The Effects of Selective Oestrogen Receptor Modulators in the Uterus

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Abstract.

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Tamoxifen, a selective oestrogen receptor modulator, is an effective treatment for breast cancer. However, women taking this drug have an increased risk of developing pathological changes in the endometrium including adenomyosis and cancer. The mechanisms by which tamoxifen promotes these changes in unknown. The effects of tamoxifen on uterine gene and protein expression have been examined in both women and mice treated with this drug. In the endometrium of women taking tamoxifen for up to 4 years, analysis of gene expression using cDNA arrays, indicated that tamoxifen increased expression of genes that may be involved in cell proliferation. In the endometrium of post-menopausal women, expression of the paracrine signalling protein Nerve Growth Factor (NGF) was increased, although real time PCR showed no increase in *NGF* gene expression. Mouse models were used to compare the long-term effects of tamoxifen, oestradiol and 4-hydroxyoestradiol on uterine pathology and gene expression. In uterotrophic assays, oral dosing of oestradiol, tamoxifen or 4-hydroxyoestradiol, administered to immature CD-1 mice on 3 days all led to increases in uterine weight, although not necessarily by cell proliferation. In newborn CD-1 mice the maximal uterotrophic dose of oestradiol (100 μg/kg), 4-hydroxyoestradiol (386 μg/kg) or tamoxifen (250 μg/kg) were given orally on days 1 to 5 after birth and gene and pathological changes examined in the uterus 3 months after dosing. Tamoxifen but not 4-hydroxyoestradiol or oestradiol led to adenomyosis. At this time, the expression of >250 genes were significantly altered by each treatment but changes in only twelve genes were common to all three oestrogens. It is concluded that not all oestrogen agonists are the same in this model and while oestrogenicity is needed for the development of adenomyosis additional, unidentified factors, are required.

Table of Contents.

Figures and Tables.

Thesis Associated Publications.

Gray, D.T., White, I.N., Thompson, J., Guzail. M., and AL-Azzawi, F. (2005) Nerve Growth Factor Expression in the Human Uterus during the Menstrual Cycle and the Effects of Tamoxifen Treatment. *Submitted.*

Green, A.R., Styles, J.A., Parrott, E.L., Gray, D.T., Edwards, R.E., Smith, A.G., Gant, T.W., Greaves, P., Al-Azzawi, F., and White, I.N.H. (2004) Neonatal Tamoxifen Treatment of Mice Leads to Adenomyosis but Not Uterine Cancer. *Experimental and Toxicological Pathology.*, **56**, 255-263.

Gray, D.T., Greaves, P., Styles, J., and White, I. (2004) Pathology and Gene Expression in the Uteri of Mice Dosed with Oestradiol and Tamoxifen. *Toxicology.*, **202**, 114. (Abstract).

Gray, D.T. Effects of Estrogen Receptor Modulators on Uterine Pathology and Gene Expression. (2005) *The Toxicologist* CD — An official Journal of the Society of Toxicology., **84**, Number S-1, (Abstract no. 1110).

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List of Commonly Used Abbreviations.

AF-1 or 2	Activation Function
βERKO	ERβ Knock Out Mouse
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
Cp	Crossing Point
CYP	Cytochrome P450
DBD	DNA Binding Domain
DNA	Deoxyribonucleic Acid
DES	Diethylstilbestrol
DEPC	Diethylpyrocarbinate
EGF	Epidermal Growth Factor
ER (α or β)	Oestrogen Receptor
ERE	Oestrogen Response Element
ERKO	ERα Knock Out Mouse
FBS	Foetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phospate Dehydrogenase
H&E	Haematoxylin and Eosin
IMS	Industrial Methylated Spirits
FSH	Follicle Stimulating Hormone
LH	Lutenising Hormone
MAPK	Mitogen-Activated Protein Kinase
mRNA	Messenger RNA
NGF	Nerve Growth Factor
PBS	Phosphate Buffered Saline

PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PgD_2	Prostaglandin D_2
PI 3-Kinase	Phosphatidylinositol 3-Kinase
PR	Progesterone Receptor
RNA	Ribonucleic Acid
RT	Room Temperature
RT-PCR	Real-Time PCR
SERM	Selective Oestrogen Receptor Modulator
T-20	Tween – 20

Chapter 1.

General Introduction.

1.1 Breast Cancer.

It is estimated that during 2001 in the UK there were 41,180 new cases of breast cancer, accounting for 30% of all cancers diagnosed in women. This compares with 5,650 cases of uterine cancer and 6,880 cases of ovarian cancer, both accounting for around 2-3% of cancers in women (www.cancerresearch.org.uk.). Around 1% of breast cancers occur in men. Breast cancer is now thought of as the most commonly diagnosed cancer in women in the Western world, with mortality rates declining over the last decade due to early diagnosis and intervention.

Up to 90% of breast cancers are sporadic, having no familial link. From the remaining 10% of breast cancers that have a hereditary link, over 80% of patients carry a mutation within the Breast Cancer Susceptibility Gene 1 (*BRCA1*) gene and less commonly in the *BRCA2* gene (Kennedy *et al.,* 2004). Women carrying mutations of the *BRCA* genes are thought to have a lifetime risk for developing breast cancer of 82% (King *et al.,* 2003b). The protein product of *BRCA1* is involved in DNA damage repair. Carriers of mutations in the *BRCA* genes are also at a higher risk of developing ovarian cancers (Metcalfe *et al.,* 2004).

In the region of 50 to 70% of all breast cancers express the oestrogen receptor (ER) and respond to oestrogen as a mitogen. 50 to 60% of these cases respond to endocrine therapy (Duffy, 2005; Scott *et al.,* 1991). Determination of total ER and progesterone receptor (PR) levels by radiolabelled ligand binding assay showed that co-expression of both the oestrogen and progesterone receptors improved responsiveness of tumours to endocrine therapy than tumours expressing ER alone (Bardou *et al.,* 2003).

1.2 Breast Cancer Treatments and Prevention.

1.2.1 Tamoxifen.

The most common first line treatment for breast cancer involves endocrine therapy that aims to deprive or block the mitogenic action of estrogens in breast cancer cells. Tamoxifen has been successfully used in the treatment of ER positive breast cancer and more recently the prophylactic use of tamoxifen as a preventative against breast cancer has proved promising (Fisher *et al.,* 1999). Acute toxicity from tamoxifen treatment is low (Powles *et al.,* 1989) and there is no significant impact on patients quality on life including depression or sexual functioning (Ganz, 2001).

1.2.2 Aromatase Inhibitors.

More recently, inhibitors of the enzyme aromatase such as anastrozole, letrozole and the steroidal exemestrane, have been used in breast cancer treatment (Janicke, 2004). Aromatase is a cytochrome P450 enzyme (CYP19) that converts testosterone or androstenedione to oestradiol or oestrone respectively. Aromatase is found in many tissues including adrenal glands, ovaries and adipose tissue. Though aromatase inhibitors are generally more expensive as a form of treatment, they are thought to overcome some of the side effects of tamoxifen described later. They are at present only approved for use in postmenopausal women.

1.2.3 Tyrosine Kinase Receptor Inhibitors.

A subset of breast cancers has been shown to over express the tyrosine kinase receptor, HER2. This receptor is within the same class as the HER1 epidermal growth factor receptor (EGFR). A natural ligand for HER2 has not been identified to date and therefore it referred to as an orphan receptor, which preferentially forms heterodimers with other members of the

HER family. Inhibitors of HER2, such as the antibody trastuzimab may be used in the treatment of HER2 over expressing tumours.

1.2.4 The Use of Tamoxifen for the Prevention of Breast Cancer.

There has been interest in the prophylactic use of tamoxifen to prevent breast cancer in women considered to be at high risk. Studies into the benefits of prophylactic tamoxifen use found convincing results as to the efficacy of tamoxifen in preventing breast cancer in healthy 'at risk' women. The United States National Surgical Adjuvant Breast and Bowel Project (NSABP- Breast Cancer Prevention Trial P-1) found that breast cancer incidence was reduced by 49-50% in women treated with tamoxifen over 5 years (Fisher *et al.,* 1999). Interestingly, tumours which were ERα positive were reduced by 69%, whereas there was no significant difference in the incidence of ER negative tumours. However, the prophylactic use of tamoxifen has been called into question due to an increased incidence in new primary malignancies, particularly endometrial cancers in women receiving the drug. The first report where concerns were raised saw a near six-fold increase in endometrial cancers, and three-fold increase in gastrointestinal cancers in tamoxifen treated patients (Rutqvist *et al.,* 1995). Another study showed that the rate of endometrial cancer was increased from 0.91 to 2.30 per 1000 women, an increase in relative risk of 2.53 (Osborne, 1999). In the general population, the increased risk of developing endometrial cancer is reported to be predominately in postmenopausal women over the age of 50 (Fisher *et al.,* 2001).

More recently, the International Breast Cancer Intervention Study (IBIS-I), demonstrated a significant reduction in breast cancer incidence in women taking 20 mg/day tamoxifen over a period of five years. Factors such as age, degree of risk and the use of hormone replacement therapy did not appear to influence the effects of the treatment, and although there was a

slight increase in the incidence of endometrial cancer, this was not statistically significant (Cuzick *et al.,* 2002). Despite this, it is widely accepted that tamoxifen treatment is associated with an increase in endometrial pathologies and monitoring of endometrium is recommended in postmenopausal women receiving the drug.

1.3 Endometrial Cancer.

Endometrial cancer is the most common malignancy of the female genital tract, with between 10 and 25 women per 100,000 developing the condition in Western countries. The lifetime risk in the USA for women to develop endometrial cancer is 1 in 38. The majority of patients, around 20 to 25%, are postmenopausal. Prognosis for patients diagnosed in the early stages of the disease is generally very good, and may be treated surgically by hysterectomy. Continuous exposure to oestrogens without the regulatory effects of progestins may promote type-1 endometrial cancers, characterised by endometrial hyperplasia and high ER expression (Emons *et al.,* 2000). Historically this has been most commonly associated with women taking the oestrogen only contraceptive pill.

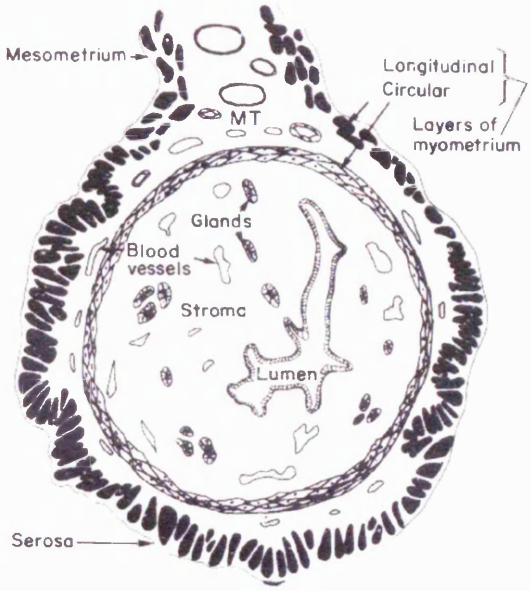
Endometrial cancers associated with tamoxifen use include endometrioid adenocarcinoma, mucinous, clear cell and serous carcinomas and adenocarcinomas. The American College of Obstetrics and Gynaecology (1996) reported tamoxifen related endometrial changes of benign polyps and hyperplasia, but stated that the grade, stage, histological appearance and prognosis in tamoxifen associated endometrial cancers was not significantly different from those found in the normal population. Uterine pathology related to tamoxifen treatment is further outlined later in this Chapter.

1.4 Uterine Structure.

The uterus is divided into two steroid responsive compartments. The inner endometrial layer comprises of blood vessels, glandular epithelial cells and the stroma which includes lymphocytes and fibroblasts. A luminal epithelial layer lines the uterine lumen (Figure 1.1). It is the endometrial layer that in humans, undergoes the monthly effects of the menstrual cycle in response to altering concentrations of oestrogens and progestins.

The larger part of the mouse uterine body is a myometrial layer consisting of smooth muscle cells. These cells are grouped into bundles and separated by collagenous connective tissue. In the mouse uterus, bundles of smooth muscle cells are arranged in two layers: within the outer layer, bundles are arranged longitudinally, with contraction shortening the length of the uterus, whereas the inner layer smooth muscle cells are arranged concentrically around the uterus with contraction leading to constriction of the uterine lumen (Finn.CA *et al.,* 1975).

a



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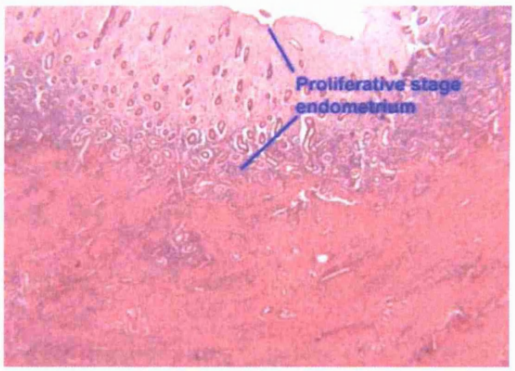


Figure 1.1: Uterine Structure.

a. Cross Section of a Mouse Uterus. The lumen is lined with a layer of luminal epithelial cells. The endometrium contains stromal cells, blood vessels and epithelial glands which are responsible for secreting mucus into the lumen. The two layers of the myometrium are shown. (Finn.CA *et al.,* 1975). **b**. Histological section of human endometrium from the proliferative stage of the menstrual cycle (http://w3.ouhsc.edu/histology/glass%20slides/19_01.jpg). Figure shows proliferative endometrium containing epithelial glands. Underneath the endometrial layer is the myometrium containing smooth muscle fibres.

1.5 Normal Female Reproductive Endocrinology.

The reproductive tract of women, namely the uterus and ovaries, undergoes complex hormonally-induced changes during the menstrual cycle (Brook *et al.,* 1996). At the beginning of the menstrual cycle, follicles containing an oocyte develop in the ovary in response to Follicle Stimulating Hormone (FSH) and Lutenising Hormone (LH) released from the hypothalamus – pituitary axis. Granulosa cells of the follicle produce oestradiol during this phase. Eventually one follicle, termed the Graafian follicle, will erupt releasing the oocyte. Cells within the ruptured follicle then proliferate, forming the Corpus Luteum, which is responsible for secretion of oestradiol and progesterone during this stage of the cycle. Secretion of oestradiol and progesterone has a negative feedback effect on the production of LH and FSH.

Varying levels of progesterone and oestrogen primarily act to alter the physiology of the endometrium. At the beginning of the cycle, high levels of oestrogen stimulate endometrial stromal and epithelial cells to proliferate and repair the endometrium after shedding from the previous cycle. Oestrogen also induces expression of the receptors for both oestrogen (ER) and progesterone (PR) in the uterus. During the second half of the menstrual cycle, a rise in progesterone from the corpus luteum stimulates endometrial glandular cells to secrete factors that will be required if a fertilised ovum was to implant. In the absence of implantation, the corpus luteum stops releasing hormones and the endometrium breaks down and is lost as menstrual shed.

1.6 Oestrogens in the Female Reproductive Tract.

Oestrogens have a wide range of actions in female mammals depending upon the tissue, the age of the woman and stage of the menstrual cycle. The primary oestrogen responsive tissues

are the breast and the uterus. Oestrogens stimulate growth of the breast and uterus and affect lipid deposition during puberty. In the uterus they cause proliferation of the endometrium during the menstrual cycle and increase uterine blood flow during pregnancy. In response to oestrogens, FSH receptor numbers within the ovarian follicle increase and FSH and Lutenising Hormone production from the pituitary is stimulated. Effects of oestradiol are also seen in the bone, blood, colon and central nervous system.

Oestrone (E1) and 17β-oestradiol (E2) (Figure 1.4, Page 24), the main endogenous oestrogens in women, are mainly synthesised in the ovaries but may be formed in other tissues such as adipose tissue. In common with all steroids, oestrogens are derived from cholesterol via androgens in a pathway involving the cytochrome P450 enzyme, aromatase (CYP19). Synthesis of oestradiol precursors, androstenedione and testosterone, occurs in the theca interna cells of the ovarian follicle. These oestrogen precursors are then transported to the granulosa cells where aromatase cleaves at carbon-19 producing an oestrogen with a phenolic A-ring. Following menopause, primary ovarian production of steroids diminishes. In this circumstance, the main source of oestrogens is from the aromatisation of androstenedione from the adrenal gland to produce oestrone. In postmenopausal women, oestrone and oestrone sulphate are the most abundant circulating oestrogens. These have weaker oestrogenic effects than oestradiol. Reduced production of oestradiol causes atrophy of the breast and vaginal mucosa, and bone mass may decline leading to an increased risk of osteoporosis (Berne *et al.*, 1998).

1.7 The Oestrogen Receptor.

The effects of oestrogens on target cells and tissues are primarily mediated by the oestrogen receptors ERα and ERβ, also known as ESR1 and ESR2. Both receptors belong to the nuclear

steroid receptor family and act as ligand activated transcription factors. Both receptors show differences in cell and tissue expression and downstream cellular effects upon ligand binding. Further isoforms of the ER have been reported to occur by alternative mRNA splicing, for example, truncated or exon-deleted transcripts of ERα mRNA have been reported in breast cancers (Murphy *et al.,* 1996).

1.7.1 Expression and Localisation.

ERα and ERβ expression has been reported in many tissues including the breast, uterus and ovaries (Pelletier *et al.,* 2000), the heart, brain, lung, bone, liver, kidney and CNS (Diel, 2002; Nilsson *et al.,* 2000; Taylor *et al.,* 2000). The ratio between ERα and ERβ levels are thought to be important in determining the proliferative response to oestrogens within the uterus (Wang *et al.,* 2002a). In the human endometrium, both ERα and ERβ have been localised to the nuclei of stroma and luminal epithelial cells (Taylor *et al.,* 2000), and levels of both receptors have been shown to alter during the menstrual cycle, with expression dependent on cell type (Lecce *et al.,* 2001). Although circulating levels of oestradiol decline after menopause expression of the ER seems to persist in the endometrium (Noci *et al.,* 1996).

ER protein levels decrease in response to oestrogen in the glandular epithelium and stroma, however, it increase in the luminal epithelium and myometrium (Tibbetts *et al.,* 1998). Research suggests that endometrial stromal ER mediates the proliferative response of glandular cells in response to oestradiol (Cooke *et al.,* 1997).

1.7.2 Genomic Organisation.

The ERα gene was assigned to chromosome 6q24-q27 in 1986 (Gosden *et al.,* 1986) and was found to be greater than 140 kb, comprising of eight exons (Ponglikitmongkol *et al.,* 1988).

The gene is under the influence of multiple promoters, regulation of which is tissue dependent.

Alternative splicing patterns of primary mRNA transcripts of the human ERα gene have been detected in human breast tissue. Levels of these alternative transcripts are thought to be higher in breast carcinomas than in normal tissue (Leygue *et al.,* 1996). Splice variants leading to altered ER proteins have also been implicated in uterine adenocarcinomas (Horvath *et al.,* 2000). Somatic point mutations within the ERα gene leading to impaired protein function have also been implicated in uterine adenomyosis (Oehler *et al.,* 2004).

Human ERβ shows approximately 89% identity to rat ERβ, 88% to mouse ERβ and 47% to human ERα, in its translated portion. The gene was assigned to 14q22–24, and the translated exons shown to span 40kb (Enmark *et al.,* 1997).

1.7.3 Protein Structure.

ERα protein is 66 kDa and ERβ is 58 kDa, and the proteins share structural homology (Figure 1.2). Both receptors contain a DNA-binding domain with two zinc fingers. Towards the N-terminal is an agonist-independent transcriptional activation function (AF-1). AF-1 activation is largely cell, ligand and receptor subtype specific. An agonist-dependent transcription activation function (AF-2) that lies in the ligand-binding domain towards the C-terminus, expresses oestrogen dependent transcriptional activation. AF-1 and AF-2 transcription activation is largely dependent on the promoter to which they bind, with some promoters requiring both AF-1 and AF-2 activation, whilst other promoters require only AF-1 activation (Tzukerman *et al.,* 1994). Analysis of the protein crystal structure shows that ERα comprises 12 α-helices and one β-turn (Wurtz *et al.,* 1998). Helix number 12 of ERα is vital for its

transcriptional action. Amino acid residues within the ligand binding domain and helix 12 are required for ligand mediated regulation of gene transcription (Lonard *et al.*, 2000).

A number of ERα and ERβ isoforms have been identified in various tissues (Matthews *et al.*, 2003). Isoforms of ERα include a full length 66 kDa variant that differs in the 5' untranslated region (UTR), and a 46 kDa isoform that lacks AF-1 activity. Isoforms of ERβ include a 495 amino acid variant that is thought to be functionally equivalent to wild type ERβ. In addition, ERβ2 is identical to ERβ except for 26 amino acids which renders ERβ2 incapable of binding ligand or initiating transcription. ERβ2 however, forms a dimer with ERα, thus inhibiting DNA binding of ERα / ERβ2 complex.

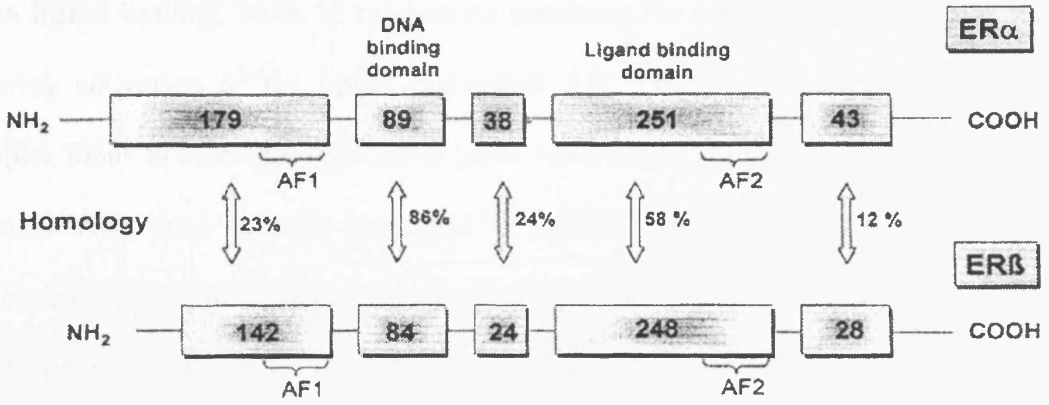


Figure 1.2: Structural Comparison of ERα and ERβ.

Numbers in boxes represent the number of amino acid residues. Homology is represented as a percentage. Homology between the two DNA binding domains is very high (Taken from Diel, 2002).

