PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS IN ENDOMETRIAL CANCER

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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SCHOOL OF MEDICINE
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<td>15d-PGJ2</td>
<td>15-Deoxy-Delta-12,14-prostaglandin J2</td>
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<td>AF</td>
<td>Activation function domain</td>
</tr>
<tr>
<td>AH</td>
<td>Atypical hyperplasia</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
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<tr>
<td>APC</td>
<td>Adenomatosis polyposis coli</td>
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<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BrDU</td>
<td>5-bromo-2'-deoxy-uridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CDK</td>
<td>Cyclin-dependent kinases</td>
</tr>
<tr>
<td>CER</td>
<td>Cytoplasmic extraction reaction</td>
</tr>
<tr>
<td>CLA</td>
<td>Conjugated linoleic acid</td>
</tr>
<tr>
<td>Cig</td>
<td>Ciglitazone</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
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<tr>
<td>DAB</td>
<td>Diamino-benzidine</td>
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<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
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<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
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<td>E-cadherin</td>
<td>Epithelial calcium-dependent adhesion</td>
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<td>EBCTCG</td>
<td>Early breast cancer trialist’s collaborative group</td>
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<tr>
<td>EC</td>
<td>Endometrial Cancer</td>
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<tr>
<td>EC₅₀</td>
<td>Half maximal effective concentration</td>
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<tr>
<td>ECM</td>
<td>Extra-cellular matrix</td>
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<td>ECL</td>
<td>Electrochemiluminescence</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EIC</td>
<td>Endometrial Intraepithelial Carcinoma</td>
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<td>ELISA</td>
<td>Enzyme-linked immunabsorbant assay</td>
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<td>ER</td>
<td>Oestrogen Receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Oestrogen response element</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>Fen</td>
<td>Fenofibrate</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FIGO</td>
<td>International Federation of Gynaecology and Obstetrics</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FLIP</td>
<td>FLICE-inhibitory protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<td>HCL</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<td>HEC-1A</td>
<td>Human endometrial cancer-1A</td>
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<td>HER 2/neu</td>
<td>Human Epidermal growth factor receptor 2</td>
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<td>Hypoxia-inducible factor 1</td>
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<td>HMG-CoA</td>
<td>3-hydroxy-3-methyl-glutaryl-CoA reductase</td>
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<tr>
<td>HNPCC</td>
<td>Hereditary nonpolyposis colorectal cancer</td>
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<td>HODE</td>
<td>Hydroxyoctadecadienoic acid</td>
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<td>HRE</td>
<td>Hormone response element</td>
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<td>HTERT</td>
<td>Human telomerase reverse transcriptase</td>
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<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
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<td>ID</td>
<td>Inhibitory domain</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>IGFBP-1</td>
<td>Insulin-like growth factor binding protein 1</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitrous oxides</td>
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<td>IUCD</td>
<td>Intra-uterine contraception device</td>
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<tr>
<td>KO</td>
<td>Knock-out</td>
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<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<td>LHRH</td>
<td>Leutenising Hormone releasing hormone</td>
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<td>LOH</td>
<td>Loss of heterozygosity</td>
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<td>MAP</td>
<td>Mitogen activated protein</td>
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<td>Macrophage colony stimulating factor</td>
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<td>MCF-7</td>
<td>Michigan cancer foundation 7 breast cancer cells</td>
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<td>Microsatellite Instability</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>mRNA</td>
<td>Messenger ribose nucleic acid</td>
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<td>MLH1</td>
<td>MutL homolog</td>
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<td>NaOH</td>
<td>Sodium hydroxide</td>
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<td>NER</td>
<td>Nuclear extraction reagent</td>
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<td>NH$_3$</td>
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<td>NHR</td>
<td>Nuclear Hormone receptors</td>
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<td>NIDDM</td>
<td>Non-insulin dependent diabetes mellitus</td>
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<td>Non-steroidal anti-inflammatory drugs</td>
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<td>OxLDL</td>
<td>oxidized low density lipoproteins</td>
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<td>PI3</td>
<td>Phosphoinositide 3-kinase/</td>
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<td>p53</td>
<td>Tumor protein 53</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCOS</td>
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<td>PG</td>
<td>Prostaglandin</td>
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<tr>
<td>PIP3</td>
<td>Phospholipid phosphatidylinositol-(3,4,5)-triphosphate</td>
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<td>PP</td>
<td>Peroxisome Proliferator</td>
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<td>Peroxisome Proliferator-activated receptor</td>
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<td>Retinoblastoma</td>
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<td>RGZ</td>
<td>Rosiglitazone</td>
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<tr>
<td>RLU</td>
<td>Reactive light unit</td>
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<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RTK</td>
<td>Receptor tyrosine kinases</td>
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<td>RT-PCR</td>
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<td>RXR</td>
<td>Retinoid X Receptor</td>
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<td>Selective oestrogen receptor modulators</td>
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<td>siRNA</td>
<td>Short interfering ribose nucleic acid</td>
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<td>Triglitazone</td>
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<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>Transcription regulating domain</td>
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<td>TNF-related apoptosis inducing ligand</td>
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<td>TZD</td>
<td>Thiazolidinedione</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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</table>
ABSTRACT

Endometrial cancer is a common gynaecological cancer. Improving outcomes for women with advanced disease remains a challenge and there is also a need to develop preventative strategies in those women at highest risk of developing disease. Peroxisome proliferator-activated receptors (PPARs) comprise of a group of transcription factors belonging to the nuclear hormone receptor subfamily. PPAR sub-types are involved in metabolic homeostasis and have been implicated in malignancy, particularly breast and colo-rectal malignancies both of which are associated with obesity. Endometrial cancer is also closely associated with both obesity and insulin resistance. The work described in this thesis examined the expression of PPARs in endometrioid endometrial cancer and investigated their effects on key pathways implicated in this disease.

Immunoblotting revealed over expression of PPARα and loss of PPARγ in human endometrioid endometrial cancer tissues. Pull-down assays also demonstrated differential selectivity of different PPARs for heterodimerisation with different isoforms of the RXR family of transcription factors. PPARα was localized to tumour cells and vascular endothelium and ELISA demonstrated an increase in VEGF-A in PPARα silenced cells suggesting that PPARα may promote tumour angiogenesis. PPARγ was largely seen in epithelial cells and also macrophages within benign endometrium. Reduction of PPARγ expression in cultured endometrial cells led to increased proliferation and decreased apoptosis. Loss of PPARγ was correlated with a loss of the tumour suppressor PTEN in endometrial tissues. Furthermore, PPARγ silencing led to diminished expression of PTEN and a concomitant increase in phosphorylated AKT suggesting that PPARγ is protective against deregulated growth within the endometrium. Synthetic PPAR-specific ligands reduced proliferation and increased apoptosis in endometrial cell lines. These effects were present in PPAR-silenced cells too although reduced in magnitude, indicating that the actions of specific PPAR ligands are mediated via both receptor dependent and receptor independent pathways.

In conclusion, this work has demonstrated the differential expression of PPARs and RXRs in endometrial cancers and identified possible mechanisms, both direct and indirect, by which these may modulate endometrial cancer growth. Different PPAR family members may provide targets for therapeutic intervention in endometrial cancer care and require further study in this regard.
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ACKNOWLEDGEMENTS

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CHAPTER ONE
INTRODUCTION

1.0 Introduction
Endometrial cancer is the most common gynaecological cancer in developing countries (Ronnett et al., 2002). The incidence in the UK is increasing, with as many as 7,536 cases recorded per year (Office for National Statistics, Series MB1 no.38. 2010). The incidence of endometrial cancer peaks between 70-75 years of age (Ball and Elkadry, 1998). Women presenting below the age of 45 represent only 2-14% of cases (Vinker et al., 1999). Most women present early with post-menopausal bleeding and the overall 5-year mortality rate is lower than that reported for other malignancies e.g. ovarian and cervical cancer (Amant et al., 2005). It is for this reason that endometrial cancer has received less publicity than other more “lethal” cancers. Endometrial cancer is however, a serious health problem. In more advanced stages of the disease, 5-year mortality rates are similar to that of cervical cancer (Creasman et al., 1998). Furthermore with a rising incidence, more women can be expected to die from this disease. It is increasingly important that the biology of endometrial cancer is understood if preventive strategies are to be designed and improved treatments are to be developed.

1.1 Normal endometrium

1.1.1 Embryonic origins of the uterus
Sexual development of the human embryo starts with an indifferent stage, in which bipotential gonads and anlagen for both the male and female reproductive tract, the Wolffian and Mullerian ducts respectively, are present (Swain and Lovell-Badge, 1999). In females, the Wolffian ducts regress in the absence of testosterone, while the Mullerian ducts develop into the fallopian tubes, the uterus and the upper part of the vagina. In the ninth week of development, the uterus is formed by fusion of the caudal tips of the Mullerian duct into a single-lumen tube, the uterovaginal canal. This canal fuses with the sinovaginal bulb, resulting in formation of the uterus, cervix and vagina. At birth, the uterus of a female baby is temporarily enlarged by maternal oestrogens, which have crossed the placenta during pregnancy. This may result in vaginal bleeding. The uterus including the endometrium is
mesodermal in origin, and several weeks after birth, the uterus shrinks and remains dormant until puberty (Larsen, 1993).

1.1.2 Structure of normal endometrium

The endometrium has three histologically and functionally different layers, the basal, intermediate and superficial layers (figure 1.1) (Young et al., 2006). The basal layer (stratum basalis) directly contacts the myometrium (muscular layer) and is not shed during menstruation (Young et al., 2006). The broad intermediate layer has a characteristic spongy stroma, and is hence called stratum spongiosum (Young et al., 2006). The thinner superficial layer, with compact stroma, is called the stratum compactum (Young et al., 2006). The stratum compactum and spongiosum undergo changes during the menstrual cycle and are jointly referred to as stratum functionalis (Young et al., 2006). The functional layer surrounds the lumen of the uterine cavity and undergoes cyclic regeneration under the influence of the ovarian hormones. Within these layers, the endometrium is composed of three main elements; the surface epithelial cells that form glands, the connective tissue stroma and blood vessels (Ludwig and Spornitz, 1991).
Figure 1.1: Histological appearance of proliferative endometrium. C represents the stratum compactum, S represents the stratum spongiosum, B represents the stratum basalis and M represents the myometrium. (Taken from Wheater’s Functional Histology, 4th Edition, 2000).
1.1.3 Endometrial changes during the menstrual cycle

During the reproductive years, the uterus undergoes monthly cyclical changes caused by differential production and secretion of the ovarian steroid hormones, oestrogen and progesterone (Johannisson et al., 1987) (figure 1.2). These bind to steroid hormone receptors (oestrogen and progesterone receptors), which are transcription factors. During the proliferative phase, oestrogen receptor concentration is highest in the glandular epithelium (Lessey et al., 1988), resulting in increased proliferation, gland formation and vascular growth of the functional layer of the endometrium (Johannisson et al., 1987). The glands appear straight and thin amongst a compact and cellular stroma (figure 1.2) (Johannisson et al., 1987). The proliferative phase is followed by ovulation. Thereafter the secretory phase begins, characterised by high progesterone, produced by the corpus luteum, stimulating differentiation of the glandular cells of the endometrium (Young et al., 2006). Progesterone receptors are present in both epithelial and stromal cells but are scant in epithelial cells during the secretory phase (Critchley et al., 2002). If no pregnancy occurs, the corpus luteum regresses, which leads to a drop in circulating oestrogen and progesterone (Young et al., 2006). In response to the decreased hormonal levels, the spiral arteries in the endometrium constrict, resulting in ischaemia of the tissue, which eventually leads to sloughing of the functional layer of the endometrium (menses) (Young et al., 2006). The entire sequence of events from onset of one menstruation to the next lasts approximately 28 days although regular cycles of 21-35 days duration are also normal (Young et al., 2006).
Figure 1.2 The menstrual cycle: Levels of gonadotrophins (LH and FSH) and ovarian steroid hormones in relation to endometrial morphology (Image taken from Wheater’s Functional Histology, Fourth Edition, 2000).
1.1.4 The menopausal endometrium
Towards the end of reproductive life, there is a progressive failure of ovarian function associated with anovulation (Sherman and Korenman, 1975). Subsequently, there is a steep decline in the production of ovarian oestrogen production. Following menopause, the main source of oestrogens is androgenic adrenal steroids, which are converted by aromatase enzymes in peripheral fat tissues, to oestrone (Gusberg, 1994). Oestrone is a weaker oestrogen compared to oestradiol, thus resulting in myometrial and endometrial atrophy. In postmenopausal women, oestrogen-induced proliferation is no longer halted by progesterone-induced secretory change and differentiation and this may lead to endometrial hyperplasia and eventually endometrial cancer. Women are considered to be postmenopausal when they have had no menses for at least 12 months.

1.2 Endometrial cancer-clinical aspects
1.2.1 Epithelial endometrial cancer
Endometrial cancer is the most common gynaecological malignancy and the fourth most common malignancy in women in the developed world after breast, colorectal and lung cancer (Ronnett et al., 2002). Endometrial cancer develops mainly in postmenopausal women (Ball and Elkadry, 1998). Risk factors for the development of endometrial cancer are mostly related to increased oestrogen levels, either endogenous (such as late onset of menopause or oestrogen-producing tumours) or exogenous (oestrogen-only hormone replacement therapy) (Gitsch et al., 1995). Other risk factors include obesity, polycystic ovarian syndrome, nulliparity and diabetes mellitus. These factors are also associated with increased oestrogen levels (Akhmedkhanov et al., 2001). Furthermore, women who use tamoxifen, a selective oestrogen receptor modulator with oestrogenic effects on the uterus, have a 2-7 fold increased risk for the development of endometrial cancer (Cohen, 2004).

The prognosis of endometrial cancer is generally favourable. This is largely due to a majority of patients presenting with early stage disease. Particular tumour types, however, are characterised by aggressive behaviour and poor prognosis. Furthermore, later stage tumours with spread beyond the uterus have a much poorer outcome. The molecular pathogenesis of endometrial cancer remains incompletely understood. As in other
malignancies, however, the transition from normal endometrium to invasive carcinoma is thought to involve a stepwise accumulation of alterations in genes favouring cell proliferation, the inhibition of apoptosis, and promotion of angiogenesis (Enomoto et al., 1991).

1.2.2 Classification of Endometrial Cancer

Most endometrial cancer cases (90%) are sporadic (Berchuck and Boyd, 1995). Approximately 10% of endometrial cancer cases are familial, many of these being associated with hereditary nonpolyposis colorectal cancer (HNPCC), a dominantly inherited syndrome. Germ-line abnormalities in one of five DNA-mismatch repair genes result in micro-satellite instability and impaired DNA repair (Watson, 1994). Females with HNPCC have a ten-fold increased lifetime risk of endometrial cancer compared with that of the general population and the lifetime risk of endometrial cancer (40-60%) is higher than that for colorectal carcinoma (30%) (Dunlop et al., 1997). In fact 50% of women with HNPCC present with endometrial cancer as their index cancer (Lu et al., 2005). Hereditary endometrial cancer is more likely to occur at a younger age and is characterized by high FIGO (International Federation of Gynecology and Obstetrics) stage and grade, cribriform growth pattern, mucinous differentiation and necrosis (Parc et al., 2000).

Endometrial cancer is classified according to histological type, histological grade and stage.

Histological type

Several distinct histological types of primary endometrial cancer exist recognised by differing morphology and listed as follows (The Royal College of Pathologists 2010):

- Endometrioid adenocarcinoma
- Adenocarcinoma with squamous differentiation
- Mucinous adenocarcinoma
- Serous adenocarcinoma
- Clear cell carcinoma
- Undifferentiated carcinoma (large and small cell type)

- Mixed carcinoma

Over 80% of endometrial cancers are endometrioid adenocarcinomas. These often develop from endometrial hyperplasia on a background of excess oestrogen exposure (Emons et al., 2000).

**Histological grade**

Histological grade refers to the degree of architectural and nuclear abnormality and is based on the internationally recognised International Federation of Obstetrics and Gynaecology (FIGO) grading. Low grade tumours (FIGO grade 1) more closely resemble benign endometrium than those of high grade (FIGO grade 3). Tumours showing an intermediate degree of differentiation are FIGO grade 2. Tumour grade is also dependent upon indicators of nuclear abnormality such as high nuclear to cytoplasmic ratio, number of mitotic figures and nuclear pleomorphism (The Royal College of Pathologists, 2010). Cellular atypia takes precedence over architectural disruption as it is more predictive for a poorer outcome (The Royal College of Pathologists, 2010).

**Stage**

Tumour stage refers to the degree of tumour spread. Staging of endometrial cancer is based upon surgical findings together with subsequent histopathological reports. The FIGO staging criteria are shown in Table 1.1. FIGO staging criteria were updated in 2009. The pre-2009 and updated versions are shown in table 1.1. Biological samples that were used in this thesis were staged according to pre-2009 FIGO staging.

Classifying endometrial tumours by stage, grade (Creasman et al., 1987) and histological type (Christopherson and Gray, 1982) is important because these features together with vascular space invasion are used both for assessing prognosis and for determining the need for adjuvant treatment. The most important prognostic indicators are stage, depth of myometrial invasion and grade (The Royal College of Pathologists, 2010).
### Table 1.1: FIGO Staging of Endometrial Cancer.

A complete overview of staging for gynecological cancers can be found at [http://www.figo.org](http://www.figo.org).

<table>
<thead>
<tr>
<th>FIGO 2000</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage</strong></td>
<td><strong>Features</strong></td>
</tr>
<tr>
<td>I</td>
<td>Tumour confined to the corpus uteri</td>
</tr>
<tr>
<td>Ia</td>
<td>Tumour confined to the endometrium</td>
</tr>
<tr>
<td>Ib</td>
<td>Myometrial invasion &lt;50%</td>
</tr>
<tr>
<td>Ic</td>
<td>Myometrial invasion &gt;50%</td>
</tr>
<tr>
<td>II</td>
<td>Cervical involvement</td>
</tr>
<tr>
<td>IIa</td>
<td>Endocervical glandular involvement only</td>
</tr>
<tr>
<td>IIb</td>
<td>Cervical stromal invasion but not extending beyond the uterus</td>
</tr>
<tr>
<td>III</td>
<td>Extension beyond myometrium</td>
</tr>
<tr>
<td>IIIa</td>
<td>Carcinoma involves serosa of uterus or adnexae, or positive ascites, or positive peritoneal washings</td>
</tr>
<tr>
<td>IIIb</td>
<td>Vaginal involvement, either direct or metastatic</td>
</tr>
<tr>
<td>IIIc</td>
<td>Para-aortic or pelvic node involvement</td>
</tr>
<tr>
<td>IV</td>
<td>Further spread</td>
</tr>
<tr>
<td>IVa</td>
<td>Carcinoma involving the mucosa of the bladder or rectum</td>
</tr>
<tr>
<td>IVb</td>
<td>Distant metastases and involvement of other abdominal or inguinal lymph nodes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FIGO 2009</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stage</strong></td>
<td><strong>Features</strong></td>
</tr>
<tr>
<td>I</td>
<td>Tumour confined to the corpus uteri</td>
</tr>
<tr>
<td>Ia</td>
<td>No or less than half myometrial invasion</td>
</tr>
<tr>
<td>Ib</td>
<td>Invasion equal to or more than half of the myometrium</td>
</tr>
<tr>
<td>II</td>
<td>Tumour invades cervical stroma, but does not extend beyond the uterus</td>
</tr>
<tr>
<td>III</td>
<td>Local and/or regional spread of the tumour</td>
</tr>
<tr>
<td>IIIa</td>
<td>Tumour invades the serosa of the corpus uteri and/or adnexae</td>
</tr>
<tr>
<td>IIIb</td>
<td>Vaginal and/or parametrial involvement</td>
</tr>
<tr>
<td>IIIc</td>
<td>Metastases to pelvic and/or para-aortic lymph nodes</td>
</tr>
<tr>
<td>IIIC(1)</td>
<td>Positive pelvic nodes</td>
</tr>
<tr>
<td>IIIC(2)</td>
<td>Positive para-aortic lymph nodes with or without positive pelvic lymph nodes</td>
</tr>
<tr>
<td>IV</td>
<td>Tumour invades bladder and/or bowel mucosa, and/or distant metastases</td>
</tr>
<tr>
<td>IVa</td>
<td>Tumour invasion of bladder and/or bowel mucosa</td>
</tr>
<tr>
<td>IVb</td>
<td>Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes</td>
</tr>
</tbody>
</table>
1.2.3 Clinical behaviour of endometrial cancer

The recommended initial investigation is a transvaginal ultrasound for measurement of endometrial thickness and identification of ovarian masses. Increasingly, magnetic resonance imaging (MRI) is used in the preoperative imaging of women with endometrial cancer to assess the depth of myometrial invasion. Depth of invasion is related to the risk of lymph node involvement. Despite imaging as an aid to treatment planning, the staging remains surgicopathological, relying on surgical assessment of intra-abdominal disease and depth of myometrial invasion.

The conventional management of early stage endometrial cancer is surgery (extrafascial hysterectomy and bilateral salpingo-oopherectomy with peritoneal washings). Where pathological features indicate a high risk of recurrence, adjuvant radiotherapy is administered (Creutzberg et al., 2004). In cases of advanced disease in women unfit for surgery or where palliation is appropriate, radiotherapy is used. Cytotoxic chemotherapy is also used but responses are often partial and short lived (Thigpen et al., 2004). Hormonal treatment with progestogens can palliate bleeding in advanced endometrial cancer and the debate continues as to the benefit of endocrine therapy (Fiorica et al., 2004, Whitney et al., 2004).

1.2.4 The dualistic model of endometrial cancer

In the past two decades, clinico-pathological, immunohistochemical, and molecular genetic studies have provided data for the dualistic model of sporadic endometrial carcinogenesis. In this model, there are two distinct types of endometrial carcinoma, which have been designated as type I and type II carcinomas (Bokhman et al., 1985). It is thought that these are different. In this model, alteration or disruption of key stages in different pathways, lead to the development of different tumour types with differing phenotype and biological behaviour.

Much is known about the common molecular changes described but a challenge still lies in the development of a progression model for endometrial cancer to match the adenoma-
carcinoma model of colorectal carcinoma (Vogelstein et al., 1988). Two pathways of endometrial tumourigenesis provide a basic outline for such a model (figure 1.3).

Figure 1.3: Endometrial cancer progression model. This model demonstrates the potential events that may lead to the development of endometrial cancer.
1.2.5 Endometrial hyperplasia

Endometrial hyperplasia is defined as an overgrowth of both endometrial glands and endometrial stroma, and is characterised by a proliferative glandular pattern with or without different degrees of atypia (Mutter, 2000b). Increased oestrogen levels are associated with hyperplasia, as seen in polycystic ovarian syndrome (PCOS), oestrogen-secreting tumours and obesity (Montgomery et al., 2004). Treatment of atypical hyperplasia is either with progestins (antagonising oestrogen) or hysterectomy (Jadoul and Donnez, 2003). Atypical hyperplasia in at least 25% of patients without treatment will progress to endometrial cancer (Kurman et al., 1985). The widely used World Health Organization (WHO) system classifies endometrial hyperplasia according to four combinations of glandular crowding and nuclear atypia: simple (SH), complex (CH), simple atypical (SAH), or complex atypical hyperplasia (CAH), although the latter two forms are often grouped together. Diagnosis of endometrial hyperplasia can be problematic due to the following issues. First, the low inter-observer reproducibility hinders the ability of WHO-based classification to effectively guide clinical management. Second, approximately 50% of women diagnosed with AH have concurrent carcinoma (Janicek and Rosenshein, 1994). Clinically, most women with AH undergo hysterectomy as primary treatment, but non-surgical management can be effective. Third, data on progression risks for women who retain their uterus is extremely limited. These data highlight priority areas for future research, such as increasing the diagnostic reproducibility, improving the discrimination between AH and carcinoma, and identifying biomarkers to stratify risks or serve as indicators of response to clinical treatment.

1.2.6 Type I carcinoma

The majority, 70%–80% of endometrial carcinomas are designated type I carcinomas and are oestrogen-related tumours, as they seem to arise on a background of unopposed oestrogen stimulation (Ronnett et al., 2002). Type I carcinomas often develop from atypical endometrial hyperplasia and express oestrogen receptors (ER) and progesterone receptors (PR), and most patients have been exposed to prolonged, relatively low serum levels of oestrogen. The major causes of persistent exposure to oestrogen are obesity, polycystic ovary syndrome, unopposed oestrogen replacement therapy, or rarely, oestrogen-producing neoplasm such as ovarian granulosa cell tumour (reviewed by Clement and Young, 2002).
Histologically, most type I tumours are endometrioid adenocarcinomas of low histological grade (Lax, 2004). Rare mucinous adenocarcinomas are also classified as type I because they usually express ER and PR and are of low histological grade. Clinically, type I carcinomas are characterized overall by a favorable biological behaviour (Bokhman et al., 1985).

1.2.7 Type II carcinoma

Approximately 10%–20% of endometrial carcinomas designated as type II carcinomas, develop via an oestrogen independent pathway and on a background of atrophic endometrium (Ronnett et al., 2002). Hence, these tumours usually occur in older women, typically about 5–10 years older than those with type I carcinoma (Bokhman et al., 1985). Type II carcinomas are typically high-grade tumours with non-endometrioid histology, most frequently of serous type and less frequently clear cell carcinoma (Bokhman et al., 1985). Serum oesrogen levels are low and expression of ER and PR is usually negative. Serous carcinoma is frequently associated with endometrial intraepithelial carcinoma (EIC), which is considered as the putative precursor. EIC may also be found adjacent to clear cell carcinoma. Both serous and clear cell carcinomas have a more aggressive clinical course and poorer patient prognosis when compared to endometrioid tumours (Bokhman et al., 1985). Small cell carcinoma and squamous cell carcinoma are also included in type II tumours although their tumourigenesis remains largely unknown. This dualistic model of endometrial carcinoma has been widely accepted despite the fact that some carcinoma cases are not always easily classified into these two categories. For example, endometrioid adenocarcinoma of low grade can occur in the atrophic endometrium without hyperplasia (Mutter, 2000a).

This classification has also been justified at the molecular level with Type 1 tumours being more commonly associated with abnormalities of DNA-mismatch repair genes, k-ras, PTEN and beta catenin, and Type 2 tumours with abnormalities of p53 and HER2/neu (Table 2). However, these abnormalities are not present in all cases.
A: Well-differentiated endometrioid adenocarcinoma  
B: Villoglandular variant of endometrioid adenocarcinoma  
C: Serous adenocarcinoma of the endometrium

**Figure 1.4: Representative examples of differing endometrial histology** (Amant et al., 2005)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unopposed Oestrogen</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Background Endometrium</td>
<td>Hyperplastic</td>
<td>Atrophic</td>
</tr>
<tr>
<td>Morphology</td>
<td>Endometrioid</td>
<td>Serous, clear cell</td>
</tr>
<tr>
<td>Genetic Abnormalities</td>
<td>MSI, PTEN, K-ras, β-cat</td>
<td>P53, HER2/neu</td>
</tr>
</tbody>
</table>

**Table 1.2:** Clinico-pathological characteristics and genetic abnormalities in Type 1 and Type 2 endometrial carcinomas
1.3 Endometrial cancer – genetic and molecular aspects

1.3.1 Genetic abnormalities of endometrioid endometrial cancers

In addition to ER and PR signaling, other genetic alterations are reported to be (potentially) involved in the initiation and progressive development of endometrioid endometrial cancer and its precursor, endometrial hyperplasia. Microsatellite instability (MI) occurs in 20% to 30% of cases, PTEN mutations in 30% to 60%, RAS mutations in 10% to 30%, and β-catenin mutations with nuclear accumulation in 28% to 35% of endometroid cancers (Bussaglia et al., 2000; Lax and Kurman, 1997; Matias-Guiu et al., 2001; Sherman, 2000) (Fig. 1.3).

![Figure 1.3](image)

Figure 1.3 Main molecular alterations found in endometroid carcinomas. Reported frequencies of microsatellite instability (MI), and mutations in PTEN, RAS and β-catenin genes. (figure adapted from Matias-Guiu et al. 2001)

1.3.2 Microsatellite instability.

Microsatellite DNA sequences are short-tandem repeats that are distributed throughout the genome. Due to their repeating nature, these microsatellites are particularly prone to errors during replication, leading to instability of parts of the genome (microsatellite instability) (Aaltonen et al., 1993; Ionov et al., 1993). The genes shown to be responsible for Microsatellite Instability (MI) encode proteins involved in DNA mismatch repair (MLH1, MLH2, PMS1 and PMS2) (Salvesen et al., 2000). Mutations of these genes alter the ability
of the cells to repair errors during replication. In the case of DNA repair gene dysfunction, genes with microsatellite DNA sequences are easily mutated (Esteller et al., 1999, Lluis Catasus, 2000).

MI was initially observed in tumours from patients with the hereditary nonpolyposis colon cancer syndrome (HNPCC), (Ichikawa et al., 1999). Patients from HNPCC families have an inherited germ line mutation in either MLH1 or MSH2 (Aaltonen et al., 1993; Ionov et al., 1993). Endometrial cancer is the index cancer in women with HNPCC in 50% of cases (Lu et al., 2005), and the occurrence of MI in endometrial cancers from HNPCC patients is 75% compared to 25% to 30% in sporadic endometrial cancers (Aarnio et al., 1995). Mutations in the MLH1 or MSH2 DNA-repair genes themselves are rarely identified in sporadic endometrial cancers (Katabuchi et al., 1995; Kowalski et al., 1997; Simpkins et al., 1999). Therefore it is assumed that in these tumours functional loss of the MLH1 and MSH2 genes is accomplished through methylation of the promoter of these genes, which may result in MI (Salvesen et al., 2000).

