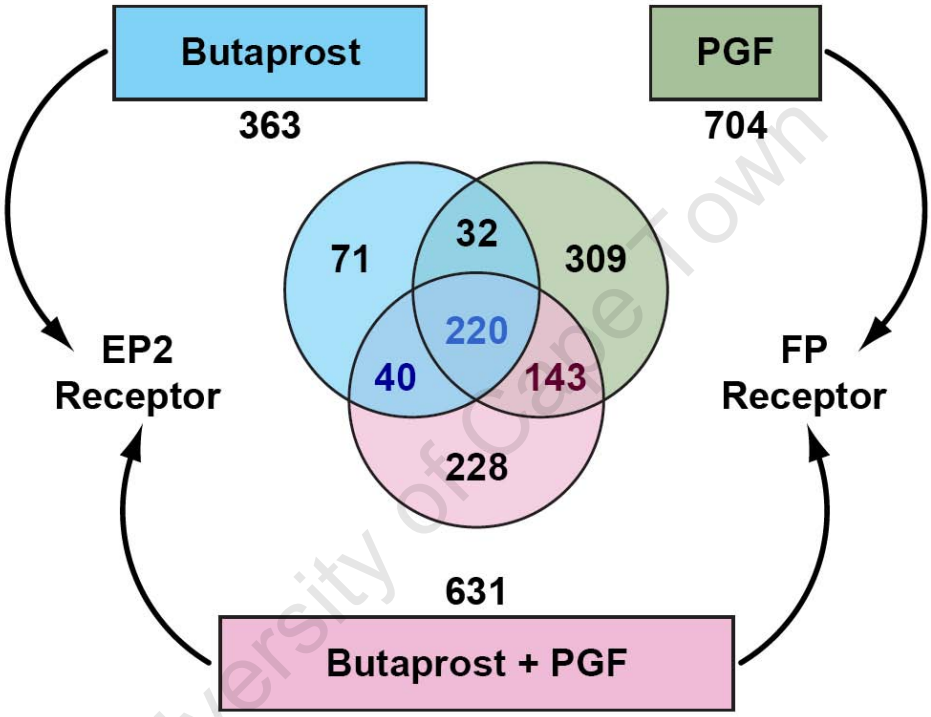

Fig. 6.2: *A Venn diagram highlighting expression profile of differently expressed genes after 8 hrs treatment with Butaprost and/or PGF in FPEP2 cells as analysed by Applied biosystems human genome survey analysis.*

Table 6.1: List of genes uniquely up-regulated by co-activation with Butaprost and PGF

Gene Symbol	Gene Name	Fold Change
C3orf23	Chromosome 3 open reading frame 23	137.68
MYL3	Myosin, light polypeptide 3, alkali ventricular, skeletal, slow	16.69
CUGBP1	CUG triplet repeat, RNA binding protein 1	9.69
TRPC6	Transient receptor potential cation channel, subfamily C, member 6	6.69
ASCC3	Activating signal cointegrator 1 complex subunit 3	5.13
C14orf28	Chromosome 14 open reading frame 28	4.58
ZNF545	Zinc finger protein 545	4.46
CAMSAP1L1	Calmodulin regulated spectrin-associated protein 1-like 1	4.32
C17orf39	Chromosome 17 open reading frame 39	4.20
RABGGTB	Rab geranylgeranyltransferase, beta subunit	4.19
PAQR9	Progestin and adipoq receptor family member IX	3.94
MFAP5	Microfibrillar associated protein 5	3.86
ISG20	Interferon stimulated exonuclease gene 20kda	3.81
NBLA00058	Asparagine synthetase domain containing 1	3.80
KIAA0020	Kiaa0020	3.77
BNC1	***Basonuclin 1***	***3.72***
IGFBP4	Insulin-like growth factor binding protein 4	3.70
RGS20	Regulator of G-protein signalling 20	3.63
ZNF503	Zinc finger protein 503	3.60
KCNK17	Potassium channel, subfamily K, member 17	3.60
GOLGA4	Golgi autoantigen, golgin subfamily a, 4	3.55
ELK4	ELK4, ETS-domain protein (SRF accessory protein 1)	3.53
C1orf107	Chromosome 1 open reading frame 107	3.52
SBDS	Shwachman-Bodian-Diamond syndrome	3.50
C6orf75	Chromosome 6 open reading frame 75	3.46
NKX2-5	NK2 transcription factor related, locus 5 (Drosophila)	3.45
TAF1A	TATA box binding protein (TBP)-associated factor, RNA polymerase I	3.44
TEX14	Testis expressed sequence 14	3.43
RPS6KA3	Ribosomal protein S6 kinase, 90kda, polypeptide 3	3.40
NAB1	NGFI-A binding protein 1 (EGR1 binding protein 1)	3.35
PTHLH	***Parathyroid hormone-like hormone***	***3.32***
IL1RAP	***Interleukin 1 receptor accessory protein***	***3.29***
HSD11B2	***Hydroxysteroid (11-beta) dehydrogenase 2***	***3.27***
NPAL1	NIPA-like domain containing 1	3.27
GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (galnac-T3)	3.27
ASB2	Ankyrin repeat and SOCS box-containing 2	3.26
PVR	Poliovirus receptor	3.26
CPOX	Coproporphyrinogen oxidase	3.25
SLC6A15	Solute carrier family 6, member 15	3.25
ZRF1	Zuotin related factor 1	3.17

Gene Symbol	Gene Name	Fold Change
PAK1IP1	PAK1 interacting protein 1	3.16
CAMK2N1	Calcium/calmodulin-dependent protein kinase II inhibitor 1	3.15
PHF2	PHD finger protein 2	3.14
TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kda	3.14
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	3.11
TRIM51	Tripartite motif-containing 51	3.10
C9orf72	Chromosome 9 open reading frame 72	3.10
WDR36	WD repeat domain 36	3.10
USP15	Ubiquitin specific peptidase 15	3.09
PLEKHC1	Pleckstrin homology domain containing, family C (with FERM domain) member 1	3.08
CD2AP	***CD2-associated protein***	***3.08***
SLC20A1	Solute carrier family 20 (phosphate transporter), member 1	3.08
BTG1	B-cell translocation gene 1, anti-proliferative	3.07
KIFC3	Kinesin family member C3	3.06
GAN	Giant axonal neuropathy (gigaxonin)	3.06
CD3EAP	CD3E antigen, epsilon polypeptide associated protein	3.05
TTC6	Tetratricopeptide repeat domain 6	3.04
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	3.04
FAT	FAT tumor suppressor homolog 1 (Drosophila)	3.04
BCL10	B-cell CLL/lymphoma 10	3.03
NARG1	NMDA receptor regulated 1	3.02
LARP2	La ribonucleoprotein domain family, member 2	3.01
MALL	Mal, T-cell differentiation protein-like	3.00
LAMB3	Laminin, beta 3	3.00
BXDC2	Brix domain containing 2	2.99
PTDSR	Phosphatidylserine receptor	2.98
RELB	V-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	2.97
SERPINB1	Serpin peptidase inhibitor, clade B (ovalbumin), member 1	2.96
BXDC1	Brix domain containing 1	2.96
C10orf47	Chromosome 10 open reading frame 47	2.96
MAPK8	Mitogen-activated protein kinase 8	2.95
C1orf27	Chromosome 1 open reading frame 27	2.95
LYAR	Ly1 antibody reactive homolog (mouse)	2.93
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	2.93
RPL22	Ribosomal protein L22	2.93
USP16	Ubiquitin specific peptidase 16	2.92
IPLA2(GAMMA)	Patatin-like phospholipase domain containing 8	2.92
CCDC41	Coiled-coil domain containing 41	2.91
GADD45B	Growth arrest and DNA-damage-inducible, beta	2.91
PDLIM2	PDZ and LIM domain 2 (mystique)	2.90
USP1	Ubiquitin specific peptidase 1	2.90

Gene Symbol	Gene Name	Fold Change
GLT28D1	Glycosyltransferase 28 domain containing 1	2.89
UBE2H	Ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)	2.88
ARID5B	AT rich interactive domain 5B (MRF1-like)	2.88
UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1	2.88
ICHTHYIN	Ichthyin protein	2.87
PRPF38B	PRP38 pre-mrna processing factor 38 (yeast) domain	2.87
MAPKBP1	Mitogen activated protein kinase binding protein 1	2.86
ARHGAP21	Rho gtpase activating protein 21	2.85
JOSD3	Josephin domain containing 3	2.85
C3orf26	Chromosome 3 open reading frame 26	2.85
CLK1	CDC-like kinase 1	2.85
SBDSP	Shwachman-Bodian-Diamond syndrome pseudogene	2.84
C21orf91	Chromosome 21 open reading frame 91	2.84

Table 6.2: List of genes uniquely down-regulated by co-activation with Butaprost and PGF

Gene Symbol	Gene Name	Fold Change
INMT	Indolethylamine N-methyltransferase	0.35
C17orf62	Chromosome 17 open reading frame 62	0.35
P2RY6	Pyrimidinergic receptor P2Y, G-protein coupled, 6	0.35
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs	0.35
UBIAD1	Ubia prenyltransferase domain containing 1	0.35
SLC27A1	Solute carrier family 27 (fatty acid transporter), member 1	0.35
PXDN	Peroxidasin homolog (Drosophila)	0.35
EHHADH	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	0.35
CRISP1	Cysteine-rich secretory protein 1	0.35
CASP8	Caspase 8, apoptosis-related cysteine peptidase	0.35
OR1D2	Olfactory receptor, family 1, subfamily D, member 2	0.35
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8	0.34
WDR71	WD repeat domain 71	0.34
CCL3L1|CCL3 L3	Chemokine (C-C motif) ligand 3-like 1|chemokine (C-C motif) ligand 3-like 3	0.34
PRSS33	Protease, serine, 33	0.34
PCYT1B	Phosphate cytidylyltransferase 1, choline, beta	0.34
CYB561D1	Cytochrome b-561 domain containing 1	0.34
TNK2	Tyrosine kinase, non-receptor, 2	0.34
KCND3	Potassium voltage-gated channel, Shal-related subfamily, member 3	0.34
EXTL1	Exostoses (multiple)-like 1	0.34
DNAH3	Dynein, axonemal, heavy polypeptide 3	0.34
ULBP3	UL16 binding protein 3	0.34
C21orf87	Chromosome 21 open reading frame 87	0.34
FHL5	Four and a half LIM domains 5	0.34
ZNF404	Zinc finger protein 404	0.34
TRFP	Trf (TATA binding protein-related factor)-proximal homolog (Drosophila)	0.34
SLC16A13	Solute carrier family 16 (monocarboxylic acid transporters), member 13	0.34
THSD1P	***Thrombospondin type I, domain containing 1 pseudogene***	***0.34***
C7orf31	Chromosome 7 open reading frame 31	0.34
C20orf175	Chromosome 20 open reading frame 175	0.33
LEFTY1	Left-right determination factor 1	0.33
DUSP26	Dual specificity phosphatase 26 (putative)	0.33
OR52K2	Olfactory receptor, family 52, subfamily K, member 2	0.33
ACBD4	Acyl-Coenzyme A binding domain containing 4	0.33
KIAA1618	Kiaa1618	0.33
PSORS1C2	Psoriasis susceptibility 1 candidate 2	0.33
VIL1	Villin 1	0.33
IFT81	Intraflagellar transport 81 homolog (Chlamydomonas)	0.33

Gene Symbol	Gene name	Fold Change
PLEC1	Plectin 1, intermediate filament binding protein 500kda	0.33
TNFAIP8L1	Tumor necrosis factor, alpha-induced protein 8-like 1	0.33
PKP3	Plakophilin 3	0.33
FGF6	Fibroblast growth factor 6	0.33
TCL6	T-cell leukemia/lymphoma 6	0.33
HIF3A	Hypoxia inducible factor 3, alpha subunit	0.33
RAB11FIP4	RAB11 family interacting protein 4 (class II)	0.33
C14orf78	Chromosome 14 open reading frame 78	0.33
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	0.32
STEAP2	Six transmembrane epithelial antigen of the prostate 2	0.32
SUHW1	Suppressor of hairy wing homolog 1 (Drosophila)	0.32
ZP1	Zona pellucida glycoprotein 1 (sperm receptor)	0.32
H1F0	H1 histone family, member 0	0.32
FXYD2	FXYD domain containing ion transport regulator 2	0.32
TP73	***Tumor protein p73***	***0.32***
NOD9	NLR family member X1	0.32
CNAP1	Non-SMC condensin I complex, subunit D2	0.32
MAN2C1	Mannosidase, alpha, class 2C, member 1	0.32
SERINC4	Serine incorporator 4	0.31
DEPDC4	DEP domain containing 4	0.31
APOL2	Apolipoprotein L, 2	0.31
IGFL2	IGF-like family member 2	0.31
APOBEC3A	Apolipoprotein B mrna editing enzyme, catalytic polypeptide-like 3A	0.31
EME1	Essential meiotic endonuclease 1 homolog 1 (S. Pombe)	0.31
TRPV6	Transient receptor potential cation channel, subfamily V, member 6	0.30
GML	GPI anchored molecule like protein	0.30
PLXDC2	Plexin domain containing 2	0.30
PARP4	Poly (ADP-ribose) polymerase family, member 4	0.30
C14orf133	Chromosome 14 open reading frame 133	0.30
SMP3	Phosphatidylinositol glycan anchor biosynthesis, class Z	0.30
ZNF610	Zinc finger protein 610	0.30
ATCAY	Ataxia, cerebellar, Cayman type (caytaxin)	0.29
KIF20A	Kinesin family member 20A	0.29
CDKN2C	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.29
PBX4	Pre-B-cell leukemia transcription factor 4	0.29
KLC3	Kinesin light chain 3	0.29
SYNJ1	Synaptojanin 1	0.29
FERD3L	Fer3-like (Drosophila)	0.29
PLEKHG1	Pleckstrin homology domain containing, family G (with rhogef domain) member 1	0.29
NFAM1	NFAT activating protein with ITAM motif 1	0.28
PSRC1	Proline/serine-rich coiled-coil 1	0.28
C11orf16	Chromosome 11 open reading frame 16	0.28
CPN1	Carboxypeptidase N, polypeptide 1, 50kd	0.27

Gene Symbol	Gene name	Fold Change
SCML4	Sex comb on midleg-like 4 (Drosophila)	0.27
TLX1	T-cell leukemia homeobox 1	0.27
SPRR4	Small proline rich protein 4	0.27
GPR68	G protein-coupled receptor 68	0.27
USP54	Ubiquitin specific peptidase 54	0.26
MBL1P1	Mannose-binding lectin (protein A) 1, pseudogene 1	0.26
ASPA	Aspartoacylase (Canavan disease)	0.26
KLHDC1	Kelch domain containing 1	0.26
RTP2	Receptor (chemosensory) transporter protein 2	0.25
C10orf33	Chromosome 10 open reading frame 33	0.25
C9orf98	Chromosome 9 open reading frame 98	0.25
ARID3A	AT rich interactive domain 3A (BRIGHT- like)	0.25
C8orf45	Chromosome 8 open reading frame 45	0.24
BAIAP2	BAI1-associated protein 2	0.24
PDK2	Pyruvate dehydrogenase kinase, isozyme 2	0.24
C2orf21	Chromosome 2 open reading frame 21	0.24
CA1	Carbonic anhydrase I	0.24
OR2C1	Olfactory receptor, family 2, subfamily C, member 1	0.24
OKL38	Oxidative stress induced growth inhibitor 1	0.24
C1orf145	Chromosome 1 open reading frame 145	0.23
ATOH7	Atonal homolog 7 (Drosophila)	0.23
C9orf150	Chromosome 9 open reading frame 150	0.23
TNRC6B	Trinucleotide repeat containing 6B	0.22
REG3G	Regenerating islet-derived 3 gamma	0.22
TSSK3	Testis-specific serine kinase 3	0.22
PFN4	Profilin family, member 4	0.22
RAI1	Retinoic acid induced 1	0.22
FOXB1	Forkhead box B1	0.21
Sept9	Septin 9	0.21
UGT2A1	UDP glucuronosyltransferase 2 family, polypeptide A1	0.21
PHIP	Pleckstrin homology domain interacting protein	0.21
MRVI1	Murine retrovirus integration site 1 homolog	0.20
GJE1	Gap junction protein, epsilon 1, 29kda	0.19
TLN2	Talin 2	0.18
TMEM37	Transmembrane protein 37	0.18
C3orf18	Chromosome 3 open reading frame 18	0.14
OR52I2	Olfactory receptor, family 52, subfamily I, member 2	0.14
MS4A3	Membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	0.13
SPATA6	Spermatogenesis associated 6	0.12
WNT1	Wingless-type MMTV integration site family, member 1	0.10
LRP12	Low density lipoprotein-related protein 12	0.07
IRGQ	Immunity-related gtpase family, Q	0.07
KLHDC6	Kelch domain containing 6	0.06
ZNF483	Zinc finger protein 483	0.06
DEFB119	Defensin, beta 119	0.05

Gene Symbol	Gene name	Fold Change
RGR	Retinal G protein coupled receptor	0.04
RASGEF1B	Rasgef domain family, member 1B	0.019
PRSS3	Protease, serine, 3 (mesotrypsin)	0.019
C20orf59	Chromosome 20 open reading frame 59	0.010
GLIS2	GLIS family zinc finger 2	0.008
MPO	Myeloperoxidase	0.006
PRSS12	Protease, serine, 12 (neurotrypsin, motopsin)	0.004
NKX6-1	NK6 transcription factor related, locus 1 (Drosophila)	0.004

Table 6.3: List of genes differentially expressed by Butaprost treatment only and modulated in combination with PGF treatment

Gene Symbol	Gene Name	Fold change	
		Butaprost	**PGF + Butaprost**
PGF enhanced modulation of gene expression			
SAT1	***Spermidine/spermine N1-acetyltransferase***	***5.94***	***7.47***
RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5	3.48	4.05
FRAS1	Fraser syndrome 1	3.46	6.41
KCNK5	Potassium channel, subfamily K, member 5	3.20	3.71
ATP1B3	Atpase, Na+/K+ transporting, beta 3 polypeptide	3.19	4.28
AQP3	Aquaporin 3	3.03	3.57
CDC42EP2	CDC42 effector protein 2	3.02	4.16
LIMS3	LIM and senescent cell antigen-like domains 3	2.98	3.81
FAM100A	Family with sequence similarity 100, member A	2.87	3.55
ZNF323	Zinc finger protein 323	0.16	0.12
NR3C2	Nuclear receptor subfamily 3, group C, member 2	0.18	0.14
SPAG8	Sperm associated antigen 8	0.24	0.15
C10orf91	Chromosome 10 open reading frame 91	0.27	0.14
DEFB1	Defensin, beta 1	0.32	0.29
KIAA1305	Kiaa1305	0.32	0.23
ALDH3B2	Aldehyde dehydrogenase 3 family, member B2	0.33	0.29
RNF144B	Ring finger protein 144B	0.33	0.27
SYNM	Synemin	0.34	0.26
MS4A2	Membrane-spanning 4-domains, subfamily A, mem 2	0.34	0.29
RLN1	Relaxin 1	0.35	0.32
OR6W1P	Olfactory receptor, family 6, subfamily W, member 1 pseudogene	0.35	0.29
TRIM6	Tripartite motif-containing 6	0.35	0.30
SAMD13	Sterile alpha motif domain containing 13	0.35	0.31
PGF repressed modulation of gene expression			
CYP26A1	Cytochrome P450, family 26, subfamily A, polypeptide 1	18.03	12.28
RPRM	Reprimo, TP53 dependent G2 arrest mediator candidate	11.90	7.76
IL1R2	Interleukin 1 receptor, type II	3.98	3.62
BTBD3	BTB (POZ) domain containing 3	3.30	3.02
ZNF703	Zinc finger protein 703	3.28	3.04
ADA	Adenosine deaminase	3.15	2.87
C1orf168	Chromosome 1 open reading frame 168	0.23	0.31
DDIT4	DNA-damage-inducible transcript 4	0.22	0.26
LIPC	Lipase, hepatic	0.19	0.22
VWA5A	Von Willebrand factor A domain containing 5A	0.17	0.23
KCNJ5	Potassium inwardly-rectifying channel, subfamily J, member 5	0.16	0.22

6.4.3 Gene Ontology Analysis and Classification of Genes

Gene ontology classifies genes in terms of their function into three categories namely, cellular component, biological process and molecular function. The cellular component ontology describes where the gene is located at the level of subcellular structure and macromolecular complexes (e.g. endoplasmic reticulum, nucleus or cytoplasm). Biological process ontology is a category that shows a series of events by one or more ordered assemblies of molecular function (e.g. cellular physiological process). The molecular function ontology describes catalytic or binding activities that occur at a molecule level. The difference between biological process and molecular function is that a biological process must have more than one distinctive step while the later is an activity performed by individual gene products (www.geneontology.org). All the 1045 genes that are regulated by Butaprost and/or PGF were subjected to gene ontology analysis. Application of gene ontology annotation for biological processes to Butaprost and/or PGF regulated genes revealed two significant themes of cell motility/chemotaxis (5.8%) (shown in red in Table 6.4) and blood vessel development/morphogenesis (4.6) (shown in green in Table 6.4).