1.8 Classical Transcription Activation through the Oestrogen Receptor.

As with other steroids, oestrogens act as transcription activators via the ER in a classical model of gene transcription. Lipophilic oestrogens pass through the extracellular membrane to bind ER localised either within the nucleus or cytosol (Yamashita, 1998). In the absence of ligand, ER may be bound to chaperone proteins such as HSP90. These proteins regulate ER folding at the ligand-binding domain acting to maintain the ER in a state ready to bind ligand (Fliss *et al.,* 2000). Upon ligand binding and subsequent dissociation from chaperone proteins, ER dimerises forming a DNA binding complex. Dimerisation of ER is thought to be essential as mutations inhibiting dimerisation result in transcriptionally inactive receptors (Lees *et al.,* 1990). Oestrogen binding decreases the stability of ERα dimers, although greater stability is associated with ER antagonists such as tamoxifen (Tamrazi *et al.,* 2002).

Upon ligand binding, helix 12 repositions providing the surface for co-activator interaction allowing activation of the ligand dependent AF-2. In the nucleus the ER dimer-ligand complex binds to oestrogen responsive genes containing a short palindromic sequence spaced by three base pairs in their promoters – 5'GGTCAnnnTGACC3'- termed an oestrogen response element (ERE). When bound to an ERE, the ER induces DNA bending of around 56° facilitating ER induced transcription activation (Nardulli *et al.,* 1993). The requirement for correct positioning of AF-2 has been shown experimentally. Planar oestrogens such as oestradiol allow the correct positioning of AF-2 co-activator binding leading to transcription. However, AF-2 is unable to position correctly when ERα is bound to a triphenylethylene derivative of tamoxifen, though it may still activate oestrogen responsive gene transcription through a putative AF-2b domain (Jordan *et al.,* 2001).

1.8.1 Regulation of Transcription.

The available helix 12 surface within AF-2 allows binding of co-activator complexes via their nuclear receptor-binding domain (NID) or NR boxes. Coactivators such as members of the p160 family SRC-1/TIF-2, SRC-2/GRIP-1 or SRC-3/pCIP complexes bind the ER to form a pre-initiation complex. The final ER/co-activator complex may recruit the CBP/p300/pCAF interrogator molecule that possess histone acetyltransferase activity (HATs) required for chromatin remodelling and eventual transcription activation (Moggs *et al.,* 2001; Shang *et al.,* 2000). AF-1 shows little independent transcriptional activity; in conjunction with AF-2 it may bind the C-terminus of p160 (Webb *et al.,* 1998).

Transcriptional activation is aided by structural remodelling of chromatin by members of the Swi-1/Snf-2 ATPases. One of these, BRG-1, was shown experimentally to be required for efficient ER mediated transcription involving the SRC-1/CBP co-activator. Oestrogen binding of ER promotes an association between BRG-1 and ER leading to the recruitment of BRG-1 to genes containing a functional ERE (DiRenzo *et al.,* 2000).

In the absence of ligand, nuclear receptors may recruit a number of corepressor factors that inhibit proper assembly of a preinitiation complex, the function of which is to prevent any possible transcription activity via an unliganded receptor. The two main corepressor molecules identified to date are NcoR (nuclear corepressors) and SMRT (silencing mediator for retinoid and thyroid hormone receptor).

1.8.2 Oestrogen Receptor Cycling.

During ER-mediated transcription, both unliganded and liganded receptors cycle on oestrogen-responsive promoters. Ubiquitination and the cyclic recruitment of E3 ligases and the 19S regulatory component of the proteasome are inherently linked. These protein degradation pathways act continuously to turn over ER on oestrogen responsive promoters, where cyclic turnover of ER permits continuous responses to changes in the concentration of oestradiol (Reid *et al.,* 2003) and where ubiquitin mediated ER degradation is required for ER transcriptional activation (Lonard *et al.,* 2000).

1.8.3 Different Roles of ERα and ERβ.

ERα and ERβ do not generally act in isolation or have the same cellular effects; in fact ERβ may play a role in antagonising the effects of ERα. For example, ERα and ERβ have opposing effects on cyclin D1 expression (Liu *et al.,* 2002). ERβ has also been shown to inhibit ERα transcriptional activity upon oestrogen stimulation (Hall *et al.,* 1999).

In the uterus the agonist effect of oestradiol is mediated by ERα (Hillisch *et al.,* 2004); ERβ expression is relatively low (Wang *et al.,* 1999a), and is not thought to be responsible for the oestrogenic response. Furthermore, when mice that do not express ERβ (βERKO mice) are dosed with oestradiol, the oestrogenic effect in the uterus is greater than in mice expressing both receptors (DeMayo *et al.,* 2002; Weihua *et al.,* 2000), suggesting that ERβ suppresses the oestrogen agonist action of oestradiol mediated through ERα. A regulatory role for ERβ is also seen in breast cancer cells where over-expression of ERβ reduces the proliferative response to oestradiol.

1.9 Non-Genomic Pathways of Oestrogen Receptor Signalling.

1.9.1 ERE Independent Gene Transcription.

Not all oestrogenic responses occur via an ERE. Signalling through non-ERE sites is thought to be as important as ERE signalling itself (Jakacka *et al.,* 2002). In ERE independent signalling, ER may alter transcriptional levels indirectly via alternative promoter sites, for example via binding to a GC nucleotide rich site in the SP1 promoter (Wang *et al.,* 1999b). Induction of gene transcription by ER may also occur in the absence of direct DNA binding. ERs carrying a mutation within the DBD are unable to bind to classical EREs, but are still able to activate via AP-1 sites (Jakacka *et al.,* 2001). This is likely to occur via protein-protein interactions enhancing the activity of hetrologous transcription factors. The AP-1 site, a TTAGTCAG sequence (Ugwumadu *et al.,* 1998) is the cognate binding site for the early response transcription factors Jun and Fos (Angel *et al.,* 1991). Activation via the AP-1 promoter is tissue and cell specific. For example, oestrogen may induce expression of c-*Jun* in the myometrium but represses c-*Jun* expression in the luminal epithelium (Bigsby *et al.,* 1994; Nephew *et al.,* 1994).

Further evidence that direct binding of the ER to DNA may not be required for transcriptional response, is supported by the ability of both ERα and ERβ to induce phosphorylation of Stat proteins (Signal transducers and activators of transcription), via the JAK proteins in the absence of direct DNA binding (Bjornstrom *et al.,* 2002). This was shown to require at least three cellular pathways including the Mitogen Activated Protein Kinase (MAPK), Src-kinase and PI3 kinase pathways.

Interactions with co-activator proteins are required for complete activation of the AP-1 pathway, for example recruitment of p160 co-activators required in classical ERE activation

(Webb *et al.,* 1999). The ligand binding domain and AF-2 within the ER is also critical for ER's ability to activate transcription at AP-1 sites (Webb *et al.,* 1999). ERβ has stronger activation through AP-1 sites than ERα (Kushner *et al.,* 2000; Weatherman *et al.,* 2001). Also oestradiol and tamoxifen have differential effects through AP-1 depending on cell type and protein-protein interactions (Jones *et al.,* 1999; Paech *et al.,* 1997). Other compounds may also activate the ER in the absence of oestrogens. For example, the effects of oestrogen on the mouse uterus may be mimicked by EGF, mediated through the ER (Ignar-Trowbridge *et al.,* 1995), which leads to relocalisation of ER to the nucleus and transcription activation at ERE sites.

1.9.2 Membrane Bound Oestrogen Receptor.

ER is not only confined to the nucleus or cytoplasm, around 3% of ERα and ERβ are localised in the cell membrane (Razandi *et al.,* 1999), anchored to the membrane via interactions with proteins such as the IGF-1 receptor (Santen *et al.,* 2005). Oestradiol binding of membrane localised ER may activate membrane bound receptors possessing tyrosine kinase activity or coupled to heterotrimeric G-proteins such as the MAPK pathway and phosphatidylinositol 3-kinase (PI-3) inositol 1,4,5-triphosphate (IP_3) pathway (Figure 1.3). Effects of membrane localised ER include induction of DNA replication via the MAPK protein ERK (extracellular regulated kinase). Similar to nuclear ER, cellular effects vary between ERα and ERβ, where ERβ has been shown to induce c-Jun N-terminal kinase activity, which was inhibited by binding of ERα (Razandi *et al.,* 1999). Oestradiol may also stimulate nitric oxide synthesis through activation of the PI 3-kinase / AKT pathway via membrane bound ER, inhibited by both PI-3 kinase inhibitors and oestrogen receptor antagonists (Haynes *et al.,* 2000).

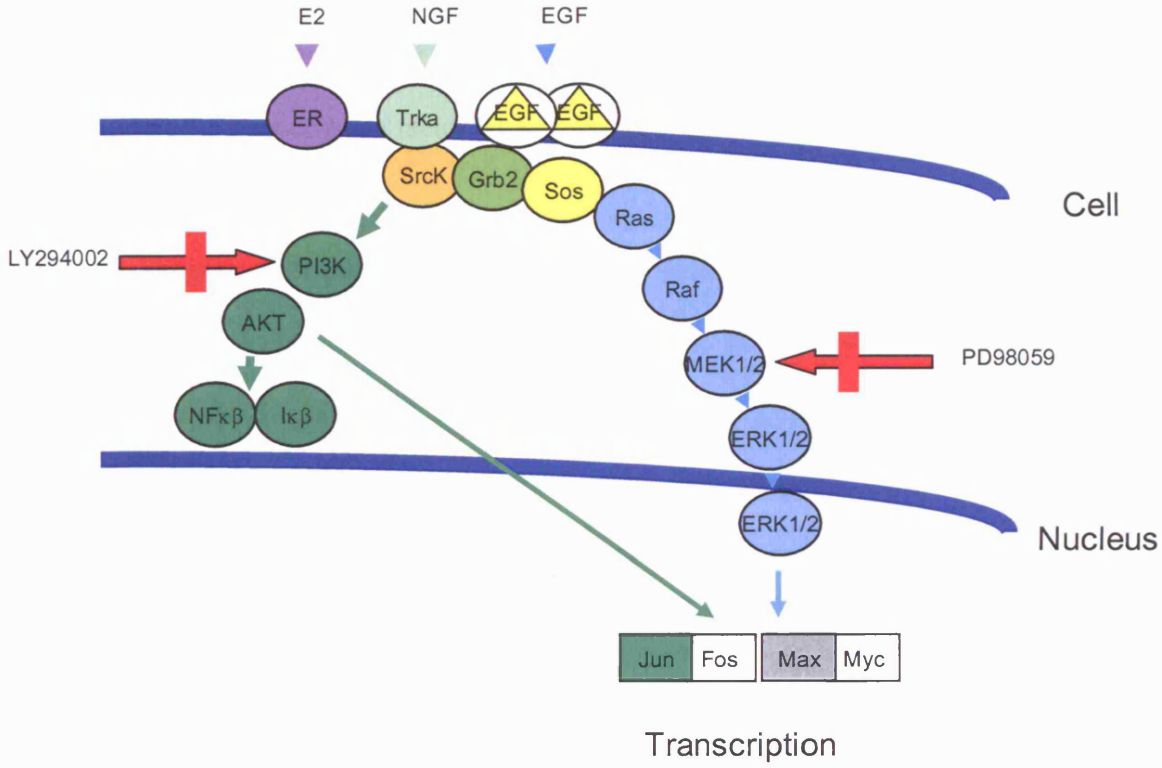


Figure 1.3: Non-Genomic Signalling Pathways: ERα, NGF and EGF.

Oestradiol (E_2) is able to activate gene transcription indirectly in some cell types via activation of the EGF stimulated MAPK pathway. This pathway may also be activated via the Nerve Growth Factor high affinity receptor, TrkA. LY294002 and PD98059 are specific inhibitors of PI 3-K and MEK1/2 phosphorylation respectively. Figure modified from Bjornstrom *et al.*, (2002).

Evidence for a non-classical response in the absence of a fully functional ER has been illustrated in the human breast cancer derived cell line, MCF-7. Oestradiol can lead to MAPK stimulation via an association between ERα bound to the membrane and SHC (Src-homology and collagen homology) phosphorylation. Interaction is dependent upon the SH2 domain of SHC and AF-1, but not AF-2 of ERα. This response is transcriptionally active, shown by the activation of the transcription factor ELK-1 (Song *et al.,* 2002).

Research suggests that oestradiol may not only induce a transcriptional response without a fully functional ER, but, ER may not be required at all. In neuroblastoma cells, oestradiol was shown to activate the MAPK pathway and subsequent phosphorylation of ERK1 and ERK2 and to induce transcription of the *c-FOS* gene. This response was not inhibited by ER antagonists suggesting that ER was not involved (Watters *et al.,* 1997). To further exemplify the importance of ER independent mechanisms, Wnt signalling is though to be critical for oestrogen-mediated uterine growth in mice, a process that occurs in an ER-independent manner (Hou *et al.,* 2004).

Evidence for ER independent, oestrogen induced transcription, is further supported by oestrogen induced activation of MAPK signalling via transactivatioin of the Epidermal Growth Factor Receptor (EGFR). Oestrogen activates a G-protein coupled receptor pathway, GPR30, by inducing the Gβγ-subunit / SRC kinase family. This in turn promotes the release of the cell surface localised complex proHB-EGF. The free HB-EGF complex is then able to activate EGFR, promote dimerisation and induce the MAPK pathway in an autocrine fashion (Filardo, 2002).

1.10 Oestrogen Receptor Modulators, Anti-Oestrogenic Compounds and Endocrine Disrupters.

Tamoxifen may display either oestrogen agonist or antagonist properties depending upon the target tissue, and is therefore referred to as a Selective Oestrogen Receptor Modulator (SERM). 'Selective' is often used interchangeably to refer to selectivity for either ligand, cell or tissue type, cellular effect or many other variables. Using the term 'oestrogen receptor modulator' may be more appropriate and less confusing phrase. However, due to the general acceptance of the acronym 'SERM', this will be used here within.

1.10.1 SERMs.

SERMs may have oestrogenic and anti-oestrogenic effects depending on their target tissue type. Tamoxifen (Figure 1.4) is one of the most successful anti-cancer drugs used to date; the standard dose being 20 mg per person per day. A number of analogous compounds are also available such as toremifene and raloxifene. These compounds are non-steroidal partial oestrogen agonists. In contrast, the steroidal fulvestrant ('faslodex') is a pure anti-oestrogen and has no oestrogenic activity. It is thought that uterine pathology following tamoxifen treatment may be partially related to its oestrogen agonist actions in this tissue.

1.10.2 Uterine Pathology Related to Tamoxifen.

a. Non-Neoplastic Uterine Changes.

Around 40% of postmenopausal women receiving tamoxifen develop some sort of endometrial abnormality. The most common is endometrial atrophy, although tamoxifen may also cause hypertrophy of the myometrium (Ugwumadu *et al.,* 1998). Ultrasonography shows

up to 75% of tamoxifen users have an irregular pseudo-thickening of the endometrium. However, as tamoxifen causes irregularity of the endometrial / myometrial junction, accurate measurement of the endometrium is often difficult (Cohen *et al.,* 1993). In one study, adenomyosis occurred in *circa* 60% of postmenopausal women on chronic tamoxifen treatment (Cohen *et al.,* 1997). Endometrial polyps may also occur with varying frequency, which are reported to have abnormal proliferative activity and aberrant epithelial differentiation (Neven *et al.,* 1997).

b. Uterine Cancers.

Endometrial cancers associated with tamoxifen use include endometrial adenocarcinomas, mucinous, clear cell and serous carcinomas and adenocarcinomas. An association between tamoxifen treatment and an increase in carcinosarcomas, very aggressive tumours containing a mixture of epithelial and mesenchymal cells, has been suggested (Evans *et al.,* 1995). However, these observations have not been confirmed by data from randomised comparisons (Ugwumadu *et al.,* 1998). Significant thickening and irregularities of the endometrium have been reported in postmenopausal women receiving standard tamoxifen therapy for more than six months (Juneja *et al.,* 2002).

In a mouse model, uterine tumours were found in neonatal CD-1 mice exposed to tamoxifen via subcutaneous injections on days one to five after birth. The uteri of all animals exposed to the drug were found to be hypoplastic and under-developed. Abnormalities included atypical, squamous and hypoplastic metaplasia, cystic endometrial hyperplasia. Uterine adenocarcinomas were found in 50% of dosed animals. These effects were not seen in control animals (Newbold *et al.,* 1997). These results suggest the developing reproductive tract is very sensitive to oestrogenic compounds with hormonal activity.

1.10.3 Endocrine Disrupters.

There is potential for every day exposure to many compounds that exert oestrogenic activity in the uterus, commonly referred to as xenoestrogens. These compounds may act via the ER to disrupt normal hormonal homeostasis which lead to them being referred to as endocrine disrupters. This class of chemicals include genistein, diethylstilbestrol (DES) and bisphenol A.

Genistein (Figure 1.4) belongs to the isoflavone class of phytoestrogens found in relatively high concentrations in soya based products. Concern has been raised over exposure of infants to isoflavones that are present in soy based infant formula at concentrations of up to 32 to 47 mg/L compared to 5.6 μg/L in human breast milk. This relates to steady state circulating plasma levels of isoflavones up to 22,000-fold higher than plasma oestradiol concentrations in four-month old infants fed a soy-based milk substitute. Up to 65% of the isoflavone content was genistein (Setchell *et al.,* 1998). In an immature mouse uterotrophic assay, genistein increased uterine weight at doses around 10,000 fold higher than oestradiol, and increased endometrial gland cell number (Jefferson *et al.,* 2002). In a human ovarian cell line stably transfected with a luciferase reporter construct, the EC_{50} dose for genistein was around 80,000 fold higher than for oestradiol. The requirement of higher doses of genistein to induce uterine weight may partially be explained by a limited ability to induce expression of genes involved in proliferation (Diel *et al.,* 2004). Neonatal exposure to high doses of genistein (50 mg/kg) may induce uterine adenocarcinomas in mice (Newbold *et al.,* 2001a).

From the 1940's to the 1970's diethylstilbestrol (DES) (Figure 1.4), a synthetic oestrogen agonist, was prescribed during pregnancy to reduce miscarriage and pregnancy related problems. Daughters whose mothers took DES during pregnancy were found to have an

increased chance of developing vaginal clear cell carcinomas, amongst other dysfunctions of the reproductive tract. Male offspring were also found to have developmental abnormalities of the reproductive tract, and DES became one of the first examples of a toxicant that could cross the placental barrier (Newbold, 2004).

DES, though not used clinically, is used widely as a model synthetic xenoestrogen. In mice neonatally exposed to DES, the uterotrophic response to oestrogen is altered, an effect that carries on into adult life, illustrating that hormone programming may be altered (Newbold *et al.,* 2004). However this effect differed depending on the level of dose received, a low dose (0.01 μg/kg) enhanced the oestrogenic response and increased ERα expression. The involvement of ERα in DES induced pathology is supported by the lack of reproductive tract abnormalities in female oestrogen receptor α knock-out mice (ERKO) exposed neonatally to DES (Couse *et al.,* 2004).

Using gene expression profiling in an immature mouse uterus model, Moggs *et al.* (2004), recently tried to distinguish fundamental differences in gene expression induced by three classes of oestrogens: oestradiol, a physiological oestrogen; genistein, a phytoestrogen; and DES a synthetic oestrogen. Previously, synthetic oestrogens have been regarded as being potentially more hazardous to human health than either physiological oestrogens or phytoestrogens. In this study, it was shown that at doses of the chemicals that produced a similar uterotrophic responses in mice after 14 days, these three oestrogens induced similar changes in gene expression profiles, though some genes were regulated at different magnitudes.

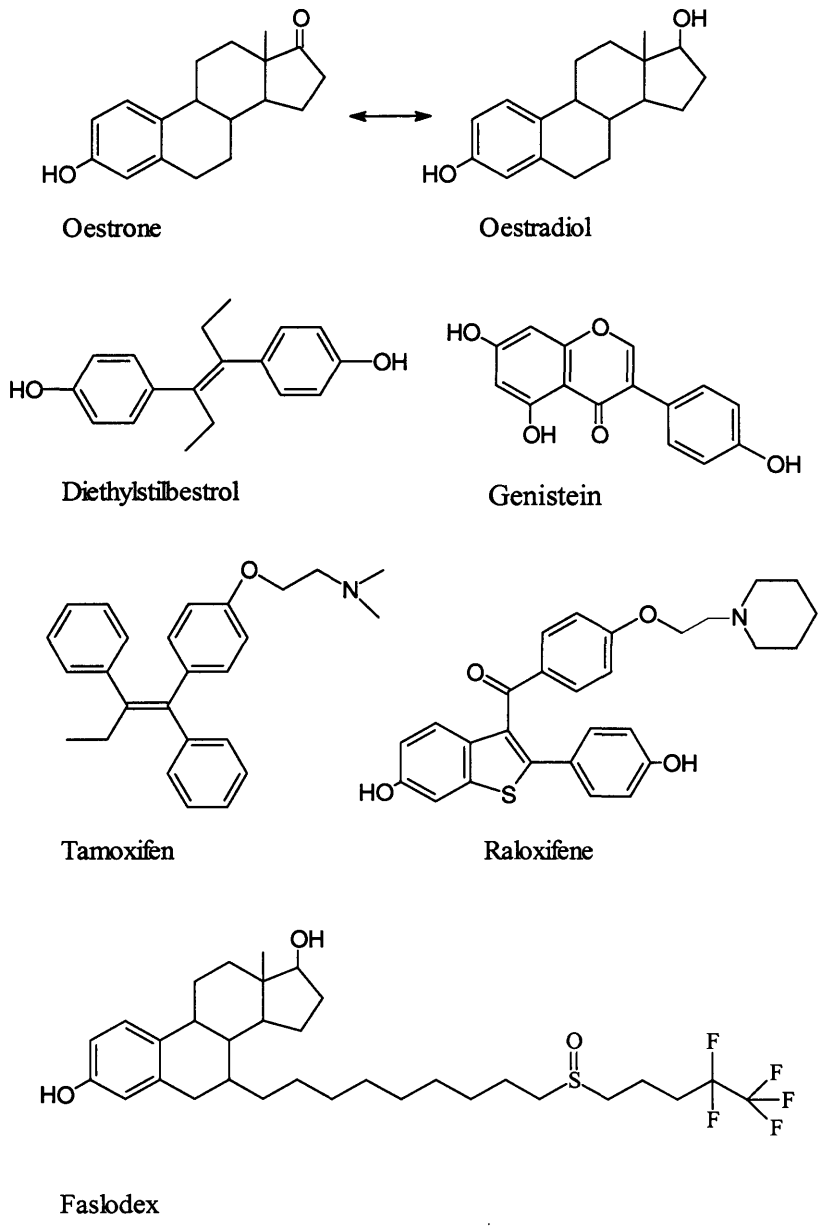


Figure 1.4: Chemical Structures of Common SERMs and Circulating Oestrogens.

1.11 Models used for the Study of SERMs and Endocrine Disrupters.

During everyday life we are continuously exposed to a range of chemicals that mimic the actions of oestrogens. There is concern that these exogenous oestrogens may have adverse effects on reproductive health in humans and animals.