1.3.3. PTEN mutations

The tumour suppressor gene PTEN (phosphatase and tensin homologue deleted from chromosome 10) is located on chromosome 10q23 (Di Cristofano and Pandolfi, 2000). PTEN encodes a member of the tyrosine phosphatase family and inhibits the PI3K/AKT signaling pathway (Mutter et al., 2000). Because of this, activated PTEN is involved in inducing apoptosis. Loss of PTEN function results in less apoptosis, and therefore PTEN may stimulate tumour progression (Downward, 2004). Loss of PTEN function (either partial or complete) seems to be an early event in carcinogenesis, since it is detected in both endometrial hyperplasias as well as endometrial cancers (Mutter, 2000a, Kinzler and Vogelstein, 1997).

In endometrial cancers loss of heterozygosity (LOH) at the locus of the PTEN gene, is found in 40% of tumours (Jones et al., 1994; Peiffer et al., 1995). Besides loss of PTEN expression due to LOH, somatic PTEN mutations are also found in endometrial tumours. The occurrence is 37% to 60%, and almost exclusively in endometroid tumours (Tashiro et al.,
Loss of expression of PTEN in the absence of LOH on locus 10q23.3 may be caused through aberrant promoter methylation (Matias-Guiu et al., 2001; Salvesen et al., 2001).

1.3.4 RAS mutations

The proto-oncogene RAS is involved in cell proliferation. RAS activates the MAP kinase signaling pathway and subsequently several growth factor systems are activated (Hill et al., 1993). Mutations of the k-ras gene have been identified in 10% to 30% of endometrial cancers, mainly in tumours of endometroid type (Matias-Guiu et al., 2001). Since RAS mutations are, like PTEN mutations, found in endometrial hyperplasias and endometrial cancers, these mutations might also be an early step during carcinogenesis (Lagarda et al., 2001).

1.3.5 β-catenin mutations

Mutations in the β-catenin gene have been reported in 28%-35% of endometroid carcinomas (Matias-Guiu et al., 2001). β-catenin complexes together with E-cadherin. In this form, it is associated with the cell membrane, playing a major role in cell-cell adhesion. Another function of β-catenin is that it is a key component in the Wnt signaling pathway. This pathway is important in embryogenesis but is also implicated in carcinogenesis (Pennisi, 1998).

As shown in figure 1.5, in many endometroid tumours, MI, PTEN and RAS mutations coexist. Mutations in the β-catenin gene, however, are less generally associated with the other alterations (Matias-Guiu et al., 2001).

1.3.6 Genetic alterations in non-endometroid tumours

For the initiation and development of non-endometroid endometrial cancer, mutations in the p53 gene and LOH on several chromosomes seem more important (Tashiro et al., 1997b, Sherman and Korenman, 1975). The tumour suppressor gene p53 functions as a G1-S cell cycle checkpoint. It induces a G1 arrest thereby creating extra time for DNA repair mechanisms. If DNA-repair fails, p53 can initiate cell death via apoptosis (Lane, 1992). If p53 is inactivated through mutations or deletions, DNA repair will not proceed as planned, and mutations may be introduced that can lead to a more aggressive cancer type, while the
second line in defense, apoptosis, is not induced. Mutations in the p53 gene are described in up to 90% of non-endometrioid tumours (Tashiro et al., 1997b). It has also been suggested that development of a non-endometrioid tumour can also arise as a result of de-differentiation of a pre-existing endometrioid tumour, since tumours are described which contain both endometrioid as well as non-endometrioid features (Tashiro et al., 1997b).

Although the classification of endometrial cancers into endometrioid (type I) tumours and non-endometrioid (type II) tumours is relevant to most cases, it is nonetheless artificial and exceptions do occur. In some endometrial cancers overlap is observed in clinical, morphological, immunohistochemical and molecular features between endometrioid and non-endometrioid tumours.

1.4 Malignancy, metastasis and the cell cycle

Tumours have been defined as “an excessive, pathological proliferation of cells that continues indefinitely in the absence of pathological stimuli without regard to the effect on the surrounding tissues or the requirements of the individual” (Anderson, 1985). Tumours that are malignant also penetrate the basement membrane, enter the bloodstream, and hence, metastasise. Cancer development is a multi-step and complex event, requiring:

1. Deregulation of cell cycle control/growth transformation
2. Dissociation of tumour cells
3. Degradation of the extracellular matrix
4. Angiogenesis
5. Metastasis

Proto-oncogenes, tumour suppressor genes and growth factors all contribute to the above processes (Evan and Vousden, 2001). As the cell progresses into malignancy, accelerated proliferation is seen and this leads to genomic instability (Hoeijmakers, 2001). This is known as tumour progression.
1. De-regulation of the cell cycle/growth transformation

Howard and Pelc, 1951, were the first to describe the normal cell cycle, following studies of radiolabelled phosphorus uptake of bean roots (Howard and Pelc, 1951). Control of cell proliferation is highly complex and involves checkpoints, which regulate the transition of each cell through different stages of the cell cycle. The G1 to S phase checkpoint is the most frequently deregulated in human cancers (Bartek et al., 1999). Critical cell cycle regulators are D-class cyclins, cyclin-dependant kinases (CDKs) and CDK inhibitors. These are controlled by regulatory elements including the E2F proto-oncogene and the retinoblastoma tumour suppressor (Bartek et al., 1999).

Proto-oncogenes encode proteins that are normally present in host cells. They form signal transduction pathways e.g. growth factor receptors, extracellular growth factors (Wallis and Macdonald, 1999). Oncogenes are generated by activation of their corresponding proto-oncogene during tumour development (Wallis and Macdonald, 1999). Oncogene activation can be quantitative with an increased production of normal protein (Wallis and Macdonald, 1999). Qualitative activation involves secretion of altered protein product by point mutations (Wallis and Macdonald, 1999). Both quantitative and qualitative activations lead to increased cellular proliferation and therefore increased susceptibility to cancer (Wallis and Macdonald, 1999).

Proto-oncogenes encode tyrosine kinases, intracellular signalling molecules and nuclear transcription factors (Wallis and Macdonald, 1999). The receptors for vascular endothelial growth factor family (VEGFs) are receptor tyrosine kinases (RTKs) (Ferrara et al., 2003). VEGF signalling through these receptors is implicated in the development of tumour blood vessels (Ferrara et al., 2003).

In endometrial cancer, VEGF receptors are abundantly expressed in blood vessels adjacent to endometrial cancers but not in the tumour cells themselves (Guidi et al., 1996). The role of VEGF in the development and/or growth will be discussed later in this thesis.

Tumour suppressor genes encode proteins that exert cellular growth inhibition (Bandera and Boyd, 1997). A loss in function of a tumour suppressor gene occurs when one allele is mutated and the other allele is deleted (Bandera and Boyd, 1997). Common tumour
suppressor genes are p53 and PTEN. Abnormal cell cycling due to oncogene activation and/or loss of tumour suppressor function increases the opportunity for further defects in the cell process (Hoeijmakers, 2001).

2. Dissociation of tumour cells
Malignant cells need the potential to be able to detach from surrounding cells, in order to spread (Hart and Saini, 1992). Cell-adhesion molecules, including integrins and cadherins, mediate cell matrix and cell-cell attachment. Cadherins, not only influence cell shape and motility (Takeichi, 1992) but also disrupt cell-cell attachments, through an invasive phenotype (Hart and Saini, 1992).

3. Degradation of the extracellular matrix (ECM)
In order for migration of tumour cells to occur, disruption of the ECM and entry into the vascular and lymphatic circulation is necessary, in addition this process needs to happen again for malignant cells to enter the new organ/tissue (Hart and Saini, 1992). Proteolytic enzymes are essential as they digest cell-cell and cell-matrix proteins. These include matrix metalloproteinases (MMPs), cathepsins and serine proteases (Chang and Werb, 2001). Tumour and stromal cells can excrete these proteolytic enzymes, in order to increase their invasiveness (reviewed in Chang and Werb, 2001).

4. Angiogenesis
Angiogenesis, the development of new vessels from existing vessels, appears to be a requirement early in tumour growth and for metastasis (Folkman, 1995). Several angiogenic factors have been identified. Some are produced by tumours, whilst others recruit and activate inflammatory cells and macrophages to produce angiogenic factors (Abulafia et al., 1995). High intra-tumour microvessel density in EC is associated with advanced clinical stage and increased risk of recurrent disease and therefore, poor prognosis (Kirschner et al., 1996). Furthermore, a progressive increase in microvessel density from benign endometrium
Figure 1.6: The cell cycle

Cell division occurs in the M phase, through mitosis. The cell then enters the resting phase (G0). When growth signal or mitogens appear, the cell enters G1 and cell division and protein synthesis occur. Prior to the S phase there is a checkpoint, at which if the cell arrests it will be committed to apoptosis. A second checkpoint lies between the G2 and M phases. Its purpose is to ensure that successful DNA replication and chromosome segregation occurs. Damaged cells may enter apoptosis at this stage.
through atypical complex hyperplasia to invasive disease has been reported suggesting that this may be a target/time at which to interrupt the process of cancer formation (Abulafia et al., 1995).

Hypoxia-inducible factor, HIF-1, is the key regulator of the angiogenic factor vascular endothelial growth factor (VEGF). The vascular endothelial growth factor family comprises four members, VEGF-A, VEGF-B, VEGF-C and VEGF-D. The most potent angiogenic factor is VEGF-A. Increased VEGF-A expression is found in many tumours including EC (Doldi et al., 1996). Whereas, VEGF-A is highly expressed in EC, it is only rarely identified in benign endometrium from postmenopausal women (Holland et al. 2003).

VEGF-A appears to be an important indicator of poor prognosis in patients with EC. Another member of the VEGF family, VEGF-B, forms homodimers and can heterodimerise with VEGF-A. In contrast to VEGF-A, VEGF-B expression in EC has been found to be significantly lower than in benign endometrium (Holland et al. 2003). Loss of VEGF-B expression might contribute to endometrial tumourigenesis, although this remains to be clarified. Other studies have shown that the presence of VEGF-C or VEGF-D appear to be associated with lymphangiogenesis rather than angiogenesis. VEGF-C or VEGF-D and its receptor VEGFR-3 may predict myometrial invasion and lymph node metastasis in EC and may prospectively identify patients who are at increased risk for poor outcome (Hirai et al., 2001, Yokoyama et al., 2003).

5. Metastasis

Malignant cells, after circulating in the bloodstream, arrive at their target organs (Hart and Saini, 1992). Cells must attach to the vascular endothelium and invade the vessel wall of the target organ. If the metastasised tumour is to survive, the malignant cells must establish themselves within the stroma and develop an independent microvascular network (Hart and Saini, 1992).
1.5 Hormonal aspects of endometrial cancer

Steroid hormones are central to endometrial physiology, regulating the menstrual cycle and controlling the regular shedding of endometrium until the time of menopause. It has been recognised that there is an association between the steroid hormone, oestrogen, and the development of endometrial cancer. (Gusberg, 1989). As mentioned previously, risk factors include early menarche and late menopause, nulliparity and polycystic ovarian syndrome, all of which are characterised by prolonged periods of unopposed oestrogen stimulation on the endometrium. Retrospective studies have shown an increased risk for the development of endometrial cancer in women taking exogenous oestrogens (Ziel and Finkle, 1975). Women taking unopposed oestrogen are at highest risk. It is for this reason that the addition of progestogens for days 10-12 is recommended (Voigt et al., 1991).

1.5.1 General structure of oestrogen and progesterone receptor

Oestrogen and progesterone exert their actions by binding to receptors. The oestrogen receptor (ER) and progesterone receptor (PR) are members of the steroid hormone receptor family, and act as hormone-dependent activators of transcription. Two ERs are described, ERα and ERβ (Green et al., 1986, Kuiper et al., 1996). ERα (60-66kD protein) and ERβ (51-61kDa protein) are encoded by two different genes (located at chromosome 6 and chromosome 14, respectively). They display considerable homology at the protein level: 96% in the DNA-binding domain and approximately 60% in the ligand-binding domain (Kuiper et al., 1996) (Figure.1.7).

The progesterone receptor exists as two isotypes, PRA and PRB, that are transcribed form two distinct promoters in the same gene, located on chromosome 11 (Kastner et al., 1990). PRA (94kDa protein) is a truncated form of PRB (114kDa protein), lacking the first 164 amino acids at the N-terminus (Kastner et al., 1990).

In both ERs and PRs several functional domains can be distinguished. These are the transcription regulating domain (TRD), DNA binding domain (DBD), hinge region (H), ligand binding domain (LBD), activation function domain (AF1-3) and an inhibitory domain (ID) (Figure.1.7).
The TRD, located in the amino-terminal (NH3) part of the receptor, is involved in modulation of transcription. The DBD contains two zinc finger motifs, and binds to specific hormone-response elements (HRE) in the genome, and the LBD, located at the carboxyl terminal (COOH) part of the receptor, is responsible for ligand binding (McKenna and O’Malley, 2000). Between the DBD and LBD lies the hinge region, which contains a nuclear localization signal, and is involved in receptor dimerization. Several activation function (AF) domains are located within these receptors. These domains act as docking sites for interaction with several cofactors and transcription factors. Upon ligand binding in the LBD, the family of SRC cofactors (steroid receptor co-activators), interact with AF-2 to stimulate transcription (McKenna and O’Malley, 2000). AF-1, however, is involved in ligand-independent activation of transcription. AF-1 can act either dependent or independent of AF-2; but after binding of ligand, these two AF domains act synergistically (Giangrande and McDonnell, 1999).

1.5.2 Mode of action

In the absence of ligands, the oestrogen and progesterone receptors are associated with heat shock proteins. After ligand binding, the conformation of the receptor changes and a
dissociation of heat shock proteins occur. The receptor-hormone complex forms dimers, of which both homodimers (for example ERα-ERα) as well as heterodimers (for example ERα-ERβ) can be formed. In the absence of ligands, both ERα and ERβ are located in the nucleus of the cell, while un-liganded PR can be present in either the cytoplasm or the nucleus (Lim et al., 1999). After binding of ligand, however, the receptors are activated (mainly through phosphorylation) and both receptors (ER and PR) predominantly localize to the nucleus. In the nucleus the dimers bind to specific hormone response elements (HREs, oestrogen response element (ERE) or progesterone response element (PRE)) on the DNA. After binding of the receptor-dimer complex to the DNA, several co-activators, co-repressors and/or transcription factors bind, after which target genes are transcribed.

Approximately one third of genes in humans that are regulated by ERs do not contain ERE-like sequences (O’Lone et al., 2004). Besides the classical transcription route, ERs can regulate transcription via modulation of other transcription factors through protein-protein interactions in the nucleus, e.g., activator protein 1 (AP-1) transcription complex (Gottlicher et al., 1998). Data has accumulated indicating that the effects of oestrogen and progesterone are not only a result of transcription activation through ERα or ERβ or PRA or PRB respectively, but also through cross-talk of the ERs or PRs with several intracellular signaling pathways, like the MAP-kinase/RAS/PI3-kinase and IGF1 receptor signal transduction pathways (Zhang et al., 2004b). In these, so called non-genomic effects, oestrogen or progesterone bind to receptors that are located in or near the membrane. It has been postulated that in this situation the ERs and PRs themselves translocate to the vicinity of the plasma membrane. Although evidence indicates the presence of membrane-linked receptors, these studies suffer from technical limitations. In most studies transient transfection systems are used, in which, due to the high amounts of molecules in the cell, the receptors may artificially be localized at non physiological sites in the cell (Simoncini and Genazzani, 2003).

1.5.3 Expression of ER and PR in the endometrium

Endometrioid type tumours are oestrogen-related tumours, since risk factors for the development of endometrioid-cancer can all be related to oestrogen exposure. Most
endometrioid tumours express both oestrogen receptors (ERα and ERβ) and progesterone receptors (PRA and PRB) (Lax, 2004). In the normal endometrium, ERα is much more highly expressed compared to ERβ (approximately 100:1). During the menstrual cycle the expression of both ERα and ERβ are decreased in the late secretory phase compared to the proliferative phase (Mylonas et al., 2004). Expression of ERα and ERβ also decreases with age in the endometrium of postmenopausal women but it does remain expressed at lower levels (Koshiyama et al., 1996), but expression in endometrial polyps and in well-differentiated endometrial cancers remains high (Sivridis and Giatromanolaki, 2004). Expression patterns of PRA and PRB also vary during the menstrual cycle. Highest levels of expression are found during the second half of the proliferative phase, although variation in expression is much higher for PRB (20-fold) than for PRA (5-fold) (Arnett-Mansfield et al., 2001).

In endometrial cancer, a selective loss of ERα is typically observed going from low grade tumours (G1 and G2) to high-grade tumours (G3) (Fujimoto et al., 2002). It is possible that with loss of ERα, other factors become more important for tumour growth in high grade disease (Utsunomiya et al., 2000). Since high-grade endometrial tumours still express ERβ, signalling via ERβ may be an important factor (Utsunomiya et al., 2000).

It is generally agreed that expression of PRA and PRB is usually lost during progression of the endometrial cancer towards a more poorly differentiated phenotype (Arnett-Mansfield et al., 2001). However, it is still a matter of debate whether the altered expression ratio between PRA and PRB affects the behaviour of the tumour. Hanekamp et al. have shown that endometrial cancer cell lines containing only PRB are more invasive in vitro and in vivo compared with PRA-only or PRA/PRB cell lines suggesting PRB is more important than PRA in endometrial cancer development (Hanekamp et al., 2005).

1.6 Oestrogen, SERMs and anti-oestrogens

Oestrogens (mainly 17β-estradiol) are major regulators of many important biological functions (Hess et al., 1997). The production of 17β-estradiol takes place mainly in the ovaries, with concentrations varying throughout the menstrual cycle (Sherman and
Korenman, 1975). At the end of fertile life (post menopause), no growing follicles are left in the ovary and a decline in circulating oestrogens is found. Reduced oestrogen levels may cause hot flushes and negatively affect cognitive functioning and bone homeostasis and are difficult to correct for because substitution with exogenous oestrogens will cause a significant increment in endometrial cancer incidence (Berger and Fowler, 1977). Therefore, development of synthetic ER ligands displaying only the beneficial effects of estradiol (E2), has intensified over the last decades. These compounds are called Selective Oestrogen Receptor Modulators (SERMs). Tamoxifen and raloxifene are the most widely used (Berger and Fowler, 1977).

The actions of SERMs may be partly explained by differential action on the AF-1 and AF-2 transactivation function ER domains. Experiments in cell lines show that when both activation functions are required for transcriptional activity, tamoxifen acts as an antagonist on ERE-reporter gene constructs (Katzenellenbogen et al., 1996). In contrast, where only AF-1 is required for transcription, tamoxifen acts as an agonist. This has led to the conclusion that tamoxifen is an agonist of AF-1 but an antagonist of AF-2 (Katzenellenbogen et al., 1996).

1.6.1 Tamoxifen and the endometrium

Tamoxifen is a commonly used treatment for women with breast cancer. The 1998 EBCTCG (Early Breast Cancer Trialists’ Collaborative Group) overview, showed that tamoxifen-use for 5 years in women who had an oestrogen receptor positive breast cancer reduced the risk of relapse by 37-54% depending on age. Unfortunately, tamoxifen use is associated with an increased incidence of endometrial pathology, including endometrial cancers.

Tamoxifen inhibits the growth of breast cancer cells by competitive antagonism of oestrogen at ERα or Erβ (Bryant et al., 2005). In the endometrium, tamoxifen has an effect that varies with the ambient concentration of oestrogen. In premenopausal women (high oestrogen levels) tamoxifen displays an oestrogen antagonistic effect, while in postmenopausal women (low oestrogen levels) tamoxifen displays an oestrogen agonistic mode of action (Bergman et al., 2000, Chang et al., 1998). Because of this, in postmenopausal tamoxifen-treated
breast cancer patients, the total incidence of endometrial pathologies is as high as 36% (Cohen, 2004). The most common endometrial pathologies seen with tamoxifen treatment are hyperplasias (incidence 2.15 to 30.3%) and polyps (incidence 5.38% to 36%). For the development of endometrial cancer in postmenopausal breast cancer patients using tamoxifen, the relative risk is estimated at 2-7 fold compared to non-users (Bergman et al., 2000).

1.7 Oestrogen and endometrial cancer
The theory that describes the relationship between endogenous steroid hormones and endometrial cancer is the "unopposed oestrogen" hypothesis (Siiteri, 1978). This states that the risk of endometrial cancer is increased in women with high bioavailable plasma oestrogen and/or low progesterone. In the absence of progesterone, oestrogen increases the mitotic activity of endometrial cells and promotes increased DNA replication and subsequent replication errors. Uncontrolled proliferation increases the likelihood that mutations accumulate in both proto-oncogenes as well as tumour suppressor genes. If apoptosis is also impaired, cells with these mutations can survive and expand clonally, thus allowing the mutations to accumulate until hyperplasia or malignancy is reached.

1.8 Obesity and endometrial cancer risk
Endometrial cancer is mainly a disease of the affluent, developed world. Epidemiological studies have shown that > 40% of endometrial cancers may be attributed to excess body weight (Bergstrom et al., 2001). Lack of physical activity may also play a part (Ball and Elkadry, 1998). Alterations in endogenous hormone metabolism may link endometrial cancer risk, excess body weight and physical inactivity (Akhmedkhanov et al., 2001). Increased endometrial cancer risk is seen in both pre- and postmenopausal women who have elevated plasma androstenedione and testosterone, and postmenopausal women who have increased levels of oestrone and oestradiol (Tornberg and Carstensen, 1994). Adiposity is inversely related to testosterone concentrations in men, but positively related in women suggesting sex differences may be relevant in the association between BMI and cancer risk.
(Key et al., 2003). Chronic hyperinsulinaemia is also a risk factor (Potischman et al., 1996). The relationship between excess weight and cancer risk is not fully understood, though three hormonal systems have been implicated; insulin and insulin-like growth factor axis, sex steroids and adipocytokines (Renehan et al., 2008a). Adiponectin is the most abundant adipocytokine. It is secreted mainly from visceral fat adipocytes, and is inversely correlated with BMI (Renehan et al., 2008a). Mean circulating concentrations are higher in women than men. Adiponectin is an insulin-sensitising agent which has anti-angiogenic and anti-inflammatory properties and has been shown to inhibit tumour growth (Rose et al., 2004).

1.9 Insulin and endometrial cancer

Many epidemiological studies have shown an increased risk of endometrial cancer in women with non-insulin dependent diabetes mellitus (NIDDM) (O'Mara et al., 1985). This disease occurs through many years of increasing insulin resistance, elevated fasting and non-fasting plasma insulin (O'Mara et al., 1985). Many mechanisms may link elevated insulin levels to endometrial cancer. For example, there is evidence that insulin can act as a growth factor, with effects similar to Insulin-like growth factor-1 (IGF-1) (Renehan et al., 2006). Tumour tissues, including endometrial tumours, have increased levels of IGF-1 receptors as well as insulin receptor content (Talavera et al., 1990). There is evidence that insulin increases IGF-1 activity in endometrial tissue by suppressing the expression of endometrial insulin growth factor binding protein-1 (IGFBP-1) (Irwin et al., 1993). Insulin also stimulates ovarian (and possibly adrenal) androgen synthesis, especially in women with polycystic ovarian syndrome (PCOS) with resulting conversion to oestrogen in excess (Franks et al., 1991). Furthermore, chronic hyperinsulinaemia may promote tumorigenesis in oestrogen-sensitive tissues, since it reduces blood concentrations of sex-hormone-binding globulin, and in turn, increases bio-available oestrogen (Calle and Kaaks, 2004).
1.10 Peroxisome proliferator activated receptors (PPARs)

1.10.1 PPARs as nuclear hormone receptors

The increasing prevalence of obesity and diabetes, and the steady increase in incidence of endometrial cancer suggests that the biological activity of adipose tissue is an important factor in determining endometrial cancer risk. A genome wide analysis of the endometrial cancer transcriptome identified that differential expression of members of the peroxisome proliferator-activated receptor (PPAR) transcription family in endometrial cancers (Holland et al., 2004). This is of interest as PPAR function is linked with both fat metabolism and insulin regulation and may therefore be relevant in the development of endometrial cancer (Berger and Moller, 2002).

PPARs are members of the nuclear hormone receptor superfamily, of which ER and PR are members (Berger and Moller, 2002). They are generally involved in the regulation of inflammation and energy homeostasis and are implicated in obesity, obesity-induced inflammation, and the metabolic syndrome of insulin resistance (Berger and Moller, 2002). Currently, synthetic PPAR agonists are used for the treatment of insulin resistance and dyslipidaemia (Rubenstrunk et al., Brown and Plutzky, 2007).

The PPARs are orphan members of the nuclear receptor family (Desvergne and Wahli, 1999). Nuclear hormone receptors comprise a large superfamily of ligand-modulated transcription factors that mediate response to steroids, retinoids, thyroid hormones and vitamin D (Desvergne and Wahli, 1999). These receptors play key roles in development, cell differentiation, and organ physiology (Beato et al., 1995). Unlike water-soluble peptide hormones and growth factors, which bind to cell surface receptors and activate a cascade of second messengers, fat soluble steroid hormones enter the cell by simple or facilitated diffusion and transduce their signals to the genome via these intracellular receptors (Mangelsdorf and Evans, 1995a). After binding to its receptor, the hormone-receptor complex translocates from the cytoplasm to the nucleus, inducing an allosteric change that enables the complex to bind to high affinity sites in the chromatin, and modulate
transcription (Forman et al., 1995). Nuclear receptors can also bind to DNA in the absence of a ligand (Mangelsdorf and Evans, 1995a).

The superfamily of nuclear hormone receptors is often divided into the steroid receptor family and the thyroid/retinoid/vitamin D (or non-steroid) receptor family (Desvergne and Wahli, 1999). Each type of receptor constitutes a subfamily (eg. PPAR subfamily). Receptor subtypes are the products of individual genes (eg. PPARα), and receptor isoforms are the products of alternate splicing, promoter usage, or both (eg., PPARγ1, PPARγ2) (Mangelsdorf and Evans, 1995b). The nuclear receptor superfamily can be broadly grouped into four classes based on their dimerisation and DNA binding properties (Mangelsdorf and Evans, 1995b). Class I receptors include the steroid hormone receptors which function as ligand induced homodimers and bind to DNA half-sites organised as inverted repeats. Class II receptors heterodimerize with the retinoid X-activated receptor (RXR) and characteristically bind to direct repeats. Class III receptors bind primarily to direct repeats as homodimers. Class IV receptors typically bind to extended core sites as monomers (Mangelsdorf and Evans, 1995b). PPARs are class II receptors (Mangelsdorf and Evans, 1995b).

1.10.2 PPAR structure

To date, three different types of PPARs have been identified in vertebrates, PPARα, PPARβ (also known as PPARδ) and PPARγ (Berger and Moller, 2002). They are encoded from separate genes and have distinct tissue distribution and function. In 1987, a peroxisome proliferator-binding protein in rat liver was identified and characterised (Lalwani et al. 1987). Subsequently, the first peroxisome proliferator-activated receptor (PPAR) was cloned from mouse liver (Isseman and Green, 1990), followed by other PPAR homologs in several species (Tontonoz et al., 1994). The potency of ligands and chemicals to activate PPARs is subtype specific and expression varies widely in a tissue-specific manner, as demonstrated in Table 3. PPARs are normally localised in the nucleus and upon ligand binding conformational changes occur which promote the transactivation of target genes. Like other nuclear receptors, PPARs have a structure consisting of six functional domains, A/B, C, D, and E/F similar to steroid receptors (figure 1.7) (Evans, 1988).
1.10.3 Mechanism of action of PPARs

PPARs generally regulate the expression of genes involved in metabolic homeostasis, specifically the regulation of fat and carbohydrate metabolism (Riesset et al., 1999). Naturally occurring PPAR ligands include fatty acids and eicosanoids. A number of pharmacological compounds also activate PPARs, including fibrates (which bind preferentially to PPARα receptors) and thiazolidinediones (TZDs); which bind preferentially to PPARγ receptors (Lee et al., 1995, Berger and Moller, 2002). These physiologic and pharmacologic ligands activate PPARs by binding to specific pockets within the receptor, allowing it to recruit and form a heterodimer with the retinoid X receptor (Yki-Jarvinen, 2004).