Table 6.4: Gene Ontology Classification of the genes that are regulated by Butaprost and/or PGF showing biological process and molecular function (Red: Cell motility/chemotaxis, Green: blood vessel development/morphogenesis)

PGF	Butaprost	Butaprost & PGF
Biological Process		
Locomotory behavior Cell motility Localization of cell Chemotaxis Cell-cell signalling Cell growth Circulation Regulation of body fluids Blood coagulation Response to wounding	Cell-cell adhesion Cell motility Localization of cell Locomotory behavior Chemotaxis Tissue development Excretion Regulation of body fluids Blood coagulation Response to wounding	Cell motility Localization of cell Cell growth Morphogenesis Regulation of body fluids Blood coagulation Physiological response to wounding
Regulation of GPCR protein signalling pathway Cell morphogenesis Lipid catabolism Leukocyte migration Regulation of vasoconstriction Wound healing Physiological response to wounding	Regulation of vasoconstriction	Regulation of cell cycle Regulation of cell growth Cell migration Cell motility Cell morphogenesis Vasculature development Regulation of vasoconstriction Wound healing Physiological response to wounding
Neutrophil chemotaxis Retinol metabolism	Negative regulation of cell proliferation Transcription Epidermis development Blood coagulation	Regulation of progression through cell cycle Regulation of cell size Negative regulation of cell proliferation Cell migration Blood vessel morphogenesis Blood coagulation
Molecular Function		
Calmodulin binding		
Actin binding Cytokine activity	Cytokine activity	Gap-junction forming channel activity Insulin-like growth factor binding Cytokine activity
Heparin binding Rhodopsin-like receptor activity	Steroid hormone receptor activity	Heparin binding

6.4.4 PGF Potentiates Butaprost-Regulated SAT1 Gene Expression in FPEP2 Cells

Since PGF-potentiation of Butaprost stimulated-cAMP increase was observed, I was primarily interested to identify genes that are transcriptionally induced by Butaprost and enhanced in combination with PGF treatment while PGF on its own has little effect. This array identified 23 genes that are induced by Butaprost and are further enhanced by co-treatment with PGF. It is logical to assume that the enhanced transcription of these is a consequence of the $G\alpha_s$-$G\alpha_q$ cross-talk which results in potentiation of cAMP production. One of the genes identified is Spermidine/spermine N1-acetyltransferase (SAT1) which is known to have roles in growth and proliferation of mammalian cells (Pegg, 2008). Interestingly, the SAT1 gene is known to be regulated by cAMP via the cAMP response element binding protein (CREB) that is located on its promoter region and this can explain its augmentation by co-treatment with Butaprost and PGF. I next decided to study the temporal regulation of the SAT1 gene by treating FPEP2 Ishikawa cells with vehicle, Butaprost and/or PGF for 4, 6 and 8 hrs. As shown in figure 6.3, Butaprost treatment alone significantly increased expression of SAT1 at all time points reaching a maximum level at 8 hrs compared to vehicle treated (3.48 ± 0.34, 3.9 ± 0.15 and 4.61 ± 0.15 vs. 1, respectively). No significant elevation of SAT1 gene expression was shown by treatment with PGF alone. However, co-stimulation of FPEP2 cells with Butaprost and PGF potentiated Butaprost stimulated expression of SAT1 significantly at all time points with the following fold increase: 4 hrs (5.29 ±0.22; $p<0.05$), 6 hrs (6.05 ± 0.3; $p<0.001$) and 8 hrs (8.28 ± 0.5; $p<0.001$). These data demonstrate that PGF augments Butaprost-stimulated SAT1 gene expression.

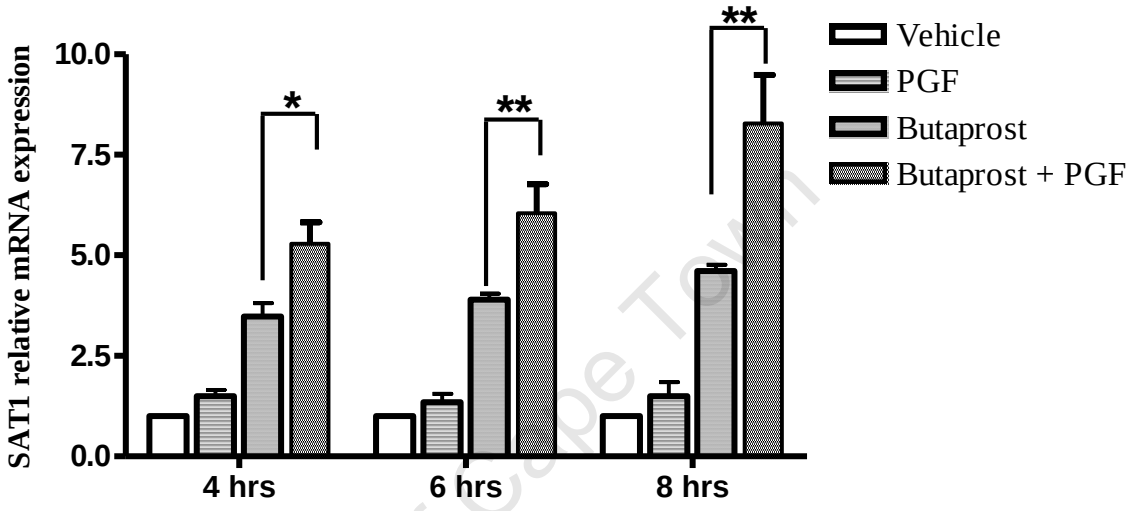

Fig. 6.3: *Relative temporal expression of SAT1 in FPEP2 cells after 4, 6 and 8 hrs treatment with vehicle, Butaprost (5μM) and/or PGF (100nM) as determined by Real-Time RT-PCR. Data are presented as mean ± SEM from four independent experiments (*, p < 0.05; **, p<0.001).*

6.4.5 PGF-Induced SAT1 Potentiation is Mediated by FP Receptor in FPEP2 Cells

To determine whether the PGF-mediated potentiation of SAT1 expression is mediated by FP receptor, the cells were co-treated with a specific antagonist for FP receptor (50µM of AL8810 with 3min pretreatment) in the presence of Butaprost (5µM) and/or PGF (100nM) for 6 hrs. As shown in figure 6.4, antagonism of the FP receptor completely abolished the potentiation of SAT1 expression by PGF without altering Butaprost-stimulated expression of SAT1 (5.82 ± 0.25 vs. 3.00 ± 0.39; $p<0.001$). These data demonstrate that Butaprost-regulated expression of SAT1 is augmented by PGF-FP receptor coupling.

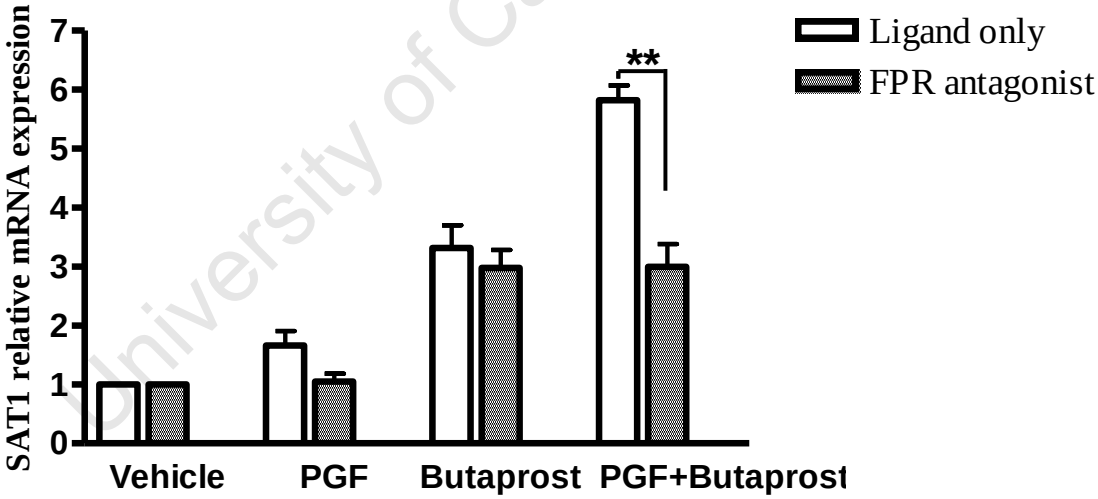

Fig. 6.4: *Relative expression of SAT1 in FPEP2 cells after 6 hrs treatment with vehicle, Butaprost (5µM) and/or PGF (100nM) in the presence/absence of the FP receptor antagonist (AL8810; 50µm) as determined by Real-Time RT-PCR. Data are presented as mean ± SEM from four independent experiments (**, p<0.001).*

6.4.6 siRNA Knockdown of AC3 Abolishes PGF Potentiation of Butaprost-Stimulated SAT1 in FPEP2 Cells

In the previous chapter, I have shown that AC3 is involved in the $G\alpha_s$-$G\alpha_q$ cross-talk in FPEP2 cells. In order to determine if AC3 isoform is involved in the mediation of SAT1 expression potentiation by PGF, AC3 siRNA-transfected FPEP2 cells were subjected to vehicle, Butaprost (5μM) and/or PGF (100nM) treatment for 6 hrs. As shown in figure 6.5, ablation of AC3 endogenous expression reduced SAT1 mRNA in FPEP2 cells treated with the combination of Butaprost and PGF to the level of Butaprost stimulation only (5.26 ± 0.36 vs. 3.46 ± 0.37; $p<0.001$) but had no effect on Butaprost treatment alone (3.46 ± 0.34 vs. 3.25 ± 0.26). These data demonstrate that PGF mediated potentiation of SAT1 mRNA expression is regulated by the EP2-FP receptor cross-talk via the calcium-sensitive AC3 isoform.

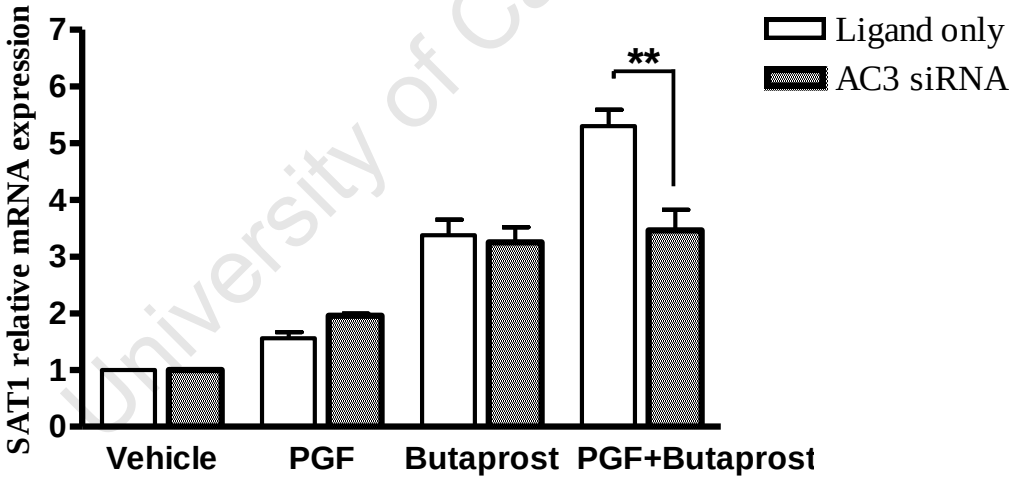

Fig. 6.5: *Relative mRNA expression of SAT1 in control and AC3 knockdown FPEP2 cells after 6 hrs treatment with vehicle, Butaprost (5μM) and/or PGF (100nM) as determined by Real-Time RT-PCR. Data are presented as mean ± SEM from four independent experiments (**, $p<0.001$).*

6.5 Discussion

In the previous chapter it has been shown that co-activation of the EP2 and FP receptors can lead to an integrative signalling pathway. Co-activation of both receptor resulted in a potentiation of Butaprost-stimulated cAMP by PGF via the calcium-calmodulin pathway through the calcium-sensitive AC3 isoform in FPEP2 cells. There has been other cAMP mediated $G\alpha_s$-$G\alpha_q$ cross-talk reported (Zhang *et al*., 1997; Beazely and Watts 2005; Ostrom *et al.,* 2003). The $G\alpha_s$-$G\alpha_q$ cross-talk reported by Ostrom *et al.* (2003) had an important physiological consequence on regulation of the extracellular matrix in myocardium. The authors showed ISO-mediated inhibition of collagen synthesis was further decreased by co-stimulation with ANG II suggesting the cross-talk plays a role in inhibition of fibrosis in heart. In order to determine the integrative effects of receptor co-activation on gene expression, human genome profiling was performed in FPEP2 cells in response to Butaprost, PGF or the combination of Butaprost and PGF.

Prior to gene array analysis the samples were validated using genes that have previously been shown to be regulated by prostaglandins using Real-Time RT-PCR. Our analysis confirmed the temporal up-regulation of COX-2, VEGF and IL-8 mRNA expression. COX-2 expression was up-regulated by Butaprost and PGF to the same level while co-activation of the two receptors showed an even higher enhancement in gene expression. VEGF mRNA expression was also up-regulated with all sets of treatments however there was not any statistical significance between the treatments. IL-8 expression by Butaprost was lower than PGF treatment while co-activation by Butaprost and PGF gave a higher expression of the gene, validating the cell treatment experiments and samples were sent for gene array analysis.

In the gene array analysis, numerous differentially regulated genes in response to treatment of FPEP2 Ishikawa cells with Butaprost and PGF were observed. The gene array revealed 1045 differentially expressed genes of which 71 were uniquely regulated by Butaprost and 309 were PGF-regulated. Co-treatment with Butaprost and PGF regulated 228 unique genes that have two major themes. These two themes were cell morphogenesis/cell motility and blood vessel development/morphogenesis. These

processes are highly significant in the development and growth of tumours and play a major role in development of endometrial carcinoma.

The gene array analysis revealed 48 genes that are regulated by Butaprost but are further modulated by co-stimulation with Butaprost and PGF. I focused on this set of genes since I have demonstrated a novel signalling cross-talk between the EP2 and FP receptors involving a potentiation of Butaprost stimulated-cAMP by PGF. Analysis of the gene list for Gene Ontology annotations indicated functions in cellular metabolism, immune response and excretion. One of the genes identified, SAT1 is a rate-limiting enzyme of polyamine catabolism. The enzyme adds an acetyl group to aminopropyl ends of spermidine and spermine to reduce the charge of polyamines, thus hindering their ability to bind acidic macromolecules and influencing their function. Acetylated polyamines are then either oxidized which leads to the back-conversion of higher polyamines to lower polyamines or they are excreted out of the cell as a means of lowering intracellular polyamine pool (Tucker *et al.*, 2005; Pegg, 2008). The promoter region of SAT1 lacks a TATA box but has multiple binding sites for transcriptional factors including cAMP response element binding (CREB), suggesting it is cAMP pathway regulated (Fogel-Petrovic *et al*., 1993). The integrative signalling mediating the role of prostanoids on SAT1 expression in FPEP2 cells was investigated. I found that SAT1 is regulated by the cAMP mediated $G\alpha_s$-$G\alpha_q$ cross-talk via AC3, such that siRNA knockdown of the AC3 isoform completely inhibited the potentiation of Butaprost-stimulated SAT1 expression by PGF. SAT1 is a highly regulated enzyme and it is inducible by polyamines and has been shown to be involved in carcinogenesis (Pegg, 2008). Transgenic mice harbouring the SAT1 gene showed a variety of defects like hair loss, female infertility, lipid metabolism and predisposition to develop pancreatitis (Janne *et al.,* 2004). Tucker *et al.* (2005) showed that SAT1-over producing transgenic mice bred with $Apc^{min/+}$ mice (mice predisposed to intestinal tumor formation) had an increase in incidence of intestinal tumors while crosses with SAT1 knockout mice led to 75% reduction in tumour load. Another study on mice over-expressing SAT1 using the keratin 6 promoter showed an increased susceptibility to incidence and development of skin tumor in response to a tumorigenesis protocol (Coleman *et al.,* 2002).

All these studies strongly suggest that SAT1 might have a key role in promoting tumor growth. Although the role for SAT1 in endometrial pathologies with dysregulated prostanoid synthesis is unclear, SAT1 has been shown to have a direct effect on cell migration by binding with α9β1-integrin (Chen *et al*., 2004). Moreover, in a recent study, Vlahakis *et al.* (2007) have demonstrated that α9B1-integrin can bind to VEGF to promote angiogenesis and lymphangiogenesis. In light of these studies, SAT1 might play a role in the pathology of the endometrium by directly promoting cell migration and an indirect enhancement of angiogenesis via VEGF-α9β1-integrin.

Interestingly, we found that co-activation of FPEP2 cells with Butaprost and PGF activated a unique subset of genes (228 genes) with known roles in regulating cell morphology, proliferation and differentiation (Table 6.1 and 6.2). Seven genes that are only regulated by co-activation of the EP2 and FP receptors will be discussed with a review of their functions.

Basonuclin-1

Basonuclin-1 is one of the genes that is uniquely up-regulated by Butaprost and PGF. It is a cell-specific transcription factor mainly found in cells of the basal layer and it has been shown to be involved in re-epithelisation (Matsuzaki *et al.*, 2004) and cell adhesion (Wang *et al.*, 2006). Basonuclin-1 has been also shown to be involved in cell proliferation (Tseng, 1998). Cell proliferation and adhesion are amongst the many characteristics of growing tumours. Up-regulation of Basonuclin by EP2 and FP receptors activation might exacerbate pathologies of endometrial adenocarcinoma by regulating genes that are involved in cell proliferation and adhesion.