A number of tests have been developed to assess oestrogenicity of organic compounds and it is important that general agreement be reached on which tests should be used. These tests might then be applied to both man-made and naturally occurring chemicals. At least five such assays are employed for a comprehensive approach to screening chemicals for suspected oestrogen activity, although none of these short-term tests is likely to be able to predict the long-term endocrine disrupting effects on the uterus such as adenomyosis or uterine tumours described in this Chapter.

1.11.1 *In Vivo* Models.

In vivo models for the study of endocrine disrupters include the uterotrophic assay in mice or rats and vitellogenin production in juvenile rainbow trout. Small rodents have probably been the most extensively used in the study of oestrogenic compounds. Classically rats, such as the Wistar rat have been used in toxicological studies, though due to the ease of use and cheaper costs, mice such as the CD-1 mouse have been used (Padilla-Banks *et al.,* 2001). In the rodent uterotrophic assay, potential oestrogen agonists are identified by their ability increase uterine weight and are often compared to oestradiol as a reference oestrogen. This is classically done in either immature or ovariectomised adult rodents to avoid interference with endogenous oestrogens.

Interpretation of data from mammalian systems may however be complicated since between strains of mice there may be over 16 fold difference in susceptibility to disruption of juvenile male reproductive development by 17β-oestradiol. Spermatid maturation was eliminated by low doses of oestradiol in the C57BL/6J strain while the widely used CD-1 line, which has been selected for large litter size, showed little or no inhibition of spermatid maturation even in response to 16 times as much oestradiol (Spearow *et al.,* 1999). There is no direct evidence that such strain difference exist in the uterotrophic effects of oestradiol in female mice (Scullion, 2003).

1.11.2 Oestrogen Receptor Knock-Out Mouse.

Both oestrogen receptor α (ERKO) and β knock out-mice (*β*ERKO) models have been studied (Couse *et al.,* 1999; Das *et al.,* 1997; Korach, 2000). Abolition of ERα leads to hypoplastic uterine tissues with decreased expression of oestrogen responsive genes. Abolition of ERβ reduces fertility but produces no obvious alteration in uterine morphology, suggesting that ERα plays a more salient role in the uterus. Interestingly in *β*ERKO mice oestrogen stimulation leads to increased uterine hyperplasia and oestrogen responsive gene expression leading to the suggestion that ERβ may play some role in antagonising the effects of ERα (DeMayo *et al.,* 2002). These data have led to the conclusion that ERα is the dominant mediator of the oestrogenic response in the mouse uterus.

Recently an isoform of ERα has been identified in the uteri of the ERKO mouse, which is 61 kDa in comparison to 66 kDa for the wild type ERα (Kos *et al.,* 2002). Named E1 ERα, this receptor has a reduced ability to induce oestrogen responsive gene expression and was shown to have impaired AF-1 activity, but retains AF-2 activity. This may have significant implications in the study of tamoxifen, which has been shown to induce transcription through

the AF-1 domain (Wakeling, 2000). In fact, tamoxifen does not have an agonist effect on the ERKO uterus (Korach *et al.,* 1996), which may be explained by the loss of AF-1 activity.

1.11.3 *In Vitro* Models.

There are many *in vitro* tests available for the screening of endocrine disrupters, the most common of which are mainly suitable for the detection of classical receptor mediated effects (Baker, 2001). *In vitro* endocrine disrupter screening assays carry obvious advantages over *in vivo* assays, primarily due to accessibility and increasing regulation involved in animal studies. Types of *in vitro* screening tests used include receptor binding assays, cell proliferation assays, and reporter gene and hormone responsive gene assays.

Receptor binding assays for oestrogenicity involve the measurement of a compounds ability to bind the ER, and have been successfully used in the screening of ER binding compounds (Baker, 2001). A major limitation to this assay is the inability to distinguish between oestrogen agonists and antagonists as either action requires ER binding.

The most commonly used cell proliferation assay for oestrogenicity is the E-SCREEN, which involves measurement of a compounds ability to induce proliferation of an oestrogen dependent cell line such as MCF-7 (Soto *et al.,* 1995). Unlike receptor binding assays, cell proliferation assays are able to distinguish between oestrogen agonists and antagonists. However, concern has been raised over inter-laboratory variation in MCF-7 cell response (Payne *et al.,* 2000). Other established cell culture lines derived from uterine tissue have proved a useful model for the study endocrine disrupters. Other oestrogen responsive cell lines include the Ishikawa cell line derived from uterine adenocarcinoma which express both the ERα and β and progesterone receptors (Lessey *et al.,* 1996), and HEC1A cell line derived

from endometrial cancer, both of which proliferate in response to both oestradiol and tamoxifen (Anzai *et al.,* 1989; Castro-Rivera *et al.,* 1998).

Reporter gene assays involve measuring the ability of a compound to activate transcription of an oestrogen-responsive promoter, usually in mammalian cell line such as human embryonal kidney cells or yeast (Baker ,2001; Gaido *et al.,* 1997; Ramamoorthy *et al.,* 1997). For this, cells are transfected with an ER together with an oestrogen-responsive promoter and reporter gene construct such as luciferase, a protein easily quantifiable by measuring luminescence.

Other *in vitro* assays developed to screen for oestrogen agonists involve the induction of *PRL* gene expression, release, and cell proliferation in anterior pituitary cells (Steinmetz *et al.,* 1997). Also, the measurement of oestrogen-responsive genes such as vitellogenin (Kloas *et al.,* 1999) from eukaryote cell lines in response to a test compound.

A model increasing being used in the study of the effects of SERMs is primary uterine cell culture. Primary cell cultures from the uterus have been established for the study of a range of cell roles in normal and disease physiology including embryo implantation and the uterine cell types role in pregnancy (Chan *et al.,* 1997) and endometriosis (Overton *et al.,* 1997). Isolated endometrial stromal and glandular epithelial cells have proved a useful model for the study of normal cellular interactions, demonstrating for example, that epithelial cell proliferation is mediated by stromal cells (Arnold *et al.,* 2001), and highlighting a role for EGF in the uterus (Tomooka *et al.,* 1986).

In terms of the study of SERMs, primary endometrial stromal and glandular epithelial cultures have not been fully utilised. Previous studies were limited to the effects of tamoxifen on

aromatase activity (Tseng *et al.,* 1986), proliferation (Zhang *et al.,* 2003) and apoptosis (Stackievicz *et al.,* 2001). More recently tamoxifen was shown to alter expression in endometrial epithelial and stromal cells of genes involved in regulation of cell cycle, transcription and signal transduction (Pole *et al.,* 2005). This has given interesting insight into the mechanisms of action of tamoxifen in a highly relevant model, with great potential for further research.

1.11.4 Transcription Profiling for the Study of Oestrogen Receptor Modulators.

Recent advances in experimental techniques that utilise nucleic acid hybridisation, such as cDNA microarrays, have allowed researchers to build a profile of gene transcription in a cell or tissue of interest. In cDNA microarrays, RNA from tissue or cells of interest is reverse transcribed into cDNA whilst fluorescent dyes are incorporated. The relative expression of a control and test sample are then analysed after competitive hybridisation to a complementary sequence. This can be done for many individual sequences simultaneously, allowing analysis of differences in gene expression between control and test samples of many different genes. Relevant applications to date include the study of gene expression involved in mouse uterine development (Hu *et al.,* 2004), gene expression involved in ageing of the uterus (Khalyfa *et al.,* 2003), and differences in gene expression between genotoxic and non-genotoxic carcinogens (van Delft *et al.,* 2004).

In terms of the study of the oestrogenic response in the mouse uterus, large scale transcription profiling have allowed identification of genes that contain either an ERE, AP-1 or SP1 site (Khalyfa *et al.,* 2003). In women, differences in gene expression during different stages of the menstrual cycle have also been analysed identifying a number of steroidal responsive genes

containing classical response sites and also many genes previously not associated with normal endometrial physiology (Borthwick *et al.,* 2003).

Recently, using a mouse model, microarrays have been used to correlate changes in gene expression with phenotypic changes involved in uterine growth (Moggs *et al.,* 2004b). Compiling phenotypic characteristics with gene expression data, four fundamental phases were identified in the oestrogenic response: 1. rapid induction of transcriptional regulators and signalling components by oestradiol; 2. induction of genes required for mRNA, DNA and protein synthesis; 3. regulation of genes controlling chromosome replication and the cell cycle; 4. induction of genes involved in uterine cell differentiation and the defence response. Genes involved in phase 1 led to fluid imbibition, and phase 2 and 3 were involved in cell proliferation.

1.12 Mechanisms of SERM Toxicity.

SERMs have different effects depending on the tissue, cell type, promoter and co-activator context. Tamoxifen causes uterine Ishikawa cancer cells to proliferate (Anzai *et al.,* 1989) and is oestrogenic in the uterus (Green *et al.,* 2001) whilst it inhibits growth of breast cancer cells (Rochefort *et al.,* 1984). For this reason the tissue specificity of SERMs has attracted a great deal of research.

There is variability between SERMs and their partial oestrogenic activities. Tamoxifen and its structurally related analogue toremifene share similar oestrogenic effects on the mouse and human uterus (Parrott *et al.,* 2001; Tomas *et al.,* 1995). Raloxifene has little proliferative effect on the endometrium and is classed as a complete antagonist in breast cancer cells.

However raloxifene has oestrogen agonist activity on the bone and it is used to treat and prevent osteoporosis in postmenopausal women (McClung, 2002; Siris *et al.*, 2002).

1.12.1 *In Vivo* Toxicity Studies.

It is generally agreed that tamoxifen does not induce uterine abnormalities by a simple oestrogenic action alone. Although a positive uterotrophic response is seen after tamoxifen administration in neonatal rodents, this does not correlate with cell proliferation (Carthew *et al.*, 1999) and endometrial cancers may develop without evidence of epithelial hyperplasia (Carthew *et al.*, 2000).

Studies on the rat liver show that long-term exposure to high doses of tamoxifen, but not toremifene, results in liver tumours (Greaves *et al.*, 1993; Hard *et al.*, 1993). Within the rat liver tamoxifen is activated mainly by CYP3A4 to an electrophile that binds irreversibly to DNA (White *et al.*, 1995). An increased frequency of gene mutations, caused by DNA damage, is seen in livers of transgenic rats dosed with tamoxifen but not in toremifene dosed rats (Davies *et al.*, 1997). Though there have been reports of low level DNA damage occurring in the uteri of patients receiving tamoxifen or toremifene (Hemminki *et al.*, 1996; Martin *et al.*, 2003), adduct levels are much lower than those seen in the livers of tamoxifen treated rats that develop liver tumours (Carthew *et al.*, 1995). Low levels of adducts were found in the livers of mice dosed with either oral (Martin *et al.*, 1997) or subcutaneous tamoxifen but not in the uterus (Hellmann-Blumberg *et al.*, 2000). Neither tamoxifen nor toremifene induced adducts in the uterus after chronic administration to Fischer rats, though tamoxifen significantly enhanced endogenous DNA damage (Li *et al.*, 1997a). It is not generally thought that DNA damage is a major contributing factor for tamoxifen toxicity in the uterus.

1.12.2 *In Vitro* Studies.

a. Mechanistic Studies.

In the breast, coactivators may not interact with ERα when tamoxifen or raloxifene binds (Norris *et al.,* 1999). Tamoxifen binding rotates helix 12 of the ER by 110° where it recognises a sequence that mimics the NR box within the hydrophobic cleft so that the p160-binding domain is blocked (Brzozowski *et al.,* 1997). AF-2 silencing is the primary mechanism of action for SERMs. However, AF-1 remains activated when ER is tamoxifen bound resulting in partial agonism, though the response to this is largely dependent on cellular and promoter context (Wakeling, 2000).

It is thought that AF-1 is the major transcriptional activator of ER and AF-2 acts as a transcriptional facilitator (Tzukerman *et al.,* 1994). As mentioned, the oestrogenic effects of tamoxifen may occur through partial agonism of AF-1, and are dependent on its dimethylaminoethoxy side-chain. If changed to an allylcarboxylic acid side chain the surface charge is displaced and D351 is repelled, therefore AF-1 is allosterically silenced and gene transcription is inhibited (Liu *et al.,* 2001; Jordan *et al.,* 2001).

Interaction of the antioestrogenic side chain with amino acid D351 of the ER is essential for the activity of raloxifene (Levenson *et al.,* 1998; MacGregor Schafer *et al.,* 1999). The interaction between the basic amines that interact with amino acid D351 on helix 3 of the ligand-binding domain of ER differ between raloxifene, a hydrogen bond, and tamoxifen, a salt bridge (Liu *et al.,* 2001; Jordan *et al.,* 2001). Considering raloxifene has little oestrogenic effect on the uterus, this supports the involvement of an oestrogen agonist effect mediated by AF-1 in the toxic effects of tamoxifen in the uterus.

b. Difference in roles of ERα and ERβ.

17β-Oestradiol binding of ERα activates transcription of AP-1 responsive genes whereas transcription is inhibited when bound to ERβ. Conversely, when bound to SERMs such as tamoxifen transcription is increased via ERβ but not ERα (Jones *et al.,* 1999; Paech *et al.,* 1997). This SERM activation of ERβ is completely independent of AF-2 which is usually required for co-activator dependent transcription activation (Webb *et al.,* 1999). However tamoxifen has no agonist effect in the uteri of ERKO mice suggesting that tamoxifen agonism is not exclusively through ERβ activation (Kuiper *et al.,* 1997).

c. Difference in Response between Breast and Uterine Derived Cell Lines.

In MCF-7 breast cancer derived cells and Ishikawa uterine cancer derived cells treated with tamoxifen or raloxifene, co-activator recruitment is inhibited, and tamoxifen or raloxifene bound ER may recruit co-repressors NCoR and SMRT and histone deacetylases (HDACs) to ERE containing promoters. However, in Ishikawa but not MCF-7 cells, tamoxifen recruits coactivators such as SRC-1 to target genes that do not contain a classical ERE such as the c-Myc gene. This effect was not seen in raloxifene treated cells (Shang *et al.,* 2000; Shang *et al.,* 2002). Furthermore, the difference in action of tamoxifen in breast and uterine tissues, has been attributed to the expression levels of SRC-1; with lower expression levels in the breast than in the uterus (Shang *et al.,* 2002).

Within Ishikawa cells, MAPK shown to stimulate AF-1 transcriptional activity, is constitutively activated in contrast to breast cancer cells (Sakamoto *et al.,* 2002). Tamoxifen stimulation of Ishikawa proliferation is thought to be due partly to the enhancement of ERα AF-1 function via the constitutive activity of the MAPK pathway.

1.12.3 Metabolism of SERMs to Toxic Intermediates.

It is well established that oestrogenic compounds may undergo metabolism by a range of cytochrome P450 enzymes to form potentially genotoxic reactive intermediates (Joosten *et al.,* 2004). A role for these metabolites and the enzymes involved has been implicated in the toxicity of both oestrogen and tamoxifen in a range of tissue including the uterus (Tsuchiya *et al.,* 2004) and the breast (Modugno *et al.,* 2003; Russo *et al.,* 2003).

a. Metabolic Activation of Oestradiol.

Oestradiol itself induces tumours in various organs of rats, mice and hamsters, and unopposed oestrogen therapy in women increases the risk of developing breast and uterine cancer (Liehr, 2000). Several epigenetic mechanisms of tumour induction have been proposed. However, it is known that oestradiol may undergo metabolism by CYP1B1 to form either 2 or 4-hydroxyoestradiol which may be further oxidised to form semiquinones or quinones, a process that may form harmful free radicals (Figure 1.5). It is generally accepted that 4-hydroxyoestradiol is more carcinogenic than 2-hydroxyoestradiol (Newbold *et al.,* 2000). CYP1B1 has been localised to both normal and tumour tissue of the breast (Modugno *et al.,* 2003), and polymorphisms of the gene leading to hyperactivation of the protein have been associated with increase risk of endometrial cancer (Sasaki *et al.,* 2003). CYP1B1 may be involved in oestrogen dependent carcinogenesis through increased expression induced by oestrogen via ERα (Tsuchiya *et al.,* 2004).

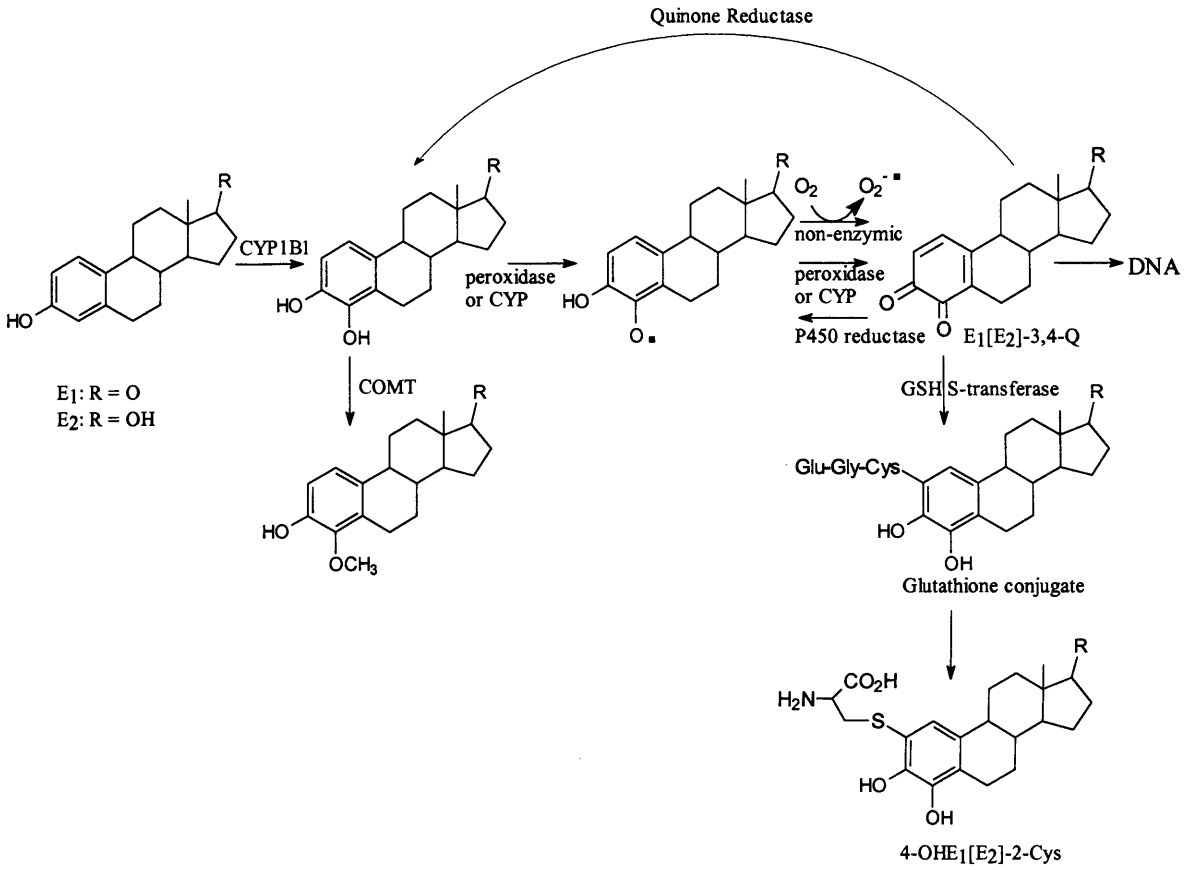


Figure 1.5: Metabolism of Oestradiol by P450 Enzymes.

CYP1B1 and CYP1A1 catalyse the oxidation of E_2 to the catechol estrogens 2-OHE_2 and 4-OHE_2, which may be further oxidised by CYP enzymes to semiquinones and quinones. These quinones are either conjugated by phase II metabolising enzymes such as glutathione S-transferase, or may form DNA adducts or oxidative DNA damage through quinone-semiquinone redox cycling (Devanesan *et al.*, 2001).

In the immature mouse uterus, 4-hydroxyoestradiol increases uterine weight and has been directly linked with the formation of uterine adenocarcinomas (Newbold *et al.,* 2000). This carcinogenic effect is not seen with 17β-oestradiol (Newbold *et al.,* 1990), suggesting that the metabolism of oestrogens may be a significant factor to be considered in the development of pathology. As outlined previously, it is likely that oestrogenic effects are not all directly regulated by the ER. 4-Hydroxyoestradiol is still able to induce lactotransferrin expression and increase water uptake in the ERKO uterus (Das *et al.,* 1997).

In terms of other oestrogen responsive tissues, it is still unclear whether 4-hydroxyoestradiol is able to induce mammary tumours in rats (Turan *et al.,* 2004), although it has been implicated as contributing to genomic instability in human breast cell lines (Rajapakse *et al.,* 2005). 4-hydroxyoestradiol was also found to bind protein at relatively high levels in the ovary (Philips *et al.,* 2004), it would not be unexpected therefore to find effects of 4-hydroxyoestradiol in this tissue.

b. Metabolic Activation of Tamoxifen.

Tamoxifen may also readily undergo metabolism by the cytochrome P450 pathway to form 4-hydroxytamoxifen and α-hydroxytamoxifen (Figure 1.6). CYP3A4 catalyses the formation of α-hydroxytamoxifen (Boocock *et al.,* 2002). Sulfation of α-hydroxytamoxifen is thought also to lead to a DNA reactive metabolite (Shibutani *et al.,* 1998). There is strong evidence for the role of DNA adducts in the development of liver tumours in rats, though the formation of DNA adducts and their role in uterine pathology is still questionable (Beland *et al.,* 2004; Carmichael *et al.,* 1996). Using the highly sensitive method accelerator mass spectrometry (AMS), low levels of DNA adducts in the uteri of women who received a single oral dose of tamoxifen were identified (Martin *et al.,* 2003). In addition, (Shibutani *et al.,* 2000) found

very low but significant levels of α-hydroxytamoxifen DNA adducts accumulate in the endometrium of many, but not all, women receiving tamoxifen.

In neonatal rats, tamoxifen administered orally on days two to five after birth caused a significant increase in uterine adenocarcinomas at 36 months after dosing. In addition squamous cell carcinomas of the vagina and cervix were increased without evidence of an oestrogen agonist effect in the uterus, suggesting that a complete oestrogen agonist effect is not required for the carcinogenic actions of tamoxifen on the female reproductive tract (Carthew *et al.,* 2000). It is possible that the formation of DNA adducts may be involved in tamoxifen-induced carcinogenesis in the uterus.