PPAR/retinoid X receptor heterodimers activate transcription of genes by binding to peroxisome proliferator response elements (PPREs) found in the promoters of a number of genes involved in lipid and glucose metabolism (Chinetti et al., 1998b, Berger and Moller, 2002). This process is called transactivation. Alternatively, PPARs may also down-regulate the expression of genes involved in the inflammatory response. This process, called transrepression, occurs by recruitment of transcription factors necessary for gene expression (Berger and Moller, 2002).
Table 1.3: An overview of the metabolic roles of the 3 PPAR isoforms

<table>
<thead>
<tr>
<th></th>
<th>PPAR α</th>
<th>PPAR γ</th>
<th>PPAR β/δ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sites of highest</strong></td>
<td>Liver, kidney and heart</td>
<td>Adipose tissue, macrophages</td>
<td>Adipose tissue, skin, brain, but widespread</td>
</tr>
<tr>
<td><strong>expression</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cellular processes</strong></td>
<td>Fatty acid β-oxidation, lipoprotein synthesis, amino acid metabolism</td>
<td>Adipocyte differentiation, triglyceride synthesis</td>
<td>Fatty acid β-oxidation</td>
</tr>
<tr>
<td><strong>activated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Physiological</strong></td>
<td>Coordination of metabolic response to fasting</td>
<td>Differentiation of adipocytes</td>
<td>Muscle fibre type determination</td>
</tr>
<tr>
<td><strong>function</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Examples of target</strong></td>
<td>HMG CoA synthase</td>
<td>Fatty acid-binding protein 4, lipoprotein lipase, adiponectin</td>
<td>Acyl-CoA oxidase</td>
</tr>
<tr>
<td><strong>genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Metabolic phenotype</strong></td>
<td>Fasting hypoglycaemia, hypothermia, hypoketonemia and hepatic steatosis</td>
<td>-/- Lethal, +/- more insulin sensitive at baseline</td>
<td>Reduced baseline adipocity; increased obesity on high fat feeding</td>
</tr>
<tr>
<td><strong>of knockout mice</strong></td>
<td></td>
<td></td>
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</tbody>
</table>
Figure 1.8: Basic mechanism of PPARs as ligand-activated transcription factors. Upon ligand binding, PPARs form heterodimeric complex with RXR, that binds to the PPRE and drives the transcription of target genes (Berger and Moller, 2002).
1.11 PPARα

PPARα was the first to be cloned among the PPAR family (Isenberg et al., 1997). It is expressed in tissues with a high metabolic rate such as liver, heart, skeletal muscle and brown adipose tissue (Isenberg et al., 1997). Such distribution is consistent with its role as a regulator of energy expenditure (Isenberg et al., 1997). PPARα is critical in mobilising energy resources by activating fatty acid catabolism and stimulating hepatic gluconeogenesis (Kersten et al., 2000). Other effects include attenuation of the inflammatory response and regulation of metabolism and urea synthesis (Kersten et al., 2000).

PPARα was identified in mice as a nuclear receptor homologue capable of inducing peroxisomes in response to a class of carcinogens (Issemann and Green, 1990). Its critical role in mediating peroxisome proliferator effects was later established using PPARα-null mice, in which targeted disruption of the isoform abolished the effects of peroxisome proliferators, including the development of hepatocarcinomas (Lee et al., 1995). To date, the mechanism(s) underlying PPARα mediated tumourigenic effects in rodents remain unclear, but suppression of apoptosis has been strongly implicated (Bayly et al., 1994). Peroxisome proliferators repress hepatocyte apoptosis in vitro and in vivo (Bayly et al., 1994) and PPARα dominant negative manipulations confer a protective advantage in response to various apoptotic stimuli (Roberts et al., 1998).

Of note, elevated expression of cell cycle regulators such as cyclin-dependent kinases and the c-myc oncogene have also been associated with the effects of peroxisome proliferators, suggesting that deregulation of cell cycle control may be involved (Peters et al., 1998). No related direct transcriptional targets of PPARα have however been identified. The generation of reactive oxygen species (ROS), a byproduct of β-oxidation in response to peroxisome proliferators, has been implicated as a factor in PPARα agonist-mediated hepatocarcinogenesis in rodent models, but the direct involvement of PPARα is yet to be proven (Rusyn et al., 2000).

Despite its demonstrated tumour-promoting effects in rodents, humans may be protected from the adverse effects of PPARα activation, although they retain the accompanying metabolic advantages (Berger and Moller, 2002). Activation of PPARα has in fact proven
therapeutic benefits in humans, particularly in patients with dyslipidemias and cardiovascular
diseases (Kersten et al., 2000). Fibrate treatment decreases triglycerides in plasma and
increases high density lipoprotein levels (Brown and Plutzky, 2007). PPARα also has an
overall anti-inflammatory effect and, therefore, is promising for the treatment of inflammatory
diseases such as atherosclerosis (Schoonjans, 1996). Tumourigenic effects may not occur
in humans, possibly because PPARα agonists do not elicit significant peroxisome
proliferation as is the case in rodents (Bentley et al., 1993). Protective advantages may also
reflect differential expression or activation of PPARα among species (Schoonjans, 1996).
Alternatively, a limited role for PPARα, or its transcriptional co-regulators, in mediating
peroxisome proliferator response in humans is possible and perhaps epigenetic variants are
involved (Roberts et al., 1998).

1.12 PPARγ

The expression of PPARγ is more restricted than that of PPARα. It is found at high levels in
brown and white adipose tissues and to a lesser extent in the intestinal epithelium, skeletal
muscle, the retina and the lymphoid organs (Mueller et al., 2002). PPARγ has an important role in adipocyte differentiation and lipid metabolism (Lowell, 1999). Importantly, it is
involved in improving insulin sensitivity and, like PPARα, in attenuating inflammatory
reactions (Kersten et al., 2000; Lowell, 1999). The role of PPARγ in tumour development
remains the subject of debate.

An anti-proliferative effect of PPARγ would be consistent with an established role in
promoting adipocyte differentiation (Lowell, 1999). Studies, using tumour cell lines derived
from breast, colon, liposarcoma and prostate carcinomas as well as organ cultures have
reported growth arrest, differentiation or apoptosis upon ligand induced PPARγ activation
(Elstner et al., 1998, Tontonoz et al., 1997, Kubota et al., 1998). Similar effects have been
reported in vivo (Kubota et al., 1998, Mueller et al., 2000, Sarraf et al., 1998). In breast
carcinogenesis, PPARγ ligand causes inhibition of chemically induced mammary tumours in
rats (Elstner et al., 1998). Also, combination therapy using troglitazone, a PPARγ agonist,
induces apoptosis and reduces tumour development derived from the MCF-7 breast cell line
in nude mice (Elstner et al., 1998).
In colon cancer, troglitazone, a PPARγ ligand, inhibits tumour development in xenograft models (Sarraf et al., 1999a) and reduces chemically induced colitis and formation of aberrant crypt foci, a potential indication of development of colon carcinoma (Tanaka et al., 2001). Similar inhibitory effects of troglitazone have been reported in prostate cancer (Mueller et al., 2000) and liposarcoma (Tontonoz et al., 1997). PPARγ has also been shown to have growth inhibitory effects on multiple myeloma (Wang et al., 2004). Furthermore, supporting a potential role for PPARγ in tumour suppression is the identification of hemizygous loss-of-function mutations in the PPARγ gene in some colon and breast cancers (Sarraf et al., 1999b). One should note however, that while such mutations may be significant in some contexts, they are rare overall and the loss of both alleles has not been found in any tumour thus far (Ikezoe et al., 2001).

Paradoxically, over-expression of PPARγ in various colon and breast carcinomas has also been reported (DuBois et al., 1998). In addition some evidence exists that questions the growth inhibitory effects of PPARγ. For example, in a recent phase II clinical trial, rosiglitazone was not effective as an anti-tumour drug in the treatment of liposarcomas, as increased PPARγ activity did not correlate with clinical response (Debrock et al., 2003). Moreover, another clinical trial reported that PPARγ activation by troglitazone had little therapeutic value among patients with metastatic breast cancer, and the trial was concluded after troglitazone was withdrawn from commercial availability following US Food and Drug Administration warnings on hepatic toxicity (Burstein et al., 2003). Importantly, no increase in tumours arose after ablating PPARγ in the epithelium of the mammary gland of cancer prone animals (Cui et al., 2002), questioning its involvement in anti-carcinogenic activities. Possibly more alarming is a study in colon cancer, which suggested that increased dietary fat intake and activation of PPARγ increases the polyp count and size in mice genetically predisposed to intestinal tumour development (Wasan et al., 1997). Taken together, these results appear to contradict the reported beneficiary anti-proliferative effects of PPARγ on colon cancer (Sarraf et al., 1999a).
1.13 PPARβ/δ

PPARβ/δ is ubiquitously expressed. Its role has not been entirely appreciated, as it has been the subject of limited investigation in the past (Berger and Wagner, 2002). Nevertheless, critical and diverse functions for PPARβ/δ in embryonic development, fatty acid metabolism, wound healing and inflammation control are beginning to emerge (Barak et al., 1999b, Wang and Kilgore, 2002). Recent investigations also implicate PPARβ/δ in tumour development, particularly in colorectal cancers (Krey et al., 1993, Berger and Wagner, 2002). However, the involvement of PPARβ/δ in cancer promotion, like PPARγ, is still controversial (Gupta et al., 2001). Work, mainly in keratinocytes, has shown that PPARβ/δ increases keratinocyte migration, survival, and resistance to apoptosis (Peters et al., 1998, Clay et al., 2002). Paradoxically, PPARβ/δ participates in anti-carcinogenic functions such as inhibiting proliferation and promoting inflammation-induced differentiation (Michalik et al., 2001). Additional work is needed to comprehend the role of PPARβ/δ in tumour development.

1.14 PPARs and endometrial cancer

There is limited information regarding the expression and function of PPARs in relation to endometrial cancer. The first description of PPARs in endometrial cancer comprised a small study of eleven endometrial cancers in which PPAR δ/β was identified in the nuclei of endometrial cancer cells. An association was noted with increased expression of cyclo-oxygenase 2 (Cox 2) (Tong et al., 2000). The expression of Cox 2 has been linked with inflammation and angiogenesis and Cox 2 is also over-expressed in endometrial cancers (Hasegawa et al., 2005). Cox 2 catalyses the production of fatty acid derivatives which are endogenous activators of PPARs (Ledwith et al., 1997). Therefore, Cox 2 may modulate inflammatory pathways and angiogenesis via indirect activation of PPARs.

A gene profiling study did not identify altered expression of PPARβ/δ but did indicate differential expression of PPARα and PPARγ (Holland et al., 2004). This was confirmed with quantitative real time PCR (Holland et al., 2004). Treatment of PPARα expressing endometrial cancer cell lines with a PPARα-specific agonist increased apoptosis and decreased proliferation (Holland et al., 2004). Retinoic acid, a ligand for RXR, potentiated
the effects on cell growth (Saidi et al., 2006). These studies were restricted to the level of the transcriptome and did not explore protein expression. More recently, PPARγ has been identified by immunohistochemistry in sections of endometrial carcinoma, although the intensity of staining was reported to be higher in benign tissues than cancers (Ota et al., 2006). In addition a naturally-occurring PPAR gamma ligand induced apoptosis and reduced proliferation in endometrial cancer cell lines (Ota et al., 2006). Very limited information is available regarding RXR expression in endometrial cancer although Holland et al confirmed that RXRβ was down-regulated at the RNA level in endometrial tumours (Holland et al., 2004).

It is striking that differential expression of PPARs is most evident in tumours that are associated with obesity i.e. breast, colon and prostate cancers (Suchanek et al., 2002b, Lefebvre et al., 1998, Collett et al., 2000). Given the role of PPARs in fat metabolism and the known strong association between obesity and endometrial cancer, it is also of interest that PPARs may be differentially expressed in endometrial cancers. It is plausible that PPARs may be linked to the development of endometrial cancers. If this were the case, there may be opportunities to manipulate this biological pathway for benefit.

1.15 Aim of the study

The aim of this study is to test the hypothesis that the PPAR/RXR pathway plays a role in the development of endometrioid endometrial cancer. this will be achieved by completing the following objectives:-

1. Examining the expression of members of the PPAR and RXR transcription factor families in endometrioid endometrial cancers (Chapter 3).
2. The effect of PPAR silencing and activation on endometrial cancer cell growth (Chapter 4).
3. Determining the response of cell-autonomous (PTEN and P-Akt expression) and non-cell autonomous (VEGF secretion) pathways to PPAR loss and activation in endometrial cancer cells (Chapter 5).
CHAPTER TWO

MATERIALS AND GENERAL LABORATORY METHODS

2.1 Reagents and equipment

Most laboratory reagents used, were supplied by BDH chemicals (Poole, Dorset, UK).
Specialist hardware and reagents that were used are also listed. Buffers were prepared as a 10x stock, autoclaved and then diluted to the required concentration with sterile water, prior to use.

Phosphate Buffered Saline (PBS)

1x PBS: 150mM sodium chloride; 20mM di-sodium hydrogen phosphate; 3mM sodium di-hydrogen phosphate.

Tris buffered saline (TBS), pH 7.5

1x TBS: 10mM Tris; 150mM sodium chloride

Citrate Buffer, pH 6.0

1x Citrate buffer: 2.1g citric acid, 1000mls sterile water.

Freezing buffer

95% fetal calf serum (FCS), 5% Dimethyl Sulfoxide (DMSO)

Western blocking buffer

3% (w/v) Marvel™ milk powder in TBS-0.05% Tween 20

Stripping buffer

100mM β-mercaptoethanol, 2% (w/v) SDS, 62.5mM Tris-HCL, pH 6.8
<table>
<thead>
<tr>
<th>Chemical/Reagent</th>
<th>Manufacturer/Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioRad dye reagent concentrate</td>
<td>BioRad Laboratories Ltd, Hertfordshire</td>
</tr>
<tr>
<td>Kaleidoscope pre-stained standards</td>
<td>BioRad Laboratories Ltd, Hertfordshire</td>
</tr>
<tr>
<td>DMEM cell culture medium</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Foetal Bovine Serum</td>
<td>GibcoBRL Life Technologies</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>Sigma-Aldrich, Dorset</td>
</tr>
<tr>
<td>ECL western blotting detection reagents</td>
<td>Amersham Biosciences, Buckinghamshire</td>
</tr>
<tr>
<td>Marvel dried skimmed milk powder</td>
<td>Premier brands, Lincolnshire</td>
</tr>
<tr>
<td>Hyperfilm ECL chemiluminescence</td>
<td>Amersham Biosciences, Buckinghamshire</td>
</tr>
<tr>
<td>Ultrapure Protogel (Solution A)</td>
<td>National Diagnostics, Hull</td>
</tr>
<tr>
<td>3,3’-Diaminobenzidine (DAB) tetrahydrochloride tablets</td>
<td>Sigma-Aldrich, Dorset</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Sigma-Aldrich, Dorset</td>
</tr>
<tr>
<td>Goat Serum</td>
<td>Sigma-Aldrich, Dorset</td>
</tr>
<tr>
<td>Rabbit Serum</td>
<td>Sigma-Aldrich, Dorset</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Sigma-Aldrich, Dorset</td>
</tr>
<tr>
<td>Mouse Serum</td>
<td>Sigma-Aldrich, Dorset</td>
</tr>
<tr>
<td>Mayers Haemalum</td>
<td>BDH Chemicals</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denley Spiramax 5</td>
<td>Denley Instruments, Billingham</td>
</tr>
<tr>
<td>Grant SUB 39 waterbath</td>
<td>Grant Instruments Ltd, Cambridge</td>
</tr>
<tr>
<td>Benchtop Centrifuge</td>
<td>MSE Equipment</td>
</tr>
<tr>
<td>VersaMax automated plate reader</td>
<td>Molecular Devices, Wokingham</td>
</tr>
<tr>
<td>BioRad power Pac 300</td>
<td>BioRad Laboratories Ltd, Hertfordshire</td>
</tr>
<tr>
<td>BioRad transblot semi-dry transfer cell</td>
<td>BioRad Laboratories Ltd, Hertfordshire</td>
</tr>
<tr>
<td>HyBond ECL nitrocellulose membrane</td>
<td>Amersham Biosciences, Buckinghamshire</td>
</tr>
<tr>
<td>96 well microplates</td>
<td>Fisher Scientific, Leicestershire</td>
</tr>
<tr>
<td>Techne dri-block DB 3A</td>
<td>Techne, Cambridge</td>
</tr>
<tr>
<td>Hypercassette autoradiography cassettes</td>
<td>Amersham Biosciences, Buckinghamshire</td>
</tr>
<tr>
<td>CO₂ Incubator</td>
<td></td>
</tr>
<tr>
<td>Hybaid shaker HB-SHK-1</td>
<td>Thermo Hybaid, Middlesex</td>
</tr>
<tr>
<td>Olympus Microscope</td>
<td>Olympus</td>
</tr>
<tr>
<td>Microflow Class 2 safety cabinet</td>
<td></td>
</tr>
</tbody>
</table>
2.2 Cell Culture

2.2.1 Cell lines and maintenance

The Ishikawa cell line was established from an endometrial adenocarcinoma from a 39-year-old Asian woman. These cells induce well-differentiated adenocarcinomas. Ishikawa cells are ER and PR positive, and PTEN null. The HEC-1A cell line was established from a human endometrioid adenocarcinoma (G2) from a Japanese female. HEC-1A cells are ER negative and PTEN wild type.

Ishikawa cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco BRL, UK). Human endometrial cancer (HEC-1A) cells were maintained in 1:1 of DMEM and Hams F12 (Invitrogen, Paisley, UK) medium supplemented with 10% FBS. The medium was sterilised using 0.22µm filter (Millipore). Cells were incubated at 37°C in 5% CO$_2$ and passaged at 70-80% confluence using 0.05% trypsin with 0.53mM EDTA (Invitrogen, UK) and neutralised in equal volume of DMEM culture medium containing FBS. Cells were grown in 75-cm$^2$ and 25cm$^2$ culture flasks (Corning, UK) for cell line maintenance and collecting cell lysates and supernatants.

2.2.2 Freezing and retrieving cells

Cells once 70-80% confluent, were trypsinized and the pellet resuspended into 1 ml of freezing buffer. The resulting cell suspension was transferred into a cryovial (Nunc, UK), stored overnight at -80°C and the next day transferred into a liquid nitrogen container for long term storage.

Frozen cells removed from liquid nitrogen were thawed at room temperature and incubated at 37°C in 5% CO$_2$ in appropriate culture medium. The following day, medium was replaced with fresh culture medium to remove DMSO from the culture and the cell line maintained as shown above.
2.2.3 Drug assays

The drugs listed in table 2.2 were used in several assays. For this application, each was dissolved in DMSO to a concentration of 50µM as stock solution. Cells were seeded and the following day medium was changed and supplemented with drug to the relevant experimental dilutions and cells cultured for the required time.

Table 2.2 Table of drugs used and their properties

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Action</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenofibrate</td>
<td>PPARα agonist</td>
<td>Sigma</td>
</tr>
<tr>
<td>GW6471</td>
<td>PPARα antagonist</td>
<td>Sigma</td>
</tr>
<tr>
<td>Ciglitazone</td>
<td>PPARγ agonist</td>
<td>Sigma</td>
</tr>
<tr>
<td>GW9662</td>
<td>PPARγ antagonist</td>
<td>Sigma</td>
</tr>
<tr>
<td>All-trans retinoic acid (ATRA)</td>
<td>RXR ligand (non-specific)</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

2.3 Tissue collection and processing

2.3.1 Formalin-fixed tissue

A cohort of archived, paraffin-embedded endometrial tissues was identified from histopathology archives with appropriate ethical permission (North Manchester REC 06/Q1406/29). These were five benign, endometrial samples (all post-menopausal), five severe atypical hyperplasia’s, five FIGO G1 and five FIGO G3 endometrial cancers (table 2.4). Sections were cut 6µM thick in the Department of Histopathology at Central Manchester and Manchester Children’s Hospital NHS Trust. These were assessed by a specialist Consultant Gynaecological Histopathologist in order to select sections representative of each tissue type.
2.3.2 Frozen tissue

A separate cohort of fresh endometrial tissue was collected with relevant ethical permission as above. Women undergoing hysterectomy for benign indications, known atypical endometrial hyperplasia or endometrial malignancy were included in the study. Twenty patient samples were collected, comprising five benign, endometrial samples and five each of the different tumour grades (G1, n=5; G2, n=5; G3, n=5) as classified by FIGO (see table 2.3). I would attend theatre sessions and take the fresh uterus to the pathology department, once the hysterectomy was complete. A Consultant Gynaecological Histopathologist with specialist expertise in gynaecological cancers examined all samples. Selected areas of viable tissues were cut by the Histopathologist, and immediately snap frozen by me in liquid nitrogen. These tissues were stored at -80°C until required. As this was a blind study, the grade of the tumour was unknown to me during experimentation; therefore, some discrepancy may exist between the grade of tissue used for research, and the final diagnosis made by Histopathologists. The median age of the women donating the cancer samples was 66 years. The median age of the women donating benign endometrium was 48 years. Three of the latter were pre-menopausal.

2.4 Protein localisation by immunohistochemistry

2.4.1 Pre-treatment of slides

Formalin-fixed paraffin-embedded sections were cut 6µm thick onto APES-treated slides by staff at the Department of Histopathology, Central Manchester Hospital NHS Trust. Sections were placed in metal racks and de-paraffinised in xylene, graduated alcohols and sterile water. In order to reduce non-specific staining with the detection reagents, endogenous peroxidases in the tissue were quenched with 0.3% hydrogen peroxide in methanol for 15 minutes. Due to formalin fixation it was frequently necessary to retrieve tissue antigens. Different antigen-unmasking protocols were used depending on the best method for each primary antibody (table 2.5). The different methods are listed below:

1) Microwave antigen retrieval for 10 minutes in 0.01M sodium citrate (pH 6.0).
2) Digestion with 0.1% trypsin in 0.1% CaCl₂ (pH 8.0) for 15 minutes at 37°C.
Table 2.3: Demographics of patients recruited in the study. Details of the patient diabetic status, height, weight, BMI and medications.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Tissue</th>
<th>Grade</th>
<th>Stage</th>
<th>Hormones</th>
<th>Diabetes</th>
<th>Medication</th>
<th>Height</th>
<th>Weight</th>
<th>BMI</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>73</td>
<td>endometrioid endometrial carcinoma</td>
<td>1</td>
<td>2A</td>
<td>No</td>
<td>No</td>
<td>atorvastatin</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>69</td>
<td>endometrioid endometrial carcinoma</td>
<td>2</td>
<td>1B</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>endometrioid endometrial carcinoma</td>
<td>3</td>
<td>3A</td>
<td>No</td>
<td>No</td>
<td>lipitor, amlodipine</td>
<td>5'5&quot;</td>
<td>13st</td>
<td>30.4</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
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<td>1</td>
<td>3A</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td>1.56</td>
<td>86</td>
<td>35.3</td>
</tr>
<tr>
<td>5</td>
<td>92</td>
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<td>1</td>
<td>1B</td>
<td>No</td>
<td>No</td>
<td>statin, clopidogrel</td>
<td>155</td>
<td>60</td>
<td>24.9</td>
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<tr>
<td>6</td>
<td>56</td>
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<td>1</td>
<td>1B</td>
<td>No</td>
<td>NIDDM</td>
<td>Metformin</td>
<td>5'7&quot;</td>
<td>120</td>
<td>41.4</td>
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<td>7</td>
<td>54</td>
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<td>2</td>
<td>1B</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td>159</td>
<td>98</td>
<td>38.7</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>endometrioid endometrial carcinoma</td>
<td>3</td>
<td>1C</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td>1.56</td>
<td>66</td>
<td>27.1</td>
</tr>
<tr>
<td>9</td>
<td>61</td>
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<td>3</td>
<td>1C</td>
<td>Medroxyprogesterone acetate</td>
<td>No</td>
<td>Frusemide, inhalers</td>
<td>1.58</td>
<td>140</td>
<td>56</td>
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<tr>
<td>10</td>
<td>38</td>
<td>Late secretory endometrium</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td>104</td>
<td>38</td>
<td></td>
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<tr>
<td>11</td>
<td>69</td>
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<td>3</td>
<td>1B</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td>41</td>
<td></td>
<td></td>
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<tr>
<td>12</td>
<td>65</td>
<td>endometrioid endometrial carcinoma</td>
<td>3</td>
<td>1C</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>70</td>
<td>endometrioid endometrial carcinoma</td>
<td>2</td>
<td>3A</td>
<td>No</td>
<td>No</td>
<td>Ramipril, statin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>41</td>
<td>Late secretory endometrium</td>
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<td>N/A</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>63</td>
<td>Benign-evidence of oestrogen influence</td>
<td>N/A</td>
<td>N/A</td>
<td>No</td>
<td>Yes</td>
<td>Insulin</td>
<td>31.5</td>
<td></td>
<td></td>
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<td>3A</td>
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<td>No</td>
<td>Anxiolytic</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>17</td>
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<td>2</td>
<td>1C</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18</td>
<td>45</td>
<td>Benign - pre-menopausal</td>
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<td>N/A</td>
<td>No</td>
<td>No</td>
<td>Nil</td>
<td>22.3</td>
<td></td>
<td></td>
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<tr>
<td>19</td>
<td>54</td>
<td>Benign</td>
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<td>N/A</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>62</td>
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<td>1</td>
<td>1B</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4 Details of paraffin-imbedded samples for immunohistochemistry

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>5</td>
</tr>
<tr>
<td>Atypical hyperplasia</td>
<td>5</td>
</tr>
<tr>
<td>FIGO G1</td>
<td>10</td>
</tr>
<tr>
<td>FIGO G3</td>
<td>10</td>
</tr>
</tbody>
</table>

2.4.2 Immunohistochemical procedure

After the appropriate pre-treatment, tissue sections were isolated by drawing around them, with a wax pen (Dako Ltd., Ely, UK). Non-specific binding sites were blocked with 20% of the appropriate serum in TBS and 0.1% Tween for 30 minutes at room temperature. This was necessary in order to reduce the potential for interaction with the secondary antibody. After shaking the serum from the section, 100µL of primary antibody, at the relevant dilution, was added to the tissue and incubated at 4ºC overnight in a humidified chamber. Concentrations or dilutions for each antibody are given in table 2.5. As a negative control, the primary antibody was replaced with the normal IgG from the same species. Following incubation, the slides were washed in TBS for ten minutes at a time. Biotinylated secondary antibody was added to each section at the appropriate dilution in TBS/0.1% for 1 hour at room temperature. The secondary antibody was directed against the species in which the primary antibody had been raised. After washing the sections in TBS, 3 times for 5 minutes each, an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Peterborough) was added to the tissues for 30 minutes at room temperature. The slides were washed in TBS, and the colour reaction developed with diamino-benzidine (DAB) at 1mg/ml in distilled water for 5 minutes at room temperature. The cell nuclei were counterstained with Mayer’s haematoxylin before washing in running water and dehydrating in graduated alcohols and xylene. Sections were mounted for viewing under a glass coverslip using DEPEX.
2.4.3 Immunofluorescence

An immunofluorescent immunohistochemistry technique was used to co-localise antigens of interest to specific cell types within the tissues examined. Paraffin-embedded sections were de-paraffinsed and rehydrated as above. Sections were incubated for 1 hour with both primary antibodies of interest, diluted in 10% relevant blocking serum, 2% human serum and TBS with 0.1% Tween for one hour at 37°C. Slides were washed in TBS (3 times for 5 minutes each) and TBS/0.6% Tween (3 times at 5 minutes each). The appropriate secondary antibodies were made up in 10% blocking serum, 2% human serum and TBS/0.1% Tween before incubating for 1 hour at room temperature in the dark. The slides were washed as before and sections mounted using Vectashield (Vector Laboratories) and counterstained with DAPI, a nuclear stain. Sections incubated with normal IgG’s provided negative controls.

2.5 Western blotting for protein expression

Western blotting was used to quantify protein expression in fresh tissues and endometrial cells.