Interleukin 1 receptor accessory protein (IL1RAP)

Another gene that is uniquely up-regulated by co-treatment with Butaprost and PGF is IL1RAP. IL-1 is an essential inflammatory protein that binds to its cognate receptors (IL-1R type I and type II) to initiate numerous biological processes. This cytokine is involved in several reproductive functions and play a role in numerous dynamic changes occurring in the endometrium through menstrual cycles (Bigonnesse *et al.,* 2001). IL1RAP, which by itself does not recognize the IL-1 but increases the

affinity of the ligand binding to its type II receptor (Greenfeder *et al.,* 1995). Studies have shown I1RAP is constitutively expressed in endometrium primarily in epithelial glands and luminal epithelium and less markedly in the stroma (Bigonnesse *et al.,* 2001). Singer *et al.* (2002) have shown the expression of the IL-1 system in endometrial malignancies and IL-1 involvement in an invasive nature of the endometrial cancer. Taking these studies into account, up-regulation of IL1RAP by Butaprost and PGF might play a role in the pathology of endometrium by increasing the affinity of IL-1R type II receptor to IL-1.

CD2 associated protein (CD2AP)

CD2AP was uniquely activated by co-stimulation of the EP2 and FP receptors. CD2AP is a widely expressed adaptor protein that facilitates receptor patterning by linking specific adhesion receptors to the cytoskeleton (Dustin *et al.,* 1998). Kirsch *et al.* (2001) have shown CD2AP can interact with the proto-oncogene c-Cbl. c-Cbl is a substrate of protein tyrosine-kinases that is rapidly phosphorylated by activation with growth factors. Other studies have also shown activation of the c-Cbl can in turn regulate formation of lamellipodia and cell morphology (Scaife and Langdon, 2000; Lehtonen *et al.,* 2002). Collectively, up-regulation of CD2AP by Butaprost and PGF can enhance various signalling pathways that regulate cellular adhesion, motility and morphology that all play important roles in endometrial pathologies by regulating cell movement and metastasis to distant sites of the body.

11β-hydroxysteroid dehydrogenases-2 (11βHSD-2)

Another gene that has been shown to be uniquely up-regulated by co-activation of the EP2 and FP receptors is 11βHSD-2. The family of 11βHSD are known to catalyse glucocorticoids metabolisms that are involved in tissue remodelling. As a dynamic and constantly remodelling tissue the endometrium expresses the 11βHSD-2 gene (McDonald *et al.,* 2006; Manolis *et al.,* 2006). A recent study has shown an up-regulation of 11βHSD-2 gene and down-regulation of the antiangiogenic factor thrombospondin-1 (TSP-1) in endometrium from women having heavy menstrual bleeding (Rae *et al.,* 2009). Interestingly, in our array study a down-regulation of thrombospondin, type I, domain containing 1 gene (THSD1) a similar gene to TSP-1

was observed by co-stimulation with Butaprost and PGF and the function of the gene will be discussed below. TSP-1 is regulated by cortisol suggesting the role of 11βHSD-2 to reduce glucocorticoids and then in turn enhance endothelial cell dysfunction and promote angiogenesis (Rae *et al.,* 2009). 11βHSD-2 up-regulation by Butaprost and PGF may contribute to down-regulation of antiangiogenic factors and can promote angiogenesis in endometrial pathologies.

Parathyroid hormone like hormone (PTHLH)

The other gene to up-regulated by co-treatment with Butaprost and PGF is parathyroid hormone like hormone (PTHLH). PTHLH is an autocrine/paracrine factor that binds to its cognate receptor to activate bone formation and resorption (Juppner *et al.,* 1991). An *in vitro* study using breast cancer cell lines has shown the role of PTHLH in development of breast cancer by inhibition of apoptosis and regulation of cell cycle (Tovar Sepulveda *et al.,* 2002). Dougherty *et al.* (1999) have shown expression of PTHLH in prostate cancer cell lines and its involvement in tumour growth and anti-apoptotic effect *in vitro*. These data may suggest a role for PTHLH in endometrial carcinoma pathology by increasing tumour growth and inhibiting apoptosis.

Thrombospondin type I, domain containing 1 gene (THSD1)

THSD1 gene was shown to be down-regulated by co-stimulation with Butaprost and PGF compared to basal level and single ligand treatments. THSD1 is a newly indentified gene and was mapped to chromosome 13q14 recently by Ko *et al.* (2008). The authors identified several tumour suppressor genes that are associated with esophageal squamos cell carcinoma (ESCC), one of them being THSD1 gene. Transfection of the THSD1 gene to a human esophageal cancer cell line (SLMT-1) resulted a reduction in colony formation ability suggesting a role for the gene in growth suppression. In six ESCC cell lines that showed a down-regulation of the gene compared to NE1 (immortalized normal esophageal epithelial cell line), methylation of THSD1 gene was highly observed. The function of this gene is not well elucidated but the gene encodes for a transmembrane molecule containing a thrombospondin type I repeat (Ko *et al.,* 2008). Another study has shown that protein containing the

thrombospondin type I motif have antiangiogenic activities (de Fraipont *et al.,* 2001). Hence, up-regulation and activation of the EP2 and FP receptors in endometrial carcinoma can lead to a reduced expression of the tumour suppressor THSD1 gene.

Tumor protein p73

Tumor protein p73, a protein that shares considerable homology with p53, was mapped to chromosome 1p36 and the region is frequently deleted in neuroblastoma and most tumors (Kaghad *et al.,* 1997). Soon after its mapping, Jost *et al.* (1997) showed that over-expression of p73 can activate p53-mediated genes and acts as a growth suppressor. Another study has shown that expression of p73 can be induced by the transcription factor E2F-1 which in turn induces apoptosis. The authors used p73 homozygous knockout mice to show that apoptosis was reduced in embryonic fibroblasts cells derived from these mice compared to wild-type mice (Irwin *et al.,* 2000). In this current study p73 was down-regulated by co-activation of EP2 and FP receptor suggesting a role for p73 down-regulation in development of endometrial pathologies by inhibiting apoptosis.

In conclusion, I have identified sets of genes that are uniquely activated by co-activation of the EP2 and FP receptors that are involved in cell morphology, migration, invasion and angiogenesis. Moreover, I demonstrated that AC3 mediated $G\alpha_s$-$G\alpha_q$ cross-talk can activate a unique gene, SAT1, which is involved in cell migration and angiogenesis. Therefore the data suggest that in pathologies where both EP2 and FP receptor are elevated, co-activation of prostanoid receptors and alteration in integrative signalling may alter the pathophysiological outcome of the cell via a molecular switch which promotes the activation of a subset of genes uniquely different from that observed when the cell is presented with and activated by a single ligand only.

Chapter VII

General Discussion and Conclusions

7.1 General Discussion

Uterine fibroids are the most common tumors found in women in the reproductive age group. Despite their frequent occurrence, little attention has been given to the cause and pathology of uterine fibroids because of the rarity of their transformation to malignant tumors. Due to their local mass, uterine fibroids can cause excessive uterine bleeding, pregnancy related complications such as infertility and repetitive pregnancy loss (Flake *et al.,* 2003; Haney, 2000). Ovarian hormones (estrogen and progesterone) play a major role in early tumorigenesis of uterine fibroids by increasing mitotic rate and the possibility of somatic mutations while several growth factors have been implicated as effectors of uterine fibroids once they are formed (Rein, 2000; Flake *et al*., 2003).

There is mounting evidence to support a role for COX enzymes, prostaglandins and their cognate receptors in reproductive pathologies such as dysmenorrhoea, endometriosis, menorrhagia, cervical cancer and endometrial adenocarcinoma (Lumsden *et al*., 1983; Karck *et al*., 1996; Rees *et al.,* 1984; Smith *et al.,* 1981; Sales *et al*., 2002; Ryu *et al*., 2000; Tong *et al*., 2000; Jabbour *et al.,* 2006b). However, their role in uterine fibroids is not well studied.

In this study, expression of COX enzymes and prostaglandin receptors was assessed in fibroids and adjacent myometrium tissue samples from women with fibroids. The data obtained showed that expression of the EP3 receptor was down-regulated in fibroids compared to adjacent myometrium samples. These findings were in agreement with observations from gene array analyses on tissues from uterine fibroid samples in which the EP3 subtype receptor was found to be down-regulated in fibroids while all the other prostanoids and their cognate receptors were not altered compared to patient-matched myometrium samples (Tsibris *et al*., 2002; Wang *et al*., 2003; Quade *et al.,* 2003; Catherino *et al.,* 2004).

Down-regulation of the EP3 prostanoid receptor could play a key role in development of fibroids since the receptor has been shown to have a protective role in tumor growth. Over-expression and activation of the EP3 subtype receptors have been shown to decrease proliferation in human colon cancer cell lines (Shoji *et al.,* 2004). Another study has shown enhanced expression of aromatase in fibroids compared to adjacent myometrium samples suggesting a role for this enzyme and the estrogen synthesized *in situ* by the enzyme in tumorigenesis of uterine fibroids (Sumitani *et al.,* 2000). PGE_2 has been shown to increase aromatase activity in human breast stromal cells via the EP1 and EP2 receptors. However, co-treatment of cells with a selective EP3 agonist completely inhibited PGE_2-induced aromatase activity suggesting an inhibitory effect of the EP3 receptor pathway on aromatase activity (Richards and Brueggemeier, 2003). These data suggest that EP3 down-regulation in uterine fibroids might result in tumor growth of uterine fibroids possibly by dysregulation of the aromatase-estrogen pathway.

Despite several studies showing over-expression of prostaglandins and their cognate receptors in numerous tumors, in this current study expression of COX enzymes, EP1, EP2, EP4 and FP receptors were not significantly altered between fibroids and adjacent myometrium samples. These data led me to evaluate a possible paracrine regulation of prostaglandin receptors from tissues surrounding myometrium such as the endometrium on uterine fibroids. This was done by assessing the expression of COX enzymes, prostaglandin receptors and COX pathway-regulated genes in endometrium from women with and without uterine fibroids. Interestingly, up-regulation of COX-2, EP2 and FP receptors, VEGF, IL-8 and IL-11 were observed in endometrium samples from women with fibroids.

7.1.2 Roles for Epithelial Cell-Derived COX-2, EP2 and FP Receptors in Uterine Fibroids

The progression of every tumor needs activation of the so-called "angiogenic-switch". This molecular switch involves over-expression of pro-angiogenic factors or down-regulation of anti-angiogenic mediators. COX-2 is one of the main factors that regulate the angiogenic pathway suggesting that over-expression of COX-2 in endometrium from women with fibroids could serve as the "angiogenic-switch" for uterine fibroid progression (Chang *et al.*, 2004; Hanahan and Folkman, 1996). Over-expression of COX-2 in endometrium from patients with uterine fibroids can trigger up-regulation of prostaglandin synthesis mainly PGE_2 and PGF which act on EP2 and FP receptors in an autocrine manner. Indeed studies in our laboratory have shown up-regulation of the EP2 and FP receptors and their interactions with their respective ligands can lead to activation of angiogenic genes in endometrial adenocarcinoma cells (Sales *et al.*, 2004; Sales *et al.*, 2005). Elevation of prostaglandins and their cognate receptors might not be sufficient to have a direct influence on the regulation of uterine fibroids but can play major roles by inducing mitogenic and angiogenic genes such as VEGF, IL-8 and IL-11 to act in a paracrine manner on neighbouring myometrial/fibroid tissue.

7.1.3 Paracrine Role of VEGF, IL-8 and IL-11 in Uterine Fibroids Development

Once the "angiogenic-switch" is triggered by the up-regulation of COX-2 and prostaglandins the resulting angiogenesis is brought about by the action of several regulatory factors. VEGF is one of the main regulatory factors since it is a potent angiogenic and mitogenic growth factor. Charnock-Jones *et al.* (1993) showed VEGF expression in endometrial glandular epithelial and stromal cells with strong expression observed at the myometrium/endometrium boundary in the proliferative stage of menstrual cycle suggesting a possible paracrine role for VEGF in tumor development. Albrecht *et al.* (2003) showed that estrogen can up-regulate VEGF expression in cultured human endometrial glandular epithelial cells. In addition when these cells were co-

cultured with human myometrial microvascular endothelial cells (HMMECs), it can regulate HMMEC tube formation and thus angiogenesis in a paracrine manner.

IL-8, a chemoattractant chemokine, has been shown to induce proliferation, chemotaxis and angiogenesis in endothelial cells (Koch *et al.,* 1992). It also has been shown to stimulate migration and proliferation in vascular smooth muscle cells (Yue *et al.,* 1994). Senturk *et al.* (2001) showed the expression of IL-8 and its receptors to be higher in the myometrium adjacent to fibroid tissue compared with fibroid itself. This study was in agreement with the observation from Kumar *et al.* (1998) in experimental tumor animal models in which they showed the expression of IL-8 in tumors were restricted to the periphery of large tumors. This high level of IL-8 expression in the peripheral regions, where cells are actively dividing and are in contact with normal cells might explain the paracrine role of IL-8 in mitogenesis (Senturk *et al.*, 2001).

IL-11 is a hematopoietic growth factor and can affect cell growth, differentiation and proliferation (Du and Williams, 1994; Leng and Elias, 1997). Tang *et al.* (1996) used the CC10 promoter to over-express IL-11 in the murine airway and showed that IL-11 can induce airway remodelling with subepithelial fibrosis and local accumulation of fibroblasts, smooth muscle cells and myofibroblasts, suggesting that IL-11 is a fibrogenic cytokine. Similar studies by Zhu *et al.* (2001) also showed that mice over-expressing IL-11 had an enhancement in deposition of type I and type III collagen that caused airway fibrosis in these mice. Nakayama *et al.* (2007) showed a correlation between higher expression of IL-11 receptor and invasive grade tumors in places with venous invasion in gastric carcinomas. In vitro administration of recombinant human IL-11 to gastric cancer cells (MKN-28 and SCH) enhanced cell migration and invasion in these cells suggesting a role of this chemokine in promoting tumorigenesis.

Taken together, these data suggest that the process of tumorigenesis in uterine fibroids may be facilitated following over-expression of COX-2 in neighbouring epithelial cells of endometrium, which can act an autocrine manner to up-regulate PGE_2 and PGF.

PGE_2-EP2 and PGF-FP receptor interactions can in turn up-regulate expression of angiogenic and mitogenic factors such as VEGF, IL-8 and IL-11 that can act on paracrine manner on adjacent myometrium/fibroid tissue to initiate neovascularisation, angiogenesis, cell migration and proliferation and formation of fibrosis as depicted schematically in figure 7.1.

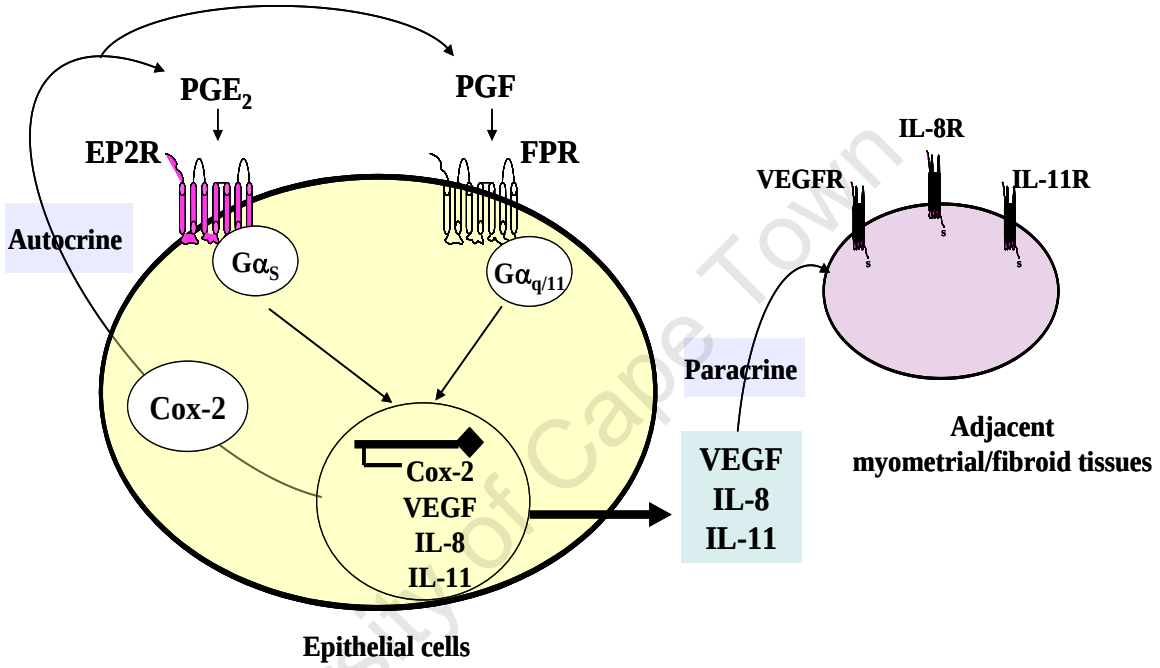

Fig. 7.1: *Autocrine/Paracrine regulation of COX-2 and prostaglandin receptors and their possible downstream effects on uterine fibroids development. PGE_2 and PGF synthesized by the COX-2 enzyme pathway activate their cognate receptors EP2 and FP receptors in epithelial cells from endometrium to initiate target gene expression such as COX-2, VEGF, IL-8 and IL-11 genes. COX-2 can in turn auto-regulate a positive feedback loop to activate prostaglandin signalling while VEGF, IL-8 and IL-11 can couple to their specific receptors on myometrial or fibroids tissue to promote angiogenesis, cell migration and tumour growth in a paracrine manner.*

7.1.4 EP2 and FP Receptors Cross-Talk and its Proposed Role in Uterine Pathology

Interestingly, EP2 and FP receptors up-regulation was also observed in the proliferative stage of endometrium samples from women with fibroids. The expression of these prostanoid receptors has also shown to be up-regulated in endometrial adenocarcinoma (Sales *et al.,* 2004; Sales *et al.,* 2005). EP2 and FP receptors are co-expressed in Ishikawa cells. This observation suggests that co-activation of these prostaglandin receptors in the same cell can lead to heterologous interactions that could alter the physiological/pathophysiological gene expression profile and outcome.

Heterologous interactions or cross-talks in the eicosanoid family have been shown in several studies. Walsh and Kinsella (2000) showed a cross-talk between thromboxane A_2 (TPα and TPβ) and EP1 receptors in HEK-293 cells that leads to desensitization and inhibition of signalling of the TP receptors in a PKC-dependent manner. The same group further indentified that the activation of the FP receptor by PGF can also mediate desensitization of the TPα and TPβ receptors via the PKC pathway (Kelley-Hickie and Kinsella, 2004). In another study, Wilson *et al.* (2004) showed that heterodimerization can occur between the human receptors for prostacyclin (IP) and TPα that leads to augmentation of TPα receptor-mediated accumulation of cAMP by IP receptor when they are co-expressed in HEK-293 cells.

Although EP2 and FP receptors are found to be co-expressed in numerous cell types such as Ishikawa cells, there have been no studies addressing the integrative signalling effects of these two receptors on signal transduction and gene expression (Sales *et al.,* 2004; Sales *et al.,* 2005). In order to investigate prostanoid integrative signalling, Ishikawa cells expressing both the EP2 and FP receptors (FPEP2 cell line) were successfully established. Expression level of the EP2 receptor in FPEP2 cells was compared to the parental FPS32 cells using Real-Time RT-PCR, Western blot analysis and immunofluorescence microscopy confirming stable expression of the EP2 receptor in FPEP2 cells localised to the perinuclear and plasma membrane.