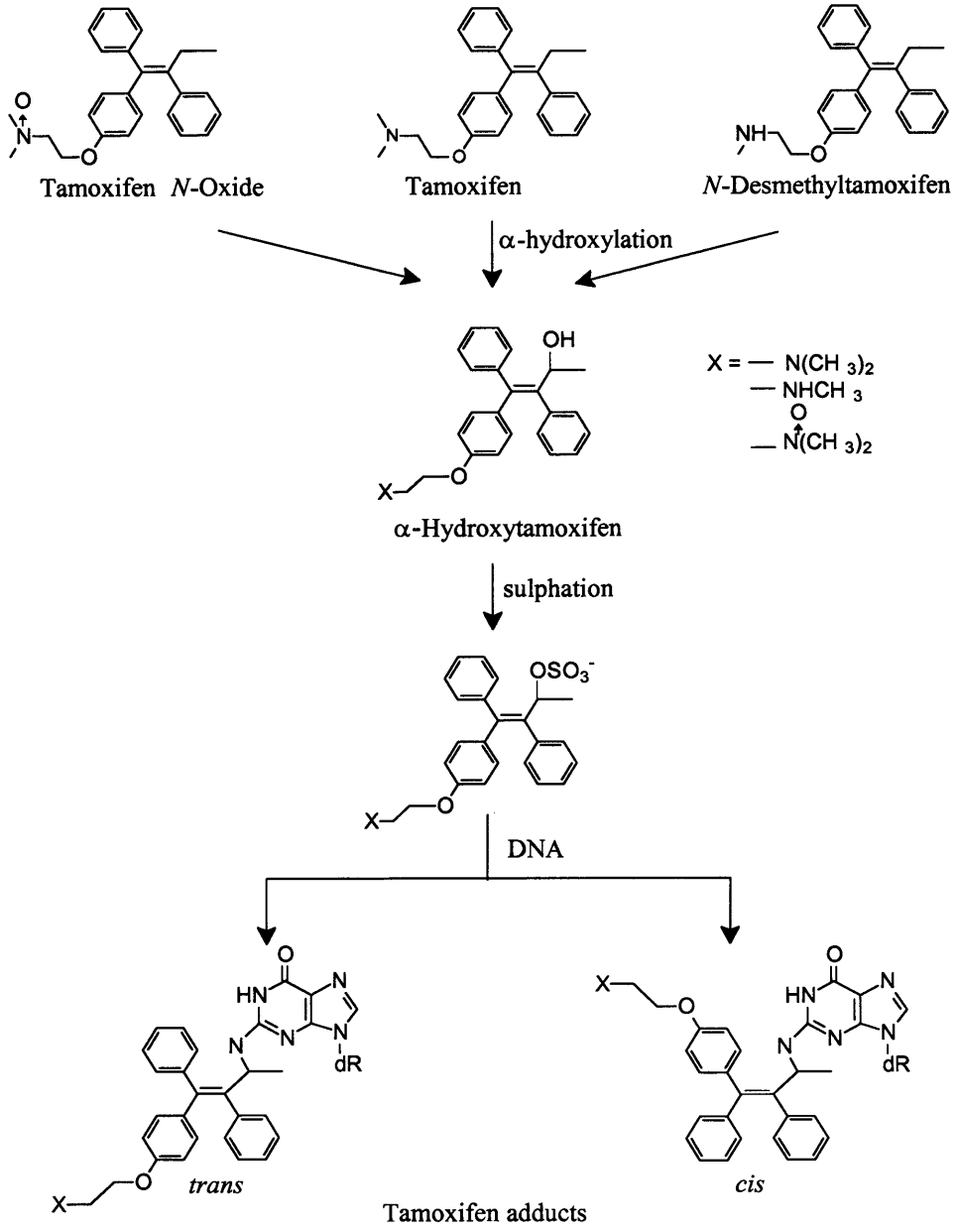


Figure 1.6: Metabolism of Tamoxifen by P450 Enzymes.

CYP3A4 catalyses the oxidation of tamoxifen to α-hydroxytamoxifen, that binds to DNA (Dasaradhi *et al.,* 1997; Osborne *et al.,* 1996). However, the addition of a sulphate group at the α-position creates a compound that may form up to 1600-fold more DNA adducts that the α-hydroxytamoxifen species alone (Dasaradhi *et al.,* 1997). Picture from Shibutani *et al.,* (2000).

1.12.4 Candidate Genes Involved in Uterine Carcinogenesis.

Many candidate genes have been identified as being involved in uterine carcinogenesis. Emphasis has been placed on the role of growth factors as being involved in the indirect oestrogenic response. The TGFα gene, encoding the transforming growth factor α protein, is oestrogen responsive. Both EGF and TGFα are stimulatory growth factors and may be involved in oestrogen stimulated carcinogenesis. However, oestradiol may also suppress expression of the inhibitory growth factor TGFβ. Oestradiol may induce expression of Vascular Endothelial Growth Factor, VEGF, involved in vascularisation of the endometrium. Control of the inhibitory growth factor TGFβ by tamoxifen or oestradiol are tissue specific. In the breast tamoxifen stimulates expression of TGFβ, where as in the uterus both tamoxifen and oestradiol inhibit expression (Butta *et al.,* 1992; Gong *et al.,* 1992). These growth factors may be involved in tamoxifen induced uterine carcinogenesis. Other genes implicated in tamoxifen induced pathology include human telomerase reverse transcriptase (hTERT) (Wang *et al.,* 2002b), Wilm's tumour (WT1) and retinoblastoma gene (Rb) (Green *et al.,* 2001), IGF-2 and NGFα (Green *et al.,* 2003; Parrott *et al.,* 2001).

1.13 Aims.

There is clear therapeutic justification for the use of tamoxifen in the treatment of breast cancer. However, evidence has now been outlined showing that tamoxifen can cause uterine pathologies. The precise mechanisms involved in this are still unclear and are likely to be complex. There is evidence to show that tamoxifen acts as an oestrogen agonist and may activate non-classical gene transcription or may bind to DNA, all of which may contribute to the development of uterine disease.

In the endometrium of women, there is evidence to suggest that alteration in gene expression may mediate the effects of tamoxifen. In this Thesis it was aimed to investigate gene expression changes in the endometrium of women receiving long term tamoxifen treatment using microarrays. Recently, NGF was identified as gene that may potentially mediate the effects of tamoxifen in the uteri of mice (Green *et al.,* 2005; Parrott *et al.,* 2001). Continuing from this work, it was aimed to further investigate NGF expression in the uteri of women and the effects of tamoxifen on this.

Research shows that endometrial cancers develop in mice dosed neonataley with both tamoxifen and 4-hydroxyoestradiol (Newbold *et al.,* 1997; Newbold *et al.,* 2000). Adenomyosis, and not endometrial tumours, developed after oral dosing with tamoxifen (Green *et al.,* 2005; Parrott *et al.,* 2001). The effects of 4-hydroxyoestradiol on uterine pathology after oral dosing are not known. Both of these compounds may be activated to form DNA reactive intermediates, a mechanism that may be involved in the toxicity of these compounds in the endometrium.

To investigate further the mechanisms of SERM toxicity, the effects of tamoxifen were compared with those of 4-hydroxyoestradiol. Oestradiol was used as a reference oestrogen agonist. The aims of this work were to investigate and compare the uterotrophic effects of these SERMs using a 14-day old mouse model where the effects of oestrous cycling are minimised. Maximal uterotrophic doses of each compound were then to be used to investigate long term changes in gene expression in relation to phenotypic changes in mice after neonatal dosing.

Chapter 2.

Materials and Methods.

2.1 Primary Cell Culture of Human Endometrial Cells.

2.1.1 Ethical Approval.

Uterine tissue samples were obtained following ethical approval from the University Hospitals Leicester NHS Trust from women undergoing hysterectomies. Participants were not known to have uterine cancers at the time of operation.

2.1.2 Cell Culture Materials.

Ficoll-Paque Plus (10-15% Diastrizoic acid dehydrate + 5 – 10% Ficoll 400) [Amersham Biosciences, Chalfont, St Giles, Buckinghamshire, UK]. 40 μm nylon cell strainers [BDFalcon, Cowley, Oxfordshire, UK]. Human recombinant Nerve Growth Factor-β (reconstituted in sterile PBS at a final concentration of 5 μg/ml) [Calbiochem, Beeston, Nottingham, UK]. Dulbecco's Modified Eagle Medium: Hams Nutrient Mixture F-12 (D-MEM/F-12 media, phenol red free), heat inactivated Foetal Bovine Serum (FBS) [Invitrogen, Paisley, UK]. Charcoal stripped FBS [Hyclone-Perbio Sciences, Cramlington, Northumberland, UK]. Phosphate Buffered Saline (Dulbecco A, pH 7.3, prepared from tablets to a 1 x solution and autoclaved) [Oxoid, Basingstoke, Hampshire UK]. Deoxyribonuclease-1 (Dnase), 2000 units/mg [Roche, Lewes, East Sussex, UK]. Bovine Serum Albumin (BSA), L-glutamine 200 mM, antibiotic antifungal solution (100x: 10,000 units/ml penicillin G, 10 mg/ml streptomycin sulphate and 25 μg/ml amphotericin B), collagenase type I-AS, trypsin-EDTA solution (T/E: A solution (10 ml) contains: trypsin (50 mg), EDTA (20 mg), NaCl (85 mg)), Trypan Blue solution, 17β-oestradiol, 4-hydroxytamoxifen, prostaglandin D2, and progesterone [Sigma, Poole, Dorset, UK]. 250 μm and 40 μm nylon filters [BD-Falcon, Franklin Lakes, NJ, USA]. All cell culture vessels, flasks and dishes were poly-lysine coated [Nunc, obtained through the University of Leicester]. All cell culture materials were phenol red free to avoid any possible oestrogenic interference (Hubert *et al.,* 1986).

2.1.3 Cell Culture Solutions.

Cell Dissociation Solution: Collagenase type I-AS (0.25%), Dnase type 1 (0.5 mg/ml) made up in sterile PBS. Cell Culture Media: D-MEM/F-12 containing FBS (10%) or charcoal stripped FBS (10%), L-glutamine (1%) and antibiotic-antimycotic solution (1%). Low Serum Media: D-MEM/F-12 containing charcoal stripped FBS (5%), L-glutamine (1%) and antibiotic-antimycotic solution (1%).

Stock solutions of SERMs were made up in sterile glass containers dissolved in ethanol to give the final concentrations of; 17β-oestradiol (10 nM), 4-hydroxytamoxifen (1 μM), progesterone (10 nM) or prostaglandin D_2 (PgD_2, 100 nM). For vehicle controls, ethanol was diluted 1:1000 in dosing media. In human breast cancer cell lines, it has been reported that > 20 mM ethanol results in a dose-dependent increase of up to 10- to 15-fold in the transcriptional activity of the liganded ER-α, but did not activate the non-liganded receptor (Fan *et al.,* 2000). For the present studies, it was calculated that the final ethanol concentration (~17 mM), was below that known to cause transcriptional activation.

2.1.4 Primary Human Endometrial Stromal and Glandular Epithelial Cell Isolation.

For isolation of primary endometrial cells, tissues were obtained from women who were still regularly menstruating and within the proliferative stage of the menstrual cycle. The endometrial layer removed by the surgeon (approximately 0.5 to 0.7 g), was collected from theatres in 10 ml ice-cold PBS and kept on ice until processing, within 15 to 30 minutes of collection. Primary cell isolation was based on published methodology (Arnold *et al.,* 2002). In a sterile laminar flow cell culture hood, tissue was washed 2 to 3 times in PBS to remove excess blood. The endometrium was centrifuged (600 x g, for 5 min, at 4 °C) to aid removal of excess mucus and blood and the supernatant discarded. Tissue was minced using sterile

scalpels and then incubated at 37 °C on a rotating platform for 2 to 2½ h in the collagenase containing cell dissociation solution in a 75 cm^2 tissue culture flask using 1 ml/g tissue. After incubation, tissue was pipetted vigorously using a sterile pasture pipette to dissociate clumps of cells.

The cell suspension was passed through a 250 μm nylon mesh to remove undigested fragments. The digest was then passed through a 40 μm cell strainer; single stromal cells passed through the sieve whilst uterine epithelial glands were retained on the membrane.

a. Stromal Cell Isolation.

The filtrate containing stromal cells was washed with 10 ml isolation media to dilute the collagenase and cells were collected by centrifugation (400 x g, for 5 min, at 4 °C) and re-suspended in 5 ml of isolation media. To further purify the stromal cells, the filtrate was then carefully layered over the top of an equal volume of Ficoll-Paque in a sterile 15 ml polycarbonate tube and centrifuged (400 x g, for 5 min, at 4 °C) to pellet blood cells present in the cell preparation. The top layer of Ficoll / medium containing stromal cells was removed and made up to 10 ml with isolation medium. Cells were pelleted by centrifugation (400 x g, for 5 min, at 4 °C) and re-suspended in 10 ml low serum media. Cell number and viability were assessed using a haemocytometer and Trypan blue exclusion assay. For this 20 μl cell suspension was mixed with an equal volume of Trypan blue solution and loaded onto a haemocytometer. The total cell number in 10 ml of cell suspension was calculated by:

$$\text{mean cell count} \times 2 \times 10 \times 10^4$$

Where 2 is to correct for the 1 : 1 dilution of cell suspension in Trypan blue, 10 is the total cell suspension volume (10 ml) and 10^4 is the haemocytometer multiplication factor.

b. Glandular Epithelium Isolation.

Epithelial glands, retained on the 40 μm sieve, were washed with 50 ml PBS before being backwashed from the sieve with 20 ml of isolation media. Medium containing glands was plated into a 75 cm^2 tissue culture flask and incubated at 37 °C with 5% CO_2 in air for 30 min to allow any remaining stromal cells to adhere. Medium removed from the 75 cm^2 culture flask contained epithelial glands in suspension which were collected by centrifugation (400 x g, for 5 min, at 4 °C) and re-suspended in 10 ml low serum culture medium.

2.1.5 Cell Culture and 24 h Dosing.

For 24 h dosing, all cell culture vessels were coated with 1% gelatine for 30 min, and excess removed prior to the addition of cells. Primary stromal cell were seeded in Petri dishes at *ca* $4x10^6$ cells per dish in 10 ml stripped serum medium. Glandular epithelial cells were seeded into 24 well plates, using 0.5 ml of the gland suspension per well (for 0.5 g starting tissue weight, 10 wells were used). Stroma and epithelial cells were cultured in humidified incubator at 37 °C in 5% CO_2 for 24 to 48 h before dosing. To dose cells, medium was aspirated and relevant dosing medium added (refer to Section 2.1.3). Cells were cultured as before for 24 h before RNA extracted (see Section 2.7).

2.1.6 NGF ELISA.

After dosing, 1 ml of medium from cell cultures was mixed with 10 μl complete mini protease inhibitor cocktail [Roche, Lewes, East Sussex, UK] and stored at -80 °C. NGF protein was measured in cell culture medium (100 μl) of 24 h dosed cell cultures using a DuoSet human β-NGF ELISA Development System [R&D Systems, Abingdon, Oxfordshire, UK. Cat. No. DY256] following the manufacturers protocol. NGF standards ranging from 0.2 to 1 ng/ml were loaded in duplicate from a stock of 60 ng/ml (supplied with kit). Substrate solution was

a 1:1 mixture H_2O_2 and tetramethylbenzidine obtained from R&D Systems [Abingdon, Oxfordshire, UK. Cat. No. DY999]. Stop solution was 2 N H_2SO_4 [Fisher Chemicals, Loughborough, Leicestershire, UK]. Optical density was assessed for each well using a FlourStar Optima plate reader set at 450 nm [BMG Labtech, Aylesbury, Buckinghamshire, UK]. To increase sensitivity of the ELISA a QuantaBlu Flourogenic Peroxidase Kit [Pierce (PerbioBioscience), Tattenhall, Cheshire, UK] was also used to assess NGF in cell culture medium following the manufacturer's protocol (Cat. No. 15169 15162).

2.2 Immunocytochemical Staining of Cell Cultures.

2.2.1 Materials.

Phosphate Buffered Saline (PBS) (Dulbecco A, pH 7.3, diluted to a 1x solution) [Oxoid, Basingstoke, Hampshire UK]. Normal goat serum (Cat. No. X0907), fluorescent mounting medium, smooth muscle α-actin mouse IgG_{2a} (Cat. No. Mo851) diluted 1:100 and mouse IgG_{2a} [DakoCytomation, Cambridgeshire, UK]. Fibroblast Antigen (Ab-1 / Thy-1) mouse anti-human monoclonal IgG_1 (Cat. No. CP28) diluted 1:100 [Oncogene, San Diego, CA, USA]. Propidium iodide (1 mg/ml), goat-anti mouse or rabbit FITC-conjugated secondary antibody (Cat. No. F5897 or F0382) diluted 1:100, bovine serum albumin (BSA) [Sigma, Poole, Dorset, UK]. Vimentin mouse anti-human IgG_1 (Cat. No. MCA862HT) diluted 1:100, cytokeratin 5/6/18 mouse anti-human IgG_1 (Cat. No. MCA1867T) diluted 1:100 [Serotec, Kidlington, Oxfordshire, UK]. Smoothelin mouse anti-chicken IgG1 (Cat. No.OMA1-06020) was used at 4 μg/ml [ABR Affinity BioReagents, Golden, CO, USA].

2.2.2 Cell Fixation and Staining.

For immunocytochemical staining, cells were grown on plastic in 24 well cell culture plates. Primary cells were grown until they were 70 to 80% confluent (3 to 4 days after isolation) and

then fixed with pre-chilled ice-cold methanol and maintained at -20 °C for 30 min. Methanol acts to precipitate proteins and fixing cells at -20 °C ensures even precipitation throughout cells. After fixation, cells were washed and re-hydrated three times for 5 min in wash buffer (PBS containing BSA 0.1% and sodium azide 0.01%). Non-specific binding of antibodies was blocked by incubating cells at room temperature for 30 min with blocking buffer (PBS containing BSA 4% and normal goat serum 2%). Excess blocking buffer was aspirated and cells were incubated at room temperature for 1 h with primary antibody diluted in blocking solution at the appropriate dilution factor (given in Section 2.2.1). Negative controls were included where normal sera from the primary antibody host species or a relative IgG type were diluted at the same concentration as the primary antibody. Cells were washed with wash buffer three times for 5 min each on a rocking platform at room temperature. A goat-anti mouse or rabbit (dependent upon primary antibody host species) FITC-conjugated secondary antibody was then applied for 1 h at room temperature diluted 1:100 in blocking buffer. Excess antibody was removed and cell nuclei were counter stained using propidium iodide for 1 min. Cells were washed in wash buffer for 10 min then twice for 5 min, air dried briefly then covered with fluorescent mounting medium to preserve fluorescence. Stained cells were viewed using a B2A filter block (Ex 450 – 490 nm, Em 505 – 520 nm) in a Nikon inverted camera. Assessments of cell purity were made by counting the number of red fluorescent stained nuclei in comparison to the number of positively stained cells (green).

2.3 NGF Immunohistochemistry In the Human Endometrium.

In this section, tissues were collected from women at different stages of the menstrual cycle, from post-menopausal women and those receiving tamoxifen for the treatment of breast cancer. Uterine tissues were fixed in 10% formalin for 24 h before being paraffin embedded. Tissues were dehydrated and paraffin embedded at the University Hospitals Leicester, Royal

Infirmary Pathology Department. Immunohistochemical staining of NGF in human endometrium was carried out by Dr. M. Guzail of the Menopause Research Group, University of Leicester.

2.3.1 NGF Immunohistochemistry.

Formalin fixed paraffin embedded slides were de-waxed in xylenes and re-hydrated through a series of alcohol concentrations. Slides were heated in a microwave at 700 w for 15 min in 10 mM citrate buffer pH 6.0 for antigen retrieval. After cooling and washing in tap water, slides were blocked against endogenous peroxidase activity with 6% hydrogen peroxidase for 10 to 20 min and then washed first in distilled water, then 1 x PBS for 5 min and finally twice in PBS 0.1% TWEEN-20 (T-20) for 5 min each. Unspecific antigen binding slides were blocked with 10 % normal goat sera and 3% bovine serum albumin in PBS for 1 h. Primary rabbit anti-NGF polyclonal antibody (H-20) [Santa Cruz Biotechnology, Santa Cruz, CA, USA] was applied diluted 1:200 in 3% BSA / PBS overnight at 4 °C. A no primary antibody negative control was also included. To remove unbound antibody, slides were washed for 30 min in PBS / T-20. Secondary goat anti-rabbit biotin conjugated antibody [DakoCytomation, Cambridgeshire, UK] was applied diluted 1:400 in PBS for 30 min, and then slides washed for 3 x 10 min in PBS. Staining sensitivity was enhanced by applying Avidin Biotin Complex (ABC) [Vector Laboratories, USA] for 30 min and excess washed off with PBS / T-20 for 3 x 10 min. Staining was developed with diaminobenzidine (DAB) for 5 min, and excess washed off in PBS T-20 for 5 min. DAB staining was enhanced by immersing slides in $CuSO_4$ (25 mM $CuSO_4$, 123 mM NaCl) for 5 min then washing in running tap water for 5 min. Slides were counterstained with haematoxylin for 30 s and rinsed in tap water for 5 min. Finally slides were dehydrated through graded alcohol concentrations and then cleared in xylene and then mounted in XAM.

2.3.2 NGF Staining Quantification and Statistical Analysis.

NGF stained slides were viewed at either x 100 (stroma) or x 200 (glands) under a Zeiss microscope using Zeiss Macros software. Per slide, 10 to 15 frames were taken for counting. NGF stained stromal cells were counted to give a total number of stained cells per frame. NGF staining in glands was visually assessed to give a percentage staining per frame. NGF counts and NGF percentages were averaged over the frames to obtain a single value for either glands or stroma for each slide. The average values were compared across phases of the menstrual cycle using one way analysis of variance with Bonferroni adjustment for multiple comparisons. Data were analysed by Professor J. Thompson of the Department of Health Sciences, University of Leicester, using Stata [StataCorp 2003 Release 8].

2.4 Animal Studies.

All rodent studies were conducted with Home Office approval in accordance to the Use of Animals in Scientific Procedures Act (1986).

2.4.1 General Materials.

Peanut oil, L-α-phosphatidylcholine (lecithin), 17β-oestradiol benzoate and 4-hydroxyoestradiol [Sigma, Poole, Dorset, UK]. Tamoxifen (base) was a gift from Dr. Terry Orton [AstraZeneca, Macclesfield, Cheshire, UK].

2.4.2 Animal Source and Housing.

For the work described in this thesis, the day of birth will be referred to as day 1. Pregnant CD-1 dams were obtained from Charles River [Kent, UK]. All male offspring were culled. Animals were kept on a 12 h light / dark cycle and had free access to food (RM pellets) and water. Mice were sacrificed by cervical dislocation. Mice did not receive anaesthesia prior to

dosing because this was thought to be more stressful than receiving the dose alone. Day-to-day animal welfare was maintained by staff of the University of Leicester Biomedical Services. For uterotrophic assays, litters of immature mice (4 to 6 pups) were housed with dams. For neonatal dosing studies, mice were housed with dams until weaning (21 days), at which point dams were sacrificed and pups assigned to cages dependent upon dose in groups of 4.