2.5.1 General considerations in the preparation of nuclear and cytoplasmic extracts

A NE-PER® nuclear and cytoplasmic extraction kit was used for all extractions (PIERCE Biotechnology, Rockford). The kit contains 3 reagents; cytoplasmic extraction reagent I (CER I), cytoplasmic extraction reagent II (CER II) and nuclear extraction reagent (NER). These reagents enable stepwise separation and preparation of cytoplasmic and nuclear extracts from cultured cells or tissue. Use of nuclear extracts was preferred to whole cell lysates as the proteins of interest are reported to be mainly nuclear with some cytoplasmic expression. Cellular components in whole cell lysates can adversely affect nuclear protein interactions and stability, and nuclear proteins are more dilute in whole cell lysates than nuclear extracts.
Table 2.5 Dilutions and applications of primary antibodies

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Isotype</th>
<th>Stock Concentration</th>
<th>Dilution</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>IgG</td>
<td>200µg/ml</td>
<td>1:100</td>
<td>IHC, IF, Co-IP, WB</td>
<td>Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>PPARβ</td>
<td>IgG</td>
<td>200µg/ml</td>
<td>1:100</td>
<td>IHC, Co-IP, WB</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>PPARγ</td>
<td>IgG</td>
<td>200µg/ml</td>
<td>1:100</td>
<td>IHC, IF, Co-IP, WB</td>
<td>Santa Cruz, CA, USA</td>
</tr>
<tr>
<td>RXRα</td>
<td>IgG</td>
<td>500µg/ml</td>
<td>1:200</td>
<td>IHC, Co-IP, WB</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>RXRβ</td>
<td>IgG</td>
<td>500µg/ml</td>
<td>1:200</td>
<td>IHC, Co-IP, WB</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>RXRγ</td>
<td>IgG</td>
<td>200µg/ml</td>
<td>1:100</td>
<td>IHC, Co-IP, WB</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>PTEN</td>
<td>IgG</td>
<td>1mg/ml</td>
<td>1:500</td>
<td>IHC, WB</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>P-AKT</td>
<td>IgG</td>
<td>500µg/ml</td>
<td>1:200</td>
<td>WB</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>CD31</td>
<td>IgG</td>
<td>100µg/ml</td>
<td>1:200</td>
<td>IHC, IF</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>CD68</td>
<td>IgG</td>
<td>40µg/ml</td>
<td>1:200</td>
<td>IHC, IF</td>
<td>Abcam, Cambridge, UK</td>
</tr>
</tbody>
</table>
Table 2.6: Details of blocking sera and controls used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Pre-treatment</th>
<th>Blocking serum</th>
<th>Negative Control</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>Trypsin</td>
<td>Rabbit</td>
<td>Goat IgG</td>
<td>Murine Kidney</td>
</tr>
<tr>
<td>PPARβ</td>
<td>Citrate Buffer</td>
<td>Swine</td>
<td>Rabbit IgG</td>
<td>Human fetal omentum</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Citrate Buffer</td>
<td>Goat</td>
<td>Mouse IgG</td>
<td>Human fetal omentum</td>
</tr>
<tr>
<td>RXRα</td>
<td>Citrate Buffer</td>
<td>Rabbit</td>
<td>Goat IgG</td>
<td>Human fetal omentum</td>
</tr>
<tr>
<td>RXRβ</td>
<td>Citrate Buffer</td>
<td>Rabbit</td>
<td>Goat IgG</td>
<td>Human fetal omentum</td>
</tr>
<tr>
<td>RXRγ</td>
<td>Citrate Buffer</td>
<td>Swine</td>
<td>Rabbit IgG</td>
<td>Human fetal omentum</td>
</tr>
<tr>
<td>PTEN</td>
<td>Citrate Buffer</td>
<td>Swine</td>
<td>Rabbit IgG</td>
<td>Benign endometrium</td>
</tr>
<tr>
<td>CD31</td>
<td>Citrate Buffer</td>
<td>Goat</td>
<td>Mouse IgG</td>
<td>Benign endometrium</td>
</tr>
<tr>
<td>CD68</td>
<td>Citrate Buffer</td>
<td>Goat</td>
<td>Mouse IgG</td>
<td>Human tonsil</td>
</tr>
</tbody>
</table>
### Table 2.7 Dilution and applications for secondary antibodies

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Dilution</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-goat Biotinylated</td>
<td>1:200</td>
<td>IHC</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>Rabbit anti-goat HRP</td>
<td>1:2000</td>
<td>WB</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>Goat anti-mouse Biotinylated</td>
<td>1:200</td>
<td>IHC</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>Goat anti-mouse HRP</td>
<td>1:2000</td>
<td>WB</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>Swine anti-rabbit Biotinylated</td>
<td>1:200</td>
<td>IHC</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>Swine anti-rabbit HRP</td>
<td>1:2000</td>
<td>WB</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>Rabbit anti-mouse FITC</td>
<td>1:200</td>
<td>IF</td>
<td>Dako, UK</td>
</tr>
<tr>
<td>Goat anti-mouse Alexofluor 568</td>
<td>1:200</td>
<td>IF</td>
<td>Invitrogen, UK</td>
</tr>
<tr>
<td>Donkey anti-goat Alexofluor 488</td>
<td>1:200</td>
<td>IF</td>
<td>Invitrogen, UK</td>
</tr>
<tr>
<td>Goat anti-rabbit Alexofluor 568</td>
<td>1:200</td>
<td>IF</td>
<td>Invitrogen, UK</td>
</tr>
</tbody>
</table>
Table 2.8: Composition of solutions used for Western blotting

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A (Protogel)</td>
<td>37.5:1 ratio of acrylamide: bis-acrylamide</td>
</tr>
<tr>
<td>Solution B (for 100ml)</td>
<td>22.72g tris 0.5g sodium dodecysulphate pH to 8.8 with HCL</td>
</tr>
<tr>
<td>Solution C (for 100ml)</td>
<td>6.06g tris 0.4g SDS pH to 6.8 using HCL</td>
</tr>
<tr>
<td>Sample buffer (2x) Heat reducing (for 90ml)</td>
<td>25ml Solution C 4g SDS (154mM) 24g deionised urea (4.4M) 20ml glycerol (22.2%) 2ml bromophenol blue-1mg/ml (0.002%) 43ml dH₂O 10% v/v 2-mercaptoethanol</td>
</tr>
<tr>
<td>Non-boil Sample buffer (for 10 ml)</td>
<td>4.81g urea (8M) 0.5g SDS (5%) 0.004g bromophenol blue (0.04%) 0.7g DTT (455mM) Tris-HCL (50mM) pH to 6.9 using HCL</td>
</tr>
<tr>
<td>Electrode buffer (5x) (for 5 litres)</td>
<td>151.5g tris 50g SDS 144g glycine</td>
</tr>
<tr>
<td>Semi-dry transfer buffer (for 5 litres)</td>
<td>29g tris 14.5g glycine 1.85g SDS 1000ml methanol</td>
</tr>
<tr>
<td>TBS (for 5 litres)</td>
<td>6.057g tris (10mM) 43.83g NaCl (150mM) pH to 8.0 with HCL</td>
</tr>
</tbody>
</table>
2.5.2 Preparation of nuclear and cytoplasmic extracts from cultured cells

Once packed cell volumes were determined, cells were isolated in a 1.5ml micro-centrifuge tube by centrifugation at 500 x g for 2-3 minutes. The supernatant was carefully removed with a pipette and discarded, leaving the cell pellet as dry as possible. The appropriate volume of ice-cold CER I (table 2.9) was then added to the cell pellet. The tube was vortexed vigorously at the highest setting for 15 seconds to ensure the cell pellet was fully re-suspended. The tube was then incubated on ice for 10 minutes. The indicated volume of CER II (table 2.9) was added to the tube, vortexed for 5 seconds and allowed to incubate on ice for 1 minute. The tube was centrifuged at 16,000 x g for 5 minutes. Following this, the supernatant (cytoplasmic extract) fraction was immediately transferred to a clean pre-chilled tube and stored at -80°C until use. The insoluble pellet (containing nuclei) was resuspended in the indicated volume of ice-cold NER (table 2.9). The sample was then vortexed for 15 seconds every 10 minutes, for a total of 40 minutes. The tube was centrifuged at 16,000 x g in a micro-centrifuge for 10 minutes. The supernatant (nuclear extract) fraction was subsequently transferred to a pre-chilled tube and stored at -80°C. All centrifugation steps were carried out at 4°C.

Table 2.9 Volumes of reagents used for nuclear and cytoplasmic extraction

<table>
<thead>
<tr>
<th>Packed cell volume</th>
<th>CER I</th>
<th>CER II</th>
<th>NER</th>
</tr>
</thead>
<tbody>
<tr>
<td>10µl (20mg)</td>
<td>100µl</td>
<td>5.5µl</td>
<td>50µl</td>
</tr>
<tr>
<td>20µl (40mg)</td>
<td>200µl</td>
<td>11µl</td>
<td>100µl</td>
</tr>
<tr>
<td>50µl (100mg)</td>
<td>500µl</td>
<td>27.5µl</td>
<td>250µl</td>
</tr>
<tr>
<td>100µl (200mg)</td>
<td>1ml</td>
<td>55µl</td>
<td>500µl</td>
</tr>
</tbody>
</table>
2.5.3 Preparation of nuclear and cytoplasmic extracts from frozen tissue samples

The snap-frozen tissue was weighed, cut into small pieces and homogenised directly in CER I. A ten-fold excess of CER I was used relative to the weight of the tissue. The tube was vortexed vigorously at the highest setting for 15 seconds to ensure the cell pellet was fully re-suspended. The tube was then incubated on ice for 10 minutes. 5.5µl of CER II was used per 100µl of CER I. The tube was centrifuged at 16,000 x g for 5 minutes. The resulting supernatant (cytoplasmic extract) fraction was immediately transferred to a clean pre-chilled tube and stored at -80°C until use. The insoluble pellet (containing nuclei) was re-suspended in the indicated volume of ice-cold NER. The sample was vortexed for 15 seconds every 10 minutes, for 40 minutes. The tube was centrifuged at 16,000 x g in a micro-centrifuge for 10 minutes. The supernatant (nuclear extract) fraction was then transferred to a pre-chilled tube and stored at -80°C.

2.5.4 BioRad protein assay

The protein concentration of all samples was determined using a BioRad protein assay. Standards of 0, 0.25, 1.25, 2.5, 3.75 and 5µg were prepared using bovine serum albumin (BSA) dissolved in 0.3M NaOH. Samples were diluted (1:20 to 1:100) in distilled water to ensure that the protein content would fall within the range of the standard curve. 20µl BSA standard or diluted samples were added to wells of a 96 well microplate (in triplicate). This was neutralised by adding 180µl of neutralising solution composed of one part 0.3M NaOH: 1 part 0.3M HCL. 50µl BioRad dye reagent concentrate was then added to each well and mixed thoroughly by pipetting up and down in order for the colour to develop. Absorbance at 630nm was measured using the Versamax microplate reader. A standard curve of protein (µg) vs. absorbance at 630nm was constructed by linear regression using GraphPad Prism version 4 (figure 2.1). The protein content of each 20µl sample was then interpolated, multiplied by the dilution factor used, and divided by 20 to give a protein concentration expressed as µg/µl.
Figure 2.1 Representative graph for the calculation of total protein concentration in nuclear and cytoplasmic extracts. BSA represents bovine serum albumin.
## Table 2.10 Antibodies used for western blotting

<table>
<thead>
<tr>
<th>Protein of Interest</th>
<th>Molecular Size (kDa)</th>
<th>Cellular Localisation</th>
<th>Positive Control</th>
<th>Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>~52</td>
<td>Nuclear</td>
<td>Murine Kidney</td>
<td>Omit primary antibody</td>
</tr>
<tr>
<td>PPARβ</td>
<td>~50</td>
<td>Nuclear</td>
<td>Murine myocardium</td>
<td>Omit primary antibody</td>
</tr>
<tr>
<td>PPARγ</td>
<td>~56</td>
<td>Nuclear</td>
<td>Human Placenta</td>
<td>Omit primary antibody</td>
</tr>
<tr>
<td>RXRα</td>
<td>~55</td>
<td>Nuclear</td>
<td>Human fetal omentum</td>
<td>Omit primary antibody</td>
</tr>
<tr>
<td>RXRβ</td>
<td>~50</td>
<td>Nuclear</td>
<td>Human fetal omentum</td>
<td>Omit primary antibody</td>
</tr>
<tr>
<td>RXRγ</td>
<td>~51</td>
<td>Nuclear</td>
<td>Human fetal omentum</td>
<td>Omit primary antibody</td>
</tr>
<tr>
<td>PTEN</td>
<td>~47</td>
<td>Cytoplasmic and nuclear</td>
<td>Rat Brain</td>
<td>Omit primary antibody</td>
</tr>
<tr>
<td>P-Akt</td>
<td>~56</td>
<td>Cytoplasmic and nuclear</td>
<td>Rat Brain</td>
<td>Omit primary antibody</td>
</tr>
<tr>
<td>β-actin</td>
<td>~45</td>
<td>Cytoplasmic</td>
<td>N/A</td>
<td>Omit primary antibody</td>
</tr>
</tbody>
</table>
2.5.5 Preparation of polyacrylamide gel

A BioRad mini-Protean III system was used for polyacrylamide gel electrophoresis. A 7% resolving gel was made up as detailed in table 2.11 and loaded between the two glass plates of the gel manifold using a 21G needle and a 10ml syringe. This was overlaid with 70% ethanol to remove air bubbles to ensuring a levelled gel and to prevent gel dehydration. The gel was allowed to set for ~ 30 minutes. After this time, the ethanol was poured off, and left to evaporate for 15 minutes. A 3% stacking gel (Table 2.12) was prepared and loaded over the resolving gel. A well-defining comb was inserted into the stacking gel solution and allowed to set for ~ 30 minutes. On setting, the comb was removed from the stacking gel and the wells were rinsed with 1x electrode buffer (five-fold dilution in dH₂O of 5x electrode buffer, table 2.8). Any air bubbles were withdrawn from each well using a 25G needle and 5 ml syringe. Two gels were then mounted in each electrophoresis unit prior to loading pre-stained markers and samples.

Table 2.11. Composition of a 7% resolving gel.

<table>
<thead>
<tr>
<th></th>
<th>Per gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate</td>
<td>15mg</td>
</tr>
<tr>
<td>dH₂O</td>
<td>5.7ml</td>
</tr>
<tr>
<td>Solution B</td>
<td>2ml</td>
</tr>
<tr>
<td>Solution A</td>
<td>2.3ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>10µl</td>
</tr>
</tbody>
</table>
Table 2.12. Composition of a 3% stacking gel.

<table>
<thead>
<tr>
<th></th>
<th>Per gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate</td>
<td>10mg</td>
</tr>
<tr>
<td>dH₂O</td>
<td>6.5ml</td>
</tr>
<tr>
<td>Solution C</td>
<td>2.5ml</td>
</tr>
<tr>
<td>Solution A</td>
<td>1.0ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15µl</td>
</tr>
</tbody>
</table>

2.5.6 Preparation of protein samples for western blotting

In order to achieve the loading requirements of 60µg protein per well, samples were diluted accordingly in dH₂O. A heat reduction step was required for most samples and was carried out carried out at 95°C for 5 minutes using a Techne dri-block DB 3A followed by chilling on ice. All samples were mixed thoroughly by pipetting up and down before loading onto the gel. Kaleidoscope pre-stained markers (10µl) were loaded onto the gel alongside the protein samples. To prevent any overspill between the lanes, 1x electrode buffer was used to overlay the loaded samples and markers. Sample: buffer ratios and details of heat reduction are shown in table 2.13.

2.5.7 Gel electrophoresis and protein transfer

The electrophoresis units containing the gels were placed into electrophoresis tanks. The inner compartment of the tank was filled to the top and the outer component was filled to approximately two thirds with 1x electrode buffer. Electrophoresis was carried out at 120v using a BioRad power pack 300 for 70 minutes or until the dye front had reached the bottom of the gel.
### Table 2.13 Sample preparation for Western blotting

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Protein load (µg/sample)</th>
<th>Ratio sample:buffer</th>
<th>Heat reduction</th>
<th>Loading volume (µl/lane)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>60</td>
<td>1:1 (12µl:12µl)</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>PPARβ</td>
<td>60</td>
<td>1:1 (12µl:12µl)</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>PPARγ</td>
<td>60</td>
<td>1:1 (12µl:12µl)</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>RXRα</td>
<td>60</td>
<td>1:1 (12µl:12µl)</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>RXRβ</td>
<td>60</td>
<td>1:1 (12µl:12µl)</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>RXRγ</td>
<td>60</td>
<td>1:1 (12µl:12µl)</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>PTEN</td>
<td>60</td>
<td>1:1 (12µl:12µl)</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>P-Akt</td>
<td>60</td>
<td>2:1 (12µl: 6µl)</td>
<td>No</td>
<td>18</td>
</tr>
<tr>
<td>β-actin</td>
<td>60</td>
<td>2:1 (12µl: 6µl)</td>
<td>No</td>
<td>18</td>
</tr>
</tbody>
</table>

### 2.5.8 Protein Transfer

Once electrophoresis was complete, the proteins were transferred from the gels onto Hybond ECL nitrocellulose membrane using a BioRad trans-blot semi-dry transfer cell. For each gel, two pieces of blotting paper and one piece of Hybond ECL membrane were pre-soaked for 20 minutes in a semi-dry transfer buffer. For each gel, a transfer sandwich was stacked from bottom to top in order of blotting paper-nitrocellulose membrane-gel-blotting paper. The sandwich was pre-soaked in semi-dry transfer buffer to prevent drying out during transfer at 10v for 40 minutes at >0.2 amps (BioRad power pack 300). Following transfer, the membranes were removed and the distance between the top of the membrane and each
coloured band of the kaleidoscope marker was measured. Each colour represents a protein of known size and a plot of log molecular weight vs. distance migrated was generated by linear regression in GraphPad Prism version 4 (figure 2.2). This standard curve was used subsequently to interpolate the molecular size of the target protein.

![Graph showing standard curve](image)

**r^2 = 0.99**

Figure 2.2. Standard curve used for the calculation of protein size

### 2.5.8 Nitrocellulose membrane probing with antibodies

Membranes were then probed with antibody at 4°C overnight. Antibody dilutions are shown in table 2.5, molecular range of target proteins and positive controls are shown in table 2.10. Positive controls were kindly donated by Dr M Williams. Membranes were blocked for one hour at room temperature in 15ml Blotto (3% dried skimmed milk powder in TBS + 0.05% Tween). This was followed by 3 x 5 minute washes with 15ml Blotto. All antibody dilutions were made in 5ml Blotto. For negative controls, the primary antibody was omitted. Following primary antibody application, the membranes were washed with 15ml TBS + 0.05% Tween for 5 minutes. This step was repeated twice to ensure removal of excess primary antibody. HRP-conjugated secondary antibody incubations were then carried out for 1 hour at room temperature. The differing secondary antibodies used are shown in table. The membranes...
were then washed (3 x 5 minute washes with 15 ml TBS + 0.05% Tween) and stored in sufficient wash solution to prevent drying out prior to signal visualisation.

2.5.9 ECL visualisation

ECL visualisation was carried out under red light illumination. The wash solution was drained from each membrane and 5 ml ECL detection reagent (one part reagent one: one part reagent two) applied for one minute. This was then drained off and the membrane transferred to an autoradiography cassette and covered with plastic film. A piece of chemiluminescence film was aligned with the top of the membrane and the cassette closed for exposure. Film was exposed for varying lengths of time depending on the protein of interest. Following exposure, the film was placed in Kodak D-19 developer for one minute, rinsed in H₂O, fixed for one minute in Kodak fixer, rinsed in H₂O, and then left to air dry. The distance between the top of the film and the band visualised was measured and the molecular weight of these immunoreactive species interpolated from the standard curve.

2.6 Co-immunoprecipitation

Co-immunoprecipitation (co-IP) uses an antibody directed against a protein of interest to immobilise the target protein on a solid support on which either Protein A or Protein G has been immobilised. Interacting proteins will then bind to the target and are “co-precipitated and proteins not immobilised can be washed away. Co-IP can be used to confirm protein-protein interactions directly in cells, using cell lysates where the protein of interest should be in its “native” environment associated with interacting partners.

Three 1.5 ml tubes were set up each containing 250 µg of cell lysate made up to a volume of 500 µl ice cold NP40 lysis buffer and a dilution of 1 in 200 of a) RXRα, b) RXRβ and c) RXRγ. Following this, the tubes were incubated at 4°C on a roller for 2 hrs. Sepharose G beads (Amersham Life Sciences Ltd, UK) were added to three fresh eppendorf tubes in 30 µl aliquots, and were washed twice with 1 ml NP40 buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% NP40, 1 mM DTT, 2 mM NaOV, 5 mM EDTA, 0.2% protease inhibitor cocktail mix III (Sigma-Aldrich Co. Ltd, Dorset, UK), 10 mM NaF), centrifuging at 1000 rpm for 2 min. The
supernatant was then removed to leave the bead slurry. Next, the incubated protein/antibody mixture was added to the washed beads and incubated on a roller at 4˚C for 1.5 h. Following this, the mixture was spun at 1000 rpm for 2 min and most of the supernatant was removed. The mixture was incubated with 1 ml of NP40 buffer on a roller for 10 min at 4˚C, spun at 1000 rpm for 2 min, and most of the supernatant was removed. Next, the mixture was washed five times using 1 ml of NP40 buffer before centrifuging at 1000 rpm for 2 min. The supernatant was removed and 10 µl sample loading buffer was added to the mixture and heated at 95˚C for 5 min. Finally the mixture was spun to pellet the beads prior to loading the supernatant on a 7% SDS PAGE gel reading for the Western blotting protocol in order to be probed with the relevant antibodies.

2.7 Growth assays using cultured cells

2.7.1 Routine cell culture

Established cell lines were grown in a medium appropriate to each particular cell line supplemented with heat-treated fetal bovine serum. Cells were cultured in flasks (25 cm² or 75cm²) or flat-bottomed culture plates (96 wells) as required for each experiment. All plasticware was supplied by Becton Dickinson Labware (New Jersey, USA) unless otherwise stated. Unless experimental conditions required otherwise, cells were grown in a humidified atmosphere in 5% CO₂ at 37°C. For all cell culture work, six replicates at each dose (for relevant drug or control) were performed. Each experiment was repeated in triplicate.

2.7.2 Assay for cellular proliferation

Cells in the logarithmic growth phase, were seeded into wells of a 96-well culture plate at a density of 6 x 10³/100µL. Full medium appropriate to each cell type was used and was supplemented with fetal bovine serum. Cells were cultured for 24 hours at 37°C and 5% CO₂ in the presence of varying doses of test compound. Control cells were treated with vehicle only. After 24 hours incubation, cell proliferation was assessed by the uptake of 5-bromo-2’-deoxy-uridine (BrdU) using the BrdU labeling and detection kit III (Roche-Diagnostics, UK) according to the manufacturer's instructions. In brief, cells were incubated with 10μM BrDU
for 3 hours and then fixed with 70% ethanol in HCl at -20°C. Cellular DNA was partially
digested with nuclease at 37°C before cells were incubated with anti-BrdU-POD antibody.
After three washes with PBS, cells were incubated with ABTS®-substrate. This undergoes a
colour change upon contact with the anti-BrdU-POD antibody. Absorbance values for
cultures in each well were measured on a microtitre plate reader (Anthos Labtech
Instruments GMbH, Austria) at 405nm with a reference wavelength at 490nm. Results were
recorded as optical density measurements (OD) at 405nm/490nm.

2.7.3 Assays for cellular apoptosis

Cells in the logarithmic growth phase were seeded into a 96-well culture plate at a density of
6 x 10^5/100µL. After incubation at 37°C and 5% CO_2 for 24 hours, the medium was replaced
with fresh complete culture medium and varying doses of the experimental drug or
substance. APOPercenage™ Dye label (Biocolor UK) (5µL) was added to each well and the
cells incubated at 37°C for one hour. Control cells were treated with vehicle and dye only.
Cells were washed with PBS before the addition of APOPercenage™ Dye Release Reagent
to aid both cell lysis and release of bound dye into solution. The dye absorbance values for
each well were measured on a microtitre plate reader (Anthos Labtech Instruments) at
550nm with a reference wavelength at 620nm. Results are recorded as optical density
measurements at 550nm/620nm.

2.8 Reduction of target gene expression using short-interfering RNA (siRNA)

In order to reduce PPARα and PPARγ expression in cell lines, Qiagen Flexitube
GeneSolution (Qiagen, West Sussex, UK) was used. Flexitube GeneSolution is a gene-
specific package of 4 preselected siRNAs for the target gene of interest. siRNA was
provided in lyophilized form (Qiagen), and therefore 100µl of sterile, RNase-free water
(provided by Qiagen with each sample) was added to each 1nmol lyophilized siRNA to
obtain a 10µM solution. The target sequences for the siRNA’s used (shown below) were
provided by Qiagen, except for their “Allstars” control. One day before transfection, cells
were seeded at 6 x10^5/100µL in a 96 well plate and allowed to reach 70-80% confluence.
1.2µl of siRNA was diluted in 50µl of Opti-MEM© (Invitrogen, Paisley, UK) into Nunc© tubes
and mixed gently. 5µl of Lipofectamine 2000 reagent (Invitrogen, Paisley, UK) was added to another 50µl of Opti-MEM® in a separate tube, flicked to mix gently and incubated at room temperature for 5 minutes. After the 5 minute incubation, diluted siRNA was combined with the diluted Lipofectamine 2000 (total volume 100µl). This was mixed gently and incubated for 20 minutes at room temperature to allow the siRNA-Lipofectamine 2000 complexes to form. Following this incubation, 100µl of the siRNA-Lipofectamine 2000 complexes was added drop by drop, to each culture well containing cells and medium, whilst swirling to ensure even mixing. The solution was mixed gently by rocking the plate back and forth. The cells were then incubated at 37°C in a CO₂ incubator for 24 hours to allow transfection. After 24 hours, cells were trypsinised (100µl trypsin per well), counted and harvested until further use.

2.9 Enzyme-linked immunoabsorbant assay (ELISA)

ELISA was used to determine VEGF levels in culture supernatants. The QuantiGlo VEGF ELISA (R&D systems, UK) was used. This is a solid phase ELISA designed to measure VEGF₁₆₅ levels in cell culture supernatants. A monoclonal antibody specific to VEGF was pre-coated onto the microplate. 150µl of assay diluent RD1-8 was added to each well. 50µl of standard, control or sample was then added to the wells. The wells were covered with the adhesive slip provided and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500rpm. Each well was then aspirated and washed, using the buffer provided, three times, for a total of four washes. After the last wash, the well was aspirated and the plate inverted and blotted against clean paper towels. 200µl of VEGF conjugate was then added to each well, covered with an adhesive strip, and left to incubate for 3 hours at room temperature on the shaker. Each well was aspirated and washed with wash buffer, for a total of four times, and inverted and blotted as described previously. 100µl of working glo reagent was added to each well and incubated in the dark for 20 minutes, at room temperature. The relative light unit (RLU) of each well was determined using a luminometer set with: 1 minute lag time; 0.5 sec/well read time; summation mode; auto gain on. Separate standard curves were used for interpolating samples employing the kit cell culture
supernatant assay (intra- and inter-assay co-efficients of variation were 6.5 and 8.5% at 29.1 and 32.8 pg/ml, respectively).

Table 2.14 Target sequences for gene reduction using siRNA

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<thead>
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<th>siRNA ID</th>
<th>Product Material Number</th>
<th>Target Sequence</th>
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<tr>
<td>Hs_PPARA_5</td>
<td>SI03034108</td>
<td>AAGCTTTGGCTTTACGGAATA</td>
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<td>Hs_PPARA_6</td>
<td>SI03053757</td>
<td>CAAGAGAATCTACGAGGCCTA</td>
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<td>TCGGCGAACGATTGACTCGA</td>
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<td>GAGGCGATCTTGACAGGAAA</td>
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<td>SI00071680</td>
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<td>Ctrl_Allstars_1</td>
<td>SI03650318</td>
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</tr>
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</table>
2.10 Imaging

2.10.1 Light microscopy

For the analysis of peroxidase stained sections, light microscopy was used (Leitz Dialux 22) with a photo camera (QICAM, Q Imaging camera). Images were taken with the aid of Image Pro imaging software using various objectives (10x, 20x and 40x).

2.10.1 Widefield microscopy for viewing immunofluorescence

Images were viewed on a Olympus BX51 upright microscope using a 10x/ 0.30 Plan Fln objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Specific band pass filter sets for DAPI, FITC and Texas red were used to prevent “bleed-through” from one channel to the next. Images were processed and analysed using Image J (http://rsb.info.nih.gov/ij).

2.11 Densitometry

Protein expression was measured from bands seen on Western blots and Co-IP studies. Films were scanned, processed and analysed using Image J software to measure density of the bands seen. The final value was expressed as a ratio of protein of interest expression: β-actin.

2.12 Statistical Analysis

The statistical package GraphPad Prism 4 was used to analyse data. A non-parametric analysis of variance was performed, incorporating kruskal-wallis statistic and Dunn’s multiple comparison tests. Statistical significance was accepted as p<0.05.
CHAPTER THREE

EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS 
AND RETINOID X RECEPTORS IN ENDOMETRIAL CANCER

3.1 INTRODUCTION

Obesity and excessive fat consumption are significant risk factors for the development of endometrial cancer (Kaaks et al., 2002, Renehan et al., 2008b). Although this is mainly attributed to increased conversion of androgens to oestrogen in peripheral fat, post-menopausal women generally have much lower circulating oestrogen levels compared with pre-menopausal woman and therefore the link between oestrogen and endometrial malignancy is not completely understood (Watanabe et al., 1995). It is likely that other mechanisms and pathways are involved. One such pathway may be the PPAR/RXR pathway. As previously described, PPARs are ligand-activated transcription factors that form heterodimers with RXRs and bind to PPRE’s within the regulatory region of target genes, in order for PPAR mediated transcription to occur (chapter 1). PPRE’s are mainly located in genes involved with lipid and glucose homeostasis (Schoonjans et al., 1996). PPAR activity has been implicated in obesity and insulin resistance(Lehmann et al., 1995, Shalev et al., 1996, Itoh et al., 1999), and the systemic inflammation associated with metabolic syndrome (Fievet et al., 2006). Recent data suggests that PPARy-regulated genes are differentially expressed in morbidly obese women compared with non-obese women (Hindle et al., 2008). Endogenous ligands for PPARs (peroxisome proliferators) comprise of the prostaglandins, leukotrienes and fatty acids which specifically bind to PPAR (Berger and Moller, 2002). Interestingly, PPAR ligands also regulate cyclo-oxygenase 2 (Cox 2) which is central to tumour promotion as well as lipid metabolism and inflammation (Tong et al., 2000, Ota et al., 2002, Gilroy et al., 1999). The structure of PPARs is similar to that of other nuclear hormone receptors and there is competition between these and other PPARs for availability of RXRs within cells (Bonofiglio et al., 2005, Houston et al., 2003). It is therefore possible that within the endometrium, PPARs have an indirect effect on oestrogen activity.
In recent years there has been an increasing interest in the roles of the PPAR/RXR transcriptional apparatus in cancer following a number of publications that implicate PPARs in oncogenesis. PPAR\(_\gamma\) in particular has been found to modulate proliferation and apoptosis in several cancer cell lines as well as being expressed in many human tumours including; lung, colon (Lefebvre et al., 1998), breast (Burstein et al., 2003), bladder (Fauconnet et al., 2002) and prostate cancers (Mueller et al., 2000). Gene profiling studies also indicate that PPAR\(_\alpha\) and PPAR\(_\gamma\) are differentially expressed at the transcriptional level in endometrial cancers (Holland et al., 2004), alongside a heightened expression of PPAR\(_\beta\) (Tong et al., 2000). In addition, previous investigations reveal that synthetic PPAR\(_\alpha\) and PPAR\(_\gamma\) ligands reduce the growth of endometrial cancer cells in vitro and in murine models (Saidi et al., 2006, Ota et al., 2006, Wu et al., 2008). Therefore, it is possible that the PPAR/RXR system acts to increase the risk of developing endometrial cancer, in connection with or independent of oestrogen status. No study of endometrial cancer to date has examined the expression of members of the PPAR family together or the RXRs with which they form heterodimers. As the functions of the PPAR/RXR system may be dependent upon differential availability of the different PPAR and RXR isoforms, it is relevant to study the expression of these proteins together in the same tissues.