Using the FPEP2 cells as a model system, I showed that PGF-stimulated IP3 production was not altered by co-stimulation with Butaprost suggesting that heterologous interaction between the two receptors does not have a synergistic effect in PLC-mediated IP3 release. However, co-stimulation of FPEP2 cells with Butaprost and PGF augmented Butaprost-mediated cAMP release despite PGF-mediated FP receptor activation on its own having no effect on cAMP accumulation. Using specific receptor antagonists and small molecule chemical inhibitors of cell signalling, I dissected the signalling pathways mediating the PGF-induced potentiation of Butaprost-stimulated cAMP in FPEP2 cells and have shown that it was mediated via the FP receptor- $G\alpha_q$-mediated activation of IP3 via PLC. Subsequently, IP3 could activate its receptors (IP3R) present on the endoplasmic reticulum membrane to promote intracellular calcium release and activation of calmodulin and CaMK-II as depicted schematically in figure 7.2.

Numerous studies have shown that the CaMK-II pathway can activate calcium sensitive AC isoforms to regulate intracellular cAMP accumulation (Zhang *et al*., 1997; Beazely and Watts, 2005; Ostrom *et al.*, 2003). For example, Ostrom *et al.* (2003) showed a potentiation of β-adrenergic (β-AR) receptor induced cAMP production by activation of angiotensin II (ANG-II) receptor coupled to $G\alpha_q$ protein through calcium/calmodulin pathway demonstrating cross-talk between ANG-II and β-AR receptors in cardiac fibroblasts cells. The authors also identified AC3 to be the only calcium-stimulated isoform in cardiac fibroblast cells suggesting that this isoform could be regulating the cross-talk reported. In FPEP2 Ishikawa cells, I identified AC1 and AC3 as calcium-regulated targets by RT-PCR analysis. Transfection studies using siRNA to abolish expression of AC1 or AC3 in FPEP2 cells revealed that the calcium-sensitive AC3 isoform is responsible for PGF-mediated augmentation of Butaprost-stimulated cAMP.

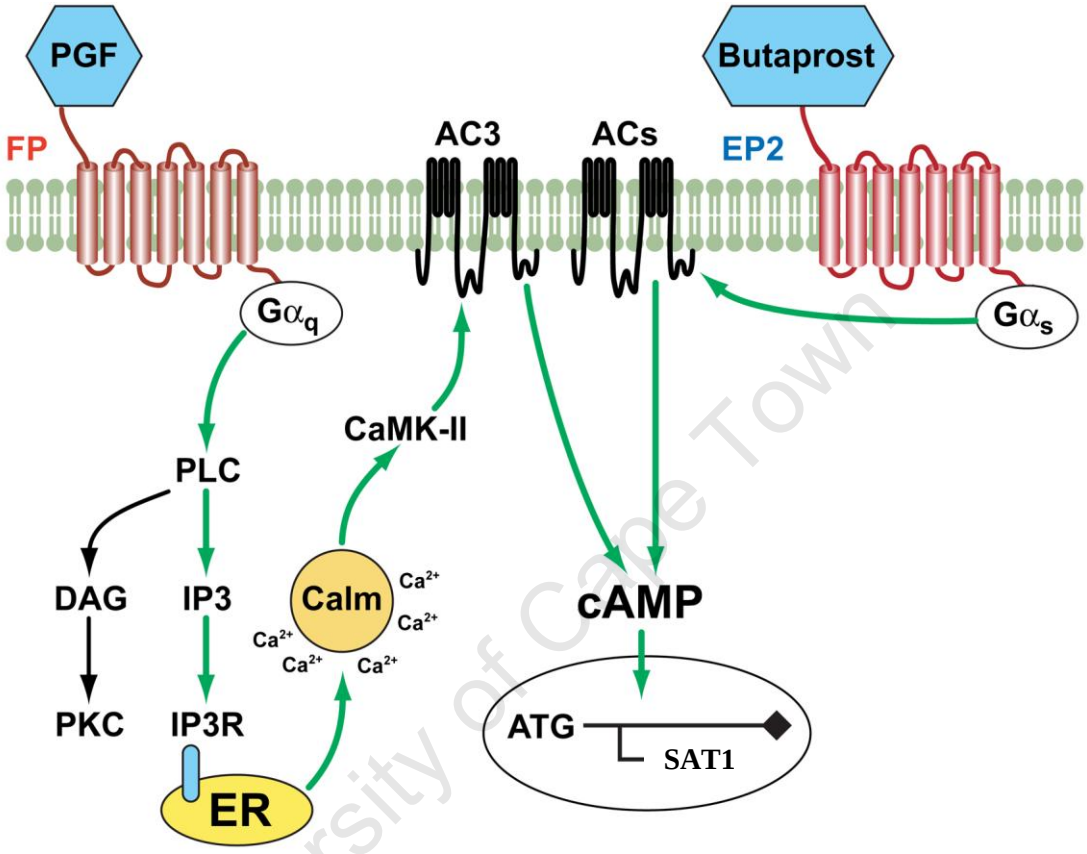

Fig. 7.2: $G\alpha_q$-mediated Potentiation of Butaprost induced cAMP release.

Butaprost activates $G\alpha_s$-coupled EP2 receptors resulting in a rapid increase in intracellular cAMP accumulation, while PGF by itself does not alter cAMP production. However, co-treatment of the cells with both ligands leads to the $G\alpha_q$-mediated activation of PLC and release of IP3 from the plasma membrane. Subsequently, IP3 via the IP3 receptor (IP3R) mediates the release of Ca^{2+} from endoplasmic reticulum (ER) leading to calmodulin-CaMK-II dependent potentiation of cAMP release via the calcium sensitive AC3 isoform and modulation of gene transcription such as SAT1.

In order to determine the integrative effects of the EP2 and FP receptors co-activation on gene expression, whole genome array profiling was performed in FPEP2 cells in response to Butaprost and/or PGF treatment. Out of 1045 genes that are regulated by PGF and/or Butaprost treatment, 228 genes are uniquely regulated by co-activation of the EP2 and FP receptors (Table 6.1 and 6.2). Gene ontology annotations revealed that the genes are involved in cell morphology, proliferation and differentiation. The list contained several genes with an important function in pathogenesis or control of cancer such as basonuclin-1, IL1RAP, CD2AP, 11βHSD-2, PTHLH, THSD1 and tumor protein p73.

Basonuclin-1, a cell specific transcription factor, was up-regulated by co-activation of the EP2 and FP receptors and it has been shown to play a role in cell proliferation and adhesion (Tseng, 1998; Wang *et al.*, 2004). IL1RAP, a receptor for a very essential inflammatory protein (IL-1), is another gene to be up-regulated by this unique pathway and over-expression of this gene has been associated with an invasive nature of endometrial cancer (Singer *et al.*, 2002). CD2AP, an adaptor protein for adhesion receptors and proto-oncogenes, was also up-regulated by co-activation of the EP2 and FP receptors and can affect cellular adhesion, motility and morphology (Kirsch *et al.*, 2001; Scaife and Langdon, 2000; Lehtonen *et al.*, 2002). 11βHSD-2 and PTHLH were also uniquely up-regulated in response to Butaprost and PGF and they have been shown to enhance angiogenesis by down-regulating anti-angiogenic factors and inhibit apoptosis and regulation of cell cycle, respectively (Rae *et al.*, 2009; Tovar Sepulveda *et al.*, 2002).

Down-regulation of two tumor suppressor genes by co-activation of the EP2 and FP receptors was also observed. THSD1 gene encodes for a transmembrane molecule containing a thrombospondin type I repeat and has anti-angiogenic activities (Ko *et al.*, 2008; de Fraipont *et al.*, 2001), while over-expression of tumor protein p73 can activate p53-mediated genes and acts as a growth suppressor (Jost *et al.*, 1997).

Taken together, these data demonstrate up-regulation of genes that are implicated in cell proliferation, cytoskeleton regulation and angiogenesis and down-regulation of genes that are involved in anti-angiogenic and apoptotic proteins by co-activation of the EP2 and FP receptors. This may exacerbate pathologies of endometrial adenocarcinoma.

Co-activation of FPEP2 Ishikawa cells with Butaprost and PGF enhanced or repressed a unique subset of Butaprost (EP2 receptor) regulated genes that are involved in cellular metabolism, immune response and excretion (Table 6.3). SAT1, an important enzyme in polyamine metabolism, was one of the genes identified and it adds an acetyl group to aminopropyl ends of spermidine and spermine that leads to lowering of intracellular polyamine pool (Casero and Pegg, 1993). The data obtained in this current study showed that SAT1 was regulated by the $G\alpha_s$-$G\alpha_q$ cross-talk between the EP2 and FP receptors via the calcium stimulated AC3 isoform.

Over-expression of this specific enzyme has been implicated in the pathology of intestinal tumors and in development of skin tumor (Pegg 2008; Tucker *et al.* 2005; Coleman *et al.*, 2002). SAT1 has also been shown to have a direct effect on cell migration by binding with α9β1-integrin (Chen *et al*., 2004). Moreover, in a recent study, Vlahakis *et al*. (2007) have demonstrated that α9β1-integrin can bind to VEGF and promote angiogenesis and lymphangiogenesis. These data suggest that SAT1 might play a role in the pathology of endometrium by directly promoting cell migration or by indirect enhancement of angiogenesis via α9B1-integrin.

Since EP2 and FP receptors are up-regulated in the proliferative phase of endometrium from women with uterine fibroids and SAT1 is regulated by the cross-talk between the EP2 and FP receptors in an endometrial epithelial cell line, I decided to assess SAT1 mRNA expression in endometrium from women with and without fibroids. Interestingly, Real-Time RT-PCR revealed that the level of SAT1 expression was significantly higher in the proliferative stage of endometrium from women with fibroids compared to control samples (3.22 ± 0.99 vs. 1.31 ± 0.19; $p<0.001$). This result validates

that the EP2 and FP receptors cross-talk reported in FPEP2 Ishikawa cells *in vitro* can also happen *in vivo* and can regulate downstream gene expression such as SAT1. It is not clear how SAT1 over-expression can cause tumor development. However, it is clear that up-regulation of SAT1 has a direct or indirect effect in development of tumors by promoting cell growth and migration. In the light of these studies, SAT1 produced by epithelial cells from endometrium might have a paracrine effect on myometrial/fibroids tissue by regulating polyamine metabolism resulting in tumor progression of uterine fibroids.

7.2 Conclusion

In conclusion, this study confirms the elevated expression of COX-2, EP2 and FP receptors, VEGF, IL-8 and IL-11 in endometrium from women with fibroids predominantly in the proliferative stage of the menstrual cycle. In addition, this study demonstrates that co-activation of the EP2 and FP receptors results in enhanced release of cAMP in a $G\alpha_q$-calcium-dependent manner in endometrial adenocarcinoma cells stably expressing the EP2 and FP receptors. Moreover, the $G\alpha_s$-$G\alpha_q$ cross-talk between the EP2 and FP receptors was mediated via the calcium-sensitive AC3 isoform and modulates expression of genes involved in regulating cell morphology, proliferation, differentiation immune response and excretion.

Taken together, these data suggest that in pathologies where both EP2 and FP receptors are elevated, such as in endometrial adenocarcinomas and endometrium from women with uterine fibroids, co-activation of these prostanoid receptors can lead to integrative signalling pathway that may change the pathophysiological outcome of the cell via a molecular switch which either alters the trans-activation of a subset of single receptor-induced genes or regulates unique genes that are involved in tumor development.

7.3 Future Studies

The whole genome profiling done on FPEP2 cells in response to Butaprost and PGF revealed interesting lists of genes that are uniquely regulated by either the EP2 or FP receptor activation. These genes will be further analyzed in our laboratory and studies on PGF-FP receptor regulated genes that are involved in endometrial pathologies are already underway.

In this current project, I have tried to study the role for SAT1 in proliferation and migration of Ishikawa cells by employing siRNA knockdown studies. These studies were not successful because knockdown studies using siRNA are transient. The role for SAT1 in endometrial pathology could be an interesting aspect to look at by employing MicroRNA (miRNA) stable knockdown techniques in FPEP2 Ishikawa cells.

In addition, studies in our laboratory are underway to determine the role for FP receptor over-expressing cell line in tumor growth *in vivo*. Another interesting aspect for a future study will be to assess the biological function of the EP2 and FP receptors in various features of endometrial cancer progression such as tumour growth, invasion, angiogenesis and metastasis by employing *in vivo* studies.

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Appendix A: List of genes differentially expressed by PGF and/or Butaprost treatment. Fold increases are above/below vehicle treated and #N/A are treatments that did not show significance difference.

Gene Symbol	Gene Name	Fold Change		
		PGF	Butaprost	PGF & Butaprost
A1BG	alpha-1-B glycoprotein	0.347	#N/A	#N/A
AADACL1	arylacetamide deacetylase-like 1	4.898	#N/A	4.162
AADAT	aminoadipate aminotransferase	0.214	0.181	0.192
ABAT	4-aminobutyrate aminotransferase	0.267	#N/A	#N/A
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	#N/A	#N/A	0.323
ABCC13	ATP-binding cassette, sub-family C (CFTR/MRP), member 13	0.327	#N/A	#N/A
ABCG8	ATP-binding cassette, sub-family G (WHITE), member 8 (sterolin 2)	0.283	#N/A	0.283
ABHD2	chromosome 11 open reading frame2|abhydrolase domain containing 2	3.667	#N/A	4.451
ACBD4	acyl-Coenzyme A binding domain containing 4	#N/A	#N/A	0.332
ACOT11	acyl-CoA thioesterase 11	#N/A	0.307	#N/A
ACTG2	actin, gamma 2, smooth muscle, enteric	7.663	4.859	7.625
ACTR2	ARP2 actin-related protein 2 homolog (yeast)	0.342	#N/A	#N/A
ACYP2	acylphosphatase 2, muscle type	0.295	#N/A	0.281
ADA	adenosine deaminase	#N/A	3.154	2.867
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	12.529	10.563	16.481
ADAMTS10	ADAM metallopeptidase with thrombospondin type 1 motif, 10	0.341	#N/A	#N/A
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	0.263	#N/A	#N/A
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)	3.622	4.033	3.769
ADM	adrenomedullin	29.454	11.512	63.131
ADORA1	adenosine A1 receptor	0.301	#N/A	0.155
ADORA2B	adenosine A2b receptor	3.100	#N/A	#N/A
ADRB2	adrenergic, beta-2-, receptor, surface	5.023	#N/A	5.602
AGPS	alkylglycerone phosphate synthase	3.283	2.834	4.630
AGRN	agrin	0.125	#N/A	#N/A
AGRP	agouti related protein homolog (mouse)	0.212	#N/A	#N/A

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
AKAP12	A kinase (PRKA) anchor protein (gravin) 12	10.525	5.446	8.782
AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	#N/A	0.334	#N/A
ALDH3A2	aldehyde dehydrogenase 3 family, member A2	0.278	#N/A	0.272
ALDH3B2	aldehyde dehydrogenase 3 family, member B2	#N/A	0.326	0.293
ALOXE3	arachidonate lipoxygenase 3	5.379	3.065	#N/A
ALPK1	alpha-kinase 1	0.144	0.272	0.131
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	46.341	5.878	40.996
ANKRD19	ankyrin repeat domain 19	0.347	#N/A	#N/A
ANKRD22	ankyrin repeat domain 22	#N/A	0.305	#N/A
ANKRD25	ankyrin repeat domain 25	0.156	0.258	0.166
ANKRD34	ankyrin repeat domain 34	0.197	#N/A	#N/A
ANKRD5	ankyrin repeat domain 5	0.317	#N/A	#N/A
APOBEC3A	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	#N/A	#N/A	0.306
APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	#N/A	0.318	#N/A
APOL2	apolipoprotein L, 2	#N/A	#N/A	0.310
AQP3	aquaporin 3	#N/A	3.025	3.567
AREG	amphiregulin (schwannoma-derived growth factor)	93.528	39.034	175.938
ARG2	arginase, type II	4.731	5.432	5.209
ARHGAP21	Rho GTPase activating protein 21	#N/A	#N/A	2.855
ARHGAP29	Rho GTPase activating protein 29	3.826	#N/A	4.344
ARHGEF15	Rho guanine nucleotide exchange factor (GEF) 15	0.228	0.321	0.296
ARHGEF2	rho/rac guanine nucleotide exchange factor (GEF) 2	3.059	#N/A	3.250
ARID3A	AT rich interactive domain 3A (BRIGHT- like)	#N/A	#N/A	0.248
ARID5B	AT rich interactive domain 5B (MRF1-like)	#N/A	#N/A	2.878
ARMC3	armadillo repeat containing 3	0.337	#N/A	#N/A
ARNTL	aryl hydrocarbon receptor nuclear translocator-like	2.920	#N/A	4.299
ARNTL	aryl hydrocarbon receptor nuclear translocator-like	2.920	#N/A	4.299
ARRDC3	arrestin domain containing 3	#N/A	0.245	#N/A
ARSJ	arylsulfatase family, member J	2.898	#N/A	3.433

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
ASB2	ankyrin repeat and SOCS box-containing 2	#N/A	#N/A	3.259
ASCC3	activating signal cointegrator 1 complex subunit 3	#N/A	#N/A	5.130
ATAD4	ATPase family, AAA domain containing 4	0.091	0.082	0.106
ATCAY	ataxia, cerebellar, Cayman type (caytaxin)	#N/A	#N/A	0.295
ATF3	activating transcription factor 3	14.512	8.033	10.936
ATOH7	atonal homolog 7 (Drosophila)	#N/A	#N/A	0.231
ATP1A2	ATPase, Na+/K+ transporting, alpha 2 (+) polypeptide	0.222	0.308	0.287
ATP1B3	ATPase, Na+/K+ transporting, beta 3 polypeptide	#N/A	3.188	4.284
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	0.331	#N/A	#N/A
ATP6V1C2	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C isoform 2	5.384	4.085	5.962
ATP6V1G2	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G isoform 2	0.261	#N/A	#N/A
AVPI1	arginine vasopressin-induced 1	3.492	#N/A	#N/A
AXL	AXL receptor tyrosine kinase	3.690	#N/A	#N/A
AXUD1	AXIN1 up-regulated 1	5.505	#N/A	5.351
BAG1	BCL2-associated athanogene	3.111	4.693	4.245
BAIAP2	BAI1-associated protein 2	#N/A	#N/A	0.244
BASP1	brain abundant, membrane attached signal protein 1	0.317	#N/A	0.285
BAZ2B	bromodomain adjacent to zinc finger domain, 2B	#N/A	0.292	#N/A
BCAR3	breast cancer anti-estrogen resistance 3	3.033	#N/A	3.829
BCL10	B-cell CLL/lymphoma 10	#N/A	#N/A	3.031
BCMP11	anterior gradient homolog 3 (Xenopus laevis)	0.200	0.330	0.228
BDNF	brain-derived neurotrophic factor	32.281	13.650	27.802
BHLHB2	basic helix-loop-helix domain containing, class B, 2	5.699	3.707	4.562
BIK	BCL2-interacting killer (apoptosis-inducing)	3.225	#N/A	#N/A
BMP2K	BMP2 inducible kinase	3.051	#N/A	#N/A
BMP4	bone morphogenetic protein 4	3.926	5.094	4.870
BNC1	basonuclin 1	#N/A	#N/A	3.719
BOC	Boc homolog (mouse)	0.232	#N/A	#N/A