2.4.3 Preparation of Oral Dosing Solutions.

In a clean glass universal, stock solutions were made up in 4 ml peanut oil containing either: oestradiol 0.25 mg/ml, tamoxifen base 1 mg/ml or 4-hydroxyoestradiol 1 mg/ml. In a separate universal, 400 mg lecithin was dissolved in 2 ml dichloromethane and this was added to the 4 ml stock dose solution. The solvents and peanut oil / dose were mixed and solvents evaporated under nitrogen at room temperature (~4 h). Evaporated unsweetened milk (6 ml, Carnation, Nestlé) was added to bring the total volume up to 10 ml, vortex mixed and the preparation placed in ultrasonic bath for 30 s at room temperature. A similar control mixture was prepared using only peanut oil. The stock dose solution was diluted with the control mixture to give a series of concentrations (Table 2.1). Dosing solutions were kept at 4 °C away from direct light until dosing. Before dosing, solutions were allowed to warm to room temperature and vortex mixed before each use.

Table 2.1: Concentrations of Dosing Compounds Used.

		Dose Group μg/kg/day				
	FW	**1**	**2**	**3**	**4**	**5**	**6**
Oestradiol Benzoate	376.5	Vehicle	1	5	10	50	100
			(0.0026)	(0.013)	(0.026)	(0.13)	(0.3)
4-Hydroxyoestradiol	288.4	Vehicle	76.3	193	386	772	1920
			(0.3)	(0.7)	(1.4)	(2.7)	(6.6)
Tamoxifen	371.5	Vehicle	250	500	1000	2500	5000
			(0.7)	(1.4)	(2.7)	(6.7)	(13.4)

FW: formula weight. Dose in μmoles/kg/day are given in parenthesis.

2.4.4 Uterotrophic Effects of SERMs in Immature CD-1 Mice.

Classical uterotrophic assays in 14 d old immature mice were carried out using subcutaneous or oral dosing (Markey *et al.*, 2001; Papaconstantinou *et al.*, 2002). Solutions were either administered by gastric tube or injected subcutaneously into mice by Leicester University Biomedical Services animal house staff using a volume of 5 μl/g body weight. Controls received vehicle only. A minimum of 4 mice per group were dosed once every 24 h for 3 days on days 14 to 16 after birth. 24 h after the final dose, the mice were weighed and then killed by cervical dislocation. The uterus was removed and weighed, and either fixed in 3.7% neutral buffered formalin for histology and PCNA staining or snap frozen in liquid nitrogen.

2.4.5 Time Course of *Ltf* Expression in Immature CD-1 Mice.

To establish if changes in gene expression following oral dosing were similar to those reported in the literature after subcutaneous dosing (Moggs *et al.*, 2004b), CD-1 mice were orally dosed with oestradiol (100 μg/kg), the maximal uterotrophic dose of this compound

established in this Thesis, on days 14, 15 and 16 after birth, as described above. In this study, 36 mice were dosed with oestradiol and 36 dosed with vehicle only. Twelve mice per group were weighed and culled at 24 h after 1, 2 or 3 doses (on days 15, 16 and 17). Uteri were removed, weighed and snap frozen in liquid nitrogen. From each time point, uteri from each group were pooled to create 4 samples (each containing 3 uteri). This was necessary to obtain quantities of RNA sufficient for reverse transcription. Uterine RNA was extracted (Section 2.7) and *Ltf* expression quantified using real time PCR (Section 2.9).

2.4.6 Effects of Short Term SERM Treatment on Gene Expression in Immature Mice.

CD-1 mice were dosed as outlined above daily on days 14, 15 and 16 after birth, with the maximal uterotrophic dose of oestradiol (100 μg/kg), tamoxifen (250 μg/kg) or 4-hydroxyoestradiol (386 μg/kg). There were 8 mice per dosed group and 24 vehicle dosed control mice. Mice were weighed and culled 24 h after the last dose (on day 17 after birth). Uteri were removed, weighed and snap frozen in liquid nitrogen for RNA extraction (Section 2.7). From each dosed group, two uteri were pooled to make a total of 4 samples. RNA was pooled from all 24 vehicle dosed controls. For all groups, gene expression of *C3*, *Ltf*, *Akp2*, *Tgfbi* and *Ngfb* was quantified using real time PCR (Section 2.9). For this, gene expression of the pooled control and for each of the four samples per dosed groups was normalised to expression of the standard.

2.4.7 The Effects of SERMs on Long Term Pathological and Gene Expression Changes in the CD-1 Mouse Uterus.

Newborn CD-1 mice were dosed orally on days 2 to 5 after birth with a maximal uterotrophic dose of SERM and a dose 4 to 8 fold higher than the maximal uterotrophic dose (for tamoxifen and 4-hydroxyoestradiol, Table 2.2). Each dose group contained 8 to 12 mice and

the control group contained > 40 mice. After dosing, mice were housed as previously described and killed by cervical dislocation at 3 months of age. Body weights were recorded before sacrifice and uterine weights recorded before processing.

From each dose group, a minimum of 4 uteri from dosed groups and 8 uteri from controls were fixed in 3.7 % formalin for histology and the remaining snap frozen in liquid nitrogen for gene and protein isolation. For histology, formalin fixed uteri were processed and H&E stained sections prepared at the MRC Toxicology Unit, Leicester, and pathology examined by Dr. Peter Greaves (Cancer Studies and Molecular Medicine, Leicester University). Unstained sections were also prepared for immunohistochemistry. For cDNA microarrays, uteri from SERM dosed mice were processed individually while uteri from vehicle dosed controls were pooled to make one sample. The same pooled control was used for all relevant microarrays. RNA was extracted (Section 2.7) and cDNA microarrays carried out using the MRC Toxicology Unit labelling protocol (Section 2.8) with HGMP oligo arrays.

Table 2.2: SERM Doses Used in Long Term Studies.

	Dose μg/kg	
	Max. Uterotrophic	**High Dose**
Oestradiol	100	-
4-Hydroxyoestradiol	386	1920
Tamoxifen	250	1000

A vehicle only dose group was also included in all studies.

2.4.8 Effects of Time of Dosing on the Development of Adenomyosis.

It has been suggested that oestrogen agonists may alter long term expression of ERα only if exposure occurs before day 4 after birth in rats (Csaba *et al.,* 1998). A study was set up to establish if the ability of tamoxifen to induce adenomyosis in mice was similarly affected by the time frame in which exposure occurred. Groups of 5 female mice were treated orally with 1000 mg/kg tamoxifen or vehicle only on either postnatal days: a. 2 to 5, b. 6 to 9, c. 10 to 13 or, d. 14 to 17. Mice were housed as previously described and culled six weeks after dosing. H&E sections of both uteri and ovaries were examined for histological changes.

2.4.9 Effects of Adenomyosis on Fertility.

During the course of this study it was confirmed that neonatal treatment with tamoxifen led to the onset of adenomyosis by three months of age (Chapter 5). This was most marked in animals receiving tamoxifen. To establish the implications that adenomyosis has on fertility, female CD-1 mice born on day 1, were dosed orally on days 2 to 5 (as described above) with 1000 μg/kg tamoxifen. This dose was established as leading to slightly more marked adenomyosis than any other dose (Chapter 5). Control animals received vehicle only. In house historical data on CD-1 mice show the litter size to be 12.5 ± 2.5 (mean ± SD, n = 33). Power calculations indicate that with this variability and for a treatment related anticipated difference in means of 60%, the sample grouped size should be 6 (Power = 95%). At 3 months mice were mated with male CD-1 mice at a ratio of 1 male to 2 females. The presence of vaginal plugs confirmed the date of mating. After mating male mice were culled. Pregnant females were kept until they gave birth. Gestation time and the litter size and sex of newborn mice were determined.

2.4.10 Effects of Neonatal Dosing with Tamoxifen in the ERKO Mouse Uterus.

A colony of heterozygous ERKO B6.129-Esr1tm1Ksk N10 mice, a model developed by Korach *et al.*, (1993), were imported from Taconic [Germantown, NY, USA] and maintained by the Laboratory Animals Centre at Leicester under quarantine. The colony was maintained by mating heterozygous ERKO mice. Offspring were genotyped by PCR for *ERα* from total DNA extracted from an ear punch. DNA was isolated using a Wizard SV Genomic DNA Purification Kit [Promega, Southampton, UK] following the manufacturer's protocol (Cat. No. A2360: Isolation of Genomic DNA from Mouse Tail Clippings or Animal Tissues). Briefly, total genomic DNA was extracted by dissociating ear punches using a solution containing proteinase K (20 mg/ml) [Sigma, Poole, Dorset, UK] overnight at 52 °C. DNA was then isolated by passing the solution through a Wizard SC Genomic DNA Purification column.

ERα was amplified by PCR using FastStart High Fidelity PCR system following the manufacturers protocol [Roche, Lewes, East Sussex, UK. Cat. No. 2032929]. A typical 20 µl reaction contained: DNA (1 µl), 2 µl 10 x FastStart PCR buffer (containing 15 mM $MgCl_2$), 0.2 mM dNTP mix (0.4 µl) [Invitrogen, Paisley. UK. 10 mM stock], forward and reverse primer (0.125 µM each), Neo F primer (0.25 µM) and FastStart Taq polymerase (0.16 units) made up to 20 µl with H_2O (14.49 µl). Primer sequences were: ERα forward; 5' - CGG TCT ACG GCC AGT CGG GCA TC - 3', ERα reverse; 5' - CAG GCC TTA CAC AGC GGC CAC CC - 3' and Neomycin resistance cassette primer; 5' - GCT GAC CGC TTC CTC GTG CTT TAC - 3'. PCR conditions were: 95 °C for 4 min: followed by 35 cycles of 94 °C for 45 s, 65 °C for 1 min and 72 °C for 1 min. A final extension at 72 °C for 5 min was included.

PCR products were electrophoresed by loading 9 µl of PCR product and running on a 1 to 2% agarose gel as outlined in Section 2.9.3 (Figure 2.1). *ERα* is disrupted in ERKO mice leading to a truncated mRNA transcript (Couse *et al.,* 1995). In homozygous wild type mice only a section of the undisrupted *ERα* is amplified leading to one amplicon of 239 bp. In homozygous ERKO mice, a combined section of both the undisrupted gene and part of the neomycin resistance cassette are amplified giving an amplicon of 790 bp. Both bands are present in heterozygotes.

Homozygous knock out mice, ERKO, were dosed orally on days 2 to 5 after birth with 1000 µg/kg tamoxifen and housed for three months as described above. Gene expression was assessed using MRC Toxicology Unit labelling protocol (Section 2.8) with MRC Toxicology Unit cDNA microarray slides.

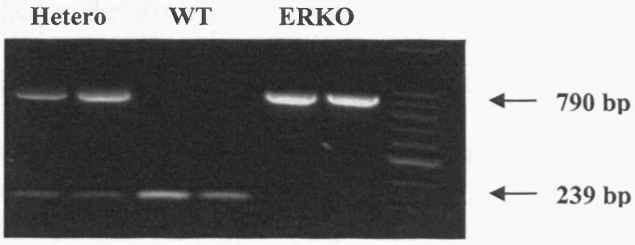


Figure 2.1: Representative ERKO Genotyping PCR Gel.

Targeted disruption of the ERα gene with a neomycin cassette leads to an amplification product of an extended product of 790 bp in homozygous ERKO mice. A 239 bp section of

the undisrupted ERα gene is amplified in WT mice. Both products are amplified in heterozygous animals.

2.5 CD-1 Mouse Uterine Immunohistochemistry.

Uteri for histology were fixed in 3.7% neutral buffered formalin (37 ml formaldehyde in 1 L PBS) at 4 °C for 1 week before being paraffin embedded. 5 μm sections were cut from paraffin blocks of formalin fixed tissues, processed and stained with haematoxylin and eosin by the staff of the MRC Toxicology Unit. Uterine pathology was examined by Dr. Peter Greaves (Department of Cancer Studies and Molecular Medicine, University of Leicester).

2.5.1 Immunohistochemical Staining for PCNA.

Immunohistochemical staining for PCNA was carried out by the staff of the MRC Toxicology Unit. From 4 animals per dose, 1 or 2 slides per animal were stained with each slide containing 2 to 4 sections of uteri. Formalin fixed 5 μm paraffin sections were de-waxed in 3 xylene changes for a total of 60 min, and through 3 changes of industrial methylated spirit (IMS) for 3 min each and finally in 70 % IMS. Sections were washed in running tap water for 5 min and then in distilled water for 3 min. For antigen retrieval, sections were transferred to citric acid (0.01 M) / NaOH pH 6.0 and heated in a microwave at 700w for 20 min. Sections were washed briefly in deionised water. Endogenous peroxidase activity was blocked by immersing the sections in 10% v/v hydrogen peroxide in deionised water for 20 min at room temperature, and then washed in 3 changes of PBS. Primary antibody was a mouse monoclonal NCL-PCNA IgG2a [NovoCastra, Newcastle upon Tyne, UK]. Antibody was applied to slides diluted 1:50 and incubated for 3 h at room temperature. For negative controls mouse IgG2a [DakoCytomation, Cambridgeshire, UK] was applied diluted 1:50 in PBS. Secondary antibody was a goat anti-mouse IgG2a HRP conjugate [Serotec, Kidlington, Oxford, UK] diluted 1:50 in PBS and incubated on slides for 1 h at room temperature. Slides

were washed in 3 changes of PBS and 180 µl per slide of DAB chromagenic substrate [also DakoCytomation] was added for 5 min. Sections were washed once in PBS, then in running tap water for 3 min. Sections were briefly counter stained with haematoxylin and then dehydrated through three changes of 70% IMS and finally cleaned in xylene before mounting.

2.5.2 Assessment of Proliferation in the Uterine Epithelium.

PCNA stained sections were viewed using a Nikon inverted microscope and digital camera at x 20 magnification. From each slide, 10 random images were captured generating 40 images per dose group. Colour levels were edited using the auto-level correction tool in Adobe Photoshop Elements 2.0. From each image, the total number of nuclei and the number of nuclei stained positive for PCNA within the glandular and luminal epithelium were counted by hand. The means from each dose group were analysed using a one-way ANOVA ($p = 0.05$) and a post-hoc Tukeys test.

2.5.3 Smooth Muscle α-Actin Immunohistochemistry in Three Month Old CD-1 Mice.

5 µm Sections were cut from paraffin blocks of formalin fixed tissues by the staff of the MRC Toxicology Unit, Leicester. Smooth muscle α-actin staining was assisted by R.E. Edwards of the MRC Toxicology Unit. Sections were prepared as described above. Primary antibody was a mouse IgG2a anti-smooth muscle α-actin [DakoCytomation, Cambridgeshire, UK. Cat. No. Mo851] a negative control mouse IgG2a was also used from the same supplier. Antibody was applied to slides diluted 1:100 in PBS and incubated for 3 h at room temperature. Secondary antibody was anti-mouse biotinylated secondary diluted 1:100 and developed using a StreptABComplex Duet HRP mouse / rabbit according to the manufacturers instructions [DakoCytomation, Cambridgeshire, UK].

2.6 Immunoblotting.

2.6.1 Immunoblotting Materials.

Bicinchinonic acid (BCA) / copper sulphate reduction protein assay kit (and 1 mg/kg BSA standard), 0.1% w/v Ponceau S in 5% acetic acid, Tween-20 (polyoxethylenesorbitan monolaurate) [Sigma, Poole, Dorset, UK]. 7.5% Polyacrylamide ready gels (50 µl well) [BioRad, Hercules, CA, USA]. Human recombinant ERα and ERβ proteins (molecular weight 66 kDa and 53 kDa, respectively) [Calbiochem, Lutterworth, Leicestershire, UK]. Nitrocellulose membrane [Amersham, Chalfont, St Giles, Buckinghamshire, UK]. SuperSignal West Pico Chemiluminescent Substrate [Pierce, Tattenhall, Cheshire, UK]. Complete mini protease inhibitor cocktail tablets [Roche, Lewes, East Sussex, UK]. Primary antibodies and dilutions used were: Anti-ERα rabbit polyclonal (SC 7207, 200 µg/ml) 1:1000, Anti-ERβ goat polyclonal (SC-6822, 200 µg/ml) 1:1000, SC-protein molecular weight marker (132 – 23 kDa), goat anti-rabbit IgG-HRP secondary (SC-2030) diluted 1:2500, goat anti-mouse IgG-HRP secondary (SC-2031) diluted 1:2500 [Santa Cruz, Santa Cruz Biotechnology. Santa Cruz, CA, USA]; Anti-GAPDH, mouse polyclonal (Cat. No. 4699-9555, 3.1 mg/ml) 1:2500 [Biogenesis, Poole, Dorset, UK]. All antibodies were diluted in 3% fat-free dried milk [Marvel] in wash buffer.

2.6.2 Immunoblotting Solutions.

H8 Protein Lysate Buffer: Tris-HCl (0.02 M), EDTA (2 mM), EGTA (2 mM), β-mercaptoethanol (0.19 M), 1 protease inhibitor tablet per 10 ml volume. Loading buffer: Tris-HCL, pH 6.8 (0.5 M), sodium dodecylsulphate (SDS) (0.14 M), glycerol (2.17 M), bromophenol blue (0.29 mM) and DTT (5 M). Running Buffer contained: Tris (0.025 M), glycine (0.192 M), SDS (0.03 M). Towbin Transfer Buffer: Tris (25 mM), glycine (192 mM) and methanol (6.24 M). Wash Buffer: PBS containing Tween-20 (0.1%).

2.6.3 Protein Lysate Preparation.

For preparation of protein from cell cultures, the medium was aspirated from the adherent cell cultures and cells were washed twice at room temperature with sterile PBS. Flasks were placed on ice and the cells solubilised with H8 protein binding buffer (1 ml) added directly on the culture vessel for 30 min. The lysed cells were transferred to Eppendorf tubes and insoluble material removed by centrifugation (14,000 x g, for 10 min, at 4 °C). The supernatant was stored at -80°C for protein determination. For the isolation of protein from tissue samples the tissue frozen in liquid N_2 was ground in a mortar and pestle whilst being maintained frozen with liquid N_2. H8 buffer (1 ml) was added and the lysate thawed on ice for 30 min. Insoluble material was collected by centrifugation (14,000 x g, for 10 min, at 4 °C). Aliquots of the supernatant were stored at -80 °C.

2.6.4 Protein Concentration Determination.

The concentration of protein lysate was determined using a bicinchinonic acid / copper sulphate reduction protein assay kit following the manufacturer's protocol (Sigma Technical Bulletin BCA-1 and B 9643). Briefly, samples were diluted between 1:5 to 1:50 in sterile water and 10 µl loaded in triplicate into wells of a 96-well plate. A range of BSA standards; 0.2, 0.4, 0.6, 0.8 and 1 mg/ml were prepared by diluting a 1 mg/ml stock solution in water and loaded in duplicate using 10 µl per well to produce a standard curve. Bicinchinonic acid – copper sulphate solution (1 part $CuSO_4$ to 50 parts bicinchinonic acid) was added (200 µl/well) and plates incubated for 30 min at 37 °C. Light absorbance was determined using a FluostarOptima plate reader [BMG Labtech, Aylesbury, Buckinghamshire, UK] set at 540 nM and protein concentrations of samples calculated using the BSA standard curve Figure 2.2.

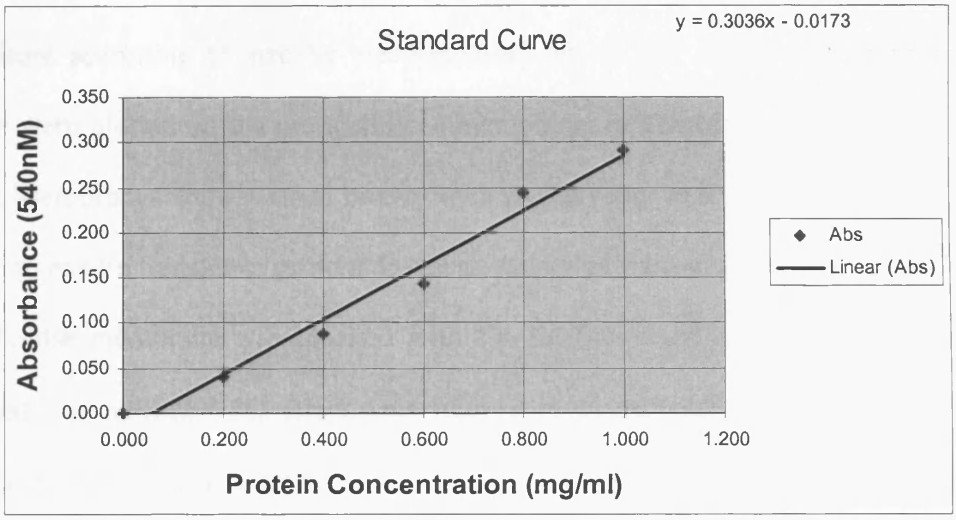


Figure 2.2: Representative Bovine Serum Albumin (BSA) Standard Curve.

Serial dilutions of BSA in water in the range of 0.2 to 1 mg/ml were prepared in duplicate and absorbance determined at 540 nm. Absorbance is corrected for the absorbance of water only. The standard curve was used to calculate the protein concentration of samples.

2.6.5 Western Blotting

Expression levels of ERα and ERß proteins were determined by Western blotting involving sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE). This method separates solubilised proteins according to their size before blotting the proteins onto a nitrocellulose membrane. The membrane is probed with antibodies specific to the ERα or ERβ, which in turn are visualised with a peroxidase linked secondary antibody and chemiluminescence.

A pre-determined concentration of protein lysate (up to 70 µg / 20 µl) was mixed with an equal volume of loading buffer (20 µL) and denatured at 100 °C for 3 min before cooling on ice. Samples were loaded into wells of pre-cast, denaturing 7.5% polyacrylamide gels. Molecular weight markers and recombinant protein standards (10 ng recombinant ERα protein, 50 µg recombinant ERβ protein) were also loaded. Proteins were separated at room temperature according to size by electrophoresis at 40 mA for 65 min in running buffer. Proteins were blotted onto a nitrocellulose membrane, in Towbin transfer buffer at 100V for 90 min. Membranes were washed briefly with sterile water and stained with 5 ml Ponceau S solution to ensure transfer of protein. Ponceau stain was washed out with wash buffer, and the nitrocellulose membrane was blocked with 5% fat free dried milk / wash buffer and were incubated overnight at 4 °C. Blots were washed in 20 ml wash buffer once for 10 min and twice for 5 min. ERα or ERβ were specifically detected by incubating with the appropriate primary antibody diluted in 3% milk / wash buffer for 1 h at room temperature with rocking. A no primary antibody control was run for each antibody used. Blots were washed as before, and incubated with a horseradish peroxidase conjugated secondary antibody diluted 1:2500 in 3% milk / wash buffer for 1 h at room temperature with rocking. Blots were washed in wash buffer as described above and then finally for 5 min in sterile water as Tween-20 can inhibit

detection reagents. Water was removed chemiluminescent substrate (3.5 ml) was added for 1 min and then drained. Blots were wrapped in cling film and exposed for 1, 5 and 20 minutes using a GeneGenome [Syngene Bio Imaging, Cambridge, UK]. Densitometry of images were captured using GeneSnap imaging software and analysed using GeneTools.