### 3.2 METHODS

#### 3.2.1 Tissue samples and specimens

Women undergoing hysterectomy for benign indications or known endometrial malignancy were included in the study. Twenty fresh endometrial samples were used, comprising five benign, endometrial samples and five each of the different endometrioid tumour grades (G1, \(n=5\); G2, \(n=5\); G3, \(n=5\)) as classified by FIGO (International Federation of Obstetrics and Gynaecology) (see table 2.1). The median age of the women donating the cancer samples was 66 years and the median age of women donating benign endometrium was 48 years. A separate cohort of archived paraffin embedded tissues was used for immunohistochemistry. These were identified and hematoxylin and eosin (H&E) sections examined by a Consultant Histopathologist before immunostaining to ensure representatives tissues.
3.2.2 Western Blotting for expression of PPARs and RXRs.

Western blotting was used to quantify PPARs and RXRs in fresh frozen endometrial tissues. Nuclear extracts were used for analysis of PPARα, PPARγ, PPARβ/δ and each RXR isoform. In addition, cytoplasmic extracts were subsequently investigated for PPARα and PPARγ expression. In brief, nuclear and cytoplasmic extracts were prepared as described in chapter 2, and used for Western blotting. The protocol and antibodies used are described in chapter 2.5. Protein expression was measured as bands seen on western blots. Films were scanned, processed and analysed using Image J software to measure density of the bands seen. The final value was expressed as a ratio of protein of interest expression: β-actin.

3.2.3 Immunohistochemistry and Immunofluorescence

Immunohistochemistry was used to determine the distribution of PPARs and RXRs in tissue sections using an immunoperoxidase staining method (section 2.4.2). Staining was not scored in these tissues because this technique relies on qualitative observation. In order to conclude anything by immunohistochemistry, one must score a sample manually, which involves deciding which cells in a given slide have stained. This can be especially problematic, as scoring results can vary greatly due to the quality of the dyes used, the length of time the sample cells have been exposed to the dye, as well as the judgment of scorer. As such, only a very low level scoring reproducibility exists, and for this reason the slides were not scored for quantitation. Immunofluorescence was subsequently used to examine the suspected co-localisation of PPARs to vessels and macrophages (section 2.4.3).

3.2.4 Co-immunoprecipitation

Co-immunoprecipitation was used to determine any preferential binding between PPAR and RXR isoforms. Nuclear extracts from benign endometrial tissues were used and probed for PPARα, PPARγ, PPARβ/δ and individual RXR isoforms.
3.2.5 Statistics

Image J software was used to calculate relative densities of protein expression in western blotting and co-immunoprecipitation. Non-parametric analysis of variance was performed incorporating Kruskal-Wallis statistic and Dunn’s multiple comparison tests. Statistical significance was accepted as p<0.05.

3.3 RESULTS

3.3.1 Expression of PPARs, RXRs and PTEN in human endometrial tissues using western blotting

All benign, tissues expressed PPARα, β/δ and γ (figure 3.1). There was a trend towards increasing PPARα expression from benign endometrium through malignant endometrium with highest levels of the protein demonstrated in the nucleus of G3 tumours (p<0.05, Kruskal-Wallis) (figure 3.1A and B). A similar trend in expression was seen for PPARβ/δ with greater nuclear expression in G3 endometrial cancers compared to either G3 cancers or benign endometrium (p<0.05, Kruskal-Wallis) (figure 3.1C and D). In contrast to both PPARα and PPAR β expression, PPARγ was less highly expressed in the nucleus of endometrial cancers compared to benign endometrium with levels in G3 tumours being even lower than those in G1 tumours (p<0.05) (figure 3.1E and F). Different RXR isoforms were also differentially expressed in nuclear extracts from different endometrial tissues. RXRα was most highly expressed in endometrial cancers compared with benign tissue (p<0.05, Kruskal-Wallis) although both RXRβ (p<0.05) and RXRγ (p<0.05) were less highly expressed in the nucleus of endometrial cancers with G3 tumours demonstrating least abundance (figures 3.1G-L).

Cytoplasmic expression of PPARα and PPARγ was also examined to highlight any difference between nuclear and cytoplasmic expression (figure 3.2). Results indicate that there was no difference in cytoplasmic expression between the differing endometrial histologies.
**Figure 3.1. Nuclear expression of PPARs in endometrial tissues.** A, C, E, G, I and K: Representative samples of Western blotting for PPARs and RXRs in endometrial tissues. (Pos) Nuclear lysates from positive controls, (B) benign endometrium, (G1) FIGO G1 endometrioid endometrial carcinoma, (G3) FIGO G3 endometrioid endometrial carcinoma.

**Figure 1B, D, F, H and J:** Densitometry measurements from western blots. Data is shown as box and “whiskers” (box extends from the 25th to 75th percentiles, whiskers show range with a horizontal line at the median, n= 5 for all) * = p<0.05, ** = p<0.01 (Dunn’s multiple comparison test).
Figure 3.2. Cytoplasmic expression of PPARα and PPARγ in endometrial tissues. A and C: Representative sample of Western blotting for PPARα and –γ in endometrial tissues. (Pos) Cytoplasmic lysates from positive controls, (B) benign endometrium, (G1) FIGO G1 endometrioid endometrial carcinoma, (G3) FIGO G3 endometrioid endometrial carcinoma. B and D: Densitometry measurements from western blots. Data is shown as box and “whiskers” (box extends from the 25th to 75th percentiles, whiskers show range with a horizontal line at the median, n= 5 for all).
3.3.2 Expression of PPARs and RXRs in human endometrial tissues using immunohistochemistry and immunofluorescent staining

Immunohistochemistry was performed on human endometrial tissues (n=30, benign =5, atypical hyperplasia =5, endometrial cancers =20). PPARα staining in both glands and stroma of benign endometrium was weak (figure 3.3D). In contrast, in atypical hyperplasia, strong PPARα staining was seen in glands and to a lesser extent in the stroma (figure 3.3E). This was similar to the staining seen in G1 cancers, with strong PPARα staining noted in tumour cells (figure 3.3F). Both nuclear and cytoplasmic staining was noted in malignant cells although in benign tissues staining was largely cytoplasmic. The intensity of staining was heterogeneous with some tumour cells demonstrating more intense staining than others. PPARα was also noted in blood vessels (figure 3.3F and G). This staining was seen in both large and small vessels but was more pronounced in peritumoral areas. Co-localisation using anti-CD31 (figure 3.3L) confirmed that PPARα was expressed in vascular endothelium. In FIGO G3 cancers, stromal PPARα expression was generally weaker and also appeared strongest in malignant cells (figure 3.3H).

The distribution of immunostaining and intracellular staining for PPARβ was noted to be similar to that of PPARα but not as intense. Weak PPARβ staining was seen in glands and stroma of benign endometrium (figure 3.4D) with more intense staining in the glands in atypical hyperplasia (figure 3.4E) and in tumour cells in both G1 (figure 3.4F) and G3 cancers (figure 3.4H). As with PPARα, PPARβ was also expressed in peri-tumoural vessels (figure 3.4G). Staining was not scored in these tissues because this technique relies on qualitative observation. As such, only a very low level scoring reproducibility exists, and for this reason the slides were not scored for quantitation.

As expected from the western blotting experiments, the intensity of staining for PPARγ was greater in benign endometrium compared with endometrial carcinoma and staining was most pronounced in endometrial glands in both tissues (figure 3.5D) with weaker staining in the stroma. This glandular staining was also seen in atypical hyperplasia (figure 3.5E). Intracellular staining was nuclear and cytoplasmic in benign tissue and largely cytoplasmic in malignant cells. In FIGO G1 cancers (figure 3.5F), the intensity of staining was stronger than
that in FIGO G3 cancers (figure 3.5G). Within the cancer stroma, small groups of cells and isolated cells demonstrated intense nuclear staining. Initial observations suggested these may be tissue macrophages and this was confirmed with dual immunofluorescence staining using CD68 antibody (Figure 3.5K) on serial sections.

Representative images of immunostaining for RXRα and β and γ are shown in figure 3.6 and 3.7 respectively. In benign endometrium, weak, patchy staining for RXRα was seen in both stroma and glands (figure 3.6D). Staining in atypical hyperplasia was stronger in glandular regions but remained weak in the stroma (figure 3.6E). In FIGO G1 and G3 cancers however, intense heterogeneous RXRα staining was seen mainly in the tumour cells. In contrast, weak RXRβ staining was seen in both glands and stroma in benign endometrium (figure 3.7A) with even weaker staining seen in FIGO G1 (figure 3.7C) and G3 cancers (figure 3.7D). In benign endometrium, strong RXRγ stromal and glandular staining was seen (figure 3.7E). However, RXRγ immunostaining was confined to the glands in atypical hyperplasia (figure 3.7F) and only weak RXRγ staining was seen in malignant glands in FIGO G1 cancers (figure 3.7G) and the cytoplasm of FIGO G3 cancers (figure 3.7H). Within the tissues studied, immunostaining for RXRα appeared mainly cytoplasmic in benign endometrial glands with nuclear and cytoplasmic staining evident in malignant glands. Conversely, nuclear and cytoplasmic staining for RXRβ and RXRγ was more striking in benign endometrial glands with cytoplasmic staining only in malignant glands.

3.3.3 Co-immunoprecipitation of PPAR and RXR isotypes

Co-immunoprecipitation was performed to determine the normal relationships between binding of different PPAR and RXR isotypes to each other. Nuclear extracts from benign endometrial tissue were used. The results indicate that RXRα appears to be the preferential binding partner for both PPARα and PPARγ compared with either RXRβ or RXRγ (figure 3.8). There is no clear preference for binding of PPARβ with any specific RXR isotype.
Figure 3.3: Immunostaining for PPARα (A) Murine kidney incubated with IgG (negative control); (B) PPARα staining in murine kidney (positive control); (C) Benign endometrium incubated with IgG (negative control); (D) PPARα expression in benign endometrium; (E) PPARα expression in atypical hyperplasia, i represents glandular staining; (F) PPARα expression in FIGO G1 carcinoma, ii represents endothelial staining; (G) magnified image of (F) at x40; (H) PPARα expression in FIGO G3 carcinoma, iii represents tumour staining; (I) FIGO G1 carcinoma with DAPI nuclear staining; (J) positive CD31 staining in FIGO G1 carcinoma; (K) positive PPARα staining in FIGO G1 carcinoma; (L) FIGO G1 carcinoma showing co-localisation of PPARα in endothelial cells, iv represents endothelial vessel with positive staining for both PPARα and CD31. Line represents scale bar measuring 100μM.
Figure 3.4: Immunostaining for PPARβ (A) Human omentum incubated with IgG (negative control); (B) Human omentum staining positive for PPARβ (positive control); (C) Benign endometrium (negative control for PPARβ); (D) PPARβ expression in benign endometrium; (E) PPARβ expression in atypical hyperplasia, i represents glandular staining; (F) PPARβ expression in FIGO G1 carcinoma; (G) magnified image of (F) at x40, ii represents peritumoural staining; (H) PPARβ expression in FIGO G3 carcinoma; Line represents scale bar measuring 100µM.
Figure 3.5: Immunostaining for PPARγ (A) Human omentum incubated with IgG (negative control); (B) Human omentum stained for PPARγ (positive control); (C) Benign endometrium with IgG (negative control for PPARγ); (D) PPARγ expression in benign endometrium; (E) PPARγ expression in atypical hyperplasia, i represents glandular staining; (F) PPARγ expression in FIGO G1 carcinoma, ii represents tumour staining; (G) FIGO G3 carcinoma staining negative for PPARγ (H) FIGO G1 carcinoma with DAPI nuclear staining; (I) positive PPARγ staining in FIGO G1 carcinoma; (J) positive macrophage staining in FIGO G1 carcinoma; (K) FIGO G1 carcinoma showing co-localisation of PPARγ in macrophages, iii represents macrophages with positive staining for PPARγ. Line represents scale bar measuring 100µM.
Figure 3.6: Immunostaining for RXRs (A) Human omentum incubated with IgG (negative control); (B) Human omentum stained for RXRα (positive control); (C) Benign endometrium (negative control for RXRα); (D) RXRα expression in benign endometrium; (E) RXRα expression in atypical hyperplasia, i represents glandular staining; (F) RXRα expression in FIGO G1 carcinoma; (G) RXRα expression in FIGO G3 carcinoma, ii represents tumour staining. Line represents scale bar measuring 100µM.
Figure 3.7 Immunostaining for RXRs (A) RXRβ expression in benign endometrium; (B) RXRβ expression in atypical hyperplasia; (C) RXRβ staining in FIGO G1 carcinoma; (D) RXRβ staining in FIGO G3 carcinoma; (E) RXRγ expression in benign endometrium; (F) RXRγ expression in atypical hyperplasia; (G) RXRγ expression in FIGO G1 carcinoma; (H) RXRγ expression in FIGO G3 carcinoma. Line represents scale bar measuring 100µM.
Figure 3.8: Co-immunoprecipitation for PPAR and RXR isoforms. Nuclear lysates from benign endometrial tissue. β-actin is used to demonstrate loading controls.
3.4 DISCUSSION

This study demonstrates, for the first time, the pattern of expression of each of the PPAR and RXR transcription factors together in endometrial carcinoma. Previous genome-wide analysis of endometrial cancer was restricted to analysis of mRNA and demonstrated that PPARα was up-regulated in endometrial cancers compared with benign endometrium (Holland et al., 2004). The data presented here, confirm for the first time that expression of the PPARα protein is increased in human endometrial cancers and shows a trend towards increased expression from well-differentiated through poorly-differentiated cancers. This suggests that PPARα over-production by tumour cells is a feature of endometrial tumourigenesis. Regulation of PPARα activity involves multiple protein complexes (Vanden Heuvel, 1999) and may involve modulation by proto-oncogenes e.g. c-jun (Sakai et al., 1995). Recent evidence has demonstrated that the activity of c-jun N terminal kinases is important in modulating apoptosis in endometrial cancer cells (Reno et al., 2009). The dysregulation of proto-oncogenes in endometrial cancer may therefore contribute to altered expression activity of PPARα. Increased PPARα expression has been demonstrated in other epithelial tumours such as prostate (Collett et al., 2000), bladder (Fauconnet et al., 2002) and breast cancers (Suchanek et al., 2002). This is consistent with the finding here that PPARα expression is increased in atypical hyperplasia and may therefore occur early in endometrial carcinogenesis. Nuclear staining was seen to increase with increasing degree of endometrial abnormality whilst cytoplasmic staining was unaltered. This is consistent with the known nuclear site of activity of PPARs.

There is known crosstalk between the PPAR pathway and other transcription factor families. Shipley and Waxman found bi-directional crosstalk between PPARα and members of the Jak-STAT pathway (Shipley and Waxman, 2003). PPARα has also been shown to regulate other growth factors e.g. TGFβ (Wilmer et al., 2002) and may therefore also contribute to endometrial cancer development and growth by influencing other mediators. Insulin increases the transcriptional activity of PPARα via activation of the MAP kinase pathway (Shalev et al., 1996) and it is well established that both diabetes and obesity are risk factors for the development of endometrioid endometrial cancer. Therefore it is possible that insulin
is linked to endometrial cancer development by increasing activation of PPARα with subsequent activation of relevant downstream pathways.

It is possible that the up-regulation of PPARα in endometrial cancer cells may simply reflect global disruption of cellular homeostasis in carcinogenesis i.e. a secondary bystander effect. Targeted disruption of PPARα in mice abolishes the carcinogenic effects of peroxisome proliferators in hepatocytes (Lee et al., 1995) suggesting that altered levels of PPARα may not merely be a secondary effect. This has resulted in an increased interest in the pro-carcinogenic and anti-carcinogenic effects of PPARs in humans (Vanden Heuvel, 1999) and the potential for exploiting PPAR expression for therapeutic benefit.

Localisation of PPARα to vascular endothelium has previously been reported in mouse prostate cancer xenografts and in samples of human prostate cancers (Panigrahy et al., 2008a). The data presented here confirms PPARα expression in vascular endothelium within endometrial cancers suggesting a role in endometrial cancer angiogenesis. The potent angiogenic growth factor, VEGF-A is up-regulated by PPARα in bladder cancers (Fauconnet et al., 2002) suggesting that PPARα-mediated angiogenesis may promote tumour growth. Increased VEGF-A is a feature of many solid tumours including endometrial cancer (Yokoyama et al., 2003, Hirai et al., 2001). The role of the development of new vascular networks in solid cancers is now established and their importance as therapeutic targets in cancer disease has led to the inclusion of expensive vascular targeting agents in treatment regimes. In the endometrial cancer sections examined here, endothelial cell staining for PPARα was most marked at the periphery of malignant tissue. PPARα may contribute to tumour growth in endometrial cancer via promotion of angiogenesis and this raises the possibility that targeting the PPARα pathway directly could be considered for potential therapeutic benefit by disrupting vascular development.

This study demonstrates that the expression of PPARβ in endometrial tissues was similar to the pattern of expression of PPARα with a trend toward increasing expression as tumours become less differentiated. Like PPARα, PPARβ is found in both tumour cells and peritumoural vessels. The findings in this thesis support those from a previous study that demonstrated PPARβ transcript and protein within endometrial tumour cells and correlated
this with the distribution of cyclo-oxygenase 2 (COX-2) (Tong et al., 2000). The latter study suggests that prostaglandins derived via COX-2, may activate PPARβ in an autocrine and/or paracrine manner. COX-2 is over-expressed in many cancers (including endometrial cancer) contributing to angiogenesis and COX-2 inhibitors produce anti-angiogenic effects and reduce growth in tumour xenografts (Shigeto et al., 2007, Jaeckel et al., 2001). The findings presented here support the hypothesis that PPARβ could contribute to tumour angiogenesis. As PPARβ has previously been investigated within endometrial cancer setting, and behaves similarly to PPARα, it will not be further investigated in this thesis.

Compared to PPARα and PPARβ, an inverse relationship was found between the expression of PPARγ in endometrial cancers and tumour grade in the experiments presented here. Benign endometrium expressed significantly higher levels of PPARγ than cancers. Immunohistochemistry demonstrated both nuclear and cytoplasmic PPARγ staining and western blotting confirmed that PPARγ expression is not limited to the nucleus. Nuclear staining declines with malignant progression but remains unchanged in the cytoplasm. This is supported by data in ovarian cancers which show that 60% of positive PPARγ staining is in the cytoplasmic compartment and 40% in the nucleus (Zhang et al., 2005).

Weak PPARγ expression has previously been reported in endometrial cancers although a non-quantitative method was used (immunohistochemistry) (Ota et al., 2006). A similar loss of PPARγ expression is also seen in oesophageal carcinoma (Terashita et al., 2002) and lung (Sasaki et al., 2002) carcinomas. Normal human ureter also expresses PPARγ protein, but there is a significant loss of PPARγ expression in high-grade transitional cell carcinomas (Nakashiro et al., 2001). Localisation of PPARγ to benign tissue and not malignant endometrial cells suggests that a down-regulation of the PPARγ gene is significant in endometrial cancer. These findings are all consistent with loss of PPARγ as a feature of malignant development. This data, alongside previously reported studies (Patel et al., 2001, Ali, 2000, Tashiro et al., 1997a), indicates that PPARγ may behave as a tumour suppressor gene and that dysfunction of PPARγ may contribute to tumourigenesis.

Contrary to this, several studies have shown that PPARγ expression is higher in carcinoma tissues than in normal tissues. For example, Zhang et al. analysed 56 specimens of normal
ovary and cancer using immunohistochemistry (Zhang et al., 2004a). Immunoreactive PPARγ was not detected in normal ovaries. However, PPARγ immunoreactivity in ovarian tumour tissues was significantly higher than in normal ovaries and benign ovarian tumours (Zhang et al., 2004a). Only 8/56 samples were endometrioid subtypes of ovarian cancer. The ages and BMI's of these patients are also not presented in their study and the difference in PPARγ expression may be due to the patient demographics. Ikezoe et al. examined the expression of PPARγ in 339 clinical samples and 71 various cancer cell lines, including colon cancer, breast cancer, prostate cancer, lung cancer, osteosarcoma, glioblastoma, and leukemia. All of the cell lines and clinical samples expressed PPARγ as detected by real-time PCR and/or Western blot, but their expression levels varied widely among samples (Ikezoe et al., 2001). Therefore, the results above suggest that the expression of PPARγ is dependent on tissue type and/or the mutational events that are required for cancer development.

The data presented here indicate that PPARγ is expressed in both glandular epithelium and tissue macrophages. PPARγ has been implicated in the promotion of macrophage differentiation alongside monocyte activation and the regulation of inflammatory activities (Nagy et al., 1998, Patel et al., 2002). PPARγ is also seen in peritoneal macrophages of women with endometriosis and migration of macrophages is inhibited by treatment with a PPARγ agonist (Hornung et al., 2001). In endometrial cancer as with other solid tumours, macrophages are abundant and macrophage infiltration is associated with poor prognostic features such as vascular space invasion and deep myometrial invasion (Soeda et al., 2008). Tumour-associated macrophages are known to secrete a variety of cytokines that can promote tumour cell growth and invasion (Soeda et al., 2008). The findings in this work support the hypothesis that PPARγ may exert a protective effect on the endometrium by regulating inflammatory cells in the stroma as well as via actions within the endometrial glandular cells.

PPARs must form heterodimeric complexes with RXRs, in order to bind to specific response elements within the promoter region of target genes (Latruffe and Vamecq, 1997). Ito et al. showed a wide variation of expression of RXRα and –γ, compared with other retinoid
receptor subtypes, in both neoplastic and non-neoplastic human endometrium (Ito et al., 2001). The results presented in this chapter demonstrate an increased expression of RXRα and loss of RXRβ and –γ in endometrial cancers. Furthermore it highlights preferential binding of RXR and PPAR subtypes, varies in benign endometrium. This may be relevant for the activity and action of PPARs within the endometrium. These findings suggest that within the context of endometrial cells, the formation and actions of the PPAR/RXR heterodimer complexes may be modulated by altering the availability of different PPAR and RXR isoforms. This could be effected either by up- or down-regulation of PPARs and RXRs in different cell types or perhaps by nuclear-cytoplasmic shuttling (Burgermeister et al., 2007). Co-immunoprecipitation studies presented here demonstrate the preferential binding of PPARα and PPARγ to RXRα. Therefore the up-regulation of RXRα in endometrial cancers may favour malignancy development and growth enhancing the ability of PPARα to form transcriptional complexes. Down-regulation of PPARγ with malignant change would be expected to reduce the availability of PPARγ/RXRα complexes.

A limitation of the work presented here is that a small sample of atypical hyperplasia samples was included. It would be advantageous to have a large cohort of tissues including a greater number of atypical hyperplasia in order to further the understanding of role of PPARs in the development of endometrioid endometrial cancer as this is the pre-malignant phase at which intervention may be beneficial. It has been difficult to identify larger numbers of severe atypical hyperplasia as these were more frequently upgraded to invasive cancer on the hysterectomy specimens. Nonetheless, these results together demonstrate, for the first time, the differential expression of each of the PPAR and RXR sub-types in endometrial carcinoma. These data suggest that PPAR/RXR transcription factor complexes may contribute to endometrial cancer development and growth via direct effects on glandular epithelial cells and via indirect mechanisms such as promotion of angiogenesis and modulation of macrophage function.
CHAPTER FOUR

AN INVESTIGATION OF THE EFFECTS OF PPARα AND PPARγ ON ENDOMETRIAL CANCER CELL GROWTH

4.1 INTRODUCTION

It is clear that PPARα and PPARγ transcription factors are expressed both in endometrial epithelial cells and in other cellular types within normal endometrium and tumours. The increased expression of PPARα in epithelial tumour cells suggests direct involvement with the growth of these cells. Peri-tumoural endothelial cell expression raises the possibility of indirect effects on angiogenesis. Interestingly, PPARγ appears to be diminished or lost in endometrial cancer cells suggesting a possible protective role for this transcription factor in normal cells. Direct and indirect evidence exists demonstrating that these factors affect cellular growth rather than simply being altered as “bystanders”.

The PPARα transcription factor is normally expressed preferentially in the liver and tissues with high fatty acid catabolism, such as the kidney, heart, skeletal muscle, and brown fat (Braissant et al., 1996). PPARα is activated by many natural ligands, including derivatives of fatty acids and leukotrienes B4, and synthetic ligands, including the common lipid-lowering drugs, fenofibrate and bezafibrate. Activated PPARα exerts beneficial effects on lipid metabolism, raising cardio-protective high-density lipoprotein (HDL) cholesterol and lowering cardiovascular mortality (Fievet et al., 2006). In addition, activation of PPARα may limit inflammation, both in the vessel endothelium and in other tissues as well as inhibiting the fibrotic response (Fievet et al., 2006). Several studies have examined a link between PPARα activation and epidermal differentiation. Fibrates induce differentiation and inhibit proliferation in normal and hyper-proliferating mouse epidermis and regulate apoptosis, but are inactive in PPARα-deficient mice (Komuves et al., 2000b, Komuves et al., 2000a).

Topical PPARα ligands have weak preventive effects on tumour promotion in mouse skin, despite up-regulation of PPARα in untreated tumours compared with normal epidermis (Thuillier et al., 2000). These observations suggest that the use of PPARα activators may have chemo-preventive properties in skin carcinogenesis. In addition, PPARα ligands have
been shown to suppress the growth of several cancer cell lines, including prostate (Collett et al., 2000), colon (Tanaka et al., 2001), breast (Suchanek et al., 2002b), and endometrial (Saidi et al., 2006) in vivo or in vitro. These data suggest that certain PPARα ligands may act as anti-tumour agents, although the exact mechanisms remain unclear.

PPARγ is important in the regulation of proliferation and differentiation of several cell types. PPARγ is known to be expressed in various organs, including adipose tissue (Keller et al., 1993), mammary glands (Gimble et al., 1998), small intestine (Mansen et al., 1996), and colon (Mansen et al., 1996). Natural ligands for PPARγ are polyunsaturated fatty acids (PUFA) (including arachidonic, oleic, and linoleic acid) and the cyclopentenone prostaglandin (PG). Synthetic ligands have been identified, such as the thiazolidinediones (including pioglitazone, rosiglitazone, and troglitazone). PPARγ ligands have been reported to induce cell differentiation and apoptosis in several types of cancer (Elstner et al., 1998, Tontonoz et al., 1997, Heaney et al., 2003, Ohta et al., 2001), suggesting their potential use as anti-cancer agents. Furthermore, some studies have suggested that PPARγ ligands can be used as chemo-preventive agents for colon, breast, and tongue carcinogenesis (Badawi and Badr, 2002, Brown and Lippman, 2000, Kopelovich et al., 2002).

The most commonly used synthetic agents belong to the thiazolidinedione class of anti-diabetic drugs (also referred to as glitazones). These include ciglitazone, troglitazone, pioglitazone and rosiglitazone. Pioglitazone, rosiglitazone, and troglitazone are currently in clinical use in the treatment of type 2 diabetes, utilizing the effects of synthetic PPARγ ligands to sensitize insulin and to lower blood glucose concentration (Yki-Jarvinen, 2004). Recent studies have indicated that certain thiazolidinediones, especially troglitazone and ciglitazone, exhibit moderate anti-proliferative effects against epithelial-derived human cancer cell lines, including those of prostate (Kubota et al., 1998), breast (Yin et al., 2001), colon (Kato et al., 2004), lung (Tsubouchi et al., 2000a), thyroid (Ohta et al., 2001) and pituitary carcinoma (Heaney et al., 2003). PPARγ is known to be expressed in many cancers, and the treatment of these cancer cells with PPARγ ligands exerts an anti-proliferative effects on human colon cancer (Sarraf et al., 1998), breast cancer (Elstner et
al., 1998), pituitary adenomas (Heaney et al., 2003), gastric cancer (Takahashi et al., 1999), and bladder cancer (Guan et al., 1999a).

These data suggest that PPARα and PPARγ may be potential targets for anti-cancer treatment. Data presented in chapter 3 indicate that PPARα and PPARγ are altered in endometrioid endometrial cancer suggesting that these may represent therapeutic targets in this cancer type. However, it is not clear whether the altered expression of PPARs in endometrioid endometrial cancer is fundamental to the development or growth of this disease or simply reflects global disruption of cellular pathways as cancer develops.

4.2 MATERIALS AND METHODS

In order to determine the potential action of PPARα and PPARγ on endometrial cell growth, expression of these receptors was initially reduced by siRNA in endometrial cell lines and growth affects studied. Further growth assays were then conducted using specific PPAR and RXR ligands.

4.2.1 Cell lines

Two differing endometrial cancer cell lines, Ishikawa cells and HEC-1A cells were maintained as described in chapter 2. The Ishikawa cell line was established from an endometrial adenocarcinoma from a 39-year-old Asian woman. These cells induce well-differentiated adenocarcinomas in animal models. Ishikawa cells are ER and PR positive and PTEN null. The HEC-1A cell line was established human endometrioid adenocarcinoma (G2) from a Japanese female. HEC-1A cells are ER negative and PTEN positive. Cells were grown in 75cm² and 25cm² culture flasks (Corning, UK) for cell line maintenance and collecting cell lysates and supernatants. Cells of the same passage were used for each experiment in order to reduce the amount of biological variability.