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
BOMB	WW and C2 domain containing 2	#N/A	2.954	#N/A
BTBD3	BTB (POZ) domain containing 3	#N/A	3.304	3.018
BXDC2	brix domain containing 2	#N/A	#N/A	2.993
C10orf137	chromosome 10 open reading frame 137	3.137	#N/A	#N/A
C10orf33	chromosome 10 open reading frame 33	#N/A	#N/A	0.253
C10orf47	chromosome 10 open reading frame 47	#N/A	#N/A	2.955
C10orf48	chromosome 10 open reading frame 48	4.374	3.456	4.498
C10orf63	chromosome 10 open reading frame 63	0.223	#N/A	0.315
C10orf79	chromosome 10 open reading frame 79	0.191	0.290	0.316
C10orf91	chromosome 10 open reading frame 91	#N/A	0.272	0.135
C11orf16	chromosome 11 open reading frame 16	#N/A	#N/A	0.276
C11orf2|ABH	chromosome 11 open reading	3.667	#N/A	4.451
D2	frame2|abhydrolase domain containing 2			
C14orf113	chromosome 14 open reading frame 113	0.293	0.298	#N/A
C14orf133	chromosome 14 open reading frame 133	#N/A	#N/A	0.299
C14orf139	chromosome 14 open reading frame 139	#N/A	0.200	#N/A
C14orf140	chromosome 14 open reading frame 140	0.182	#N/A	0.267
C14orf148	chromosome 14 open reading frame 148	0.337	#N/A	#N/A
C14orf28	chromosome 14 open reading frame 28	#N/A	#N/A	4.585
C14orf39	chromosome 14 open reading frame 39	0.248	#N/A	#N/A
C14orf58	chromosome 14 open reading frame 58	0.353	#N/A	#N/A
C14orf78	chromosome 14 open reading frame 78	#N/A	#N/A	0.326
C14orf93	chromosome 14 open reading frame 93	0.307	#N/A	0.324
C15orf26	chromosome 15 open reading frame 26	0.322	#N/A	#N/A
C16orf52	chromosome 16 open reading frame 52	2.870	#N/A	3.161
C17orf39	chromosome 17 open reading frame 39	#N/A	#N/A	4.203
C17orf62	chromosome 17 open reading frame 62	#N/A	#N/A	0.352
C18orf25	chromosome 18 open reading frame 25	4.258	#N/A	3.982
C18orf56	chromosome 18 open reading frame 56	0.336	#N/A	#N/A
C19orf9	chromosome 19 open reading frame 9	0.175	0.313	#N/A
C1orf101	chromosome 1 open reading frame 101	0.248	#N/A	#N/A
C1orf102	chromosome 1 open reading frame 102	0.229	#N/A	#N/A
C1orf105	chromosome 1 open reading frame 105	0.342	#N/A	#N/A
C1orf107	chromosome 1 open reading frame 107	#N/A	#N/A	3.518
C1orf116	chromosome 1 open reading frame 116	10.656	3.501	7.275
C1orf145	chromosome 1 open reading frame 145	#N/A	#N/A	0.233
C1orf168	chromosome 1 open reading frame 168	0.127	0.236	0.314

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
C1orf178	chromosome 1 open reading frame 178	0.200	#N/A	#N/A
C1orf26	chromosome 1 open reading frame 26	0.180	#N/A	#N/A
C1orf27	chromosome 1 open reading frame 27	#N/A	#N/A	2.946
C1orf38	chromosome 1 open reading frame 38	0.350	#N/A	#N/A
C20orf12	chromosome 20 open reading frame 12	0.335	#N/A	#N/A
C20orf175	chromosome 20 open reading frame 175	#N/A	#N/A	0.335
C20orf26	chromosome 20 open reading frame 26	0.332	#N/A	#N/A
C20orf59	chromosome 20 open reading frame 59	#N/A	#N/A	0.010
C20orf94	chromosome 20 open reading frame 94	0.325	#N/A	#N/A
C21orf63	chromosome 21 open reading frame 63	3.186	3.574	4.307
C21orf87	chromosome 21 open reading frame 87	#N/A	#N/A	0.338
C21orf91	chromosome 21 open reading frame 91	#N/A	#N/A	2.839
C21orf99	chromosome 21 open reading frame 99	0.336	#N/A	#N/A
C22orf15	chromosome 22 open reading frame 15	0.304	#N/A	#N/A
C2orf21	chromosome 2 open reading frame 21	#N/A	#N/A	0.240
C3orf18	chromosome 3 open reading frame 18	#N/A	#N/A	0.140
C3orf23	chromosome 3 open reading frame 23	#N/A	#N/A	137.676
C3orf26	chromosome 3 open reading frame 26	#N/A	#N/A	2.846
C3orf32	chromosome 3 open reading frame 32	3.049	3.228	3.070
C5R1	complement component 5 receptor 1 (C5a ligand)	0.209	0.258	0.255
C6orf114	chromosome 6 open reading frame 114	3.014	#N/A	3.021
C6orf117	chromosome 6 open reading frame 117	0.314	#N/A	#N/A
C6orf157	chromosome 6 open reading frame 157	3.302	3.618	4.865
C6orf165	chromosome 6 open reading frame 165	0.280	#N/A	#N/A
C6orf203	chromosome 6 open reading frame 203	0.345	#N/A	#N/A
C6orf75	chromosome 6 open reading frame 75	#N/A	#N/A	3.464
C6orf85	chromosome 6 open reading frame 85	0.333	#N/A	#N/A
C7orf31	chromosome 7 open reading frame 31	#N/A	#N/A	0.336
C8orf22	chromosome 8 open reading frame 22	0.245	#N/A	#N/A
C8orf45	chromosome 8 open reading frame 45	#N/A	#N/A	0.245
C8orf48	chromosome 8 open reading frame 48	#N/A	0.230	#N/A
C8orf51	chromosome 8 open reading frame 51	#N/A	0.320	0.332
C9orf130	chromosome 9 open reading frame 130	#N/A	0.350	#N/A
C9orf150	chromosome 9 open reading frame 150	#N/A	#N/A	0.230
C9orf72	chromosome 9 open reading frame 72	#N/A	#N/A	3.103
C9orf98	chromosome 9 open reading frame 98	#N/A	#N/A	0.251

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
CA1	carbonic anhydrase I	#N/A	#N/A	0.239
CABLES1	Cdk5 and Abl enzyme substrate 1	4.341	3.702	4.962
CACNG5	calcium channel, voltage-dependent, gamma subunit 5	0.319	0.328	0.197
CALB1	calbindin 1, 28kDa	3.211	3.182	3.966
CALCOCO1	calcium binding and coiled-coil domain 1	0.271	#N/A	#N/A
CALCRL	calcitonin receptor-like	#N/A	0.321	#N/A
CALD1	caldesmon 1	4.948	#N/A	4.933
CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1	#N/A	#N/A	3.154
CAMKK1	calcium/calmodulin-dependent protein kinase kinase 1, alpha	0.274	0.324	#N/A
CAMSAP1L1	calmodulin regulated spectrin-associated protein 1-like 1	#N/A	#N/A	4.318
CAPSL	calcyphosine-like	0.204	#N/A	#N/A
CARD15	caspase recruitment domain family, member 15	0.283	0.261	#N/A
CARD9	caspase recruitment domain family, member 9	0.345	#N/A	#N/A
CASP6	caspase 6, apoptosis-related cysteine peptidase	0.346	0.340	0.289
CASP8	caspase 8, apoptosis-related cysteine peptidase	#N/A	#N/A	0.346
CCDC11	coiled-coil domain containing 11	0.300	#N/A	#N/A
CCDC15	coiled-coil domain containing 15	0.235	#N/A	0.276
CCDC19	coiled-coil domain containing 19	0.250	#N/A	0.218
CCDC41	coiled-coil domain containing 41	#N/A	#N/A	2.915
CCK	cholecystokinin	3.292	#N/A	2.927
CCL20	chemokine (C-C motif) ligand 20	134.923	175.585	358.544
CCL23	chemokine (C-C motif) ligand 23	0.342	0.325	#N/A
CCL3L1|CCL3L3	chemokine (C-C motif) ligand 3-like 1|chemokine (C-C motif) ligand 3-like 3	#N/A	#N/A	0.343
CD2AP	CD2-associated protein	#N/A	#N/A	3.081
CD3EAP	CD3E antigen, epsilon polypeptide associated protein	#N/A	#N/A	3.045
CD44	CD44 antigen (homing function and Indian blood group system)	3.437	#N/A	3.637
CDC42EP2	CDC42 effector protein (Rho GTPase binding) 2	#N/A	3.020	4.157
CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3	4.786	#N/A	3.207

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
CDK2AP2	CDK2-associated protein 2	3.423	4.350	3.316
CDKN2C	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	#N/A	#N/A	0.291
CDR2	cerebellar degeneration-related protein 2, 62kDa	3.234	#N/A	#N/A
CDX1	caudal type homeobox transcription factor 1	#N/A	0.333	#N/A
CEBPB	CCAAT/enhancer binding protein (C/EBP), β	3.162	#N/A	#N/A
CEP1	centrosomal protein 1	0.184	#N/A	#N/A
CES7	carboxylesterase 7	3.491	3.494	#N/A
CETN1	centrin, EF-hand protein, 1	0.221	#N/A	0.238
CFHL4	complement factor H-related 4	0.278	#N/A	0.321
CGA	glycoprotein hormones, alpha polypeptide	76.564	149.509	222.226
CGN	cingulin	0.296	0.283	0.259
CHAC1	ChaC, cation transport regulator-like 1 (E. coli)	2.987	#N/A	#N/A
CHML	choroideremia-like (Rab escort protein 2)	0.318	#N/A	#N/A
CHMP1B	chromatin modifying protein 1B	3.987	4.940	5.226
CHRM3	cholinergic receptor, muscarinic 3	0.337	#N/A	#N/A
CITED1	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1	3.556	3.052	3.712
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	12.750	5.698	10.603
CKLF	chemokine-like factor	0.304	#N/A	#N/A
CLASP1	cytoplasmic linker associated protein 1	#N/A	0.323	0.337
CLCF1	cardiotrophin-like cytokine factor 1	59.310	20.474	48.934
CLCN4	chloride channel 4	0.301	#N/A	0.288
CLDN1	claudin 1	5.709	3.546	4.540
CLDN4	claudin 4	0.214	0.095	0.176
CLEC2L	C-type lectin domain family 2, member L	0.305	#N/A	#N/A
CLIC5	chloride intracellular channel 5	9.904	6.537	5.846
CLIPR-59	CAP-GLY domain containing linker protein 3	0.241	#N/A	0.234
CLK1	CDC-like kinase 1	#N/A	#N/A	2.846
CMIP	c-Maf-inducing protein	4.705	3.660	4.124
CMTM1	CKLF-like MARVEL transmembrane domain containing 1	0.325	#N/A	#N/A
CNAP1	non-SMC condensin I complex, subunit D2	#N/A	#N/A	0.316
CNFN	cornifelin	0.230	0.265	#N/A
CNN1	calponin 1, basic, smooth muscle	5.449	#N/A	#N/A

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
COL16A1	collagen, type XVI, alpha 1	0.328	#N/A	7.398
COL9A1	collagen, type IX, alpha 1	0.239	#N/A	#N/A
CORO1C	coronin, actin binding protein, 1C	4.348	#N/A	3.228
CPN1	carboxypeptidase N, polypeptide 1, 50kD	#N/A	#N/A	0.275
CPOX	coproporphyrinogen oxidase	#N/A	#N/A	3.249
CREM	cAMP responsive element modulator	3.661	6.572	8.599
CRISP1	cysteine-rich secretory protein 1	#N/A	#N/A	0.346
CRISPLD1	cysteine-rich secretory protein LCCL domain containing 1	3.101	#N/A	#N/A
CRMP1	collapsin response mediator protein 1	0.333	#N/A	0.308
CRYAA	crystallin, alpha A	#N/A	3.468	#N/A
CRYAB	crystallin, alpha B	3.631	10.143	9.950
CSMD2	CUB and Sushi multiple domains 2	0.249	#N/A	#N/A
CSRP1	cysteine and glycine-rich protein 1	3.069	#N/A	#N/A
CTAG2	cancer/testis antigen 2	0.160	#N/A	#N/A
CTGF	connective tissue growth factor	184.520	30.535	80.159
CTSG	cathepsin G	0.174	0.249	0.227
CTSO	cathepsin O	0.347	#N/A	0.280
CUGBP1	CUG triplet repeat, RNA binding protein 1	#N/A	#N/A	9.686
CX3CL1	chemokine (C-X3-C motif) ligand 1	0.228	#N/A	#N/A
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	20.981	9.825	19.383
CXCL3	chemokine (C-X-C motif) ligand 3	34.744	16.627	43.660
CXCR4	chemokine (C-X-C motif) receptor 4	9.397	10.939	11.964
CXorf23	chromosome X open reading frame 23	0.286	#N/A	#N/A
CXorf40B	chromosome X open reading frame 40B	3.142	2.865	3.181
CXXC5	CXXC finger 5	0.274	#N/A	0.251
CYB561D1	cytochrome b-561 domain containing 1	#N/A	#N/A	0.341
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	0.141	0.154	0.161
CYP26A1	cytochrome P450, family 26, subfamily A, polypeptide 1	#N/A	18.032	12.278
CYP2C8	cytochrome P450, family 2, subfamily C, polypeptide 8	#N/A	#N/A	0.345
CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1	0.326	#N/A	#N/A
CYR61	cysteine-rich, angiogenic inducer, 61	67.001	24.867	53.348

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
DCBLD2	discoidin, CUB and LCCL domain containing 2	3.151	#N/A	2.860
DDIT3	DNA-damage-inducible transcript 3	5.546	#N/A	#N/A
DDIT4	DNA-damage-inducible transcript 4	#N/A	0.224	0.260
DEFB1	defensin, beta 1	#N/A	0.323	0.287
DEFB119	defensin, beta 119	#N/A	#N/A	0.052
DEFB126	defensin, beta 126	#N/A	0.249	#N/A
DEPDC4	DEP domain containing 4	#N/A	#N/A	0.313
DEPDC6	DEP domain containing 6	0.215	0.325	0.235
DGCR5	DiGeorge syndrome critical region gene 5 (non-coding)	0.309	#N/A	#N/A
DHRS3	dehydrogenase/reductase (SDR family) member 3	0.323	#N/A	#N/A
DIRAS3	DIRAS family, GTP-binding RAS-like 3	#N/A	3.234	#N/A
DIRC2	disrupted in renal carcinoma 2	6.534	3.927	4.619
DKK1	dickkopf homolog 1 (Xenopus laevis)	333.177	299.203	639.096
DLC1	deleted in liver cancer 1	5.875	4.368	6.444
DMN	desmuslin	#N/A	0.342	0.261
DNAH3	dynein, axonemal, heavy polypeptide 3	#N/A	#N/A	0.339
DNAI1	dynein, axonemal, intermediate polypeptide 1	0.327	#N/A	0.331
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	3.954	3.745	4.353
DNAL4	dynein, axonemal, light polypeptide 4	0.346	#N/A	#N/A
DNM1	dynamin 1	0.294	#N/A	0.280
DOCK8	dedicator of cytokinesis 8	#N/A	0.339	#N/A
DOK2	docking protein 2, 56kDa	0.252	#N/A	#N/A
DPCR1	diffuse panbronchiolitis critical region 1	0.324	#N/A	0.327
DRG2	developmentally regulated GTP binding protein 2	0.312	#N/A	#N/A
DSCR8	Down syndrome critical region gene 8	0.217	#N/A	#N/A
DUSP1	dual specificity phosphatase 1	56.217	15.377	40.019
DUSP14	dual specificity phosphatase 14	3.350	#N/A	3.273
DUSP19	dual specificity phosphatase 19	0.253	#N/A	0.288
DUSP26	dual specificity phosphatase 26 (putative)	#N/A	#N/A	0.333
DUSP4	dual specificity phosphatase 4	45.065	39.449	58.084
DUSP5	dual specificity phosphatase 5	67.080	23.248	52.013
DUSP6	dual specificity phosphatase 6	4.173	5.130	3.603
DUSP7	dual specificity phosphatase 7	3.024	#N/A	#N/A

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
DYDC1	DPY30 domain containing 1	0.307	#N/A	#N/A
DZIP3	DAZ interacting protein 3, zinc finger	0.344	0.342	#N/A
EBF	early B-cell factor	0.306	#N/A	0.232
EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	3.502	4.174	3.822
EDG5	endothelial differentiation, sphingolipid G-protein-coupled receptor, 5	0.273	#N/A	#N/A
EDN1	endothelin 1	7.331	5.579	25.355
EDN2	endothelin 2	278.419	66.082	280.207
EDN3	endothelin 3	0.327	#N/A	#N/A
EFHB	EF-hand domain family, member B	0.314	0.216	0.284
EFHC2	EF-hand domain (C-terminal) containing 2	0.317	#N/A	#N/A
EGFL6	EGF-like-domain, multiple 6	0.170	0.221	0.326
EGR1	early growth response 1	9.123	#N/A	9.378
EHHADH	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	#N/A	#N/A	0.348
EIF1	eukaryotic translation initiation factor 1	2.998	#N/A	3.055
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	4.435	#N/A	#N/A
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	0.312	0.211	0.194
ELK4	ELK4, ETS-domain protein (SRF accessory protein 1)	#N/A	#N/A	3.525
ELN	elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)	0.287	#N/A	#N/A
ELSPBP1	epididymal sperm binding protein 1	0.325	#N/A	0.297
EME1	essential meiotic endonuclease 1 homolog 1 (S. pombe)	#N/A	#N/A	0.306
EMILIN1	elastin microfibril interfacer 1	0.308	#N/A	0.283
ENC1	ectodermal-neural cortex (with BTB-like domain)	3.770	#N/A	4.149
ENPP3	ectonucleotide pyrophosphatase/phosphodiesterase 3	0.353	#N/A	#N/A
EPHA2	EPH receptor A2	6.052	3.682	6.728
EPN3	epsin 3	0.278	#N/A	#N/A
EPPK1	epiplakin 1	4.886	#N/A	3.290

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ERRFI1	ERBB receptor feedback inhibitor 1	18.905	8.985	20.883
ESR1	estrogen receptor 1	0.191	0.337	0.228
ETV5	ets variant gene 5 (ets-related molecule)	3.739	#N/A	#N/A
EVC	Ellis van Creveld syndrome	0.323	#N/A	0.344
EXT1	exostoses (multiple) 1	5.025	#N/A	#N/A
EXTL1	exostoses (multiple)-like 1	#N/A	#N/A	0.339
F2RL1	coagulation factor II (thrombin) receptor-like 1	2.994	#N/A	3.368
F3	coagulation factor III (thromboplastin, tissue factor)	15.651	12.660	31.494
FABP5	fatty acid binding protein 5 (psoriasis-associated)	4.009	4.423	6.069
FABP5L3	fatty acid binding protein 5-like 3	3.170	3.495	4.462
FAM100A	family with sequence similarity 100, member A	#N/A	2.868	3.553
FAM102A	family with sequence similarity 102, member A	5.214	#N/A	3.999
FAM102B	family with sequence similarity 102, member B	0.237	#N/A	#N/A
FAM27L	family with sequence similarity 27-like	0.295	#N/A	#N/A
FAM40B	family with sequence similarity 40, member B	0.280	0.337	#N/A
FAM48A	family with sequence similarity 48, member A	0.288	0.341	#N/A
FAM60A	family with sequence similarity 60, member A	3.971	3.128	3.759
FAM62C	family with sequence similarity 62 (C2 domain containing), member C	0.330	#N/A	#N/A
FAM79B	family with sequence similarity 79, member B	3.906	#N/A	5.068
FAM81B	family with sequence similarity 81, member B	0.291	#N/A	#N/A
FAM92A3	family with sequence similarity 92, member A3	0.149	0.327	#N/A
FANCB	Fanconi anemia, complementation group B	0.200	#N/A	#N/A
FANK1	fibronectin type III and ankyrin repeat domains 1	0.303	#N/A	#N/A
FAS	Fas (TNF receptor superfamily, member 6)	2.990	#N/A	4.389
FAT	FAT tumor suppressor homolog 1 (Drosophila)	#N/A	#N/A	3.035
FAT2	FAT tumor suppressor homolog 2 (Drosophila)	0.248	0.294	0.194
FBP2	fructose-1,6-bisphosphatase 2	#N/A	0.285	#N/A
FERD3L	Fer3-like (Drosophila)	#N/A	#N/A	0.286
FGD5	FYVE, RhoGEF and PH domain containing 5	0.272	#N/A	#N/A
FGF2	fibroblast growth factor 2 (basic)	6.148	#N/A	7.736
FGF6	fibroblast growth factor 6	#N/A	#N/A	0.326
FGFBP1	fibroblast growth factor binding protein 1	2.850	4.168	4.955
FHL2	four and a half LIM domains 2	8.991	3.598	7.938