2.6.6 Expression Normalisation.

Expression of protein of interest was normalised to expression of GAPDH to correct for inconsistencies in loading. For this, blots were washed in 20 ml wash buffer as described above. GAPDH was then detected by incubating with mouse polyclonal anti-GAPDH antibody diluted 1:2500 in 3% milk / wash buffer for 30 min at room temperature with rocking and processed as described previously. Where shown, expression levels of target protein are expressed as: target / GAPDH.

2.7 Preparation of Nucleic Acids.

2.7.1 RNA Isolation from Endometrial Primary Cell Cultures.

RNA was isolated from primary cell cultures using RNeasy Mini Kit columns [Qiagen, Crawley, West Sussex, UK] following the manufacturers protocol (Qiagen RNeasy Mini Handbook, Third Edition, June 2001). To harvest RNA, medium was removed from cell cultures and cells washed twice with 1 x PBS. Cells were lysed directly while attached to the cell culture vessel using 350 to 700 µl of Qiagen buffer RLT + β-mercaptoethanol (10 µl/ml). Cells were homogenised by passing through a 20 G syringe needle 5 times to shear genomic DNA and reduce the viscosity of the lysate. RNA was then passed through a Qiagen silica-gel membrane spin column to which RNA longer than 200 bp selectively binds whilst all other cellular material is washed through. RNA bound membranes were washed using the wash buffers included in the kit and then RNA eluted in 20 to 30 µl RNase free water.

2.7.2 RNA Isolation from Uterine Tissues.

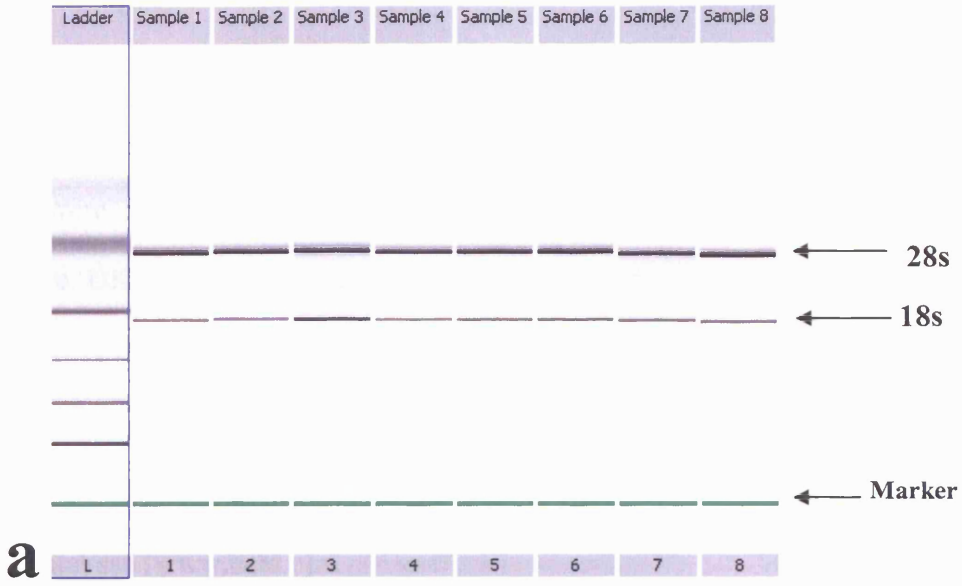
RNA was extracted from either whole mouse uteri or human endometrial tissue using TriReagent [Sigma, Poole, Dorset, UK] following the manufacturer's instructions (Sigma Technical Bulletin, MB-205, August 1999). This reagent is a mixture of guanidine thiocyanate and phenol and the method is a modification of a previously published protocol (Chomczynski, 1993). Briefly, 50 to 200 mg of tissue was homogenized in a mortar and pestle whilst maintained frozen using liquid nitrogen. TriReagent (1ml) was added per 50 to 100 mg tissue and the frozen suspension allowed to thaw to room temperature. Samples were transferred to sterile Eppendorfs and insoluble material separated by centrifugation (12,000 x g, for 10 min, at 4 °C). Supernatant was transferred to a new Eppendorf and a volume of isoamyl alcohol free chloroform equal to the initial volume of TriReagent was added. After centrifugation (12,000 x g, for 15 min, at 4 °C) the solution separated into three distinct phases: an organic red phase containing protein, a white interphase containing DNA and a clear aqueous phase containing RNA. RNA was precipitated from the aqueous phase using isopropanol (0.5 ml per 1 ml of TriReagent used), pelleted by centrifugation (12,000 x g, for 5 min, at 4°C) and the pellet washed in 75% ethanol and re-centrifuged (12,000 x g, for 5 min, at 4 °C). The pellet was allowed to air dry briefly and then re-suspended in 20 to 40 µl RNase free water and stored at -80 °C. For cDNA microarrays, 25 µg in 10 µl aliquots were made to avoid degradation due to freezing and thawing.

2.7.3 RNA Quantification.

RNA was quantified by measuring absorbance at 260 nm and 281 nm using an Amersham Biosciences GeneQuant spectrophotometer and quality assessed using the A_{260} / A_{280} absorbance ratio. Samples were diluted 1:50 – 1:100 in either water or Tris-HCl (10 mM). Diluting in water gives a more accurate reading of concentration whereas Tris-HCl allows a more accurate reading of quality (Qiagen RNeasy Mini Handbook June 2001).

2.7.4 Agilent Bioanalysis.

To further assess integrity and quality, RNA was analysed using Agilent Bioanalyser and the Agilent Technologies RNA 6000 Nano Chip system [Agilent Technologies. West Lothian. UK] following the manufacturer's protocol. Total RNA (200 – 500 ng) was electrophoresed and the ribosomal RNA units 18s and 28s component normalised against the internal RNA ladder [Ambion, Huntingdon, Cambridgeshire, UK] (Figure 2.3a). The ratio of 18s and 28s peaks is used to asses RNA quality, where the 28s peak should have an area around twice the value of the 18s peak (Figure 2.3b). Only RNA of A_{260} / A_{280} > 1.9 was used giving an Agilent trace with no evidence of RNA degradation.



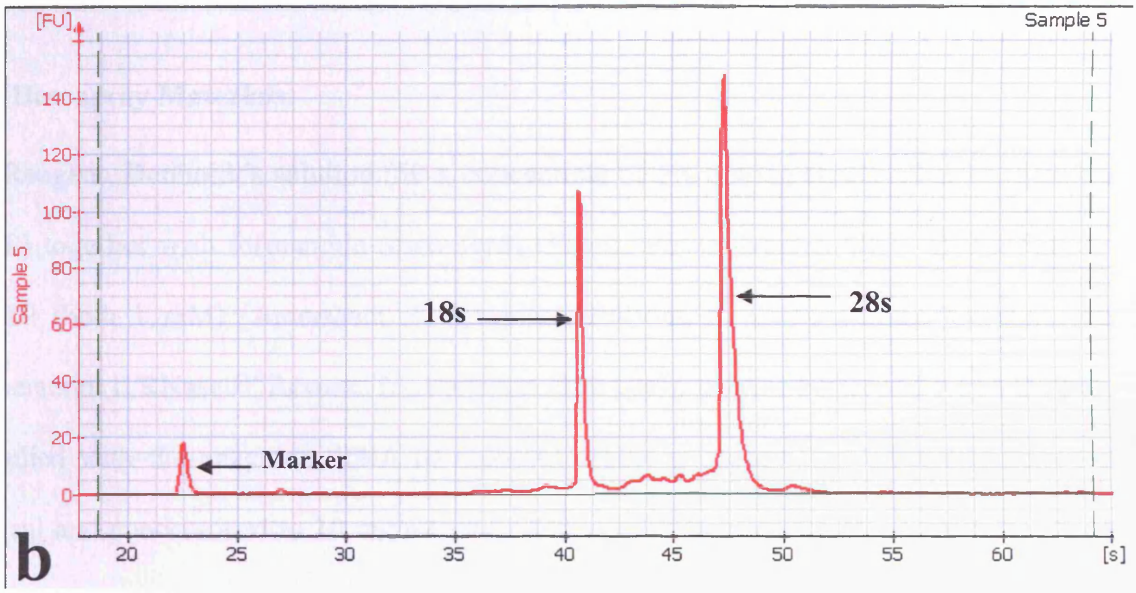


Figure 2.3: Agilent Bioanalysis of Human primary cell culture RNA.

a. Gel like summary of RNA electrophoresis showing the RNA ladder and 18s and 28s ribosomal units of sample RNA. **b.** Electrophoregram of sample no.5 RNA.

2.8 cDNA and Oligo Microarray Analysis.

Two sets of microarray platforms were used. i. 10080 mouse ESTs or 6528 human ESTs spotted on glass (a gift from T. Gant, MRC Toxicology Unit, Leicester). ii. 'Known' oligo gene arrays were provided by the MRC Human Genome Mapping Project [HGMP, Cambridgeshire, UK] and consisted of 7,455 mouse or 18,664 human oligo sequences (60 to 65mers) printed in duplicate (on two individual slides for human gene arrays). HGMP oligo arrays were supplied ready for use. Details of the genes represented on the different platforms together with raw data of individual changes in gene expression have been deposited on the GEO public database (www.ncbi.nlm.nih.gov/geo) (Appendix 1).

2.8.1 MRC Toxicology Unit Labelling Protocol.

a. Microarray Materials.

TriReagent, Denhardt's solution (50 x concentrate of 1% bovine serum albumin, Ficoll and PVP) together with formamide (deionised) [Sigma, Poole, Dorset, UK]. Cy3-dUTP, Cy5-dUTP (both 1 mM) [Amersham Biosciences, Chalfont, St Giles, Buckinghamshire, UK]. Superscript II RNase H^- Reverse Transcriptase (200 U/µl), enzyme buffer and 0.1 M DTT (both supplied with the enzyme), tRNA (4 mg/ml), human or mouse Cot 1-DNA (supplied at 1 mg/ml and concentrated to 10 mg/ml) and low T dNTP mix (100 mM) [Invitrogen, Paisley, UK]. Oligo $dT_{18\text{-}25}$ (8 µg/µl) and Oligo dA_{80} (1 µg/µl) [PNACL, University of Leicester, UK]. RNasin Ribonuclease Inhibitor (40 U/µl) [Promega, Southampton, UK]. Centri-Sep purification columns [Princeton Separations, Adelphia, N.J, USA. Supplied by Cambio, Dry Drayton, Cambridge, UK. Cat. No. CS-901].

b. Buffer solutions.

Diethylpyrocarbonate (DEPC) water (6.9 mM): was prepared by adding DEPC (1 ml) to ultra-pure water (999 ml), stirred overnight and autoclaved. Sodium Dodecylsulphate: SDS (1g) in DEPC water (10 ml). Hybridisation Buffer: was prepared by adding formamide (1 ml) to a glass vial, together with 50 x Denhardt's solution (100 µl) ultrapure water (200 µl) and SDS (100 µl). This was filter sterilised through a 0.45 µm syringe filter before use. SSPE Buffer (50 x): buffer contained NaCl (3 M), NaH_2PO_4 (1 mM), EDTA (20 mM) and was adjusted to pH 7.4 with NaOH (4 M). Array Wash Solutions: No 1 contained 20 x SSC buffer (25 ml), SDS (1.5 ml) in 500 ml ultrapure water; No 2, was 0.2 x SSC in ultrapure water; No 3, was 0.05 x SSC in ultra pure water. SSC (20 x): A 20 x stock of SSC contained sodium chloride (3 M) and trisodium citrate (0.3 M).

c. RNA Reverse Transcription and Labelling.

For each control and treated sample, 0.5 µl OligodT primer was added to 25 µg RNA in 10 µl DEPC water, mixed, and incubated at 70 °C for 8 min to denature RNA. The temperature was then reduced to 42 °C over a period of 30 min to allow primers to anneal. Whilst at 42 °C a master mix was added containing: RNasin (0.5 µL) 5 x first strand buffer (4 µL), 0.1 M DTT (2µL), dNTP mix (5µL) and 0.5 µL Superscript II and either Cy5 or Cy3 dye (2µL) added depending upon the labelling direction and mixed by pipetting. Incubation was continued at 42 °C for 1 h. In this study Cy3 when added to control samples and Cy5 to treated samples was referred to as forward labelling. During this time RNA was reverse transcribed into a complementary DNA (cDNA) probe that was labelled with the appropriate fluorescent dye. After 1 h, a further 0.5 µl Superscript was added and incubated again for 1 h. After incubation, 20.5 µL DEPC-water was added. To stop reverse

transcription, 0.5 M EDTA (1 μL) was added, followed by 10% SDS (1 μl) NaOH (3 M, 3μL). Samples were mixed, and incubated for 10 min at 70 °C to allow hydrolysis of any remaining RNA. After incubation, 3 μL 2M HCl and 10 μL 1M Tris / HCl, pH 7.5 were added to neutralise the reaction mixture. tRNA (1 μL of 4 μg/ml solution) was added to act as carrier of the labelled cDNA probe. At this stage, the total reaction volume was 60μL.

d. cDNA Probe Purification.

cDNA probes were passed through Centri Sep columns to remove any unincorporated Cy dye or dNTPs. Centri Sep columns were re-hydrated with 800 μl water for 30 min and excess liquid removed by centrifugation (750 x g, for 2 min, at 4 °C) prior to use. To the surface of individual columns, the complete reaction mixture (60 μL) was added and the columns centrifuged (750 x g, for 6 min, at 4 °C) to elute the purified probes. Cot DNA (1 μL from mouse or human) was added to treated samples and mixed well. Labelled cDNA probes were dried using a Speedvac to complete dryness (for 35 – 45 min, at 43 °C).

e. Probe Hybridisation.

To one of the control-treated probe pairs in an Eppendorf tube was added 21 μL hybridisation buffer and 9 μL 20 x SSPE. The contents were vortex mixed and the whole solution added to the partner treated pair and mixed. The mixed control-treated cDNA probe pair was denatured at 100 °C for 2 min and cooled to 42 °C.

f. Microarray Slide and Coverslip Preparation.

For MRC Toxicology cDNA Arrays, DNA was cross-linked on array slides under UV light at 650 μJ. Non-specific binding was then blocked by heating slides to 100 °C for 2 min,

washed in ultra pure water and centrifuged to dryness (350 x g, for 5 min, at room temperature). Glass cover slips were washed in 1% SDS for 30 min then in ultra pure water for 5 min, 5 times and centrifuged to dryness (350 x g, 5 min, room temperature). HGMP oligo arrays were provided ready to use.

g. Hybridisation, Post-Hybridisation Washes and Microarray Scanning.

A cover slip was placed over the printed area of the microarray slide. 15 µl of the mixed cDNA probe was pipetted onto the array slide so that it was drawn in between the slide and cover slip. Array slides were placed into a sealed humid hybridisation chamber and incubated in a water bath at 42 °C overnight. After incubation, cover slips were removed by soaking slides in No 1 wash buffer for 10 min at 50 °C. Slides were then further washed in No 2 wash buffer and No 3 wash buffer each for 10 min at room temperature. Slides were centrifuged to dryness (46 x g, for 5 min, at room temperature). Arrays were repeated using reverse labelling where control samples were labelled with Cy5 and test samples labelled with Cy3.

h. Microarray Statistical Analysis.

Array slides were scanned using an Axon A300 scanner at the MRC Toxicology Unit, Leicester, at excitation wavelengths of 532 and 635 nm to detect the Cy3 and Cy5 dyes respectively. Pixel intensity for hybridisation was determined using GenePix Pro 3.0.6 software [Axon Instruments, Union City, CA, USA]. For dual label hybridizations there is imbalance in the measured intensity between the fluorescent channels, due to different intensities of fluorescence between equal quantities of bound dyes and also non-specific binding of the probes leading to background fluorescence. To correct for imbalance in the

measured fluorescent intensities between the two fluorescent channels the data were normalized in two ways. First, global normalization in which the fluorescence levels of all features are uniformly adjusted (Gant and Zhan, 2005). Second, to correct for imbalances in each feature each array was forward and reverse labelled (Dobbin *et al.*, 2003). The data from forward and reverse labelled arrays were normalized, condensed and *t* tested to determine differential gene expression (Zhang *et al.*, 2004a) using statistical software (NorTT) hosted by the MRC Toxicology Unit, Leicester. The normalization procedure eliminates features that are morphologically abnormal, where there is failure of hybridization or where there is only fluorescence in one channel. The software allows the investigator to choose *t* test options such as the fraction of false positive features (0.05) and the cut off p value (0.05) below which a gene is regarded as having changed expression. Genes having a p value less that 0.05 in a two-tailed t test were regarded as being significantly changed in expression.

2.9 Real-Time PCR.

2.9.1 cDNA Synthesis.

Total RNA was isolated from cells or tissue as described in Section 2.7. cDNA was reverse transcribed using Superscript II Reverse Transcriptase supplied with 5 x first strand buffer (250 mM Tris-HCl, 375 mM KCl and 15 mM $MgCl_2$) and 0.1 M DTT, OligodT primer (500 μg/ml) and 10 mM dNTP mix [Invitrogen, Paisley, UK] following the manufacturer's protocol (Invitrogen Part No. 18064.pps). Briefly, 1 μg total RNA was diluted in 10.5 μl H_2O. Added to this was OligodT primer (1 μg) and dNTP mix (1 μl). RNA was denatured by heating to 65 °C for 5 min and then chilling on ice for 3 min. To each sample a master mix was added containing: 5 x first strand buffer (4 μl), 0.1 M DTT (2 μl), RNasin (20 units) [Promega, Southampton, UK] and Superscript II reverse transcriptase (200 units). To allow reverse transcription of RNA, samples were incubated at 42 °C for 50 min. Enzymes were denatured after 50 min by heating to 70 °C for 15 min.

2.9.2 LightCycler Real-Time PCR.

Gene expression was quantified using LightCycler real-time PCR (RT-PCR) and data analysed using RealQuant Relative Quantification software [Roche, Lewes, East Sussex, UK]. In principle, LightCycler PCR works by amplifying cDNA whilst a fluorescent dye (SYBR Green) is incorporated into double stranded DNA. Binding enhances the fluorescence of SYBR green, and fluorescence is measured after each amplification cycle (Figure 2.5a). The cycle number where the level of fluorescence crosses the background level is referred to as the crossing point (Cp). Therefore, the higher the concentration of gene in the sample to start with, the lower the cycle number will be when fluorescence crosses the background, and thus the lower the Cp. At the end of the amplification section, a melting curve analysis takes place. For this the PCR products are heated from 45-99 °C and fluorescence measured during this

transition (Figure 2.6a). A peak is generated when there is a drop in fluorescence from the denaturing of a PCR product (Figure 2.6b). Essentially, this is used to identify self annealed primers and target product, whilst checking for multiple amplicons.

For LightCycler PCR, initially SYBR Green Taq ReadyMix for Quantitative PCR (capillary formulation) 10x concentrate [Sigma, Poole, Dorset, UK] was used following the manufacturers technical bulletin (product code S 1816). However due to the production of a defective batch, LightCycler FastStart DNA Master SYBR Green I PCR reagents [Roche, Lewes, East Sussex, UK. Cat. No. 3 003 230] were used in latter experiments. Essentially the protocol was the same for both reagents. The reagents and components for a typical FastStart DNA Master reaction are outlined in Table 2.3 and cycle conditions are outlined in Table 2.4. Up to 45 to 50 cycles of the amplification programme were used until the PCR had entered the plateau phase (saturation). The LightCycler 2nd Derivative maximum calculation with arithmetic base line adjustment was used for calculating Cp values. For melting curve analysis, a value above or equal to 4 °C per average was used.

Table 2.3: Fast Start DNA Master RT-PCR Components.

Reagent	Components	Volume
Sybr Green 10 x PCR Mix.	Fast start Taq polymerase, reaction buffer, dNTP mix, SYBR green 1 dye and 10 mM $MgCl_2$	2µl
$MgCl_2$	25 mM $MgCl_2$ to adjust reaction concentration to 3.5 mM	2µl
Forward Primer	20 pmol – 1 nmol per primer, per reaction.	1µl
Reverse Primer		1µl
H_2O		13µl
cDNA		1µl

Table 2.4: LightCycler RT-PCR Cycle Conditions.

Cycle Programme	Section	Temp °C	Time (min / s)	Acquisition	Analysis Mode
Denature		95	10 min	None	None
Amplification	Denature	95	10 s	None	Quantification
<45-50 cycles	Primer Anneal	primer specific*	5 s	None	
	Extension	72	1s per 25 bp*	None	
	Primer Denature	primer specific*	5 s	Single	
Melting Curve		45-99	0.1 °C/s	Continuous	Melting Curve

For the amplification programme the temperature transition rate was 20.0 °C/s, for the melting curve programme the transition rate was 0.1 °C/s. * Primer specific data are included in Table 2.5.

2.9.3 RT-PCR Primer Details.

For each gene of interest a sequence specific set of primers (Table 2.5) was designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or sequences used from relevant published literature and were obtained from either PNACL [University of Leicester, UK] or from Sigma Oligonucleotides services [Sigma, Poole, Dorset, UK] and sequences were checked using BLAST. Primers were reconstituted to 1 nmol/µl stocks and then diluted to the required concentration (20 pmol to 1 nmol/µl). In each reaction 1 µl of each primer was used to give a final concentration of 20 pmol to 1 nmol per primer. For each set of primers, RT-PCR conditions were first optimised to obtain the optimal annealing temperature and $MgCl_2$ concentration if required. Amplicon size was verified using gel electrophoresis, running 5-10 µl RT-PCR product in 6 x loading buffer [Promega, Southampton, UK] on a 1 to 2 % agarose / 1 x TAE gel + 1 µl / 100 ml ethidium bromide in 1 x TAE buffer (2M Tris, 1M glacial acetic acid and 0.05M EDTA, pH 8.0) for around 45 minutes at 100 V. Products were detected (by ethidium bromide staining) under UV light and images analysed by Syngene software (Figure 2.4).

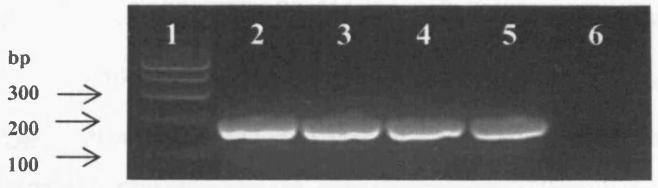


Figure 2.4: Electrophoresis of *Tpm1* to Confirm Amplicon Size.