4.2.2 Antibodies and drugs

Anti-PPARα and -PPARγ antibodies for western blotting were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PPARβ, -RXRα, -RXRβ, -RXRγ were supplied by Abcam (Cambridge, UK). HRP-conjugated anti-goat, anti-mouse and anti-rabbit
secondary antibodies were supplied by Dako (Dako Cytomation, Ely, UK). Fenofibrate (PPARα ligand), Ciglitazone (PPARγ agonist), a PPARγ antagonist (GW9662), a PPARα antagonist (GW6471) and all-trans retinoic acid (ATRA) were supplied by Sigma-Aldrich, UK. These were used in growth assays at doses ranging from 5µM and 50µM.

4.2.3 siRNA

In order to reduce expression of PPARα and PPARγ, Qiagen Flexitube GeneSolution (Qiagen, West Sussex, UK) was used. Target sequences and the protocol are described in chapter 2. Western blotting was used to confirm reduced expression of the relevant protein using the method described in chapter 2. Following confirmation of successful gene reduction, cells were harvested and used for proliferation and apoptosis assays as previously described (chapter 2). Silencing lasted up to 36 hours in the Ishikawa cell lines and up to 30 hours in the HEC-1A cell lines.

4.2.4 Proliferation assays

Cell lines were seeded and cultured as described in chapter 2. Cells were cultured for 24 hours at 37°C and 5% CO2 in the presence of varying doses of test compound. Six replicates were used for each dose of drug and control in growth assays. Each individual experiment was repeated three times to validate results and in view of biological availability (n=18 for each dose). Control cells were treated with vehicle only. After 24 hours incubation, cell proliferation was assessed by the uptake of 5-bromo-2'-deoxy-uridine (BrdU) using the BrdU labeling and detection kit III (Roche-Diagnostics, UK) according to the manufacturer's instructions. Detailed methodology is provided in chapter 2. Results were recorded as optical density measurements (OD) at 405nm/490nm. Initial experiments at 24, 48 and 72 hours treatment showed that BrDU incorporation declined at 48 and 72 hours. This is probably due to limiting conditions within the cell culture system. Therefore 24 hours was selected as the optimum incubation period for this experiment. Furthermore, cell doubling times were similar for both cell lines up to 36 hours, therefore incubation for 24 hours was also used to ensure assays were carried out at the same time point with similar doubling.
4.2.5 Apoptosis assays

Cells were seeded and cultured as described in chapter 2. After incubation at 37°C and 5% CO₂ for 24 hours, the medium was replaced with fresh complete culture medium and varying doses of the experimental drug or substance. There were six replicates for each dose of drug and each experiment was repeated 3 times in total (n=18 for each dose). APOPercentage™ Dye label (Biocolor UK) (5µL) was added to each well and the cells incubated at 37°C for one hour. Control cells were treated with vehicle and dye only. Uptake of dye into apoptotic cells were recorded as optical density measurements at 550nm/620nm.

4.2.6 Statistical analysis

All statistical analyses were performed using Prism version 4 (GraphPad Software, San Antonio, CA). Non-parametric analysis of variance was performed incorporating Kruskal-Wallis statistic and Dunn’s multiple comparison tests. Statistical significance was accepted at p<0.05.

4.3 RESULTS

4.3.1 Confirmation of silencing of PPARα and PPARγ in endometrial cancer cell lines

Short interfering RNA was used to reduce PPARα and PPARγ expression in both endometrial cancer cell lines. Western blotting was used to confirm effective knockdown (Fig 4.1). Allstars negative control (Qiagen) was used as the negative control. This siRNA has no homology to any known mammalian gene. Results from Allstars siRNA were compared to results from untransfected cells, confirming that experimental setup did not cause any non-specific effects. In Ishikawa cells, A reduction of 70% in PPARα protein expression was seen in Ishikawa cells (p<0.01) and 75% in HEC-1A cells (p<0.01). A reduction of 80% in PPARγ protein expression was seen in Ishikawa cells (p<0.01) and 90% in HEC-1A cells (p<0.01).

4.3.2 Growth effect in endometrial cells with reduced expression of PPARα

Silencing of PPARα in both Ishikawa and HEC-1A cell lines caused a decrease in proliferation and increase in apoptosis (Fig 4.2A and B). The addition of fenofibrate, a
PPARα ligand to PPARα –ve cells further decreased proliferation in a dose-dependent manner (p<0.01, ANOVA, fig 4.2A). Fenofibrate also increased apoptosis in PPARα –ve cells (p<0.01, ANOVA, fig 4.2B).

4.3.3 Growth effects in endometrial cells with reduced expression of PPARγ

Silencing of PPARγ in both Ishikawa and HEC-1A cell lines caused an increase in proliferation and decrease in apoptosis (Fig 4.3A and B). The addition of ciglitazone, a PPARγ activating ligand to PPARγ silenced cells decreased proliferation in a dose-dependent manner, with the significant effects seen at doses of 20µM and 50µM (p<0.01). Ciglitazone also increased apoptosis in PPARγ silenced cells (p<0.01).

4.3.4 Effect of PPARα ligand on endometrial cancer cell growth in non-silenced cells

Endometrial cancer cells cultured with the PPARα ligand, fenofibrate, showed a decrease in DNA synthesis as measured by BrDU uptake in both Ishikawa and HEC-1A cells in a dose-dependent manner after 24-hour incubation (figure 4.4A). The difference in BrDU incorporation across the range of drug doses is significant (p<0.001, ANOVA). Low doses of fenofibrate had no significant effect on DNA synthesis in both cell lines. A reduction in BrDU uptake was however seen at doses of 20µM and 50µM (p<0.01, Dunn’s multiple comparison test). There is a dose-dependent increase in apoptosis in both endometrial cancer cell lines treated with fenofibrate (p<0.001, ANOVA). Dunn’s multiple comparison test demonstrated a statistically significant increase of apoptosis at 20µM and 50µM (p<0.01).

4.3.5 Effect of PPARα antagonist on endometrial cell growth in non-silenced cells

A PPARα antagonist, GW6471 was added at increasing doses to endometrial cancer cells that had been incubated with 50µM fenofibrate. Addition of GW6471 significantly reduced the effects of fenofibrate on endometrial cancer cell proliferation (p<0.01, ANOVA) in a dose-dependent manner but did not reverse them entirely (Fig 4.5A). Dunn’s multiple comparison demonstrated this effect to be greatest at 50µM GW6471 (p<0.05). Addition of GW6471 significantly abrogated effects of fenofibrate on apoptosis in endometrial cancer cells (p<0.01, ANOVA). This effect was greatest when 50µM GW6471 was used (p<0.05, Dunn’s, Fig 4.5B).
Figure 4.1 The effect of PPARα and -γ siRNA on PPARα and –γ protein expression in endometrial cancer cell lines. A. Western blots demonstrating the effect of PPAR siRNA on PPAR expression. B. Densitometry results of the effects of PPARα siRNA on PPARα expression. C. Densitometry results of the effects of PPARγ siRNA on PPARγ expression. Data is shown as box and “whiskers” (box extends from the 25th to 75th percentiles, whiskers show range with a horizontal line at the median, n= 6 for all) ** = p<0.01 (Dunn’s multiple comparison test).
Figure 4.2. The effect of the PPARα ligand fenofibrate on proliferation and apoptosis in PPARα siRNA endometrial cancer cells. A. The effect of fenofibrate on Ishikawa and HEC-1A cell proliferation after 24-hour incubation. B. The effect of fenofibrate on apoptosis in Ishikawa and HEC-1A cells. Error bars represent inter-quartile ranges.
Figure 4.3. The effect of the PPARγ ligand, ciglitazone, on proliferation and apoptosis in PPARγ siRNA endometrial cancer cells.  

A. The effect of reducing PPARγ expression in Ishikawa and HEC-1A cell proliferation after 24-hour incubation.  

B. The effect of reducing PPARγ expression in Ishikawa and HEC-1A cell apoptosis. Error bars represent inter-quartile ranges.
Figure 4.4. The effect of the PPARα ligand, fenofibrate, on proliferation and apoptosis in endometrial cancer cells. A. BrDU incorporation into Ishikawa and HEC-1A cells after 24-hour incubation. B. Apoptosis in Ishikawa and HEC-1A cells measured by dye uptake method. Error bars represent inter-quartile ranges.
Figure 4.5. The effect of PPARα antagonist, GW6471, on proliferation and apoptosis in endometrial cancer cells. **A.** The effect of GW6471 on Ishikawa and HEC-1A cell proliferation after 24-hour incubation as measured by BrDU uptake. **B.** The effect of GW6471 on apoptosis in Ishikawa and HEC-1A cells using a dye uptake method. Error bars represent inter-quartile ranges.
4.3.6 The effect of All-trans retinoic acid on cellular growth

As previously mentioned, in order for PPARs to exert downstream effects, they need to heterodimerise with RXRs. The addition of a synthetic retinoid X receptor to cells already incubated with PPAR ligand, was examined. All-trans retinoic acid (ATRA) was added in a dose-dependent manner to endometrial cancer cells already incubated with 50µM fenofibrate to determine if there was synergism of effects. The addition of ATRA significantly decreased proliferation in both cell lines (p<0.01, ANOVA). The reduction seen in proliferation with Fenofibrate +ATRA was greater (Fig 4.6A) than the effect of fenofibrate alone. Addition of ATRA further increased the effects of fenofibrate on apoptosis than those of fenofibrate alone (Fig 4.6B). This indicates a synergistic effect of ATRA on PPARα ligand, fenofibrate.
Figure 4.6. The effect of ATRA addition on proliferation and apoptosis in endometrial cancer cells. A. The effect of ATRA addition to fenofibrate on Ishikawa and HEC-1A cell proliferation after 24-hour incubation. B. The effect of ATRA addition to fenofibrate on apoptosis in Ishikawa and HEC-1A cells. ATRA. Error bars represent inter-quartile ranges.
4.3.7 Effects of PPARγ ligand on endometrial cancer cell growth

Endometrial cancer cells cultured with the PPARγ ligand, ciglitazone, showed a decrease in DNA synthesis in both Ishikawa and HEC-1A cells in a dose-dependent manner after 24-hour incubation (p<0.001, ANOVA) (figure 4.7A). Low doses of ciglitazone had no significant effect on DNA synthesis although a reduction in BrDU uptake was seen at doses of 20µM and 50µM (p<0.01, Dunn’s). Conversely, a dose-dependent increase in apoptosis was noted in both endometrial cancer cell lines treated with ciglitazone (Figure 4.7B, p<0.001, ANOVA). Dunn’s multiple comparison test demonstrated an increase of apoptosis at doses of 20µM and 50µM (p<0.01).

4.3.8 Effects of PPARγ antagonist on endometrial cell growth

The PPARγ antagonist, GW6992 was added at increasing doses to endometrial cancer cells previously incubated with 50µM ciglitazone. Addition of GW6992 significantly reduced the effects of ciglitazone on endometrial cancer cell proliferation (p<0.01, ANOVA) in a dose-dependent manner (Figure 4.8A). This effect was greatest at 50µM GW6992 (p<0.05). Addition of GW6992 also reduced the effects of ciglitazone on apoptosis in endometrial cancer cells (Figure 4.8B, p<0.01, ANOVA). Again, this effect was greatest when 50µM GW6992 was used (p<0.05). Although GW6992 appeared to partially reverse the effects of ciglitazone, it did not completely eliminate these.

4.3.9 Addition of All-trans retinoic acid on cellular growth

All-trans retinoic acid (ATRA), a ligand for the RXRs, was added to endometrial cancer cells previously incubated with 50µM ciglitazone at a range of doses to determine any synergism of effect. The addition of ATRA decreased proliferation in both cell lines (p<0.01, ANOVA). The reduction seen in proliferation with ciglitazone + ATRA was greater (Fig 4.9A) than the effect of ciglitazone alone. Addition of ATRA further increased the effects of ciglitazone on apoptosis than those of ciglitazone alone (Fig 4.9B) indicating a synergistic effect.
Figure 4.7. The effects of the PPARγ ligand, ciglitazone on proliferation and apoptosis in endometrial cancer cells. A. BrDU uptake in Ishikawa and HEC-1A cells treated with ciglitazone after 24-hour incubation. B. Apoptosis in Ishikawa and HEC-1A cells treated with ciglitazone. Error bars represent inter-quartile ranges.
Figure 4.8. The effect of the PPARγ antagonist, GW6992 on proliferation and apoptosis in endometrial cancer cells. A. BrDU incorporation in Ishikawa and HEC-1A cell proliferation after 24-hour incubation with GW9662 in ciglitazone treated cells. B. The effect of GW6992 on apoptosis in Ishikawa and HEC-1A cells pre-treated with ciglitazone. Error bars represent inter-quartile ranges.
Figure 4.9. The effect of ATRA addition on proliferation and apoptosis in endometrial cancer cells. **A.** The effect of ATRA addition to ciglitazone on Ishikawa and HEC-1A cell proliferation after 24-hour incubation. **B.** The effect of ATRA addition to ciglitazone on apoptosis in Ishikawa and HEC-1A cells. Error bars represent inter-quartile ranges.
4.4 DISCUSSION

This is the first study to demonstrate the functional effects of PPARα and PPARγ silencing in endometrial cancer cell lines. PPARα loss appears to have beneficial effects on endometrial cell growth in terms of proliferation and apoptosis. This study demonstrates that reducing the expression of PPARα reduces proliferation and increases apoptosis in Ishikawa and HEC-1A endometrial cancer cell lines. Conversely, reducing the expression of PPARγ in the same cell lines leads to increased proliferation and reduced apoptosis.

The effects of PPARα knockout (KO) has been studied in mice. PPARα KO mice are healthy, fertile and viable (Lee et al., 1995). Additional studies have demonstrated that PPARα KO mice accumulate lipids in the liver and have problems regulating insulin secretion during fasting (Lefebvre et al., 2006) in addition to impaired skin wound healing (Michalik et al., 2001) and prolonged inflammatory responses (Devchand et al., 1996). In addition increased adipose tissue mass with aging is noted and the mice develop spontaneous liver tumors, have age-dependent lesions of the liver, kidney and heart, and decreased longevity (Howroyd et al., 2004).

The addition of either a PPARα activating ligand, fenofibrate, or a PPARγ activating ligand, ciglitazone had beneficial effects on endometrial cancer cell growth by decreasing cellular proliferation and increasing programmed cell death. The combination of PPAR ligand and ATRA enhanced these effects. PPARα is expressed in rat ovaries using in situ hybridisation (Braissant et al., 1996). One study reported that fenofibrate inhibited aromatase cytochrome P450 expression in mice ovaries but did not in PPARα null mice, suggesting an important role of PPARα in the ovaries (Toda et al., 2003). The effect of fenofibrate on proliferation and apoptosis in endometrial cancer cells has been demonstrated previously although no attempt was made to determine whether or not these effects were dependent on the presence of the transcription factor (Holland et al., 2004).

These data suggest that fenofibrate may have receptor-dependent and independent effects. This is supported by previous work suggesting multiple possible mechanisms of action. The lipid-lowering actions of fenofibrate are based on PPARα binding to the PPRE within
promoter regions of genes involved in lipid, lipoprotein and glucose metabolism (Lefebvre et al., 2006, Gervois et al., 2007). The anti-inflammatory and anti-atherogenic actions are thought to be mediated in part through interaction with nuclear factor-κB and activated protein 1 (AP-1) (Lefebvre et al., 2006, Zandbergen and Plutzky, 2007). In this study, fenofibrate did reduce further proliferation and further apoptosis in cells with reduced PPARα expression which suggests that the effects seen may not be dependent on the presence of PPARα. Furthermore, there have been reports indicating that fenofibrate acts in a PPARα independent manner on human endothelial cells (Kim et al., 2007b, Murakami et al., 2006).

This is demonstrated in this study, as whilst GW6471 appears to abrogate the effects of fenofibrate, it does not completely reverse the effects. This suggests that fenofibrate may have both receptor-dependent and receptor-independent effects. A limitation of the work presented here, is that fenofibrate was the only PPARα ligand used. The effects seen may indeed be through PPARα activation however, fenofibrate may be working through the other PPAR receptors or indeed through other pathways. Likewise, GW6471 was the only antagonist used and a limitation of this antagonist may be incomplete inhibition.

The expression of PPARα in tumour cells (Collett et al., 2000, Suchanek et al., 2002a, Suchanek et al., 2002b) initiated studies of the role of this nuclear receptor and its ligands on the prevention of tumour cell proliferation in vitro and in vivo. Studies have demonstrated that PPARα ligands can suppress the growth of several cancer lines including colon, liver, breast, endometrial, and skin in vitro (Tanaka et al., 2001, Marina Maggiora, 2004, Muzio et al., 2007, Saidi et al., 2006, Thuillier et al., 2000), as well as inhibit the metastatic potential of melanoma cells in vitro and in vivo (Grabacka et al., 2006). Furthermore, PPARα ligands reduce the development of ovarian and colorectal cancers in mice (Tanaka et al., 2001).

Mechanisms investigating how PPARα directly prevents tumour cell functions have not been researched in detail but potential targets have been identified. Clofibrate significantly suppresses the growth of OVCAR-3 xeno-transplanted tumours and inhibits ovarian tumour cell proliferation by increasing the expression of carbonyl reductase, an enzyme that promotes the conversion of pro-tumourigenic prostaglandin E2 to inactive PGF2α (Yokoyama et al., 2007). Moreover, clofibrate reduces levels of circulating VEGF in tumour-
bearing mice (Yokoyama et al., 2007), while bezafibrate decreased the number of intestinal polyps in Apc Min/+ mice, possibly by lowering serum level of triglycerides and up-regulating lipoprotein lipase synthesis (Niho et al., 2003, Niho et al., 2005). Finally, PPARα activation has been shown to inhibit vascular smooth muscle cell proliferation underlying intimal hyperplasia by inducing the expression of the tumour suppressor p16INK4a (Gizard et al., 2005).

Although some studies have suggested that PPARα activation might be beneficial in reducing cancer growth, others demonstrate that long-term administration of certain PPARα agonists (clofibrate and WY14643) induces liver adenoma and carcinomas in rats and mice (Corton et al., 2000, Shah et al., 2007, Hays et al., 2005). Induction of hepatocarcinoma with PPARα ligand use has been found to be PPARα-dependent (Shah et al., 2007). All together, these findings indicate that, with few exceptions, PPARα ligands can be viewed as potential anti-tumourigenic agents.

This study has demonstrated that activation of PPARα in endometrial cancer cells has been proven to be beneficial in inhibiting cancer growth; it has also shown that loss of PPARα can be advantageous as it prevents tumour growth and development. This is supported by previous work, although this was demonstrated in granulocytes rather endometrial cancer cells (Kaipainen et al., 2007). Loss of PPARα leads to an increased infiltration to the side of injury of granulocytes that suppress tumour-associated angiogenesis via excess production of the endogenous angiogenesis inhibitor thrombospondin (Kaipainen et al., 2007). Kaipainen et al concluded that both activation of PPARα in specific host cells (i.e. endothelium) and concomitant inhibition of PPARα in immune cells (i.e. granulocytes) might lead to the same effects, namely protection from tumor growth.

In this study, there is a significant dose-dependent decline in proliferation of endometrial cancer cells through the addition of increasing concentrations of ciglitazone, a PPARγ activating ligand. Studies based on tumour cell lines have previously implicated PPARγ in cell-cycle withdrawal. PPARγ activation decreases the binding of the E2F/DP heterodimers to its target genes. This is, in part mediated by PPARγ through the down-regulation of the
PP2A protein phosphatase (Altiok et al., 1997). Inhibition of E2F/DP activity can also be achieved via activation of retinoblastoma (RB). PPARγ ligands have also been shown to inhibit phosphorylation of RB in vascular smooth muscle cells (Wakino et al., 2000), therefore contributing to maintain RB in its active form. Consequently, the G1/S transition in these cells was abrogated. Morrison and Farmer suggested a role of PPARγ in up-regulating the cyclin-dependent kinase inhibitors p18 and p21 during adipogenesis (Morrison and Farmer, 1999). This has been confirmed in further studies (Atsushi Itami, 2001, Elnemr, 2000). Therefore, PPARγ could control the expression of genes involved in the acquisition of a differentiated phenotype as well as genes involved in the negative regulation of cell cycle (Elnemr, 2000). Cell-cycle arrest in the G1 phase occurs in pancreatic tumour cell lines when P21 is induced by glitazones (Elnemr, 2000). Furthermore, up-regulation of P27 but not p21 (Waf1/Cip1) is seen in pancreatic tumours after treatment with a PPAR-γ agonist (Motonura, 2000, Atsushi Itami, 2001). Cell cycle arrest in human hepatoma cells is induced through the same mechanisms (Hironori Koga, 2001). PPAR-γ agonists, therefore induce arrest of the cell cycle, by the up-regulation of CDK inhibitors (Motonura et al., 2000). The precise mechanisms of growth inhibition, however, appear to be dependent on cell type and are yet to be shown in endometrial cancer cell lines.

Despite the reported expression of PPARγ in a number of epithelial malignancies, the physiological function of PPARγ in normal epithelial cells remains unknown. PPARγ KO has been examined in the rodent embryo and is embryologically lethal as a consequence of its effects on placental development (Barak et al., 1999a). In the placenta PPARγ is involved in secretion of reproductive hormones by trophoblasts, as well as being a negative regulator of trophoblastic invasion of uterine endometrium (Barak et al., 2008, Yang et al., 2008). These studies suggest that PPARγ is important in embryo development and indeed PPARγ -/- in rodents is fatal.

PPARγ activation was reported to inhibit the proliferation of malignant cells from different lineages; liposarcoma (Tontonoz et al., 1997), breast adenocarcinoma (Elstner et al., 1998, Yin et al., 2005), prostate carcinoma (Kubota et al., 1998), colorectal carcinoma (Brockman et al., 1998), non-small cell lung carcinoma (Chang and Szabo, 2000a), pancreatic
carcinoma (Motomura et al., 2000), bladder cancer cells (Guan et al., 1999b), and gastric
carcinoma cells (Sato et al., 2000). In adipocytes, macrophages, breast, prostate and non-
small cell lung cancer cells, thiazolidinediones are reported to induce apoptosis (Chinetti et
al., 1998a, Elstner et al., 1998, Kubota et al., 1998, Chang and Szabo, 2000a, Mueller et al.,
1998). The data presented in this thesis demonstrate that not only does ciglitazone reduce
proliferation and induce apoptosis in endometrial cells, it reverses the proliferative and anti-
apoptotic effects on PPARγ silencing. These observations suggest that enhanced activation
of PPARγ by specific ligands may represent a promising novel therapeutic approach for
endometrial cancers which seem to lose PPARγ. PPARγ ligands are already used to
enhance apoptosis in liposarcoma (Demetri et al., 1999) and in xenograft models of prostate
(Kubota et al., 1998) and colon cancer (Sarraf et al., 1998).

Possible mechanisms of PPARγ ligands in apoptosis have been identified. TNF-related
apoptosis inducing ligand (TRAIL) is a member of the TNF family of cytokines (Kim et al.,
2007a). TRAIL preferentially kills tumour cells, sparing normal tissues (Kim et al., 2007a).
Interest has emerged in applying this biological factor for anti-cancer therapy. A variety of
natural and synthetic PPARγ ligands sensitise only tumour cells to apoptosis induction by
TRAIL, sparing normal cells (Kim et al., 2007a). Kim et al, also reported that PPARγ ligands
selectively reduced levels of FLICE-inhibitory protein (FLIP), an apoptosis-suppressing
protein that blocks early events in TRAIL/TNF family death receptor signalling. In addition,
PPARγ modulators induced ubiquitination and proteasome-dependent degradation of FLIP,
without concomitant reductions in FLIP mRNA (Kim et al., 2007a).

Shimada et al investigated the modulation of apoptosis related gene expression by PPARγ
ligands in HT-29 colon cancer (Shimada et al., 2002). Apoptosis was induced in a dose-
dependent manner, associated with increased fragmented DNA, sensitive to a caspase
inhibitor (Shimada et al., 2002). Down-regulation of c-myc and up-regulation of c-jun and
GADD153 expression, was associated with the addition of PPARγ ligands, 15d-PGJ2 and
troglitazone (Shimada et al., 2002). C-myc is an important target gene of the APC/catenin
pathway. Deregulation of c-myc expression, induced by mutated APC, was compensated
through PPARγ activation (Shimada et al., 2002). By regulating apoptosis, the APC tumour
suppressor gene is involved in programmed cell death. Regulation of cell proliferation may also be PPARγ related to c-myc down-regulation. When bound to β-catenin, a component of cell–cell adherent junctions, APC acts to inhibit proliferation. C-jun and GADD153 gene protein expression lead to growth arrest and apoptosis (Shimada et al., 2002). Apoptosis was also induced in HT-29 human colon cancer cells following ciglitazone treatment (Yang and Frucht, 2001). Troglitazone inhibited liver cell cancer lines by inducing apoptosis through caspase 3 activation (Toyoda et al., 2002). Similar data exist for human lung cancer cells. Troglitazone and 15d-PGJ2 treatment induced apoptosis, a result that did not occur when PPARα agonists were applied (Tsubouchi et al., 2000b) Adenocarcinoma cells underwent enhanced growth inhibition when subjected to combined treatment with histone deacetylase (HDAC) inhibitors and PPARγ ligands compared to single treatment (Chang and Szabo, 2000b).

Breast cancer growth in MCF-7 cells was inhibited through the application of troglitazone. The addition of 9 cis-retinoic acid reversibly inhibited growth and induced apoptosis, and induced a reduction of bcl-2 protein levels (Elstner et al., 1998). The addition of PPARγ ligands, 15d-PGJ2 or troglitazone, attenuated cellular proliferation of both the oestrogen receptor (ER)-negative breast cancer cell line MDA-MB-231, as well as of the ER-positive cell line MCF-7. This resulted in total cell number reduction by inhibition of cell-cycle progression and apoptosis. This suggests that the possible primary biological response from PPARγ activation is the induction of apoptosis in breast cells, indicating a potential role for PPARγ ligands in treating breast cancer (Clay et al., 1999). Clay et al., more recently reported that while 15d-PGJ2 activates PPRE-mediated transcription, PPARγ is not required for apoptosis induction in breast cancer cells, suggesting cyclopentenone prostaglandins as the inducers of apoptosis (Clay et al., 2001). This is potentially an important finding as ER is important in the development of endometrioid endometrial cancer, and it has been suggested that ligand dependent or ligand independent activation of ERβ could have an impact on progression of endometrial cancers especially those with a more aggressive phenotype that are ERα-negative (Collins et al., 2009). Collins et al only examined ER status in endometrial cancers (FIGO G1-3) and did not include benign endometrium or atypical
hyperplasia in their sample collection and this may have provided insight into the change of ER status throughout carcinogenesis.

Data concerning the putative role of PPARγ in cellular function has been mostly obtained in vitro. Silencing of PPARγ demonstrated an increase in proliferation and decreases in apoptosis, suggesting that PPARγ may have anti-proliferative properties. Its anti-proliferative properties i.e. its action on cell cycle, proliferation, differentiation and apoptosis are dependent on the target cell type and/or the mutational events predisposing tissues to tumourigenesis. A limitation of this work is that it was carried out in vitro, one would need to examine the effect of PPARγ KO in vivo in order to examine the effects of KO on endometrial cancer cells as well as any other effects on normal cells. Furthermore, the effects of PPAR ligands may not be wholly receptor dependent.

In this study, the effects of ciglitazone appear to be both receptor-dependent and receptor-independent. Direct and indirect evidence exists suggesting that select anti-tumourigenic properties of PPARγ ligands could occur independently of PPARγ. Indirect evidence includes several studies that found that selective TZDs, could inhibit cholesterol biosynthesis or affect cholesterol efflux from cells (Wang et al., 1999). Additional evidence shows that pharmacological concentrations of the PPARγ ligands are required to produce an anti-carcinogenic effect or inhibit cytokines, whereas concentrations several logs lower are required for modulation of lipid-related genes and induction of differentiation of pre-adipocytes to adipocytes.

Direct evidence also exists suggesting the actions of PPARγ ligand may be independent of PPARγ. Asou et al noted that proliferation of acute myeloid leukemia cell lines was markedly inhibited by PGJ₂ (Asou et al., 1999). Addition of PGJ2 or TZDs to normal, activated macrophages, resulted in the decreased release of inflammatory cytokines; TNF, IL-1, IL-6, inducible nitric oxide synthase, and COX-2 (Jiang et al., 1998, Ricote et al., 1998). Exposure of the PPARγ -/- macrophages to either PGJ2 or TZDs also lowered their production of these anti-inflammatory molecules (Chawla et al., 2001). This indicates that expression of PPARγ is not necessary for beneficial effects of PPARγ ligands. A limitation of this work is that the investigators did not examine if the PPARγ ligand may be working through the other PPAR
receptors in order to mediate their effects. In the second series of experiments, TZD inhibited proliferation, DNA synthesis, and caused a G1 cell cycle arrest of embryonic stem cells from both PPARγ−/− and PPARγ+/+ mice (Palakurthi et al., 2001). Likewise, TZDs inhibited cyclin D1 expression and the growth of PPARγ−/− embryonic stem cell tumours in syngeneic mice (Rossi et al., 2000) suggesting that TZD’s work independently of PPARγ receptor status.