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		PGF	**Butaprost**	**PGF & Butaprost**
FLNC	filamin C, gamma (actin binding protein 280)	2.973	#N/A	#N/A
FMO5	flavin containing monooxygenase 5	0.239	#N/A	0.316
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	5.344	4.606	5.926
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	#N/A	#N/A	3.036
FOXA1	forkhead box A1	4.333	4.093	5.096
FOXB1	forkhead box B1	#N/A	#N/A	0.214
FOXI1	forkhead box I1	0.183	0.319	0.347
FOXJ1	forkhead box J1	0.163	#N/A	0.234
FOXN1	forkhead box N1	0.351	#N/A	#N/A
FRAS1	Fraser syndrome 1	#N/A	3.461	6.408
FXR2	fragile X mental retardation, autosomal homolog 2	3.030	#N/A	3.081
FXYD2	FXYD domain containing ion transport regulator 2	#N/A	#N/A	0.318
G1P2	interferon, alpha-inducible protein (clone IFI-15K)	3.111	#N/A	3.384
GAD2	glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa)	0.332	#N/A	#N/A
GADD45A	growth arrest and DNA-damage-inducible, alpha	2.844	2.902	5.504
GADD45B	growth arrest and DNA-damage-inducible, beta	#N/A	#N/A	2.909
GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	#N/A	#N/A	3.267
GALNT8	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 8 (GalNAc-T8)	0.322	#N/A	#N/A
GAN	giant axonal neuropathy (gigaxonin)	#N/A	#N/A	3.058
GBE1	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)	4.249	#N/A	4.275
GBX2	gastrulation brain homeobox 2	0.332	#N/A	#N/A
GDF15	growth differentiation factor 15	3.652	#N/A	#N/A

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		PGF	**Butaprost**	**PGF & Butaprost**
GEM	GTP binding protein overexpressed in skeletal muscle	9.015	4.340	13.802
GIT2	G protein-coupled receptor kinase interactor 2	0.286	#N/A	#N/A
GJA1	gap junction protein, alpha 1, 43kDa (connexin 43)	0.309	#N/A	#N/A
GJA7	gap junction protein, alpha 7, 45kDa (connexin 45)	2.992	#N/A	4.180
GJB6	gap junction protein, beta 6 (connexin 30)	5.251	#N/A	5.469
GJE1	gap junction protein, epsilon 1, 29kDa	#N/A	#N/A	0.190
GLI1	glioma-associated oncogene homolog 1 (zinc finger protein)	0.145	#N/A	#N/A
GLIPR1	GLI pathogenesis-related 1 (glioma)	13.521	4.862	13.309
GLIS1	GLIS family zinc finger 1	0.346	#N/A	#N/A
GLIS2	GLIS family zinc finger 2	#N/A	#N/A	0.008
GLT28D1	glycosyltransferase 28 domain containing 1	#N/A	#N/A	2.890
GML	GPI anchored molecule like protein	#N/A	#N/A	0.304
GOLGA4	golgi autoantigen, golgin subfamily a, 4	#N/A	#N/A	3.550
GON4	gon-4 homolog (C.elegans)	#N/A	0.241	#N/A
GPBP1L1	GC-rich promoter binding protein 1-like 1	0.315	#N/A	#N/A
GPR126	G protein-coupled receptor 126	3.541	#N/A	3.343
GPR162	G protein-coupled receptor 162	0.296	#N/A	0.264
GPR19	G protein-coupled receptor 19	0.328	#N/A	#N/A
GPR68	G protein-coupled receptor 68	#N/A	#N/A	0.268
GPRC5A	G protein-coupled receptor, family C, group 5, member A	26.893	17.643	22.859
GPRC5D	G protein-coupled receptor, family C, group 5, member D	0.241	#N/A	0.331
GPX2	glutathione peroxidase 2 (gastrointestinal)	0.299	#N/A	#N/A
GRAMD2	GRAM domain containing 2	0.179	#N/A	#N/A
GRB7	growth factor receptor-bound protein 7	0.285	#N/A	#N/A
GRHL3	grainyhead-like 3 (Drosophila)	0.286	#N/A	#N/A
GRK1	G protein-coupled receptor kinase 1	0.119	0.189	0.122
GRK5	G protein-coupled receptor kinase 5	2.942	3.109	3.424
GSPT2	G1 to S phase transition 2	0.334	#N/A	#N/A
GUCY1B3	guanylate cyclase 1, soluble, beta 3	0.339	#N/A	#N/A
H1F0	H1 histone family, member 0	#N/A	#N/A	0.319
H1T2	H1 histone family, member N, testis-specific	0.326	#N/A	#N/A

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HCRTR1	hypocretin (orexin) receptor 1	0.186	#N/A	#N/A
HDAC2	histone deacetylase 2	0.305	#N/A	#N/A
HEMGN	hemogen	0.271	#N/A	#N/A
HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	3.612	#N/A	#N/A
HES4	hairy and enhancer of split 4 (Drosophila)	4.719	3.730	4.941
HIF3A	hypoxia inducible factor 3, alpha subunit	#N/A	#N/A	0.326
HINT1	histidine triad nucleotide binding protein 1	0.319	#N/A	#N/A
HIST1H2BA	histone 1, H2ba	#N/A	0.323	#N/A
HIST1H2BC	histone 1, H2bc	0.272	#N/A	#N/A
HIST1H3G	histone 1, H3g	0.264	0.288	0.347
HIST1H4A	histone 1, H4a	#N/A	0.303	#N/A
HIVEP1	human immunodeficiency virus type I enhancer binding protein 1	2.928	2.953	4.318
HK2	hexokinase 2	7.060	5.412	6.421
HNRPA3	heterogeneous nuclear ribonucleoprotein A3	0.305	#N/A	#N/A
HOMER1	homer homolog 1 (Drosophila)	3.641	#N/A	4.638
HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	0.302	#N/A	#N/A
HS6ST1	heparan sulfate 6-O-sulfotransferase 1|heparan sulfate 6-O-sulfotransferase 1 pseudogene	0.320	#N/A	#N/A
HS6ST3	heparan sulfate 6-O-sulfotransferase 3	0.352	#N/A	#N/A
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	#N/A	#N/A	3.273
HSPA2	heat shock 70kDa protein 2	3.092	2.994	#N/A
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	3.634	#N/A	#N/A
HTR4	5-hydroxytryptamine (serotonin) receptor 4	#N/A	0.352	#N/A
HYOU1	hypoxia up-regulated 1	3.287	#N/A	#N/A
HYPE	Huntingtin interacting protein E	3.075	#N/A	#N/A
IBRDC2	IBR domain containing 2	#N/A	0.335	0.274
IBRDC3	IBR domain containing 3	3.868	3.211	4.288
IBTK	inhibitor of Bruton agammaglobulinemia tyrosine kinase	5.409	4.859	8.699
ICHTHYIN	ichthyin protein	#N/A	#N/A	2.871
ID2B	inhibitor of DNA binding 2B, dominant negative helix-loop-helix protein	#N/A	2.986	2.910

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		PGF	**Butaprost**	**PGF & Butaprost**
IER2	immediate early response 2	3.407	#N/A	3.649
IER3	immediate early response 3	28.451	17.779	20.647
IFIH1	interferon induced with helicase C domain 1	0.323	#N/A	#N/A
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	0.278	0.348	0.269
IFITM5	interferon induced transmembrane protein 5	0.305	0.258	0.351
IFT74	intraflagellar transport 74 homolog (Chlamydomonas)	0.311	#N/A	#N/A
IFT80	intraflagellar transport 80 homolog (Chlamydomonas)	0.174	#N/A	#N/A
IFT81	intraflagellar transport 81 homolog (Chlamydomonas)	0.338	#N/A	#N/A
IFT81	intraflagellar transport 81 homolog (Chlamydomonas)	#N/A	#N/A	0.331
IFT88	intraflagellar transport 88 homolog (Chlamydomonas)	#N/A	0.354	#N/A
IGFBP1	insulin-like growth factor binding protein 1	4.727	#N/A	5.336
IGFBP4	insulin-like growth factor binding protein 4	#N/A	#N/A	3.702
IGFL2	IGF-like family member 2	#N/A	#N/A	0.307
IGSF9	immunoglobulin superfamily, member 9	#N/A	0.338	#N/A
IHPK3	inositol hexaphosphate kinase 3	0.221	0.127	0.245
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	#N/A	0.336	#N/A
IL11	interleukin 11	40.751	10.255	59.307
IL13RA2	interleukin 13 receptor, alpha 2	0.249	#N/A	#N/A
IL15	interleukin 15	#N/A	0.317	#N/A
IL1R2	interleukin 1 receptor, type II	#N/A	3.984	3.624
IL1RAP	interleukin 1 receptor accessory protein	#N/A	#N/A	3.295
IL3	interleukin 3 (colony-stimulating factor, multiple)	0.299	#N/A	0.161
IL6	interleukin 6 (interferon, beta 2)	70.472	14.355	29.138
IL8	interleukin 8	66.669	28.640	109.802
INMT	indolethylamine N-methyltransferase	#N/A	#N/A	0.353
IPLA2(GAMMA)	patatin-like phospholipase domain containing 8	#N/A	#N/A	2.917
IRF8	interferon regulatory factor 8	0.270	#N/A	#N/A
IRGQ	immunity-related GTPase family, Q	#N/A	#N/A	0.065

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ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	4.011	#N/A	7.875
JAK3	Janus kinase 3 (a protein tyrosine kinase, leukocyte)	0.323	#N/A	#N/A
JOSD3	Josephin domain containing 3	#N/A	#N/A	2.847
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	5.800	3.187	6.753
JUNB	jun B proto-oncogene	3.019	3.035	3.532
KATNAL2	katanin p60 subunit A-like 2	0.305	#N/A	#N/A
KCND3	potassium voltage-gated channel, Shal-related subfamily, member 3	#N/A	#N/A	0.339
KCNE3	potassium voltage-gated channel, Isk-related family, member 3	0.301	#N/A	0.322
KCNJ5	potassium inwardly-rectifying channel, subfamily J, member 5	#N/A	0.157	0.222
KCNK17	potassium channel, subfamily K, member 17	#N/A	#N/A	3.597
KCNK5	potassium channel, subfamily K, member 5	#N/A	3.204	3.713
KCNK6	potassium channel, subfamily K, member 6	3.835	2.838	#N/A
KCTD12	potassium channel tetramerisation domain containing 12	2.897	#N/A	2.900
KIAA0020	KIAA0020	#N/A	#N/A	3.770
KIAA0408	KIAA0408	#N/A	0.305	#N/A
KIAA1212	KIAA1212	0.295	#N/A	#N/A
KIAA1217	KIAA1217	0.228	0.308	0.209
KIAA1305	KIAA1305	#N/A	0.323	0.227
KIAA1328	KIAA1328	#N/A	0.281	#N/A
KIAA1618	KIAA1618	#N/A	#N/A	0.332
KIAA1622	KIAA1622	0.320	#N/A	#N/A
KIAA1949	KIAA1949	3.400	#N/A	#N/A
KIF20A	kinesin family member 20A	#N/A	#N/A	0.291
KIFC3	kinesin family member C3	#N/A	#N/A	3.061
KLC3	kinesin light chain 3	#N/A	#N/A	0.287
KLF5	Kruppel-like factor 5 (intestinal)	4.030	3.707	6.948
KLF6	Kruppel-like factor 6	3.104	#N/A	4.350
KLHDC1	kelch domain containing 1	#N/A	#N/A	0.255
KLHDC6	kelch domain containing 6	#N/A	#N/A	0.063
KRT13	keratin 13	3.437	#N/A	6.912

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KRT16|KRT14	keratin 16 (focal non-epidermolytic palmoplantar keratoderma)|keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner)	5.141	#N/A	#N/A
KRT17	keratin 17	217.379	46.443	99.745
KRT6C|KRT6A	keratin 6C|keratin 6A	37.403	22.714	79.461
KRT6E	keratin 6E	46.094	27.267	97.668
KRTAP1-3	keratin associated protein 1-3	0.341	#N/A	#N/A
KRTAP1-3	keratin associated protein 1-3|keratin associated protein 1-1	#N/A	0.191	#N/A
KRTAP2-1|KRTAP2-4	keratin associated protein 2-1|keratin associated protein 2-4	14.548	#N/A	9.717
KRTAP2-2|KRTAP2-1|KRTAP2-4	keratin associated protein 2-2|keratin associated protein 2-1|keratin associated protein 2-4	2.983	#N/A	#N/A
KRTAP6-2	keratin associated protein 6-2	0.332	#N/A	#N/A
KRTAP9-8	keratin associated protein 9-8	0.302	0.322	0.329
KRTHB5	keratin, hair, basic, 5	0.265	#N/A	#N/A
KUB3	XRCC6 binding protein 1	0.298	0.336	0.305
LALBA	lactalbumin, alpha-	0.165	#N/A	#N/A
LAMA3	laminin, alpha 3	13.742	5.586	8.519
LAMB3	laminin, beta 3	#N/A	#N/A	3.001
LAMC2	laminin, gamma 2	4.030	#N/A	3.566
LARP2	La ribonucleoprotein domain family, member 2	#N/A	#N/A	3.007
LATS2	LATS, large tumor suppressor, homolog 2 (Drosophila)	5.913	3.237	5.405
LBH	limb bud and heart development homolog (mouse)	21.975	15.981	20.322
LCP1	lymphocyte cytosolic protein 1 (L-plastin)	2.946	#N/A	#N/A
LEFTY1	left-right determination factor 1	#N/A	#N/A	0.335
LEFTY2	left-right determination factor 2	0.233	#N/A	#N/A
LIMS3	LIM and senescent cell antigen-like domains 3	#N/A	2.982	3.807
LIPC	lipase, hepatic	#N/A	0.186	0.221
LIPF	lipase, gastric	0.348	#N/A	#N/A
LMBRD2	LMBR1 domain containing 2	0.300	#N/A	#N/A
LMCD1	LIM and cysteine-rich domains 1	3.353	4.706	5.062

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LMO7	LIM domain 7	#N/A	0.312	#N/A
LOH11CR2A	loss of heterozygosity, 11, chromosomal region 2, gene A	#N/A	0.170	0.234
LOH12CR2	loss of heterozygosity, 12, chromosomal region 2	0.275	#N/A	0.309
LOH3CR2A	loss of heterozygosity, 3, chromosomal region 2, gene A	0.260	#N/A	#N/A
LOX	lysyl oxidase	3.807	#N/A	#N/A
LOXL4	lysyl oxidase-like 4	3.336	#N/A	#N/A
LRFN5	leucine rich repeat and fibronectin type III domain containing 5	0.213	#N/A	#N/A
LRP12	low density lipoprotein-related protein 12	#N/A	#N/A	0.074
LRRC50	leucine rich repeat containing 50	0.262	#N/A	#N/A
LRRC8C	leucine rich repeat containing 8 family, member C	3.874	#N/A	5.124
LTBP2	latent transforming growth factor beta binding protein 2	0.166	0.338	0.333
LYAR	Ly1 antibody reactive homolog (mouse)	#N/A	#N/A	2.934
LYZL1|LYZL 2	lysozyme-like 1|lysozyme-like 2	0.284	#N/A	#N/A
MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian)	11.846	11.478	13.376
MAK	male germ cell-associated kinase	0.294	#N/A	#N/A
MALL	mal, T-cell differentiation protein-like	#N/A	#N/A	3.002
MAN2C1	mannosidase, alpha, class 2C, member 1	#N/A	#N/A	0.316
MAP1A	microtubule-associated protein 1A	0.146	0.290	0.179
MAP1B	microtubule-associated protein 1B	2.875	#N/A	#N/A
MAP2K3	mitogen-activated protein kinase kinase 3	5.796	3.231	4.884
MAP2K7	mitogen-activated protein kinase kinase 7	#N/A	0.337	#N/A
MAPK8	mitogen-activated protein kinase 8	#N/A	#N/A	2.951
MAPK8IP2	mitogen-activated protein kinase 8 interacting protein 2	3.134	#N/A	#N/A
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2	3.748	#N/A	3.264
MAPKBP1	mitogen activated protein kinase binding protein 1	#N/A	#N/A	2.855
MBL1P1	mannose-binding lectin (protein A) 1,	#N/A	#N/A	0.262

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
MCTP2	multiple C2 domains, transmembrane 2	3.572	#N/A	#N/A
MDH1B	malate dehydrogenase 1B, NAD (soluble)	0.326	#N/A	0.262
MEIS1	Meis1, myeloid ecotropic viral integration site 1 homolog (mouse)	0.323	0.344	0.300
MEOX1	mesenchyme homeobox 1	0.067	0.052	0.059
MEOX2	mesenchyme homeobox 2	0.345	#N/A	0.286
METRNL	meteorin, glial cell differentiation regulator-like	4.234	#N/A	3.997
MFAP5	microfibrillar associated protein 5	#N/A	#N/A	3.864
MICAL1	microtubule associated monoxygenase, calponin and LIM domain containing 1	#N/A	0.342	#N/A
MICALCL	MICAL C-terminal like	5.695	#N/A	6.063
MMP10	matrix metallopeptidase 10 (stromelysin 2)	11.120	8.434	31.984
MN1	meningioma (disrupted in balanced translocation) 1	0.289	#N/A	#N/A
MPEG1	macrophage expressed gene 1	#N/A	0.325	#N/A
MPFL	MPF-like	0.329	#N/A	0.253
MPO	myeloperoxidase	#N/A	#N/A	0.006
MPPED2	metallophosphoesterase domain containing 2	0.211	#N/A	0.332
MRPS16	mitochondrial ribosomal protein S16	0.263	#N/A	#N/A
MRVI1	murine retrovirus integration site 1 homolog	#N/A	#N/A	0.204
MS4A2	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)	#N/A	0.344	0.290
MS4A3	membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	#N/A	#N/A	0.135
MTHFD1L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	3.198	#N/A	#N/A
MTHFR	5,10-methylenetetrahydrofolate reductase (NADPH)	0.277	#N/A	0.269
MXD3	MAX dimerization protein 3	0.213	0.301	0.253
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	4.273	3.425	3.883
MYH9	myosin, heavy polypeptide 9, non-muscle	3.329	#N/A	#N/A
MYL3	myosin, light polypeptide 3, alkali; ventricular, skeletal, slow	#N/A	#N/A	16.689
MYL7	myosin, light polypeptide 7, regulatory	4.930	#N/A	3.960
MYO1E	myosin IE	3.844	#N/A	3.276