Promega 100 bp DNA ladder (**1**); Standard curve of *Tpm1* using serial dilutions of mouse cDNA template ranging from neat (**2**), 1 : 10 (**3**), 1 : 100 (**4**) and 1 : 1000 (**5**); No template control (**6**). The *Tpm1* amplicon is 197 bp.

	Primer Name	Forward Sequence 5' - 3'	Reverse Sequence 5' - 3'	Temp (°C)	Amplicon Size (bp)
Mouse	*Ltf*	CGGGGGCCTTCAGACCATC	CTAAAGTGACAGCAGGGAGTG	60	49
	Akp2	CCAGCAGGTTTCTCTCTTGG	CTGGGAGTCTCATCCTGAGC	64	239
	*Ckb**	AAGTTCTCGGAGGTGCTCA	AGTTTCACTCCGTCCACCA	62	158
	C3	AAGCATCAACACACCCAAC	CTTGAGCTCCATTCGTGAC	60	173
	Ngfb	GCAGAACCGTACACAGATAGC	CAGCACTGTCACCTCCTTGC	60	428
	Cyp2e1	AGGCTGTCAAGGAGGTGCTA	GGAAGTGTGCCTCTCTTTGG	63	210
	Tpm1	CACTGAAACATCTGCCGAGA	TGGCACTCTGGCTGTTAATG	54	197
	Gja1	GAACACGGCAAGGTGAAGAT	GAGCGAGAGACACCAAGGAC	60	247
	Kifc2	TTGCTGGCACTAGGAGGAGT	AACTTGAGCGAGCAGATGGT	57	182
	Pdgfra	TGGCATGATGGTCGATTCTA	CGCTGAGGTGGTAGAAGGAG	57	152
	Tsp1	CCAAAGCCTGCAAGAAAGAC	CCTGCTTGTTGCAAACTTGA	60	192
	Col4a2	GATGCATGCAGTGTGCTTCT	ATGCTGGTGAGGGCTAGAGA	57	206
	Tgfbi	TGTCTTGTGTCCACACATGGTT	TCCCTCACACTCAGGGAATC	58	100
	Gapdh	ACCCAGAAGACTGTGGATGG	GGAGACAACCTGGTCCTCAG		300
Human	*NGFb*	CCAAGGGAGCAGCTTTCTATC--CTGG	GGCAGTGTCAAGGGAATGCTG--AAGT	65	189
	*CKB**	TTCTCAGAGGTGGAGCTGGT	TACCAAGGGTGACGGAAGTC	60	250
	PENK	GCTGTCCAAACCAGAGCTTC	CTTCTGGCTCCATGGGATAA	63	161
	IGF1	TGGTGGATGCTCTTCAGTTC	GACAGAGCGAGCTGACTTG	60	191
	ERα	ATGGCCTTGTTGGATGCTGAGC	CAGGATCTCTAGCCAGGCACATTC	57	211
	GAPDH	AGAACATCATCCCTGCCTCC	GCCAAATCCGTTGTCATACC	60	350

Table 2.5: LightCycler Primer Details.

Extension time (s) was: amplicon size / 25.

* Efficiency correction was not used for this primer set.

Temp (°C) was the optimal temperature established for primers to anneal.

2.9.4 RT-PCR Relative Quantification and Co-Efficiency Correction.

Target gene expression was normalised to the expression of a housekeeping gene where expression is assumed to be consistent through a range of samples (*GAPDH).* Our study design employed relative quantification which was calibrator normalised and included efficiency correction. Calibrator cDNA used in this study was isolated from Ishikawa cells for human primer sets, and whole mouse uterine cDNA for mouse primer sets. For each sample and the calibrator, the relative amount of a target gene and reference gene are determined [Roche Molecular Biochemicals LightCycler Relative Quantification Software Version 1.0 March 2001]. Results are expressed as the target / reference ratio (where target refers to the target gene *i.e., Ltf,* and reference refers to the housekeeping gene) of the sample divided by the target/reference ratio of the calibrator. In addition to this results are also corrected for differences in the PCR primer efficiency of the target and reference genes.

To account for differences in efficiency of amplification, a coefficient file was created for each set of target gene primers comparing efficiency to *GAPDH* RT-PCR efficiency. Serial dilutions of standard cDNA ranging from 1:0, 1:10, 1:100 and 1:1000 were created and run in duplicate (Figure 2.5b). This was repeated for each set of primers used. Using RealQuant software the standard curves for both target gene and *GAPDH* were combined and the difference in efficiency expressed as a correction value. For some primers, at low cDNA template concentrations amplification efficiency was low in which case efficiency correction could not be used.

2.9.5 Data Analysis.

When analysing data from an experiment, data were exported from the LightCycler analysis programme as a standard curve. In the RealQuant software, selecting dual colour experiment and efficiency correction, experimental data were imported for the target gene, reference gene and the coefficient file. The position of the calibrator and samples was identified in the relevant sections and then analysis run. Final data were expressed as a ratio of target gene normalised to *GAPDH* and corrected for efficiency to the calibrator. The calibrator ratio value was '1' for each experiment. For example, values less than 1 represent a decrease in expression and values more than 1 show an increase in value. For 24 h dosing of human primary cell cultures expression is expressed relative to dosed controls. Unless otherwise stated, expression for *in vivo* mouse studies is shown relative to calibrator and normalised to pooled control.

2.10 Statistical Analysis of Data.

Microsoft Excel and Minitab v14 were used to analyse data using a one-way students ANOVA (analysis of variance) with post hoc analysis using either Tukeys test or Dunnetts comparison with a control. 'Significant' refers to value $p < 0.05$ unless specified otherwise.

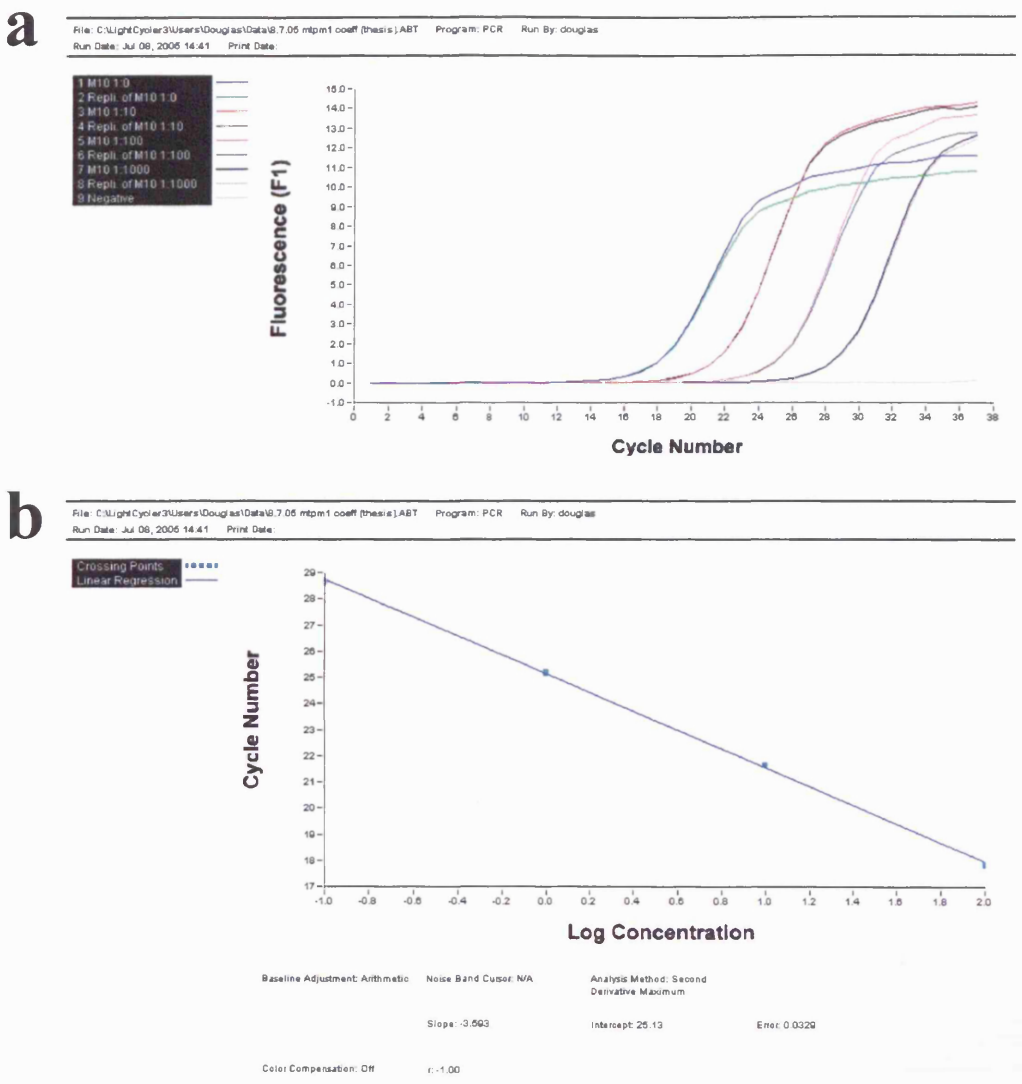


Figure 2.5: Real-Time LightCycler PCR Analysis.

a. Representative analysis of fluorescence plotted against cycle number for *Tpm1* amplified (38 cycles) from serial dilutions of cDNA. The point at which the log-linear phase of the PCR crosses the background fluorescence level (0) is referred to as the crossing point (Cp). Relative expression levels of a gene are calculated from the Cp, with a low Cp correlating to higher expression. **b**. Cp expressed as standard curve of the Log concentration of gene expression.

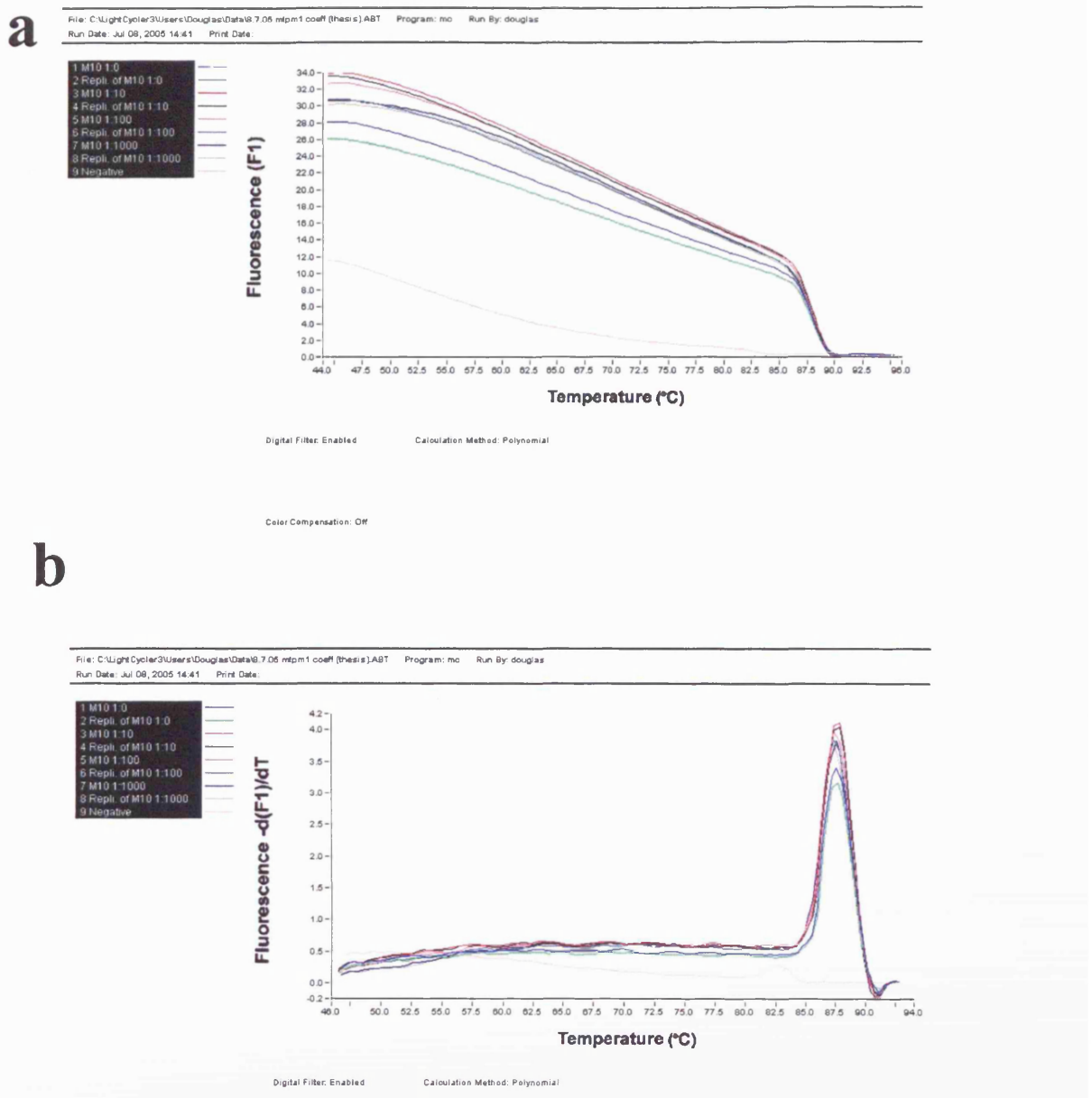


Figure 2.6: Real-Time LightCycler PCR Melting Data.

a. Representative melting curve data for *Tpm1* amplified from a serial dilution of cDNA. Figure shows fluorescence measured at increasing temperature. **b**. Peak analysis of melting curve data. Figure shows the melting peak centre at 87.5 °C for all positive samples and unspecific product in negative sample. Data from the melting peak allows the identification of multiple amplicons as gel electrophoresis does in standard PCR.

Chapter 3.

Effects of Tamoxifen on Gene and Protein Expression in the Endometrium of Women.

3.1 Introduction.

The mechanisms by which tamoxifen causes endometrial abnormalities is unclear. Suggested pathways include oestrogen agonist activity, selective induction of gene expression or genotoxicity. Reports in the literature suggest that neither genotoxicity or an oestrogen agonist action is sufficient to cause endometrial cancers (Carmichael *et al.,* 1996; Carthew *et al.,* 2000; Martin *et al.,* 2003; White, 1999) and it is likely that other mechanisms such as induction of gene expression specific to tamoxifen are also involved.

Tamoxifen has been shown to affect cell signalling via peptide growth factors such as TGF-α, IGF-I/II, EGF and adrenomedullin, and also the inhibitory growth factor TGF-β, which may contribute in the development of uterine abnormalities (Ugwumadu *et al.,* 1998; Zhao *et al.,* 1998; Ugwumadu *et al.,* 1998). Using cDNA microarrays, our laboratory recently identified that *Ngf* expression was increased in the mouse uterus in response to tamoxifen (Green *et al.,* 2003). Interest in the role of NGF outside the nervous system has increased recently (Reinshagen *et al.,* 2002).

In the human uterus, the only report of NGF expression has been in deep adenomyotic nodules, and peritoneal and ovarian endometriosis where it was associated with neurite growth and pain perception (Anaf *et al.,* 2002). In a mouse model, neonatal tamoxifen treatment led to the up-regulation of NFG protein and gene expression in the uterus over many months (Green *et al.,* 2003; Green *et al.,* 2005). Mouse muscle-derived C2C12 cells down-regulate NGF and p75 receptor during myogenic differentiation (Seidl *et al.,* 1998; Erck *et al.,* 1998), and it was proposed that in the mouse uterus, NGF may have a role in inhibiting myocyte differentiation and promoting adenomyosis (Parrott *et al.,* 2001). In other

cell types, recombinant human NGF stimulates the proliferation of both LNCaP prostate and MCF-7 breast cancer cells in culture (Chiarenza *et al.,* 2001; Sortino *et al.,* 2000).

Nerve Growth Factor (NGF) is a soluble peptide that promotes neurite survival and outgrowth of sympathetic ganglia (Birren *et al.,* 1992). More recently, three additional structurally homologous neurotrophic factors have been identified. These include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4/5), all of which promote differentiation, growth and survival of specific neurone populations (Berkemeier *et al.,* 1991; Hohn *et al.,* 1990; Klein, 1994). NGF effects are mediated through high affinity tyrosine kinase TrkA and low affinity p75 cell surface receptors (Sortino *et al.,* 2000). The low affinity p75 receptor is most closely related to the TNF-R/fas family that binds neurotrophins (Chao, 1994).

In women, during the menstrual cycle, changing levels of oestrogens and progesterone are responsible for many of the cyclic cellular changes observed in the endometrium. Oestradiol and progesterone regulate the expression of down-stream proteins acting via genomic and non-genomic signalling (Song *et al.,* 2002). ERα, ERβ and PR are detected in glandular epithelial and stromal cells and expression is highest during the proliferative stage (Lecce *et al.,* 2001; Mylonas *et al.,* 2004). Other uterine proteins that show cyclical changes in expression include uteroglobin (Tanaka *et al.,* 2004), matrix metalloproteinases and tissue inhibitors of metalloproteinases (Goffin *et al.,* 2003) and VEGF (Macpherson *et al.,* 1999).

NGF expression and its distribution patterns in the uterus may be important for understanding normal endometrial function and may have clinical implications. In a mouse model, NGF expression is up-regulated by oestradiol and progesterone in uterine tissues (Bjorling *et al.,*

2002). In this Chapter, the aim was to characterise the expression of NGF in women during the menstrual cycle, and also to compare protein expression in the uteri of women receiving tamoxifen and postmenopausal women using immunohistochemistry. In addition the effects of tamoxifen on *NGF* gene expression were examined using primary endometrial cell cultures isolated from premenopausal women. To identify genes that are potentially involved in mediating the effects of tamoxifen in the uterus, microarrays were used on postmenopausal endometrium of women receiving tamoxifen and compared with that in endometrium of age matched women not receiving tamoxifen. It was not the intention to directly compare data from pre and postmenopausal women, but to use the primary uterine cells as a model to identify the effects of SERMs on *NGF* gene expression.

3.2 Results.

3.2.1 Expression of NGF during the Menstrual Cycle.

Expression of the NGFβ protein was assessed by immunohistochemistry of formalin fixed paraffin embedded sections from the endometrium of women. Figure 3.1 shows that NGF undergoes a cyclic expression pattern in the endometrium. Expression of NGF was higher in glands than in stroma at all stages of the cycle. In both, expression was very low or absent during the proliferative stage (Figure 3.1 and 3.2a), apart from occasional staining in polymorphonuclear lymphocytes (Figure 3.2f) which strongly express NGF at this stage. Expression increased during the early and mid secretory stage, peaking at the late secretory stage, during which polymorphonuclear lymphocyte expression decreased. Stromal and glandular expression declined during the menstrual stage. During the secretory phase, NGF staining was localised primarily to the glandular epithelium with diffuse staining throughout the stroma (Figure 3.2b and c). Glandular staining during the later secretory and menstrual stage appeared to be most prominently localised to the luminal surface of the epithelium (Figure 3.2c and d) as if being secreted into the luminum by an apocrine mechanism. There was an absence of staining in the subnuclear vacuoles or nucleus. Both staining in the stroma and polymorphonuclear cells appears to be localised to either cytoplasmic or surface membrane but not the nucleus.

Figure 3.3 shows expression of NGF in the endometrium of either postmenopausal women receiving tamoxifen or age matched women not receiving this drug. NGF expression was higher in the endometrium of tamoxifen patients than in controls. In both sets of patients NGF expression was higher in glands than in stroma.

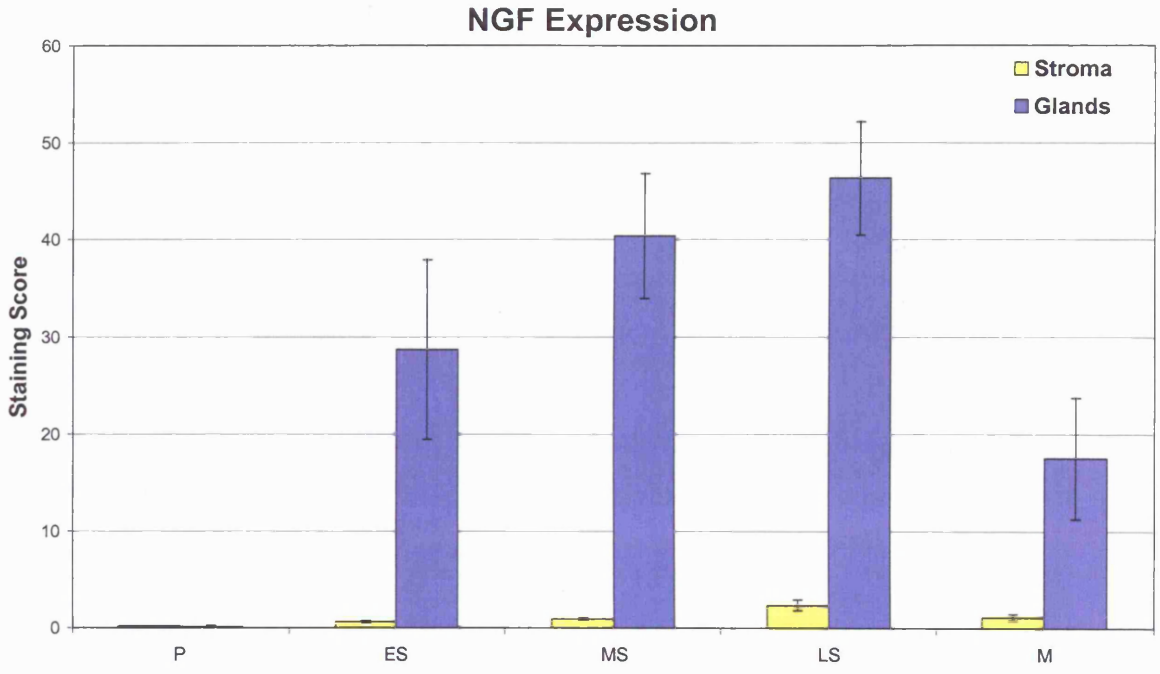


Figure 3.1: Endometrial Expression of NGF during the Menstrual Cycle.

Expression of NGFβ protein assessed in stroma and glands by immunohistochemistry. Staining score represents a count of positive stromal cells and a percentage stain for glands. Data represent the mean staining score (± SE) for a minimum of 7 patients per menstrual stage: P, proliferative (n = 8); ES, early secretory (n = 7); MS, mid secretory (n = 10); LS, late secretory (n = 8); and M, menstrual (n = 8). Data were statistically significant ($F < 0.0009$) using ANOVA with Bonferroni adjustment for multiple comparisons.