The addition of ATRA to fenofibrate or ciglitazone enhanced their growth effects in both endometrial cancer cell lines. These results are supported by previous studies. Tontonoz et al. reported that the combined use of PPARγ and RXRa specific ligands is able to trigger terminal differentiation of primary human liposarcoma cells in vitro, suggesting that the combination of these ligands may be beneficial in the treatment of liposarcoma (Tontonoz et al., 1997). Beneficial effects for the combined treatment with PPAR ligands plus retinoids are extensively reported in preclinical studies of the hematologic malignancies (Konopleva et al., 2004, Lee et al., 2007, Hirase et al., 1999). Therefore, the combination of PPARγ ligand with RXR agonist or RAR agonist can enhance the differentiating and growth-inhibitory effects in human leukemia cells (Konopleva et al., 2004). The combination of PPARγ ligand, ciglitazone, and ATRA synergistically reduces the cell growth rates and cell cycle arrest at the G1 phase in HL-60 human leukemia cells and this is associated with synergistic up-regulation of PTEN expression (Lee et al., 2007). The combination of 9-cis RA and PPARγ ligand shows significant synergistic effects for the induction of apoptosis in multiple myeloma cells (Ray et al., 2004).

Preclinical studies confirm the favourable effects of combination of PPAR ligands plus retinoids on the inhibition of cell growth in solid malignancies, especially in breast cancer (Crowe and Chandraratna, 2004, Mehta et al., 2000, Elstner et al., 2002). Rubin et al. showed that a combination of ligands for PPARγ and RXR inhibits breast aromatase expression induced by tumor-derived factors (Rubin et al., 2002). As aromatase activates oestrogen biosynthesis, the combination of these ligands may be able to find utility in the treatment of oestrogen-dependent carcinogenesis, such as breast cancer and endometrial cancer (Saidi et al., 2006). The combination of RXR ligand with ciglitazone also
cooperatively inhibits the growth of breast cancer and lung cancer cells by activating the RARE promoter activity and inducing RARβ, which plays a critical role in mediating the growth-inhibitory effects of retinoids in various cancer cells (James et al., 2003).

The synergistic effects of PPARs and RXR ligands have been studied by many authors. Yang et al. reported that the PPARγ and RXR ligands have been shown to differentially recruit subsets of transcriptional coactivators (i.e., p160 by RXR and DRIP205 by PPARγ) to the receptor complex, thus leading to an enhanced transcriptional activation and cellular effects (Yang et al., 2000). The transcriptional activity of PPRE is additively induced by treatment with a PPARγ activator plus 9-cis RA, and RXRα accumulation, by inhibiting its degradation due to the proteasome system, therefore contributing to the enhancement of PPARγ/RXR activation (Tsao et al., 2005). The transactivation of the PPRE by the PPARγ/RXR heterodimer enhances the expression of the glutathione S-transferase gene, which is responsible for the cellular metabolism as well as the detoxification of several xenobiotics and carcinogenic compounds (Park et al., 2004). The findings of these reports suggest that the accumulation of the unphosphorylated form (i.e., functional form) of RXRα activates the transcriptional activity of PPRE and thereby enhances the expression of important target genes. The significance of the restoration of RXRα by inhibiting its aberrant phosphorylation is reported in the studies using the cell lines of HCC (Matsushima-Nishiwaki et al., 2001), leukemia (Kanemura et al., 2008), and colon cancer (Yamazaki et al., 2007). This supports the work presented in this chapter in relation to ATRA but also the up-regulation of RXRα seen in endometrial cancers, alongside the preferential binding of PPARα and PPARγ to RXRα (chapter 3). The gain of RXRα may be beneficial by activating specific target genes through PPRE or indeed may be a compensatory mechanism due to the loss of RXRβ and RXRγ as PPARs need to form heterodimers with RXRs in order for transcription to occur.

In summary, this work has demonstrated that both the PPARα and PPARγ transcription factors are capable of controlling proliferation and apoptosis in endometrial cancer cells. Gene silencing experiments as well as the use of receptor antagonists has suggested that the effects of PPAR ligands can work both dependently and independently of the PPAR
receptors. Combination treatment with PPAR ligands and ATRA demonstrated an enhancement of the anti-tumourigenic effects suggesting that activation of the RXRs may be able to compensate for reduced PPAR availability. These data suggest the beneficial use of the agents as options in chemoprevention and/or treatment of endometrioid endometrial cancers, however, further research into their possible mechanisms of action is necessary before these in vitro results can be translated safely into clinical practice.
5.1 INTRODUCTION

In the previous chapter, data showed that gene silencing of either PPARα or PPARγ had effects on the growth of cultured endometrial cancer cells by decreasing proliferation and increasing apoptotic cell death. Similar effects were seen when cells were treated with PPAR specific ligands. Although these effects were not wholly dependent on the presence of the respective transcription factors, the growth effects were enhanced in cells that expressed PPARγ or PPARα respectively. It is possible that activated PPARs cause downstream growth effects via so-called cell autonomous or non-cell autonomous mechanisms. Cell autonomous is a genetic trait in multi-cellular organisms in which only genotypically mutant cells exhibit the mutant phenotype. Conversely, a non-autonomous trait is one in which genotypically mutant cells cause other cells (regardless of their genotype) to exhibit a mutant phenotype. For example, it has been established that PPARs regulate the inflammatory process (Kostadinova et al., 2005) as well as being involved in the tumour stroma, including immune and endothelial cells (Michalik and Wahli, 2006).

PPARγ is the most studied PPAR isoform and is capable of regulating differentiation and/or cell growth in differing cell types (Sarraf et al., 1998, Sarraf et al., 1999b, Elstner et al., 1998). One potentially important mechanism by which this may be achieved is via the regulation of the PTEN tumour suppressor gene. PTEN is known to modulate several cellular functions, including cellular migration, survival and proliferation (Di Cristofano and Pandolfi, 2000, Myers et al., 1997). PTEN is located on chromosome 10q23 (Myers et al., 1997) and is a lipid Phosphatase that de-phosphorylates the 3-position of phosphatidylinositol 3,4,5-triphosphate, a second messenger of phosphatidylinostol 3-kinase (PI3K) (Di Cristofano and Pandolfi, 2000, Maehama and Dixon, 1998a). PTEN antagonizes PI3K activity and negatively regulates its downstream target, the serine/threonine kinase Akt.
Phosphorylated and activated Akt modulates the activity of differing downstream proteins involved in cell survival and proliferation (Di Cristofano and Pandolfi, 2000). Some studies have suggested that PPARγ, like PTEN, can act as a tumour suppressor (Teresi et al., 2006, Patel et al., 2001, Bonofiglio et al., 2005, Zhang et al., 2006). Patel et al. demonstrated that PPARγ can be a PTEN transcription factor (Patel et al., 2001) and also demonstrated that activation of PPARγ with rosiglitazone, up-regulates PTEN expression in human macrophages, Caco2 colorectal cancer cells and MCF7 breast cancer cells (Patel et al., 2001). Using electrophoretic mobility shift assays, they demonstrated that PPARγ is able to bind two response elements in the genomic sequence upstream of PTEN (Patel et al., 2001). Furthermore, two independent laboratories confirmed Patel’s findings that PPARγ induces PTEN transcription in MCF7 breast cancer cells (Teresi et al., 2006, Bonofiglio et al., 2005). In addition to these findings, Zhang et al, reported that stimulation of hepatocarcinoma cells with rosiglitazone resulted in an up-regulation of PTEN and PTEN-dependent inhibition of cell migration (Zhang et al., 2006). These studies highlight the role of PPARγ as a possible regulator of PTEN expression. These experiments, however, may be cancer-type dependent as they were confined to breast cancer cell lines.

Loss of functional PTEN is common in endometrioid type endometrial cancers (Risinger et al., 1997, Tashiro et al., 1997a). PTEN mutations have been identified in 55% of precancerous endometrial lesions and in up to 83% of endometrioid endometrial cancers (Mutter et al., 2001). This suggests that loss of functional PTEN is an early step in the development of this tumour. The relationship between PPARγ and PTEN in the development of endometrial cancer is currently unknown although it is well established that loss of PTEN is also associated with progression to endometrial cancer (Terakawa et al., 2003).

Although, the development of a malignant tumour originates with abnormalities of cellular DNA, it is clear that this alone is insufficient to result in a clinical tumour and metastasis. Other processes and interactions between the cancer cell and other cells within the tumour micro-environment are important, so-called, non-cell autonomous mechanisms. In 1972, Judah Folkman proposed that tumour growth requires tumour angiogenesis i.e. the development of new blood vessels from an existing vessel network, and investigated the role...
of the non-neoplastic “host” cell in the tumour microenvironment (Folkman, 1972).

Angiogenesis, essential for physiological as well as pathological processes, is regulated by pro- and anti-angiogenic factors (Hanahan and Folkman, 1996). Excessive angiogenesis is closely linked to human disease, including chronic inflammatory disease, diabetic retinopathy, and cancer (Folkman, 1995, Carmeliet and Jain, 2000). Studies have demonstrated that the inhibition of angiogenesis in tumours often decreases the growth of cancer (Bergers and Benjamin, 2003). The most potent pro-angiogenic factor is VEGF-A which is up-regulated by hypoxia inducible factor (HIF1α) (Ferrara et al., 2003). Thus, tumour-associated hypoxia plays an essential role in the regulation of angiogenesis (Liao and Johnson, 2007). In addition to hypoxia, cytokines, growth factors, tumour promoters, and hypoxia all modulate the expression of VEGF (Ho and Kuo, 2007). VEGF is considered key in cancer growth contributing to tumour neo-vascularisation and dissemination.

Increased expression of VEGF has been found in most tumours, and agents neutralizing VEGF expression or activity inhibit tumour growth in vivo (reviewed in (Carmeliet and Jain, 2000, Bergers and Benjamin, 2003, Griffioen and Molema, 2000). Increased VEGF-A is a feature of endometrial cancer (Doldi et al., 1996, Fujimoto et al., 1998, Kamat et al., 2007). Microvessel density is an independent prognostic factor of disease progression and survival (Kaku et al., 1997) and progressive increase in microvessel density is seen from benign endometrium, through atypical hyperplasia to malignant disease (Abulafia et al., 1995).

In order to determine likely mechanisms of action of PPARα and PPARγ on direct and indirect cell growth in endometrial cancer, the key pathways in this disease were studied i.e. PTEN (direct) and VEGF-A (indirect). Immunohistochemistry and western blotting was used to assess PTEN and P-AKT expression in endometrial tissues.

5.2 METHODS

5.2.1 Immunohistochemistry

Immunohistochemistry was used to determine the distribution of PTEN and P-AKT in tissue sections using an immunoperoxidase staining method
5.2.2 Cell lines

Ishikawa and Human endometrial cancer (HEC-1A) cells were cultured and maintained in medium as previously described (Chapter 2). Cells were incubated at 37°C in 5% CO₂ and passaged at 70-80% confluence using 0.05% trypsin with 0.53mM EDTA (Invitrogen, UK) and neutralised in an equal volume of DMEM culture medium containing FBS. Cells were grown in 75-cm² and 25cm² culture flasks (Corning, UK) for cell line maintenance and collecting cell lysates and supernatants.

5.2.3 Gene silencing using siRNA

Gene silencing for PPARα and PPARγ was performed following the procedure described in chapter 2, section 2.8. Qiagen Flexitube GeneSolution (Qiagen, West Sussex, UK) was used. Western blotting was used to confirm adequate knock down had been achieved prior to use of samples. A reduction of 70% in PPARα protein expression was seen in Ishikawa cells and 75% in HEC-1A cells. A reduction of 80% in PPARγ protein expression was seen in Ishikawa cells and 90% in HEC-1A cells.

5.2.4 Drug Treatment

Endometrial cancer cell lines were treated with the selective PPARα agonist, fenofibrate, and selective PPARγ agonist, ciglitazone. The PPARα antagonist, GW6471 and PPARγ antagonist, GW9662 respectively, were also added. The drugs were dissolved in DMSO to a concentration of 50µM as stock solution. Cells were seeded; the following day medium was changed to the relevant experimental dilutions (0, 5, 10, 20 and 50µM) and cultured for 24 hours to observe the effects on cell growth. Ciglitazone was chosen as it is a selective PPARγ agonist (EC₅₀ =3µM) and fenofibrate as it is the commonly used PPARα agonist (EC₅₀ =30µM).

5.2.5 Western Blotting for PTEN, P-AKT, PPAR alpha and PPAR gamma.

Western blotting was used initially to confirm that siRNA had resulted in adequate reduction of PPAR alpha and PPAR gamma protein expression within the cultured endometrial cancer cells. Thereafter the quantities of PTEN and P-AKT were also analysed to determine
whether or not these were affected by PPAR alpha or gamma expression. Western blotting was also used on paraffin-embedded endometrial tissues to examine PTEN expression. In brief, nuclear extracts were prepared as described in chapter 2, and used for Western blotting. The same protocol and antibodies were used as described in chapter 2. In addition, established cultures of endometrial cancer cells which had not been treated with siRNA were used as controls and also treated with either a specific PPAR alpha or gamma ligand, PTEN and P-AKT were then determined using Western blotting. Six replicates were used for each control or each test dose and the experiments were repeated three times. Protein expression was measured as bands seen on western blots. Films were scanned, processed and analysed using Image J software to measure density of the bands seen. The final value was expressed as a ratio of protein of interest expression: β-actin.

5.2.6 ELISA

The QuantiGlo VEGF ELISA (R&D systems, UK), a solid phase ELISA, was used to measure VEGF_{165} (a soluble form of VEGF) levels in cell culture supernatants. Supernatants were acquired from the drug treatment experiments described above. Cells were incubated and grown as previously described in section 5.2.1. Supernatants were collected from control cells as well as for the drug treated cells. The ELISA protocol is described in chapter 2, section 2.9. Six replicates were used for each control or test solution and the same plate and conditions used throughout the experiment.

5.2.7 Statistical Analysis

The statistical package GraphPad Prism 4 was used to analyse the data. A non-parametric analysis of variance was performed, incorporating kruskal-wallis statistic and Dunn’s multiple comparison tests. Statistical significance was accepted at p<0.05.
5.3 RESULTS

5.3.1 Silencing of PPARα and PPARγ in endometrial cancer cell lines

Short interfering RNA was used to silence PPARα and PPARγ expression in both endometrial cancer cell lines. Western blotting was used to confirm effective knockdown (Chapter 4, Fig 4.1). In Ishikawa cells, a reduction of 70% in PPARα protein expression was seen in Ishikawa cells (p<0.01) and 75% in HEC-1A cells (p<0.01). A reduction of 80% in PPARγ protein expression was seen in Ishikawa cells (p<0.01) and 90% in HEC-1A cells (p<0.01).

5.3.2 PTEN and P-AKT expression in endometrial tissues

In order to investigate the validity for investigation of PTEN with PPARγ gene silencing, endometrial samples were probed for PTEN (figure 5.1A and B) which is commonly mutated in endometrial carcinoma. As expected, wild type PTEN was less highly expressed in the endometrial cancers (p<0.05, Kruskal-Wallis). It has been suggested that PTEN mutations exert their effects through the phosphoinositide 3-kinase and phosphorylated AKT (PI3K-AKT) pathway (Panigrahi et al., 2004). In order to investigate if this pathway was functional, western blots were also probed for P-AKT. The data indicate an increase in P-AKT expression in endometrial cancers compared to benign (figures 5.1C and 5.1D) endometrium (p<0.05, Kruskal-Wallis).

Immunostaining for PTEN is shown in figure 5.2, and as expected, intense staining for PTEN was seen in endometrial glandular cells and weaker stromal staining within benign tissues (figure 5.2B). In atypical hyperplasia, strong staining is seen in glands with minimal stromal staining (figure 5.2C). However, little or no PTEN staining is seen in the malignant tissue, particularly G3 tumours (figure 5.2D and E).
Figure 5.1 Nuclear expression of PTEN and P-AKT in endometrial tissues. A and C: Western blotting for PTEN and P-AKT respectively. (Pos) Nuclear lysates from positive controls, (B) benign endometrium, (G1) FIGO G1 endometrioid endometrial carcinoma, (G3) FIGO G3 endometrioid endometrial carcinoma. B and D: Densitometry measurements from western blots. Data is shown as box and “whiskers” (box extends from the 25th to 75th percentiles, whiskers show range with a horizontal line at the median, n= 5 for all).
Figure 5.2: Immunostaining for PTEN (A) Benign endometrium incubated with IgG (negative control); (B) Benign endometrium with PTEN antibody (positive control); (C) PTEN expression in atypical hyperplasia; (D) PTEN expression in FIGO G1 carcinoma; (E) PTEN expression in FIGO G3 carcinoma. Line represents scale bar measuring 100µM. All images at x10 magnification.
5.3.3 Effect of PPAR gene silencing on PTEN and P-AKT

PTEN and P-AKT expression was examined both in Ishikawa and HEC-1A endometrial cancer cells. Western blotting confirmed the PTEN null status of Ishikawa cells (Fig 5.3A). P-Akt was present in Ishikawa cells and silencing of PPARγ to 70% normal expression reduced P-Akt expression (Fig 5.3A and B, p<0.05). Western blotting confirmed PTEN expression in PTEN wild-type HEC-1A cells. Silencing of PPARγ reduced PTEN expression (p<0.05) and increased P-Akt expression (p<0.05) in HEC-1A cells (Fig 5.3C).

5.3.4 Effects of PPARγ agonist and antagonist on PTEN and P-Akt expression

Increasing doses of the PPAR gamma specific ligand, ciglitazone were added to Ishikawa cells and the expression of PTEN and Akt examined by Western blotting (figure 5.4). Despite an absence of PTEN in Ishikawa cells, ciglitazone decreased P-Akt expression in these cells in a dose-dependant manner. Conversely, the addition of the PPAR gamma antagonist GW9662 reversed the effects of ciglitazone in a dose-dependent manner. The action of ciglitazone was also investigated in HEC-1A cells. The addition of ciglitazone increased PTEN expression (P<0.05) and the addition of GW9662 reversed these effects (p<0.05). Both the effects of ciglitazone and GW9662 appear to be dose-dependent. The addition of ciglitazone decreased P-Akt expression and addition of GW9662 reversed these effects. Again, these effects appeared to be dose-dependent.

5.3.5 Effect of PPARα and PPARγ silencing on VEGF secretion

Median VEGF levels in normal PPARα positive Ishikawa cells were 18pg/ml. Median VEGF levels were reduced in Ishikawa cells with reduced PPARα expression. (fig 5.5A). Median VEGF levels in normal PPARα positive HEC-1A cells were 19pg/ml. Gene silencing of PPARα also reduced VEGF-A secretion in HEC-1A cells (fig 5.5A). These data indicate a statistically significant reduction in VEGF secretion in both endometrial cancer cell lines through PPAR silencing compared to controls (p<0.05). Conversely, PPARγ silencing increased median VEGF levels increased in both Ishikawa and HEC-1A cell lines (p<0.05, fig 5.5B).
Figure 5.3 The effect of PPARγ silencing on PTEN and P-Akt expression in endometrial cancer cells. A. Western blots demonstrating the effect of PPARγ silencing on PTEN null Ishikawa cells and on PTEN wild type HEC-1A cells. B. Densitometry results of the effects of PPARγ siRNA on P-Akt expression. C. Densitometry results of the effects of PPARγ siRNA on PTEN expression. Data is shown as box and “whiskers” (box extends from the 25th to 75th percentiles, whiskers show range with a horizontal line at the median, n=6 for all) * = p<0.05, ** = p<0.01 (Dunn’s multiple comparison test).
Fig 5.4. Effect of PPARγ agonist and antagonist addition on PTEN and P-Akt expression in Ishikawa and HEC-1A cells. Increasing doses of PPARγ agonist, ciglitazone and PPARγ antagonist, GW9662, were added to Ishikawa and HEC-1A cells and western blotting performed to determine PTEN and P-Akt expression.
5.3.6 Effect of PPAR ligands on VEGF secretion in PPAR positive endometrial cancer cells

The addition of a PPARα ligand, fenofibrate, reduced VEGF secretion in Ishikawa endometrial cancer cells compared to untreated cells (fig 5.7, p<0.05). Addition of all-trans retinoic acid (ATRA) and fenofibrate together further reduced VEGF secretion (p<0.05). The addition of a PPARα antagonist, GW6471, reversed the effects on VEGF secretion seen with the addition of fenofibrate (p<0.05). Similar results were noted in the HEC-1A cell line with the addition of fenofibrate also reducing VEGF secretion significantly (p<0.05). The addition of ATRA further reduced VEGF secretion (p<0.05) and the addition of GW6471 reversed the effects of fenofibrate on HEC-1A cells (p<0.05).

5.3.7 Effects of PPAR ligands on VEGF secretion in PPAR silenced endometrial cancer cells

Neither the addition of a PPARα ligand, fenofibrate, nor PPARγ ligand, ciglitazone had any significant effect on VEGF secretion in PPARα siRNA or PPARγ siRNA Ishikawa cells respectively (Fig 5.6A). Similarly, the addition of fenofibrate or ciglitazone had no significant effect on PPAR siRNA HEC-1A cells (fig 5.6B).

5.3.8 Effect of PPARγ ligands on VEGF secretion

The addition of the PPARγ ligand, ciglitazone reduced VEGF secretion in Ishikawa cells (figure 5.8). Furthermore, the combination of ciglitazone and ATRA reduced VEGF secretion even further (p<0.05). The addition of PPARγ antagonist, GW9662, appears to partially reverse these effects. The addition of ciglitazone to HEC-1A cells also reduced VEGF secretion as in Ishikawa cells, the addition of ATRA to ciglitazone further reduced VEGF secretion (p<0.05). Addition of GW9662 appears to partially reverse these effects.
Figure 5.5 The effect of PPARα and PPARγ silencing on VEGF secretion in endometrial cancer cells. A. The effect of PPARα silencing on VEGF secretion in Ishikawa (Ish) and HEC-1A endometrial cancer cells. B. The effect of PPARγ silencing on VEGF secretion in Ishikawa (Ish) and HEC-1A endometrial cancer cells. Error bars represent interquartile ranges, * represents p<0.05.
Figure 5.6. The effect of PPAR agonists on PPAR silenced endometrial cancer cell lines. A. The addition of PPARα ligand, fenofibrate, and PPARγ ligand, ciglitazone, on PPAR siRNA Ishikawa cells. B. The addition of fenofibrate and ciglitazone, on PPAR siRNA HEC-1A cells.
Figure 5.7 The effect of a PPAR\(\alpha\) ligand, fenofibrate, on VEGF secretion in endometrial cancer cells. A. The effect of fenofibrate on VEGF secretion in Ishikawa endometrial cancer cells (Ish). B. The effect of fenofibrate on VEGF secretion in HEC-1A endometrial cancer cells. Error bars represent interquartile ranges.
Figure 5.8 The effect of PPARγ ligand, ciglitazone, on VEGF secretion in endometrial cancer cells. A. The effect of ciglitazone on VEGF secretion in Ishikawa endometrial cancer cells (Ish). B. The effect of ciglitazone on VEGF secretion in HEC-1A endometrial cancer cells. Similar effects were seen in HEC-1A cells as described for Ishikawa cells. Error bars represent interquartile ranges.
5.4 DISCUSSION

Cancer development is no longer defined as the loss of growth control of a single cell; it is a developmental process involving the tumour cell as well as the host tissue. In the previous chapters, the data demonstrated the differential expression of PPARs in endometrial cancer, as well as the role of PPARα and PPARγ on cellular growth. Two possible key actions of PPARs, direct or indirect were examined in this chapter, in relation to PTEN and VEGF.

In this study, loss of PPARγ was also correlated with reduced expression of PTEN. PTEN is a known tumour suppressor gene (Di Cristofano and Pandolfi, 2000) encoding a protein that negatively regulates the functions of integrins (Tamura et al., 1998). Loss of function mutations are common in endometrioid endometrial cancer occurring in up to 83% of tumours (Tashiro et al., 1997a, Risinger et al., 1997). PTEN mutation, deletion and loss of expression have all been demonstrated in pre-malignant endometrial lesions (Ali, 2000, Mutter et al., 2000) with up to 55% of pre-malignant endometrial lesions being reported to show mutated PTEN (Mutter et al., 2001). The findings in this study indicate that PPARγ-related effects may be mediated by PTEN. Two PPRE's have been identified within the PTEN promoter (Patel et al., 2001) and it has been shown that PPARγ can upregulate the transcription of PTEN in breast cancer (Teresi et al., 2006, Bonofiglio et al., 2005). Similar to the results in this thesis, the PPARγ ligand, rosiglitazone inhibited growth in both PTEN wild-type and PTEN-null endometrial cancer cell lines in murine growth studies (Wu et al., 2008) suggesting that growth activity is not solely dependent on functional PTEN. PTEN function is mediated via the Phosphoinositide-3-kinase (PI3K)/Akt pathway and PTEN expression is inversely correlated with P-Akt expression (Maehama and Dixon, 1998b, Li et al., 1998, Dahia et al., 1999). This is confirmed in the current study, supporting the hypothesis that Akt activation and PTEN inactivation is involved in the development and/or progression of endometrial cancers.

The effect of PPARγ silencing and activation was examined in relation to PTEN and P-Akt expression. These data demonstrate that PTEN expression is reduced when PPARγ expression is reduced by siRNA in HEC-1A (PTEN wild type) cells compared to controls.
Conversely, PPARγ activation with ciglitazone, results in increased PTEN and decreased P-Akt expression. Previous studies demonstrate that PPARγ activation by rosiglitazone is associated with increased PTEN expression in colorectal, breast and pancreatic cell lines, which in turn inhibits the PI3K pathway and Akt (Patel et al., 2001, Farrow and Evers, 2003, Wu et al., 2008). These studies support the findings presented in this chapter although a different ligand was used. The expression of P-Akt was investigated in order to determine if the PTEN expressed was functional and able to inhibit PI3K activity. After treatment with ciglitazone for 24 hours, phosphorylated Akt levels decreased. This is supported by Farrow et al, who similarly demonstrated a reduction in P-Akt at the same time point as an increase in PTEN expression (Farrow and Evers, 2003). Furthermore, I have demonstrated that effects of ciglitazone on PTEN and P-Akt expression were almost completely reversed by the addition of PPARγ antagonist, GW9662, suggesting that ciglitazone increases PTEN levels through PPARγ activation. These data are also supported by Farrow et al, in pancreatic cells (Farrow and Evers, 2003). The data in this chapter therefore suggests a direct relationship between PPARγ activation and PTEN expression, although the mechanism of action was not determined. Patel et al. have shown that PPARγ can be a PTEN transcription factor (Patel et al., 2001). They observed that rosiglitazone induced PTEN protein expression in both MCF-7 breast and CoCa2 colon cancer cell lines. In addition to the increase in PTEN expression, they observed an inhibition of both Akt phosphorylation and cellular proliferation, supporting the data presented in this dissertation. They also identified two putative PPREs within the PTEN promoter approximately 15 and 13 kb upstream of the ATG site. This study was significant in demonstrating a potential link between PPARγ and PTEN, as well as a mechanism of action whereby PPARγ exerts its effects on PTEN. Two independent laboratories confirmed Patel’s suggestion that PPARγ induces PTEN transcription in a breast cancer setting (Teresi et al., 2006, Bonofiglio et al., 2005). Furthermore, Teresi et al. showed that stimulation with rosiglitazone induces a PTEN protein that is both protein- and lipid-phosphatase active, as evidenced by decreased phosphorylation of Akt and MAPK concomitant with PTEN expression. Additionally, rosiglitazone treatment induced G1 arrest that paralleled PTEN expression. Teresi et al, used a rosiglitazone analog, compound 66, which is incapable of activating PPARγ, to
confirm that rosiglitazone induced PTEN expression via a PPARγ-dependent mechanism in several reporter assays (Teresi et al., 2006).

Bonofiglio et al. also demonstrated that PPARγ could upregulate PTEN transcription in a breast cancer setting (Bonofiglio et al., 2005). Their results were similar to those reported in this chapter as ciglitazone in both endometrial cancer cell lines results in an increase in PTEN expression and an inhibition of Akt. Bonofiglio et al. reported an increase in PTEN protein expression as well as an inhibition of Akt phosphorylation and cellular growth with RGZ stimulation. In addition to confirming these results, Bonofiglio et al observed for the first time the specific binding of PPARγ to the PTEN promoter (Bonofiglio et al., 2005). Interestingly, this interaction was enhanced by rosiglitazone treatment. Further analysis indicated that PPARγ and oestrogen receptor (ER) could bind to the PPRE both independently and simultaneously (Bonofiglio et al., 2005). The association of ER with the PPRE inhibited the ability of PPARγ to induce transcription. This was demonstrated by co-treatment of MCF-7 breast cancer cells with both rosiglitazone and 17ß-oestradiol. This co-treatment inhibited the induction of PTEN protein that was observed by rosiglitazone stimulation alone (Bonofiglio et al., 2005). This cross talk between PPARγ and ER is an important observation because any therapeutic strategy based upon PPAR gamma would need to consider the effect of endogenous oestrogens. A limitation of the work presented here is that the relationship between ER/oestrogen and PPARs.

The data in this chapter indicates that ciglitazone can reduce growth in PTEN wild-type as well as PTEN null cell lines. This is important as loss of functional PTEN and PTEN mutations are very common in endometrial cancers (Mutter et al., 2001). These data are supported by Zhang et al. who showed that RGZ stimulation resulted in both increased PTEN transcription and PTEN-dependent inhibition of cell migration in hepatocellular carcinoma cells which frequently exhibit decreased or absent PTEN expression (Zhang et al., 2006). They speculated that there may be three other potential PPREs within the PTEN promoter, however, it has not yet been determined if these are functional PPREs. Interestingly, these authors did not observe an increase in transcriptional activity of the PTEN promoter in response to rosiglitazone treatment (Zhang et al., 2006). This may
suggest that elements beyond the full-length PTEN promoter are required for RGZ-mediated PTEN transcription.