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NAPSA	napsin A aspartic peptidase	0.286	#N/A	#N/A
NARG1	NMDA receptor regulated 1	#N/A	#N/A	3.021
NAT6|HYAL3	N-acetyltransferase 6|hyaluronoglucosaminidase 3	#N/A	4.549	4.564
NAT8	N-acetyltransferase 8 (camello like)	0.247	#N/A	0.240
NBLA00058	asparagine synthetase domain containing 1	#N/A	#N/A	3.797
NCF1	neutrophil cytosolic factor 1 (47kDa, chronic granulomatous disease, autosomal 1)	0.277	#N/A	0.257
NCOA2	nuclear receptor coactivator 2	0.308	#N/A	0.336
NDEL1	nudE nuclear distribution gene E homolog like 1 (A. nidulans)	3.194	#N/A	3.046
NDRG1	N-myc downstream regulated gene 1	10.275	9.694	12.636
NDRG4	NDRG family member 4	0.329	#N/A	#N/A
NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa	0.297	#N/A	#N/A
NEDD4L	neural precursor cell expressed, developmentally down-regulated 4-like	4.202	#N/A	4.766
NEIL3	nei endonuclease VIII-like 3 (E. coli)	0.347	#N/A	#N/A
NEU2	sialidase 2 (cytosolic sialidase)	0.202	#N/A	#N/A
NFAM1	NFAT activating protein with ITAM motif 1	#N/A	#N/A	0.284
NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	4.202	#N/A	3.817
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	3.194	#N/A	3.104
NID2	nidogen 2 (osteonidogen)	2.933	#N/A	#N/A
NIP	dual oxidase maturation factor 1	0.334	#N/A	#N/A
NKX2-5	NK2 transcription factor related, locus 5 (Drosophila)	#N/A	#N/A	3.447
NKX6-1	NK6 transcription factor related, locus 1 (Drosophila)	#N/A	#N/A	0.004
NMNAT1	nicotinamide nucleotide adenylyltransferase 1	0.287	#N/A	#N/A
NOD9	NLR family member X1	#N/A	#N/A	0.318
NOX1	NADPH oxidase 1	0.251	#N/A	#N/A
NOXA1	NADPH oxidase activator 1	0.297	#N/A	#N/A
NPAL1	NIPA-like domain containing 1	#N/A	#N/A	3.267
NR3C2	nuclear receptor subfamily 3, group C, member 2	#N/A	0.176	0.136

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NR4A2	nuclear receptor subfamily 4, group A, member 2	9.097	18.751	20.220
NR4A3	nuclear receptor subfamily 4, group A, member 3	23.907	9.223	14.894
NRBP2	nuclear receptor binding protein 2	0.228	#N/A	#N/A
NRCAM	neuronal cell adhesion molecule	3.397	#N/A	3.118
NRG1	neuregulin 1	3.216	#N/A	#N/A
NRIP1	nuclear receptor interacting protein 1	3.090	2.915	5.682
NRP2	neuropilin 2	4.482	7.933	10.570
NT5E	5'-nucleotidase, ecto (CD73)	11.756	7.268	13.481
NUDT13	nudix (nucleoside diphosphate linked moiety X)-type motif 13	#N/A	0.354	#N/A
NUDT7	nudix (nucleoside diphosphate linked moiety X)-type motif 7	0.174	#N/A	0.296
NUPL1	nucleoporin like 1	2.851	2.902	4.311
NXF5	nuclear RNA export factor 5	0.207	#N/A	#N/A
NYD-SP14	tetratricopeptide repeat domain 29	#N/A	0.341	#N/A
NYD-SP17	coiled-coil domain containing 54	0.016	0.022	0.010
OACT1	O-acyltransferase (membrane bound) domain containing 1	#N/A	0.342	0.353
ODF2L	outer dense fiber of sperm tails 2-like	0.308	0.270	#N/A
OKL38	oxidative stress induced growth inhibitor 1	#N/A	#N/A	0.236
OLFML3	olfactomedin-like 3	0.353	#N/A	#N/A
OR10G3	olfactory receptor, family 10, subfamily G, member 3	0.242	#N/A	#N/A
OR10W1	olfactory receptor, family 10, subfamily W, member 1	3.592	2.857	#N/A
OR1D2	olfactory receptor, family 1, subfamily D, member 2	#N/A	#N/A	0.346
OR1F2	olfactory receptor, family 1, subfamily F, member 2	0.249	#N/A	#N/A
OR2C1	olfactory receptor, family 2, subfamily C, member 1	#N/A	#N/A	0.239
OR2F1	olfactory receptor, family 2, subfamily F, member 1	0.318	#N/A	0.179
OR2F2	olfactory receptor, family 2, subfamily F, member 2	0.342	#N/A	#N/A

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OR4B1	olfactory receptor, family 4, subfamily B, member 1	0.332	#N/A	#N/A
OR51T1	olfactory receptor, family 51, subfamily T, member 1	0.343	#N/A	0.275
OR52I2	olfactory receptor, family 52, subfamily I, member 2	#N/A	#N/A	0.139
OR52K1	olfactory receptor, family 52, subfamily K, member 1	0.267	#N/A	#N/A
OR52K2	olfactory receptor, family 52, subfamily K, member 2	#N/A	#N/A	0.333
OR5AU1	olfactory receptor, family 5, subfamily AU, member 1	0.178	#N/A	#N/A
OR5B21	olfactory receptor, family 5, subfamily B, member 21	0.344	#N/A	#N/A
OR6W1P	olfactory receptor, family 6, subfamily W, member 1 pseudogene	#N/A	0.348	0.291
OR7D4	olfactory receptor, family 7, subfamily D, member 4	0.187	0.346	#N/A
OTOS	otospiralin	0.336	#N/A	#N/A
OVOS2	ovostatin 2	0.346	#N/A	0.338
P2RXL1	purinergic receptor P2X-like 1, orphan receptor	0.266	#N/A	#N/A
P2RY6	pyrimidinergic receptor P2Y, G-protein coupled, 6	#N/A	#N/A	0.352
PABPC5	poly(A) binding protein, cytoplasmic 5	0.262	#N/A	0.135
PACRG	PARK2 co-regulated	0.291	#N/A	0.352
PADI3	peptidyl arginine deiminase, type III	0.320	#N/A	#N/A
PAK1IP1	PAK1 interacting protein 1	#N/A	#N/A	3.160
PAQR5	progestin and adipoQ receptor family member V	3.695	4.058	6.337
PAQR8	progestin and adipoQ receptor family member VIII	0.165	#N/A	0.292
PAQR9	progestin and adipoQ receptor family member IX	#N/A	#N/A	3.945
PARP4	poly (ADP-ribose) polymerase family, member 4	#N/A	#N/A	0.301
PAX8	paired box gene 8	#N/A	2.945	#N/A
PBX4	pre-B-cell leukemia transcription factor 4	#N/A	#N/A	0.288

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PCDH9	protocadherin 9	#N/A	0.310	#N/A
PCDHA2	protocadherin alpha 2	#N/A	0.345	#N/A
PCDHA4	protocadherin alpha 4	#N/A	0.331	#N/A
PCDHB14	protocadherin beta 14	0.332	#N/A	#N/A
PCDHB8	protocadherin beta 8	#N/A	0.348	#N/A
PCDHGA6	protocadherin gamma subfamily A, 6	#N/A	0.293	#N/A
PCDHGB6	protocadherin gamma subfamily B, 6	0.194	#N/A	#N/A
PCNXL3	pecanex-like 3 (Drosophila)	0.301	#N/A	#N/A
PCYT1B	phosphate cytidylyltransferase 1, choline, beta	#N/A	#N/A	0.342
PDE2A	phosphodiesterase 2A, cGMP-stimulated	3.032	#N/A	#N/A
PDK2	pyruvate dehydrogenase kinase, isozyme 2	#N/A	#N/A	0.243
PDLIM2	PDZ and LIM domain 2 (mystique)	#N/A	#N/A	2.901
PDLIM5	PDZ and LIM domain 5	4.986	2.853	5.777
PDLIM7	PDZ and LIM domain 7 (enigma)	3.242	#N/A	#N/A
PDZK3	PDZ domain containing 3	0.340	0.279	0.295
PEX11G	peroxisomal biogenesis factor 11 gamma	0.335	#N/A	#N/A
PEX12	peroxisomal biogenesis factor 12	0.302	#N/A	#N/A
PFN4	profilin family, member 4	#N/A	#N/A	0.216
PGAM2	phosphoglycerate mutase 2 (muscle)	0.292	#N/A	#N/A
PHF2	PHD finger protein 2	#N/A	#N/A	3.138
PHF21A	PHD finger protein 21A	0.252	0.305	#N/A
PHIP	pleckstrin homology domain interacting protein	#N/A	#N/A	0.205
PHLDA1	pleckstrin homology-like domain, family A, member 1	4.755	5.857	6.806
PHLDB2	pleckstrin homology-like domain, family B, member 2	4.476	2.980	3.686
PI3	peptidase inhibitor 3, skin-derived (SKALP)	5.027	3.020	6.396
PIK3AP1	phosphoinositide-3-kinase adaptor protein 1	0.334	#N/A	#N/A
PIM1	pim-1 oncogene	3.229	#N/A	3.310
PIM3	pim-3 oncogene	4.051	#N/A	3.814
PIN4	protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	0.264	#N/A	#N/A
PKP3	plakophilin 3	#N/A	#N/A	0.326
PLA2G1B	phospholipase A2, group IB (pancreas)	0.262	#N/A	#N/A
PLA2G2E	phospholipase A2, group IIE	0.327	#N/A	#N/A
PLA2G4F	phospholipase A2, group IVF	0.328	#N/A	#N/A

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PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	0.265	#N/A	#N/A
PLAG1	pleiomorphic adenoma gene 1	0.348	#N/A	#N/A
PLAU	plasminogen activator, urokinase	3.776	#N/A	#N/A
PLAUR	plasminogen activator, urokinase receptor	13.711	4.154	15.528
PLEC1	plectin 1, intermediate filament binding protein 500kDa	#N/A	#N/A	0.331
PLEKHA7	pleckstrin homology domain containing, family A member 7	3.386	#N/A	#N/A
PLEKHC1	pleckstrin homology domain containing, family C (with FERM domain) member 1	#N/A	#N/A	3.082
PLEKHG1	pleckstrin homology domain containing, family G (with RhoGef domain) member 1	#N/A	#N/A	0.286
PLSCR4	phospholipid scramblase 4	0.217	0.277	0.228
PLXDC2	plexin domain containing 2	#N/A	#N/A	0.302
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	#N/A	#N/A	3.109
POLR3G	polymerase (RNA) III (DNA directed) polypeptide G (32kD)	0.288	#N/A	#N/A
PPFIBP2	PTPRF interacting protein, binding protein 2 (liprin beta 2)	#N/A	0.308	#N/A
PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A	3.151	#N/A	4.609
PPP3CC	protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform (calcineurin A gamma)	3.778	5.301	5.656
PRAC	small nuclear protein PRAC	0.348	#N/A	#N/A
PRICKLE2	prickle-like 2 (Drosophila)	0.207	#N/A	#N/A
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	5.307	3.246	4.073
PRKCG	protein kinase C, gamma	0.342	#N/A	#N/A
PRO0478	PRO0478 protein	0.318	#N/A	0.262
PROC	protein C (inactivator of coagulation factors Va and VIIIa)	0.214	#N/A	#N/A
PROM1	prominin 1	3.021	#N/A	#N/A
PRPF38B	PRP38 pre-mRNA processing factor 38 (yeast) domain containing B	#N/A	#N/A	2.869

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PRSS12	protease, serine, 12 (neurotrypsin, motopsin)	#N/A	#N/A	0.005
PRSS3	protease, serine, 3 (mesotrypsin)	#N/A	#N/A	0.019
PRSS33	protease, serine, 33	#N/A	#N/A	0.343
PRTG	protogenin homolog (Gallus gallus)	0.326	#N/A	#N/A
PSG4	pregnancy specific beta-1-glycoprotein 4	0.322	#N/A	#N/A
PSME2	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	0.243	#N/A	#N/A
PSORS1C2	psoriasis susceptibility 1 candidate 2	#N/A	#N/A	0.331
PSRC1	proline/serine-rich coiled-coil 1	#N/A	#N/A	0.280
PTDSR	phosphatidylserine receptor	#N/A	#N/A	2.975
PTGER4	prostaglandin E receptor 4 (subtype EP4)	3.238	2.837	8.337
PTGIR	prostaglandin I2 (prostacyclin) receptor (IP)	0.325	#N/A	0.295
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	6.245	3.923	10.906
PTHLH	parathyroid hormone-like hormone	#N/A	#N/A	3.316
PTPRB	protein tyrosine phosphatase, receptor type, B	2.986	#N/A	3.081
PTPRU	protein tyrosine phosphatase, receptor type, U	0.268	#N/A	#N/A
PTRF	polymerase I and transcript release factor	3.291	#N/A	3.756
PVALB	parvalbumin	0.270	#N/A	#N/A
PVR	poliovirus receptor	#N/A	#N/A	3.256
PXDN	peroxidasin homolog (Drosophila)	#N/A	#N/A	0.350
PXK	PX domain containing serine/threonine kinase	0.284	#N/A	#N/A
RAB11FIP4	RAB11 family interacting protein 4 (class II)	#N/A	#N/A	0.326
RAB26	RAB26, member RAS oncogene family	0.254	#N/A	#N/A
RAB7B	RAB7B, member RAS oncogene family	0.313	0.196	0.241
RABGGTB	Rab geranylgeranyltransferase, beta subunit	#N/A	#N/A	4.194
RAFTLIN	raftlin, lipid raft linker 1	3.144	3.422	4.340
RAG1	recombination activating gene 1	0.330	0.349	#N/A
RAI1	retinoic acid induced 1	#N/A	#N/A	0.215
RAI17	retinoic acid induced 17	0.327	0.262	0.292
RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5	#N/A	3.479	4.048
RASAL1	RAS protein activator like 1 (GAP1 like)	0.296	#N/A	#N/A
RASAL2	RAS protein activator like 2	3.538	#N/A	3.817
RASD1	RAS, dexamethasone-induced 1	27.066	11.275	14.339
RASGEF1B	RasGEF domain family, member 1B	#N/A	#N/A	0.020

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RDH12	retinol dehydrogenase 12 (all-trans and 9-cis)	0.318	#N/A	#N/A
RDHE2	epidermal retinal dehydrogenase 2	0.339	#N/A	#N/A
RDM1	RAD52 motif 1	0.215	0.286	#N/A
RECK	reversion-inducing-cysteine-rich protein with kazal motifs	#N/A	#N/A	0.350
RECQL5	RecQ protein-like 5	0.319	#N/A	0.347
REG3G	regenerating islet-derived 3 gamma	#N/A	#N/A	0.220
RELB	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	#N/A	#N/A	2.969
RGC32	chromosome 13 open reading frame 15	4.042	5.167	6.149
RGR	retinal G protein coupled receptor	#N/A	#N/A	0.041
RGS16	regulator of G-protein signalling 16	5.391	4.172	6.796
RGS20	regulator of G-protein signalling 20	#N/A	#N/A	3.633
RGS3	regulator of G-protein signalling 3	2.975	3.828	5.033
RHBDL2	rhomboid, veinlet-like 2 (Drosophila)	0.271	0.317	0.329
RHCG	Rhesus blood group, C glycoprotein	0.309	#N/A	#N/A
RHOF	ras homolog gene family, member F (in filopodia)	5.316	4.791	6.335
RHOH	ras homolog gene family, member H	0.328	#N/A	#N/A
RHOU	ras homolog gene family, member U	0.177	#N/A	#N/A
RIBC2	RIB43A domain with coiled-coils 2	0.296	#N/A	#N/A
RLN1	relaxin 1	#N/A	0.348	0.323
RLN2	relaxin 2	0.219	#N/A	#N/A
RNASEL	ribonuclease L (2',5'-oligoisoadenylate synthetase-dependent)	0.217	0.246	0.332
RND3	Rho family GTPase 3	3.132	#N/A	5.647
RNF126	ring finger protein 126	2.956	#N/A	2.909
RNF148	ring finger protein 148	0.256	#N/A	#N/A
ROBO4	roundabout homolog 4, magic roundabout (Drosophila)	0.225	#N/A	0.314
RPL19	ribosomal protein L19	0.320	#N/A	#N/A
RPL22	ribosomal protein L22	#N/A	#N/A	2.926
RPRM	reprimo, TP53 dependent G2 arrest mediator candidate	#N/A	11.899	7.764
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	#N/A	#N/A	3.401

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RSHL3	radial spokehead-like 3	0.153	#N/A	0.286
RTP2	receptor (chemosensory) transporter protein 2	#N/A	#N/A	0.255
RUNX3	runt-related transcription factor 3	#N/A	2.927	#N/A
SACS	spastic ataxia of Charlevoix-Saguenay (sacsin)	2.914	3.877	11.779
SALL2	sal-like 2 (Drosophila)	0.308	#N/A	0.130
SAMD13	sterile alpha motif domain containing 13	#N/A	0.352	0.311
SAT	spermidine/spermine N1-acetyltransferase	#N/A	5.938	7.465
SBDS	Shwachman-Bodian-Diamond syndrome	#N/A	#N/A	3.499
SBDSP	Shwachman-Bodian-Diamond syndrome pseudogene	#N/A	#N/A	2.839
SBLF	stonin 1	#N/A	0.285	#N/A
SBP1	multiple EGF-like-domains 8	0.247	#N/A	#N/A
SBSN	suprabasin	0.156	#N/A	#N/A
SCGB1D4	secretoglobin, family 1D, member 4	0.084	0.033	0.055
SCHIP1	schwannomin interacting protein 1	6.344	3.527	8.078
SCML4	sex comb on midleg-like 4 (Drosophila)	#N/A	#N/A	0.274
SCN1A	sodium channel, voltage-gated, type I, alpha	0.150	0.301	#N/A
SCN3B	sodium channel, voltage-gated, type III, beta	0.305	#N/A	#N/A
SDCBP2	syndecan binding protein (syntenin) 2	#N/A	3.640	#N/A
SDF2L1	stromal cell-derived factor 2-like 1	#N/A	2.870	#N/A
SDPR	serum deprivation response (phosphatidylserine binding protein)	23.088	22.710	25.532
SECTM1	secreted and transmembrane 1	0.286	#N/A	#N/A
SEMA3B	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	3.500	3.327	3.711
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	2.929	#N/A	3.222
Sept9	septin 9	#N/A	#N/A	0.209
SERINC4	serine incorporator 4	#N/A	#N/A	0.313
SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	#N/A	#N/A	2.964
SERPINB8	serpin peptidase inhibitor, clade B (ovalbumin), member 8	4.768	2.872	5.570
SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	7.090	7.240	9.681
SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1) 2	5.800	7.021	16.107

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		PGF	**Butaprost**	**PGF & Butaprost**
SFN	stratifin	9.903	6.957	15.545
SH3GL2	SH3-domain GRB2-like 2	3.077	#N/A	#N/A
SH3MD2	SH3 multiple domains 2	3.302	#N/A	3.759
SIAH2	seven in absentia homolog 2 (Drosophila)	3.095	#N/A	#N/A
SLC16A13	solute carrier family 16 (monocarboxylic acid transporters), member 13	#N/A	#N/A	0.336
SLC16A2	solute carrier family 16 (monocarboxylic acid transporters), member 2	0.195	#N/A	#N/A
SLC17A2	solute carrier family 17 (sodium phosphate), member 2	0.225	#N/A	#N/A
SLC19A2	solute carrier family 19 (thiamine transporter), member 2	3.682	2.831	3.855
SLC20A1	solute carrier family 20 (phosphate transporter), member 1	#N/A	#N/A	3.075
SLC20A2	solute carrier family 20 (phosphate transporter), member 2	8.839	3.029	5.453
SLC22A6	solute carrier family 22 (organic anion transporter), member 6	0.321	#N/A	0.343
SLC24A1	solute carrier family 24 (sodium/potassium/calcium exchanger), member 1	0.275	#N/A	#N/A
SLC25A25	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	4.001	3.050	4.566
SLC25A27	solute carrier family 25, member 27	0.224	#N/A	#N/A
SLC25A32	solute carrier family 25, member 32	3.526	3.342	4.787
SLC27A1	solute carrier family 27 (fatty acid transporter), member 1	#N/A	#N/A	0.350
SLC35D3	solute carrier family 35, member D3	0.314	#N/A	0.276
SLC36A2	solute carrier family 36 (proton/amino acid symporter), member 2	0.306	#N/A	#N/A
SLC38A1	solute carrier family 38, member 1	2.899	#N/A	#N/A
SLC38A2	solute carrier family 38, member 2	4.247	#N/A	3.078
SLC39A14	solute carrier family 39 (zinc transporter), member 14	3.457	#N/A	#N/A
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	0.331	0.301	#N/A

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
SLC6A1	solute carrier family 6 (neurotransmitter transporter, GABA), member 1	0.268	#N/A	#N/A
SLC6A13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	#N/A	0.340	#N/A
SLC6A15	solute carrier family 6, member 15	#N/A	#N/A	3.248
SLC6A16	solute carrier family 6, member 16	0.351	#N/A	#N/A
SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	3.874	5.601	5.950
SLC7A10	solute carrier family 7, (neutral amino acid transporter, y+ system) member 10	0.199	#N/A	#N/A
SLC7A4	solute carrier family 7 (cationic amino acid transporter, y+ system), member 4	0.296	#N/A	#N/A
SLCO4A1	solute carrier organic anion transporter family, member 4A1	5.798	4.136	5.463
SLITRK1	SLIT and NTRK-like family, member 1	0.068	0.184	0.154
SLITRK5	SLIT and NTRK-like family, member 5	#N/A	0.316	0.324
SMAD3	SMAD, mothers against DPP homolog 3 (Drosophila)	3.143	#N/A	2.899
SMP3	phosphatidylinositol glycan anchor biosynthesis, class Z	#N/A	#N/A	0.295
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	6.362	3.245	7.439
SNAI3	snail homolog 3 (Drosophila)	0.339	#N/A	#N/A
SNF1LK	SNF1-like kinase	8.053	7.748	5.656
SOCS2	suppressor of cytokine signaling 2	3.149	3.253	4.582
SORBS2	sorbin and SH3 domain containing 2	0.118	0.331	0.184
SORCS2	sortilin-related VPS10 domain containing receptor 2	#N/A	0.240	#N/A
SORCS3	sortilin-related VPS10 domain containing receptor 3	0.325	#N/A	#N/A
SOSTDC1	sclerostin domain containing 1	0.092	0.124	0.143
SOX17	SRY (sex determining region Y)-box 17	4.541	4.203	5.067
SOX4	SRY (sex determining region Y)-box 4	#N/A	2.883	#N/A
SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	#N/A	0.277	#N/A
SPAG4	sperm associated antigen 4	#N/A	3.061	#N/A
SPAG8	sperm associated antigen 8	#N/A	0.237	0.148

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
SPATA7	spermatogenesis associated 7	0.188	#N/A	0.339
SPHK1	sphingosine kinase 1	9.932	5.058	11.676
SPRR4	small proline rich protein 4	#N/A	#N/A	0.271
SPRY2	sprouty homolog 2 (Drosophila)	13.016	6.642	15.883
SRF	serum response factor (c-fos serum response element-binding transcription factor)	4.857	3.860	5.632
SSFA2	sperm specific antigen 2	4.356	3.082	4.585
SSPN	sarcospan (Kras oncogene-associated gene)	#N/A	0.315	#N/A
ST3GAL1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	5.256	3.346	5.416
STAMBPL1	STAM binding protein-like 1	5.367	4.572	9.026
STARD6	START domain containing 6	0.310	#N/A	#N/A
STAU2	staufen, RNA binding protein, homolog 2 (Drosophila)	0.329	0.262	0.322
STEAP2	six transmembrane epithelial antigen of the prostate 2	#N/A	#N/A	0.322
STK17A	serine/threonine kinase 17a (apoptosis-inducing)	3.863	5.962	10.187
STK23	serine/threonine kinase 23	0.321	#N/A	#N/A
STK38L	serine/threonine kinase 38 like	3.482	3.048	5.345
STK40	serine/threonine kinase 40	0.350	#N/A	#N/A
STX11	syntaxin 11	8.884	5.916	11.956
SUHW1	suppressor of hairy wing homolog 1 (Drosophila)	#N/A	#N/A	0.322
SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	0.277	0.346	#N/A
SULT2B1	sulfotransferase family, cytosolic, 2B, member 1	0.264	#N/A	#N/A
SUSD3	sushi domain containing 3	0.308	#N/A	#N/A
SYNE1	spectrin repeat containing, nuclear envelope 1	0.234	#N/A	0.234
SYNJ1	synaptojanin 1	#N/A	#N/A	0.287
SYT1	synaptotagmin I	0.334	#N/A	#N/A
TAF1A	TATA box binding protein (TBP)-associated factor, RNA polymerase I, A, 48kDa	#N/A	#N/A	3.438
TAF4B	TAF4b RNA polymerase II, TATA box binding protein (TBP)-associated factor, 105kDa	3.219	2.925	3.992

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
TAGLN	transgelin	11.127	#N/A	5.695
TAIP-2	family with sequence similarity 130, member A2	0.267	0.214	#N/A
TA-PP2C	PTC7 protein phosphatase homolog (S. cerevisiae)	3.496	3.525	4.636
TAS2R4	taste receptor, type 2, member 4	#N/A	0.267	#N/A
TCAM1	testicular cell adhesion molecule 1 homolog (mouse)	0.352	#N/A	#N/A
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	3.139	#N/A	3.291
TCL1A	T-cell leukemia/lymphoma 1A	0.304	#N/A	#N/A
TCL6	T-cell leukemia/lymphoma 6	#N/A	#N/A	0.326
TEAD1	TEA domain family member 1 (SV40 transcriptional enhancer factor)	5.415	4.017	5.357
TENC1	tensin like C1 domain containing phosphatase (tensin 2)	#N/A	0.343	#N/A
TEX14	testis expressed sequence 14	#N/A	#N/A	3.432
TFAM	transcription factor A, mitochondrial	#N/A	2.986	3.118
TFDP3	transcription factor Dp family, member 3	0.332	#N/A	#N/A
TFF3	trefoil factor 3 (intestinal)	0.353	#N/A	#N/A
TFPI2	tissue factor pathway inhibitor 2	8.138	5.338	6.674
TFRC	transferrin receptor (p90, CD71)	3.043	#N/A	3.366
TGFB2	transforming growth factor, beta 2	5.091	#N/A	3.451
THAP2	THAP domain containing, apoptosis associated protein 2	0.317	#N/A	#N/A
THBS1	thrombospondin 1	15.614	5.176	13.901
THNSL1	threonine synthase-like 1 (bacterial)	0.305	#N/A	#N/A
THSD1P	thrombospondin, type I, domain containing 1 pseudogene	#N/A	#N/A	0.336
THSD3	thrombospondin, type I, domain containing 3	0.314	#N/A	#N/A
TINAGL1	tubulointerstitial nephritis antigen-like 1	5.002	#N/A	4.403
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	#N/A	#N/A	2.932
TLE3	transducin-like enhancer of split 3 (E(sp1) homolog, Drosophila)	3.559	3.825	3.248
TLN2	talin 2	#N/A	#N/A	0.176
TLX1	T-cell leukemia homeobox 1	#N/A	#N/A	0.273
TM4SF1	transmembrane 4 L six family member 1	91.457	14.301	123.248

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
TMEM2	transmembrane protein 2	6.245	6.009	12.377
TMEM22	transmembrane protein 22	3.840	2.884	3.115
TMEM37	transmembrane protein 37	#N/A	#N/A	0.176
TMEM39A	transmembrane protein 39A	2.865	#N/A	#N/A
TMEPAI	transmembrane, prostate androgen induced RNA	4.280	4.543	6.928
TMPRSS11D	transmembrane protease, serine 11D	#N/A	0.339	#N/A
TMPRSS2	transmembrane protease, serine 2	0.332	#N/A	#N/A
TNFAIP1	tumor necrosis factor, alpha-induced protein 1 (endothelial)	6.059	4.807	4.617
TNFAIP8	tumor necrosis factor, alpha-induced protein 8	#N/A	0.286	#N/A
TNFAIP8L1	tumor necrosis factor, alpha-induced protein 8-like 1	#N/A	#N/A	0.330
TNFAIP8L3	tumor necrosis factor, alpha-induced protein 8-like 3	0.313	0.283	#N/A
TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a	#N/A	3.881	#N/A
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	4.112	7.628	6.237
TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	6.377	4.701	5.614
TNFRSF19	tumor necrosis factor receptor superfamily, member 19	0.323	#N/A	#N/A
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	3.609	3.061	3.726
TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	0.219	#N/A	0.295
TNFSF4	tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kDa)	0.293	#N/A	#N/A
TNK2	tyrosine kinase, non-receptor, 2	#N/A	#N/A	0.341
TNN	tenascin N	0.313	#N/A	#N/A
TNNC2	troponin C type 2 (fast)	#N/A	0.331	#N/A
TNRC6B	trinucleotide repeat containing 6B	#N/A	#N/A	0.222
TP53I11	tumor protein p53 inducible protein 11	0.274	0.306	0.235
TP73	tumor protein p73	#N/A	#N/A	0.318
TPM1	tropomyosin 1 (alpha)	3.169	#N/A	#N/A

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
TPM4	tropomyosin 4	3.925	#N/A	#N/A
TRFP	Trf (TATA binding protein-related factor)-proximal homolog (Drosophila)	#N/A	#N/A	0.337
TRIB1	tribbles homolog 1 (Drosophila)	9.643	5.502	14.502
TRIB3	tribbles homolog 3 (Drosophila)	3.340	#N/A	#N/A
TRIM38	tripartite motif-containing 38	0.301	#N/A	#N/A
TRIM45	tripartite motif-containing 45	0.307	#N/A	0.279
TRIM51	tripartite motif-containing 51	#N/A	#N/A	3.103
TRIM6	tripartite motif-containing 6	#N/A	0.352	0.298
TRPC6	transient receptor potential cation channel, subfamily C, member 6	#N/A	#N/A	6.694
TRPS1	trichorhinophalangeal syndrome I	#N/A	0.311	#N/A
TRPV6	transient receptor potential cation channel, subfamily V, member 6	#N/A	#N/A	0.305
TSGA10	testis specific, 10	#N/A	0.183	#N/A
TSNARE1	t-SNARE domain containing 1	#N/A	0.296	#N/A
TSPAN19	tetraspanin 19	0.284	#N/A	#N/A
TSSK3	testis-specific serine kinase 3	#N/A	#N/A	0.218
TTC25	tetratricopeptide repeat domain 25	0.352	#N/A	#N/A
TTC6	tetratricopeptide repeat domain 6	#N/A	#N/A	3.036
TU12B1-TY	5'-nucleotidase domain containing 3	3.361	#N/A	4.090
TUBA2	tubulin, alpha 2	0.287	#N/A	#N/A
TUBE1	tubulin, epsilon 1	3.040	#N/A	#N/A
TXLNB	taxilin beta	0.297	#N/A	0.332
TXNIP	thioredoxin interacting protein	0.179	0.119	0.101
UAP1	UDP-N-acteylglucosamine pyrophosphorylase 1	#N/A	#N/A	2.876
UBE2H	ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)	#N/A	#N/A	2.882
UBIAD1	UbiA prenyltransferase domain containing 1	#N/A	#N/A	0.350
UBXD3	UBX domain containing 3	0.282	#N/A	#N/A
UCA1	urothelial cancer associated 1	4.959	3.493	4.452
UGT1A10	UDP glucuronosyltransferase 1 family, polypeptide A10	0.312	#N/A	0.270
UGT2A1	UDP glucuronosyltransferase 2 family, polypeptide A1	#N/A	#N/A	0.208
ULBP3	UL16 binding protein 3	#N/A	#N/A	0.338

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
USP1	ubiquitin specific peptidase 1	#N/A	#N/A	2.897
USP15	ubiquitin specific peptidase 15	#N/A	#N/A	3.092
USP16	ubiquitin specific peptidase 16	#N/A	#N/A	2.921
USP54	ubiquitin specific peptidase 54	#N/A	#N/A	0.263
VAV3	vav 3 oncogene	0.119	0.168	#N/A
VCPIP1	valosin containing protein (p97)/p47 complex interacting protein 1	3.262	#N/A	#N/A
VEGF	vascular endothelial growth factor	5.351	6.048	8.186
VGF	VGF nerve growth factor inducible	4.008	7.001	6.959
VIL1	villin 1	#N/A	#N/A	0.331
VLDLR	very low density lipoprotein receptor	3.770	#N/A	#N/A
VMAC	vimentin-type IF-associated coiled-coil protein	0.275	#N/A	#N/A
VMO1	vitelline membrane outer layer 1 homolog (chicken)	8.842	19.813	0.143
WDR36	WD repeat domain 36	#N/A	#N/A	3.095
WDR65	WD repeat domain 65	0.329	#N/A	#N/A
WDR71	WD repeat domain 71	#N/A	#N/A	0.344
WDR78	WD repeat domain 78	0.296	#N/A	#N/A
WFDC10A	WAP four-disulfide core domain 10A	#N/A	0.300	#N/A
WFIKKN1	WAP, follistatin/kazal, immunoglobulin, kunitz and netrin domain containing 1	3.549	#N/A	3.755
WNT1	wingless-type MMTV integration site family, member 1	#N/A	#N/A	0.102
WWTR1	WW domain containing transcription regulator 1	3.574	#N/A	4.269
XKR5	X Kell blood group precursor-related family, member 5	0.284	#N/A	#N/A
XKR5	X Kell blood group precursor-related family, member 5	#N/A	0.322	#N/A
XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	0.305	#N/A	#N/A
YOD1	YOD1 OTU deubiquinating enzyme 1 homolog (yeast)	6.842	2.840	11.577
YRDC	yrdC domain containing (E.coli)	4.185	3.702	4.736
ZBTB1	zinc finger and BTB domain containing 1	#N/A	2.868	#N/A
ZC3H6	zinc finger CCCH-type containing 6	0.348	#N/A	#N/A
ZDHHC15	zinc finger, DHHC-type containing 15	0.261	#N/A	#N/A

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
ZHX2	zinc fingers and homeoboxes 2	3.126	#N/A	#N/A
ZIC5	Zic family member 5 (odd-paired homolog, Drosophila)	2.979	3.301	3.679
ZMYND17	zinc finger, MYND-type containing 17	0.210	#N/A	#N/A
ZNF165	zinc finger protein 165	7.478	2.906	5.551
ZNF20	zinc finger protein 20 (KOX 13)	0.232	#N/A	#N/A
ZNF214	zinc finger protein 214	0.264	#N/A	#N/A
ZNF223	zinc finger protein 223	0.325	#N/A	#N/A
ZNF23|ZNF19	zinc finger protein 23 (KOX 16)|zinc finger protein 19 (KOX 12)	#N/A	0.321	#N/A
ZNF306	zinc finger protein 306	0.274	0.225	0.288
ZNF307	zinc finger protein 307	0.310	#N/A	#N/A
ZNF323	zinc finger protein 323	#N/A	0.164	0.117
ZNF333	zinc finger protein 333	0.304	#N/A	#N/A
ZNF337	zinc finger protein 337	0.289	0.293	#N/A
ZNF396	zinc finger protein 396	0.189	#N/A	#N/A
ZNF404	zinc finger protein 404	#N/A	#N/A	0.337
ZNF418	zinc finger protein 418	0.340	#N/A	#N/A
ZNF433	zinc finger protein 433	#N/A	0.314	#N/A
ZNF483	zinc finger protein 483	#N/A	#N/A	0.059
ZNF488	zinc finger protein 488	0.199	0.321	0.227
ZNF499	zinc finger protein 499	0.303	#N/A	#N/A
ZNF503	zinc finger protein 503	#N/A	#N/A	3.599
ZNF512	zinc finger protein 512	0.302	#N/A	0.245
ZNF545	zinc finger protein 545	#N/A	#N/A	4.457
ZNF546	zinc finger protein 546	0.353	#N/A	#N/A
ZNF564	zinc finger protein 564	0.275	#N/A	#N/A
ZNF568	zinc finger protein 568	0.337	#N/A	#N/A
ZNF575	zinc finger protein 575	0.323	#N/A	#N/A
ZNF596	zinc finger protein 596	0.340	#N/A	0.345
ZNF606	zinc finger protein 606	0.258	0.200	#N/A
ZNF610	zinc finger protein 610	#N/A	#N/A	0.295
ZNF621	zinc finger protein 621	#N/A	0.348	#N/A
ZNF66	zinc finger protein 66	#N/A	0.343	#N/A
ZNF676	zinc finger protein 676	0.321	0.345	#N/A
ZNF681	zinc finger protein 681	0.228	#N/A	#N/A
ZNF681	zinc finger protein 681	#N/A	0.224	#N/A

Gene Symbol	Gene Name	Fold Change		
		PGF	**Butaprost**	**PGF & Butaprost**
ZNF702	zinc finger protein 702	#N/A	0.346	#N/A
ZNF703	zinc finger protein 703	#N/A	3.279	3.040
ZNF85	zinc finger protein 85 (HPF4, HTF1)	0.348	#N/A	#N/A
ZNRF1	zinc and ring finger 1	0.119	#N/A	0.246
ZP1	zona pellucida glycoprotein 1 (sperm receptor)	#N/A	#N/A	0.321
ZRF1	zuotin related factor 1	#N/A	#N/A	3.173
ZXDA	zinc finger, X-linked, duplicated A	0.257	#N/A	#N/A
ZYG11B	zyg-11 homolog B (C. elegans)	0.290	0.338	#N/A
ZYX	zyxin	2.952	#N/A	#N/A

APPENDIX B

BUFFERS AND SOLUTIONS

Agarose gel-loading buffer

0.25% w/v bromophenol blue

0.25% xylene cyanol FF

40% (w/v) sucrose in water

Agarose gel tracking dye:

0.25 % w/v bromophenol blue

40% w/v sucrose

20mM (pH 8.0) EDTA

Buffer I:

140mM NaCl

20mM HEPES

4mM KCL

8mM Glucose

1mM $MgCl_2$

1mM $CaCl_2$

1mg/ml bovine serum

10mM LiCl

Laemmli buffer:

125mM Tris-HCL pH 6.8

4% SDS

20% glycerol

5% 2-mercapthoethanol

0.05% bromophenol blue

Lysis Buffer:

150mM NaCl

10% Glycerol

0.6% (v/v) NP-40

50mM Tris/HCL pH 7.4

10mM EDTA (add protease inhibitor cocktail before use)

TAE electrophoresis buffer (50X 1litre):

242g Tris

57.1ml Glacial acetic acid

100ml EDTA (0.5M) adjust volume with distilled water

Transfer Buffer:

25mM Tris/HCL,

0.192M Glycine and

20% Methanol

Tris-EDTA (TE) buffer:

10mM Tris

1mM EDTA adjust to pH 7.6 with HCL