The data presented in this thesis could indicate that PPARγ acts as a tumor suppressor in an endometrial cancer setting by up-regulating PTEN transcription. These studies were performed solely in endometrial cell lines however and it is possible that this action is cell-type specific although previously noted similar results have been reported in a breast cancer setting (Bonofiglio et al., 2005, Teresi et al., 2006). Several groups have studied the ability of PPARγ to regulate PTEN levels in other cancer backgrounds. Lee et al. observed an inhibition of cellular proliferation and Akt phosphorylation in accord with an increase in G1 arrest and PTEN protein expression in A549 lung cancer cells (Lee et al., 2006). Subsequently, PPARγ has been shown to upregulate PTEN expression in non small cell lung cancer, neuroblastoma, adrenocortical, pancreatic, hepatocarcinoma, and thyroid cell lines (Zhang et al., 2006, Han and Roman, 2006, Aiello et al., 2006).

Teresi et al. demonstrated that in breast cancer, of the TZDs only RGZ was capable of inducing PTEN expression (Teresi et al., 2006). Chen et al. showed however that both ciglitazone and 15d-PG-J2 could upregulate PTEN expression in W-2 thyroid cells (Chen et al., 2006), indicating that the effects of the TZDs may also be tissue dependent. Whilst evidence suggests that TZDs may induce PTEN expression through PPARγ, further studies are warranted to determine the exact mechanism of action.

Tumour angiogenesis is a complex process implicated in the downstream signalling pathways that contribute to tumour development and metastasis. PPARs have been implicated in the angiogenesis cascade and PPARα and PPARγ have been localised to tumour endothelial cells (Panigrahy et al., 2008a, Inoue et al., 2001). In chapter 3, immunofluorescence was used to demonstrate the localisation of PPARα in endothelial cells in endometrial cancers, suggesting a role for PPARs in non-cell autonomous (indirect) mechanisms. PPARα was also expressed in tumour cells.
In this chapter, the data demonstrate that loss of PPARα is associated with a reduction in VEGF secretion and loss of PPARγ is associated with an increase in VEGF secretion. This suggests that PPARs may exert effects on angiogenic pathways and contribute to tumour growth via this mechanism. This is supported by work suggesting that the PPAR's may be involved in vascular regulation. Several groups have identified PPARγ and PPARα expression in monocytes/macrophages, vascular smooth muscle cells, and endothelial cells (Staels et al., 1998, Inoue et al., 1998, Chinetti et al., 1998a). In the endothelium, PPARγ has been identified by PCR reaction, western blot and immunoprecipitation (Itoh et al., 1999). PPARα has also been demonstrated in the vascular endothelium by immunohistochemistry (Plutzky, 2001). While PPARγ has previously been studied for its anti-angiogenic properties (Giaginis et al., 2007), recent studies now indicate that PPARα may have anti-angiogenic properties as well (Meissner et al., 2004, Varet et al., 2003), a finding with potential therapeutic implications for endometrial carcinogenesis.

The PPAR alpha ligand, fenofibrate, and the PPAR gamma ligand, ciglitazone, both decreased VEGF secretion. This effect was noted in both endometrial cell lines. These effects were partly abrogated with the use of PPAR alpha and PPAR gamma antagonists respectively. In vitro studies examining the effect of TZDs on cancer cells use a concentration of agonist higher than required to activate PPARγ, which may lead to cross-activation of PPARα and PPARβ (Berger, 2002). This may lead to differing effects at higher doses compared to lower doses where only PPARγ is activated.

The addition of fenofibrate or ciglitazone to PPARα and PPARγ siRNA cell lines did not have an effect on VEGF secretion supporting the suggestion that the effects of PPAR agonists on VEGF secretion were PPAR mediated. Indeed, it has been established that the VEGF promoter does contain a PPAR response element (PPRE) (Peeters et al., 2005). These results are further supported by several studies demonstrating the effects of PPARα ligands in VEGF-mediated signalling (Table 5). PPARα ligands can inhibit endothelial cell proliferation and migration and induce endothelial cell apoptosis in vitro, suggesting a role of PPARα in tumour angiogenesis (Panigrahy et al., 2002). These effects may partly be
mediated through the ability of PPARα ligands to interfere with VEGF-mediated signaling. At the transcriptional level, PPAR agonists have been shown to inhibit endothelial VEGFR-2 expression by repressing transactivation and binding of Sp1 to DNA (Meissner et al., 2004). Interestingly, fenofibrate induces a significant reduction of VEGF levels in serum (Blann et al., 2001).

Fenofibrate reduces adventitial angiogenesis and inflammation in a porcine model of coronary angioplasty (Kasai et al., 2006). Both Fenofibrate and Wy-14643 are able to suppress VEGF secretion in glioblastoma cells and Lewis lung carcinoma cells and to inhibit angiogenesis both in vivo and in vitro (Panigrahy et al., 2008b). Moreover, Clofibric acid inhibits VEGF expression in tumor xenografts of ovarian cancer cell lines with a reduction in angiogenesis and decreased microvessel density in solid tumors (Shigeto et al., 2007, Yokoyama et al., 2007). PPAR-α agonists have recently been shown to inhibit expression of VEGF receptor 2 (VEGFR2) up-regulation in neovascularization (Meissner et al., 2004). Varet et al. have demonstrated that fenofibrate inhibits angiogenesis in vitro and in vivo. They have also shown that fenofibrate reduces endothelial cell growth rate, endothelial cell mediated wound repair, and capillary tube formation. Interestingly fenofibrate has been shown to inhibit bFGF-induced angiogenesis in vivo (Varet et al., 2003). Simultaneous inhibition of VEGFR2, bFGF, and VEGF could in theory reduce pathological angiogenesis. Endometrial cancer cells may also contain VEGF receptors and therefore increased secretion of VEGF can have growth effects by both increasing microvessel density and stimulating tumour cell growth and/or migration by autocrine and paracrine mechanisms.

The loss of PPARγ in both endometrial cancer cell lines was associated with an increase in VEGF secretion. This suggests that the presence of PPARγ may inhibit the angiogenic cascade associated with tumour development and growth. This is supported by the data reported in chapter 3, where a loss of PPARγ was seen in endometrial cancers compared to benign endometrium. The addition of ciglitazone to both Ishikawa and HEC-1A endometrial cancer cell lines results in a dose-dependent decrease in VEGF secretion. Similar results have been reported in primary human endometrial cell cultures (Peeters et al., 2005). In the
same study, Peeters et al. demonstrated that the effects of PPARγ ligands appear to be mediated via transcriptional repression of a PPRE upstream of the VEGF gene (Peeters et al., 2005). PPARγ is expressed in endothelial cells and PPARγ ligands appear to exert direct effects on endothelial cells (Xin et al., 1999, Murata et al., 2000). PPARγ activation by either naturally occurring or synthetic ligands results in potent inhibition of growth factor-induced differentiation and proliferation in human umbilical vein endothelial cells (HUVECs) and choroidal endothelial cells (Xin et al., 1999, Murata et al., 2000). Importantly, rosiglitazone levels that can inhibit endometrial cell proliferation are readily achieved in patients undergoing standard anti-diabetic rosiglitazone treatment (Freed et al., 1999). Moreover, rosiglitazone at pharmacological concentrations, resulted in reduction of VEGF-induced tube formation and endothelial migration (Sarayba et al., 2005).

PPARγ is also reported to be expressed in tumour endothelial cells (Inoue et al., 2001) and TZDs, even at low doses can reduce growth in both endothelial and malignant cells (Panigrahy et al., 2002). Moreover, TZDs inhibited tumor cell invasion across blood vessel endothelium. In fact, rosiglitazone at concentrations close to the range of its binding affinity for PPARγ (Berger, 2002) exerted inhibitory effects on tumour angiogenesis in malignant cell lines and in immunodeficient mice with transplanted tumours (Panigrahy et al., 2002). At concentrations of 5µM and higher, rosiglitazone induced phosphorylation of eIF-2α in HUVECs, indicating that the inhibition of endothelial proliferation may also be mediated through a pathway independent of PPARγ. Although PPARγ ligands can also induce endothelial cell apoptosis, it is unlikely that they do this under physiological conditions as this would result in a severe prothrombotic state. Thus, this suggests that PPARγ ligands may be better targets to endothelial cell proliferation than apoptosis (Panigrahy et al., 2005, Freed et al., 1999).

PPARγ knockout mice embryos die on day 10 of life because of interference with the terminal differentiation pattern of trophoblasts, as well as the loss of vascular development in the placenta (Barak et al., 1999a). This data provides additional evidence that PPARγ functions as a modulator of angiogenesis.
Beyond action on the endothelium, PPAR-γ ligands have been reported to down-regulate angiogenesis via indirect mechanisms by modulating levels of the endogenous angiogenic mediators. In this context, the VEGF/VEGFR signaling pathway seems to be a key target. Xin at al. provided the first evidence that 15d-PGJ2 reduced levels of mRNA encoding VEGFRs in HUVECs (Xin et al., 1999). It has also been proposed that PPARγ ligands may have bi-functional properties in KDR gene expression that involve the enhancement of Sp1-DNA binding in the absence of ligand by PPAR-γ itself and the suppression of Sp1-DNA-binding in the presence of PPAR-γ ligands (Sassa et al., 2004). Moreover, PPAR-γ activation has been shown to down-regulate leptin and tumor necrosis factor (TNF-α), two well-known pro-angiogenic factors (Fruhbeck, 2006, Sierra-Honigmann et al., 1998). In fact, PPAR-γ activation by TZDs attenuated leptin gene expression both in vivo and in vitro (Qian et al., 1998) and blocked leptin-induced endothelial cell migration through inhibition of Akt and eNOS signaling (Goetze et al., 2002). This evidence suggests that PTEN (endothelial phosphatase and tensin homologue), a negative regulator of PI3K/Akt signaling, may be important for the anti-migratory actions of TZDs in endothelial cells (Goetze et al., 2002).

It is proposed that, PPAR-γ ligand activation blocks expression of angiogenic stimulators and subsequently suppresses tumor-associated angiogenesis. Indeed PPAR-γ activation by troglitazone or pioglitazone reduces the production of the angiogenic chemokines IL-8 (CXCL8), ENA-78 (CXCL5), and Gro-α (CXCL1) in the human non-small-cell lung cancer cell line A459 (Keshamouni et al., 2005). In addition, ciglitazone decreases PGE2 production via down-regulation of cyclooxygenase-2 (COX-2) expression in human non-small-cell lung carcinoma A427 and A549 cell lines (Hazra and Dubinett, 2007). Interestingly, use of a dominant negative PPAR-γ construct revealed that the effect of ciglitazone on both COX-2 and PGE2 was mediated through PPAR-γ independent pathways (Hazra and Dubinett, 2007). 15d-PGJ2, a naturally-occurring PPAR gamma ligand attenuates the expression of Ang-1 and hence angiogenesis via the angiopoietin-Tie2 system in the gastric cancer cell line MKN45 (Fu et al., 2006). Ang-1 is involved in the regulation of maturation and stabilization of the vascular wall, and thus it might be a potential target for inhibiting tumor angiogenesis. Moreover, in a model of human anaplastic thyroid carcinoma, RS5444, a
novel high-affinity PPAR-γ agonist exerted potent anti-angiogenic actions, in vivo, and decreased CD31, a specific molecular marker of blood vessels (Copland et al., 2006).

Recently, Ciglitazone has also been shown to produce anti-tumour effects against ovarian cancer, in vitro and in vivo, in conjunction with reduced angiogenesis and induction of apoptosis (Xin et al., 2007). Ciglitazone induced antitumor effects were comparable to that of cisplatin and were ascribed to inhibition of VEGF production in relation to PGE$_2$ reduction, an endogenous stimulator of angiogenesis and invasiveness. This study, alongside the results from the data presented in this chapter, provide evidence of the potential use of PPAR-γ ligands to inhibit pathological vascularization in endometrial adenocarcinoma (Peeters et al., 2005).

In conclusion, these data suggest that both PPARα and PPARγ may be involved in angiogenesis by regulating VEGF secretion. In addition, PPARγ appears to modulate PTEN expression, challenging the traditional thinking of one-receptor-one-effect. PPAR activation may be beneficial against endometrial cancer development/growth through inhibition of angiogenesis and up-regulation of PTEN/P-Akt. The data presented here have, however, been obtained in vitro and further clinical research is necessary to elucidate their pharmacological significance in vivo.
CHAPTER SIX

CONCLUDING REMARKS AND FUTURE WORK

During this work, the potential roles of the PPAR/RXR transcription factors in the development of endometrial cancer were investigated. Initially, the expression of PPARs and RXRs in benign postmenopausal endometrium, endometrial carcinoma and atypical hyperplasia, was determined (chapter 3). Western blotting confirmed the up-regulation of PPARα in endometrial cancer and immunohistochemistry localized it to tumour epithelial cells and vascular endothelium, particularly at the tumour periphery. The increase in PPARα was largely in the nuclear component with no change in cytoplasmic levels. Both gene silencing for PPARα and the addition of PPARα agonist, fenofibrate reduced cell proliferation and increased apoptosis in a dose dependent manner (chapter 4). Co-incubation with PPARα antagonist, GW6471, partly abrogated these effects suggesting PPARα dependent mechanisms. Knockdown of PPARα in endometrial cancer cell lines also reduced VEGF secretion in both cell lines, suggesting that PPARα may contribute to endometrial cancer through indirect mechanisms via tumour angiogenesis (chapter 5).

Western blotting confirmed the down-regulation of PPARγ in endometrial cancer and immunohistochemistry localized this transcription factor to tumour cells as well as macrophages (chapter 3). In addition to gene silencing, addition of the PPARγ agonist, ciglitazone, reduced cell proliferation and increased apoptosis in endometrial cancer cell lines (chapter 4). Addition of antagonist, GW9662, partly abrogated these effects, suggesting PPARγ dependent mechanisms. Knockdown of PPARγ also reduced PTEN and increased P-AKT expression suggesting a direct effect of PPARγ on key mechanisms of cell growth (chapter 5). The RXRs, PPAR heterodimerisation partners, were also investigated. RXRa expression was up-regulated in endometrial cancers whereas RXRβ and RXRγ were down-regulated (chapter 3). Co-immunoprecipitation demonstrated preferential binding of PPARα and PPARγ to RXRα (chapter 3). The addition of ATRA to PPAR ligands potentiates their
effects suggesting that they act synergistically (chapter 4). The significance of and future work arising from these results will be discussed below.

Previous genome-wide investigation of endometrial cancer demonstrated that transcription of PPARα was up-regulated in endometrial cancers compared with benign endometrium (Holland et al., 2004). Increased PPARα has been previously demonstrated in other epithelial tumours such as prostate (Collett et al., 2000) and bladder (Fauconnet et al., 2002) cancers. The finding of increased nuclear expression of PPARα in malignant endometrial epithelial cells and vascular endothelium, suggests that PPARα may have multiple roles within the endometrial cancer microenvironment. Localisation of PPARα to vascular endothelium has previously been reported in mouse prostate cancer xenografts and in samples of human prostate cancers (Panigrahy et al., 2008a). The exact functions of PPARα in the development and/or maintenance of endometrial cancer remain unknown although the potent angiogenic growth factor, VEGF-A is up-regulated by PPARα in bladder cancers (Fauconnet et al., 2002) suggesting that PPARα-mediated angiogenesis may promote tumour growth. Increased VEGF-A is a feature of many solid tumours including endometrial cancer. The role of the development of new vascular networks in solid cancers is now established and their importance as therapeutic targets in cancer disease has led to the inclusion of expensive vascular targeting agents in treatment regimes. Insulin increases the transcriptional activity of PPARα via activation of the MAP kinase pathway (Shalev et al., 1996) and it is well established that both diabetes and obesity are risk factors for the development of endometrioid endometrial cancer. The findings in this thesis suggest that PPARα may contribute to tumour growth in endometrial cancer via promotion of angiogenesis and raises the possibility that targeting the PPARα pathway directly could be considered for potential therapeutic benefit. Further investigation of the possible functions of PPARα within endometrial tumour cells and functional studies investigating the effects of specific PPARα ligands on vascular growth within endometrial tumours is needed.

The expression of PPARβ in endometrial tissues was similar to the pattern of expression of PPARα with a trend toward increasing expression as tumours become less differentiated.
Like PPARα, PPARβ is found in both tumour cells and peri-tumoural vessels. A previous study demonstrated PPARβ transcript and protein within endometrial tumour cells and correlated this with the distribution of cyclo-oxygenase 2 (COX-2) (Tong et al., 2000). This suggests that prostaglandins derived via COX-2, may activate PPARβ in an autocrine and/or paracrine manner. COX-2 is over-expressed in many cancers (including endometrial cancer) contributing to angiogenesis and COX-2 inhibitors produce anti-angiogenic effects and reduce growth in tumour xenografts. These findings support the hypothesis that PPARβ could contribute to tumour angiogenesis via transcriptional activation, driven by the production of COX-2-derived prostaglandins.

An inverse relationship between expression of PPARγ in endometrial cancers and tumour grade was demonstrated in this work. Benign endometrium expressed significantly higher levels of PPARγ than cancers. Weak PPARγ expression has previously been reported in endometrial cancers (Ota et al., 2006) and a similar loss of PPARγ expression is also seen in oesophageal carcinoma (Terashita et al., 2002). Loss of PPARγ was also correlated with reduced expression of PTEN. These effects were also mirrored in atypical hyperplasias. The addition of PPARγ ligand, ciglitazone, induced PTEN expression in the PTEN wild-type, HEC-1A cells. P-Akt expression was reduced in a dose-dependent manner. A surprising finding was that in the PTEN null, Ishikawa cell line, P-Akt was also reduced, suggesting that PPARγ may work through PTEN-dependent and PTEN-independent effects (chapter 5). This is an important finding given the importance of PTEN status in endometrial cancer, however, there has been much data demonstrating the PPARγ-PTEN connection, yet no in vivo study has been able to support this. The relevance of the putative PPRE in the PTEN promoter remains to be determined as it is located a long distance from the ATG site, making it unclear if this site is functional in PTEN regulation (Bonofiglio et al., 2005).

I have demonstrated that in endometrium, PPARγ is expressed in both glandular epithelium and tissue macrophages. PPARγ has been implicated in the promotion of macrophage differentiation alongside monocyte activation and the regulation of inflammatory activities (Nagy et al., 1998, Patel et al., 2002). PPARγ is also seen in peritoneal macrophages of women with endometriosis and migration of macrophages is inhibited by treatment with a
PPARγ agonist (Hornung et al., 2001). In endometrial cancer as with other solid tumours, macrophages are abundant and macrophage infiltration is associated with poor prognostic features such as vascular space invasion and deep myometrial invasion (Soeda et al., 2008). Tumour-associated macrophages are known to secrete a variety of cytokines that can promote tumour cell growth and invasion. These findings support the hypothesis that PPARγ may exert a protective effect on the endometrium by regulating inflammatory cells as well as via actions within the endometrial epithelium.

There was an up-regulation of RXRα and down-regulation of RXR-β and –γ in endometrial cancers. The possibility exists that the PPAR/RXR heterodimers regulate the expression of genes that maintain cell proliferation and apoptosis appropriate to environmental conditions. In this setting, a gain of RXRα and loss of RXR-β and –γ expression may disturb the balance in favour of cell proliferation with PPARα transcription increasing and PPARγ transcription decreasing due to compensatory mechanisms. Ito et al. showed a wide variation of expression of RXRα and –γ, compared with other retinoid receptor subtypes, in both neoplastic and non-neoplastic human endometrium (Ito et al., 2001). I have demonstrated that there is an increased expression of RXRα and loss of RXRβ and –γ in endometrial cancers. Furthermore, I have shown that the intracellular distribution of RXR and PPAR subtypes varies between benign and malignant endometrial cells. These findings suggest that within the context of endometrioid type endometrial cancer cells, the formation and actions of the PPAR/RXR heterodimer complexes may be modulated by altering the availability of different PPAR and RXR subtypes. This could be effected either by up- or down-regulation of PPARs and RXRs in different cell types or perhaps by nuclear-cytoplasmic shuttling.

The effect of PPARα and PPARγ silencing were examined (chapter 4). Silencing of PPARα decreased proliferation and increased apoptosis. Silencing of PPARγ had the opposite effect, leading to an increase in proliferation and decrease in apoptosis. This further
implicates that PPARs may be important in the development and/or growth of endometrial cancer.

The effects of synthetic ligands for PPARα and PPARγ on cellular proliferation and apoptosis were also examined (chapter 4). Both ligands had anti-proliferative and pro-apoptotic effects in both Ishikawa and HEC-1A cells. Previous studies based on tumour cell lines have implicated PPARγ in cell-cycle withdrawal. The use of PPAR antagonists and PPAR silencing demonstrated the effects of PPAR ligands may in receptor dependent as well as receptor independent.

The limitations of the work presented here on the relationship between PPARs and VEGF secretion is limited as I only examined VEGF secretion and not expression, mRNA levels or VEGFR effects. Due to time constraints, only fenofibrate and ciglitazone were used as agonists, and in order to consider utilising PPAR-mediated effects in clinical studies, it would be important to examine the use of other PPAR agonists in endometrial cancer cell lines. PPARs are pleiotropic receptors which regulate multiple cellular functions in differing cell types, and so their pharmacological properties have differing effects dependent on the tissue and dose used (Rumi et al., 2004). Four specific doses of PPAR agonist were used in this dissertation (5, 10, 20 and 50μM), although dosage of PPAR ligands in clinical trials as chemopreventative agents need to be investigated thoroughly. For example, low concentrations of PPARγ ligands increase cell proliferation whilst higher concentrations block breast cancer cell growth (Clay et al., 2001). Conversely, it has been reported that higher doses of rosiglitazone were less anti-angiogenic than the lower doses comparable to serum rosiglitazone levels in humans (Freed et al., 1999). Moreover, PPARγ agonist concentrations that lead to chemopreventative actions are higher in mice than the doses used to treat Type II diabetes (Panigrahy et al., 2002, Sarraf et al., 1999b, Heaney et al., 2003, Ohta et al., 2001). Ciglitazone is not currently in clinical use, however, rosiglitazone and pioglitazone are in use for the treatment of Type II diabetes. Current clinical dosing is 4-8mg/day (0.11/mg/kg/day) for rosiglitazone and 15-45mg/day for pioglitazone (Goldstein, 2002), however, the chemopreventative activity of rosiglitazone in mice is 100-
150mg/kg/day, which is effectively 1000 times stronger (Panigrahy et al., 2002, Heaney et al., 2003).

It is suggested that there is an inverse relationship between PPARα and ERα mRNA levels in ER-positive breast cancer cell (Faddy et al., 2006). This suggests that PPARα may be involved in the activation of breast cancer. Furthermore, given the similarity between breast and endometrial cancer, future work could include examining the relationship between ER and PPAR expression and activation within the endometrial cancer setting. Data exists indicating that PPAR/RXR heterodimers can bind to oestrogen response elements and that ER and PPAR share cofactors that mediate transcription, confirming that there may be signal cross talk between ER and PPAR (Wang and Kilgore, 2002, Jeong and Yoon, 2007, Keller et al., 1995). The response elements for both receptors share homology, an ERE is an inverted repeat containing three intervening bases (AGGTCA N3 TGACCT), whereas a PPRE is a direct repeat with a single intervening sequence (AGGTCA N AGGTCA). Both sequences contain an AGGTCA half site, which may be recognised by ER or PPARα. This signal cross-talk has been reported to occur through competitive binding to ERE (Keller et al., 1995) or through competition for co-activators, increased availability of co-repressors or through other mechanisms (Tcherepanova et al., 2000, Jeong and Yoon, 2007, Foryst-Ludwig et al., 2008). The actions of PPARα activators have been shown to be effective in treating obesity in men and postmenopausal women, but not in pre-menopausal women (Nunez et al., 1995, Nunez et al., 1997, Wang and Kilgore, 2002), indicating that the relationship between PPARs and oestrogen requires further investigation, in particular in an oestrogen dependent cancer setting.

PPARγ status has recently started being examined in the context of clinical cancer trials. The TZDs have also been used in a variety of clinical trials in benign disease, although not directly related to PPARγ activation. Polycystic ovary syndrome (PCOS) is the most commonly studied syndrome with regards to the effects of TZD treatment (Banning, 2006). Studies have demonstrated that rosiglitazone treatment raises insulin and androgen levels in the obese PCOS population, thereby inhibiting tumour progression. Yee et al. recently performed a pilot study in women with breast cancer to determine whether or not RGZ
treatment would be beneficial. Thirty-eight women with early stage breast cancer were recruited and treated with rosiglitazone for 2–6 weeks with tumour growth inhibition or progression as an end point (Yee et al., 2007). Their findings indicate that short-term rosiglitazone therapy in early-stage breast cancer patients has both local and systemic effects on PPARγ signalling (Yee et al., 2007). Both of these studies suggest that PPARγ agonists may be used clinically to benefit cancer patients. Lacking in many of these studies however is the role of PTEN. Clinical trials are necessary to determine if TZD treatment is beneficial for cancer patients and which patients it is most advantageous for. The relevance of the putative PPRE in the PTEN promoter identified by Bonofiglio et al. remains to be determined as it is located a long distance from the ATG site, thus making it unclear if this site is functional in regulating PTEN expression (Bonofiglio et al., 2005).

The work presented in this thesis suggests that PPARs and their ligands may be working through different pathways to exert their effects (Figure 6.1). This may be through inhibition of angiogenesis, anti-proliferation, pro-apoptotic, as a tumour suppressor itself or through other pathways. This challenges the concept of one drug-one target-one effect, especially within cancers. Tumour formation involves more than the cell autonomous state of uncontrolled growth. Tissue response in the tumour bed, notably angiogenesis and inflammation represent targets for the pleiotropic PPAR agonists. Secondly, not all actions of PPAR ligands are mediated by their selective receptor, indeed non-selective off target effects are still unknown. Furthermore, given the pleiotropic role of PPARs on a variety of differing cell types, the dose and regimen would need to be optimised. Moreover, a multi-target attack on tumour cells, as well as on inflammatory response and angiogenesis, maybe the most effective option for treatment. The addition on ATRA may further enhance these effects. Elucidating the individual cellular targets for PPAR agonists and defining the role of PPARs within endometrial cells will be important in designing combinational therapies, as synthetic ligands are already licensed and in common use for the treatment of dyslipidaemias and type 2 diabetes, demonstrating good safety profiles. Furthermore, obesity and insulin-resistance are risk factors for the development of endometrioid endometrial cancer. Although some of the body mass indexes (BMI) values are available for the patients recruited in this study, not all information had been recorded.
Figure 6.1: The possible sites of actions for PPARs. PPARγ may exert its effects directly through PTEN, or by negatively inhibiting P-Akt. PPARα may exert its effect on angiogenesis through VEGF. The risk factors for endometrial cancer (blue boxes) all lead to increased insulin release which directly increases oestrogen and can increase insulin growth factor-1 (IGF-1) and insulin releasing substrate-1P (IRS-1P) which may in turn affect P-Akt formation and lead to other downstream effects.
Further laboratory work

- Western blotting and immunostaining demonstrated the differential expression and localisation of PPARs and RXRs in endometrial cancer. Although two different cohorts were used for this work, the sample number was limited. Future work should include an examination of the expression of PPARs and RXRs in a larger sample of atypical hyperplasias as this may provide insight into the transition between benign to malignant endometrium. Collection of samples was difficult as there is no central bank of tissues and atypical hyperplasia is readily upgraded during classification.

- This thesis focussed on Type I endometrial cancers, as these are oestrogen related. It would also be of interest to identify whether the findings here are extended to type II cancers or not because these are non-oestrogen related with less prominent loss of PTEN.

- Chapter four covered the effects of synthetic PPAR silencing and ligand addition on cellular growth in endometrial cancer cells. It would be beneficial to examine the effect of PPARα and PPARγ silencing on other cellular growth mechanisms including invasion and migration. This may further elaborate the direct effect of PPAR loss on cellular growth.

- A range of PPAR ligands to examine if all PPAR ligands exert similar effects, if some are more selective than others and if there is cross-talk through the other PPAR isoforms.

Suggested clinical work

- It may be of interest to examine any role BMI may have on PPAR expression, in particular the relationship of adiponectin and PPARs. Adiponectin has been shown to be inversely related to endometrial cancer, and serum samples have been collected that from the patients recruited here could be examined.
• Future work may include a window of opportunity study. Ethical approval could be sought to collect fresh tissue extracts from women who were recently diagnosed with endometrial cancer. These women would then be given a PPAR agonist whilst awaiting hysterectomy. Fresh tissue would then be collected again and examined to investigate endometrial proliferative indexes, apoptosis, VEGF expression and availability and microvessel density changes as well as changes in PPAR, RXR and PTEN expression. This would provide insight into the in vivo effects of these agonists.

Taken together, the current information suggests that the PPAR/RXR transcriptional apparatus may be involved in endometrial carcinogenesis. No study prior to this has determined the role of this family of transcription factors in endometrial cancer. The beneficial effects of PPARs on the growth of endometrial cells are potentially important in developing preventative treatment for the ever-increasing population at risk.
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Agonists Differentially Alter Tumor Differentiation and Progression during Mammary Carcinogenesis