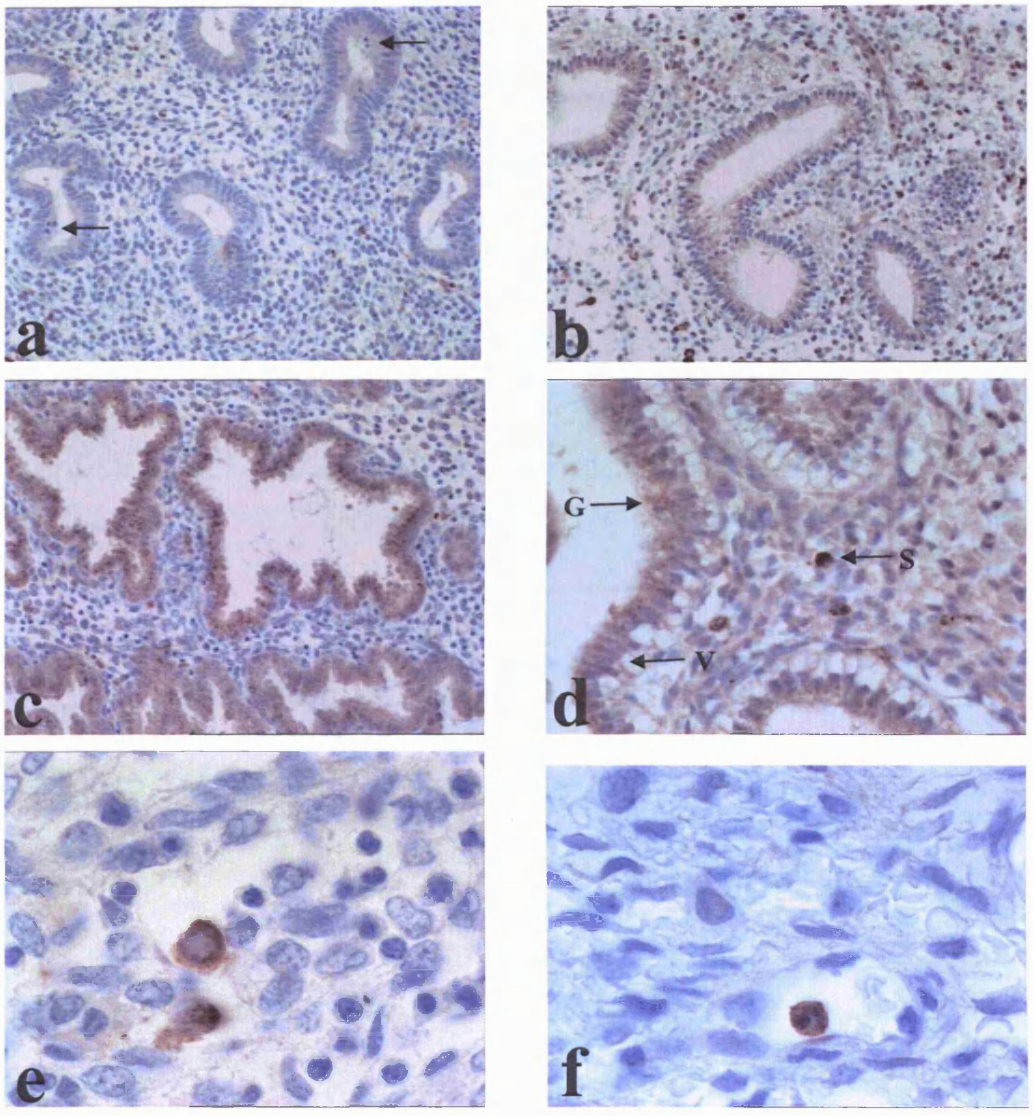


Figure 3.2: Representative Pictures of NGF Immunohistochemistry.

Figure shows representative sections of immunostaining used to assess NGF expression throughout the menstrual cycle. Pictures are: **a**. proliferative stage x 20, showing mitotic epithelial cells (arrowed) **b**. early secretory stage x 20, **c**. late secretory stage x 100, **d.** menstrual stage showing stained stromal cell (**S**), subnuclear vacuole (**V**) and luminal surface staining in glandular cells (**G**), x 20. **e**. Stromal staining during the mid secretory stage x 100. **f**. stained polymorphonuclear cell inside a blood vessel during the proliferative stage x100. This cell is most like a neutrophil in morphology.

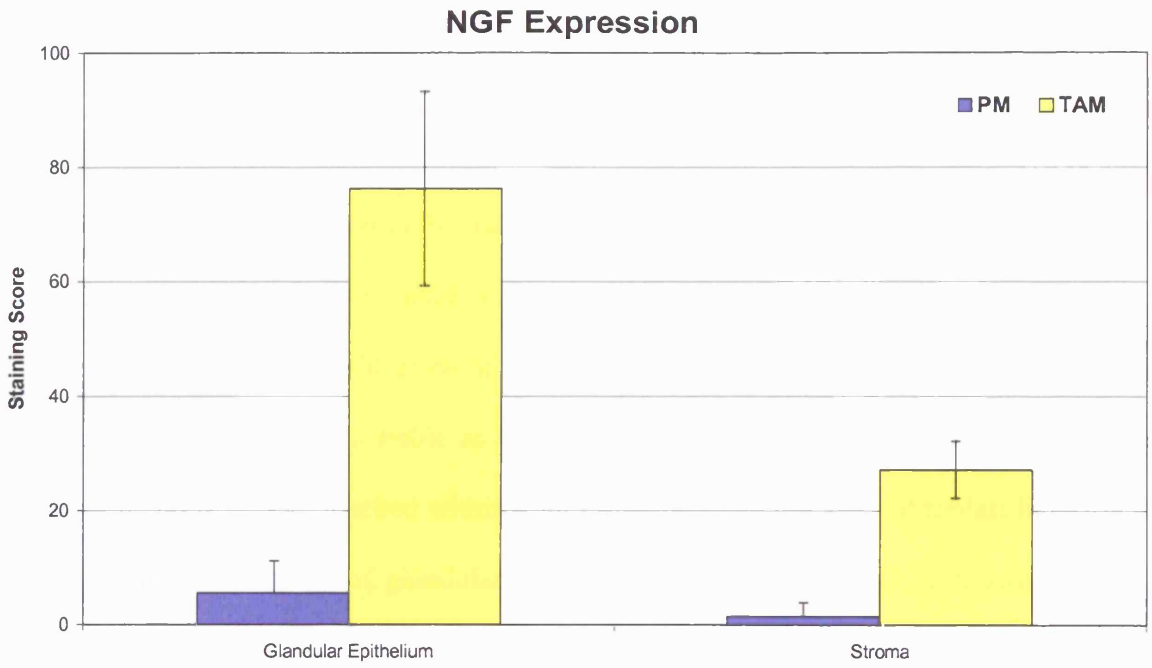


Figure 3.3: NGF Expression in Tamoxifen Exposed Endometrium.

Expression of NGFβ protein assessed in stroma and glands by immunohistochemistry. Staining score represent a count of positive stromal cells and a percentage stain for glands. Data represent the mean staining score in either stroma or glandular epithelium for patients receiving tamoxifen (TAM, n = 7) or postmenopausal women (PM, n = 4).

3.2.2 Method Development for a Primary Endometrial Cell Culture Model.

In this Section the aim was to isolate and culture primary endometrial stromal and glandular epithelial cells in order to study the effects of SERM dosing on *NGF* gene expression. A method was developed based on Arnold *et al.*, (2002), where endometrial tissue was dissociated into single stromal cells and glands using collagenase. Details of patients from which endometrial tissues were obtained are given in Table 3.1. Enzymatic dissociation of endometrial tissue gave a yield of *circa* 1 to $4x10^7$ stromal cells per g of tissue. These were routinely between 95 to 99 % viable as assessed by Trypan blue exclusion test. When plated onto plastic, stromal cells attached within 24 h, appearing flattened and fibroblast like (Figure 3.4a), and the morphology of glandular epithelial cells was consistent with Arnold *et al.*, (2001) (Figure 3.4b).

To assess purity of stromal and epithelial cell cultures, cells were immunostained for a range of cell markers, outlined in Figure 3.5. Cytokeratins 5/6/18 were used as a typical marker for cells of epithelial origin. In our study, epithelial cell cultures were found to be around 80% pure using cytokeratins as a marker. Smooth muscle α-actin was expressed in myofibroblasts and fully differentiated smooth muscle cells. Smoothelin was exclusively expressed in fully differentiated smooth muscle cells. Both epithelial and stromal cells expressed vimentin.

To characterise cell cultures, a range of preliminary experiments was carried out. Figure 3.6 shows typical proliferation of primary stromal cells in culture for 6 days. Under similar conditions it was not possible to determine if glandular epithelial cells proliferated. Western blotting identified low levels of ERα expressed in both stromal and glandular cultures (Figure 3.7). Very low levels of PR were detectable, however these were too low to quantify accurately. A suitable antibody for ERβ was not available at the time of investigation.

Experiment Number	Age	Endometrial Specimen Weight (g)	Stromal Dosing	Glandular Epithelium Dosing
H34	46	1.0	C, Pg	C, Pg
H37	40	0.96	C, P, E	_
H38	41	0.54	C, P, E	C, E
H39	45	0.89	C, P, T	C, P, T
H43	50	1.15	C, E, P, T	C, E, P, T
H44	36	0.84	C, E, P, T	C, E, P, T
H45	44	0.82	C, E, P, T	C, E, P, T
H52	71*	0.61	C, Pg	C, Pg
H53	39	0.63	C, E, P, Pg	C, Pg
H54	42	0.55	C, E, P, T, Pg	C, E, T, Pg
H55	49	1.1	C, E, T	C, E, T
H56	45	0.6	C, P, T	_

Table 3.1: Patient Details from which Primary Endometrial Cell Cultures were Isolated.

* Patient was post-menopausal but receiving hormone replacement therapy. Endometrial specimen weight is included as glands were cultured at a density of 10 wells of a 24-well plate per 0.5 g of tissue obtained. Treatments of the cells in culture were as follows: C. Control Ethanol (17 mM); E. Oestradiol (10 nM); P. Progesterone (10 nM); T. Tamoxifen (1 μM) and; Pg. Prostaglandin D_2 (100 nM). Tissue from premenopausal women was obtained during the proliferative stage.

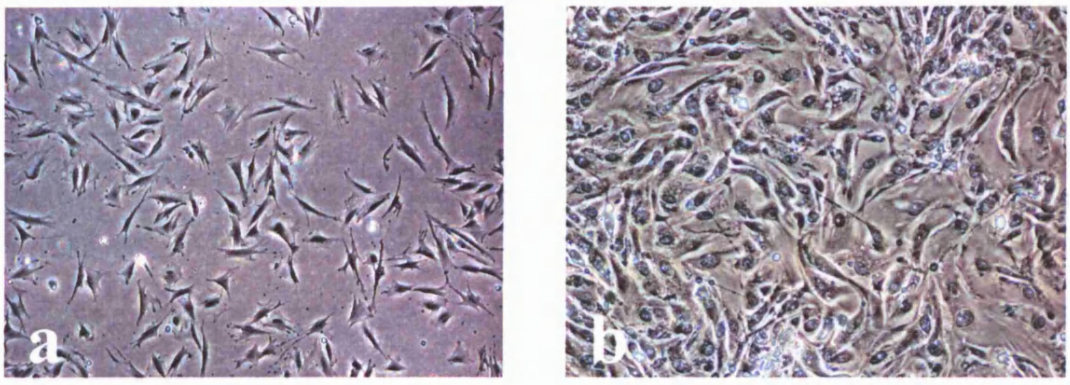
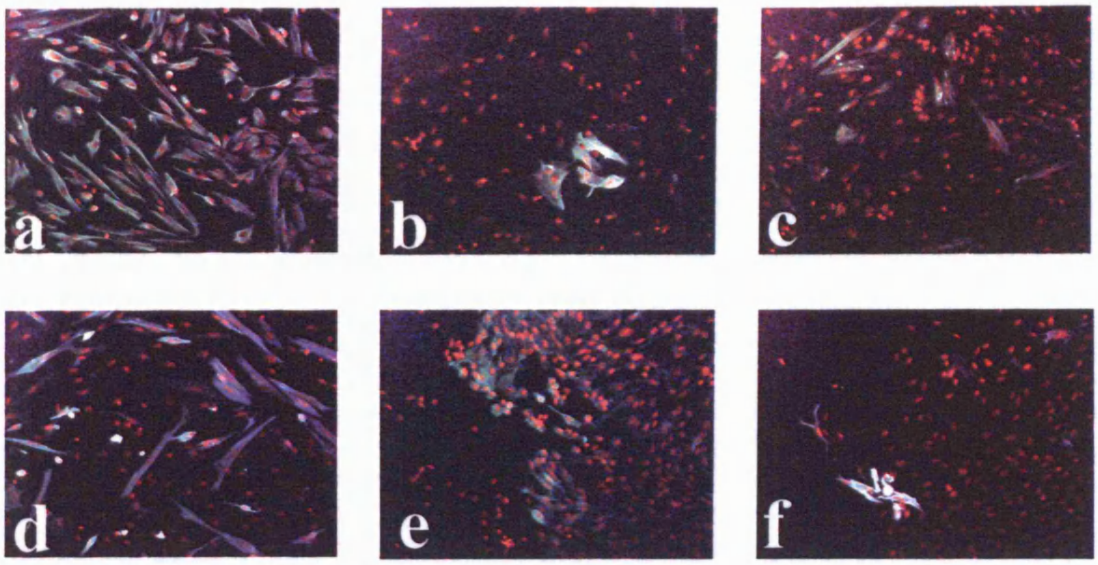


Figure 3.4: Primary Endometrial Cells in Culture.

a. Endometrial stromal cells original magnification x 40; **b**. endometrial glandular epithelial cells original magnification x 100. Cells shown were in culture for *circa* 48 h.



Marker		Cell Type	
Antibody Name	**Cell Expression**	**Stroma**	**Glandular Epithelium**
Vimentin (**a**)	FB / E	++	++
Cytokeratin 5/6/18 (**b**, **e**)	E	4%	++
Smooth Muscle α-Actin (**d**, **f**,)	MFB / SM	30%	-
Smoothelin (**c**)	SM	2%	-
Fibroblast Antigen (Thy-1)	FB	+	-

Figure 3.5: Immunocytofluorescent Analysis of Primary Endometrial Cell Cultures.

Figure shows representative primary stroma and epithelial cells grown in culture and stained for a range of cell markers. Cells and FITC-stained proteins (green) are: **a**. stroma, vimentin; **b**. stroma, cytokeratin; **c**. stroma, smoothelin; **d**. stroma, smooth muscle alpha actin; **e**. glandular epithelium, cytokeratin and, **f**. glandular epithelium, smooth muscle alpha actin. Staining intensity is represented as: strong ++, weak +, and, – no staining. Nuclei are stained with propidium iodide (red). Cell expression is: FB, fibroblasts; E, epithelial; SM, smooth muscle; and MFB, myofibroblasts. Percentages represent the percent of cells that stain for each marker in cultures.

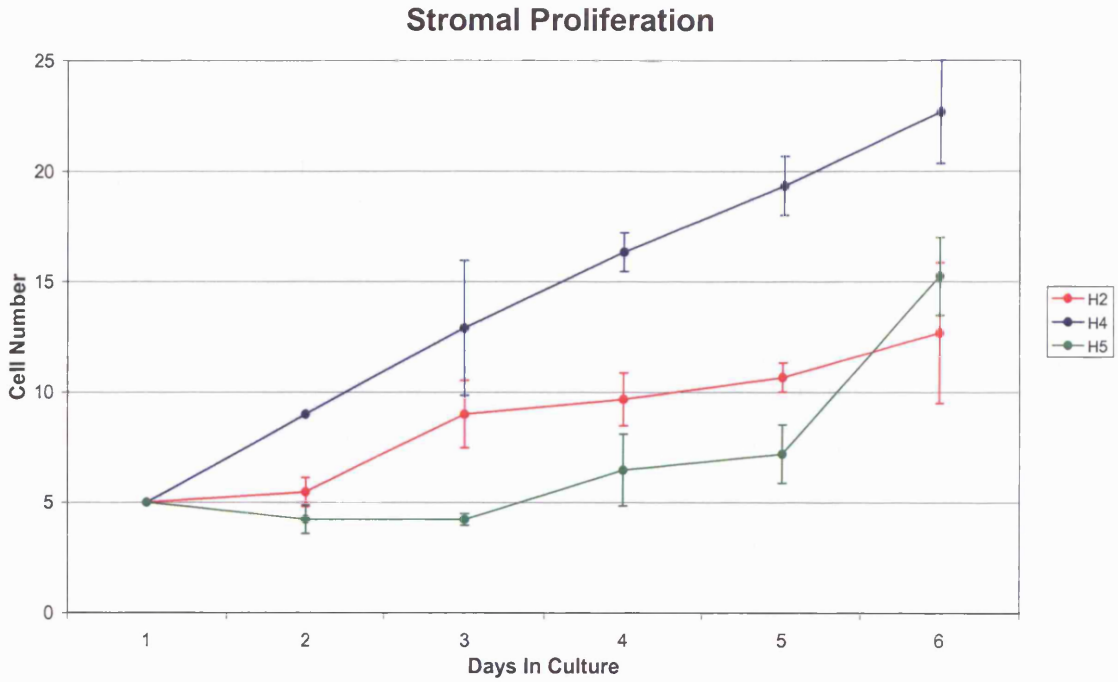


Figure 3.6: Proliferation of Stromal Cells in Culture: Individual Variation between Patients.

Stromal cells were plated at a density of $5x10^4$ cells per well of a 6-well plate in full serum media (day 1). After 24 h in culture, media was changed for low serum media and cells counted each day for 5 days using a Haemocytometer. Cells were > 95 % confluent by day 6. Cell number is $x10^4$ (± SE, n=3).

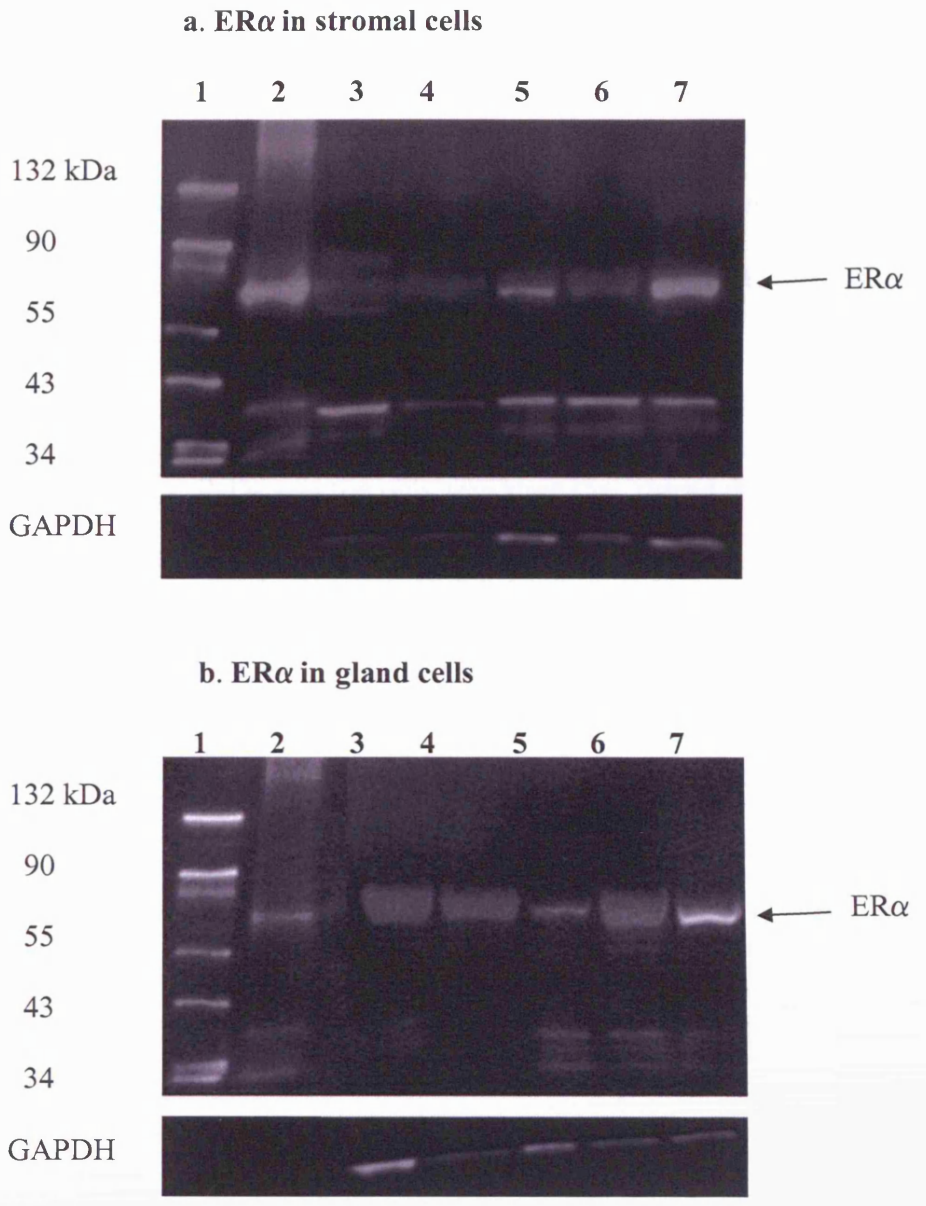


Figure 3.7: Western Blot of ERα in Human Endometrial Gland and Stromal Cells.

a. Stromal cell preparations, and; **b**. Glandular cell preparations. In both lanes A and B, lane 1 shows molecular weight markers of 132, 90, 55, 43 and 34 kDa; lane 2, 5 ng recombinant human ERα; lanes 3 to 7, ERα expression (arrowed) from five different cell preparations (30 μg protein/lane). Lower trace shows GAPDH expression. Quantitation of ERα, relative to the recombinant human standard, gave 2.44 ± 0.58 ng/lane for stroma and 4.44 ± 1.66 ng/lane for glands (mean ± SE, n = 5).

3.2.3 Regulation of NGF Protein and mRNA Expression in Primary Endometrial Cells.

Primary stromal and epithelial cells were allowed to attach for up to 48 h after isolation. Cells were then treated for 24 h with either vehicle control; oestradiol (10 nM); progesterone (10 nM); tamoxifen (1 μM) or prostaglandin D_2 (100 nM). Prostaglandins have previously been shown to be powerful inducers of NGF secretion only in cultured mouse astrocytes (Toyomoto *et al.*, 2004).

In terms of morphology, PgD_2 was the only treatment that altered the appearance of cells in culture (Figure 3.8). Control, oestradiol, tamoxifen and progesterone dosed cells attached to the vessel surface. Relative to vehicle dosed controls, PgD_2 dosed cells appeared rounded and condensed, however they were still adherent to the plate. Cell viability was not assessed at this stage, although before dosing cells appeared comparable to control cultures.

Oestradiol treatment for 24 h led to increased *NGF* gene expression in the stroma and in glands from premenopausal patients while surprisingly at this time point, tamoxifen was without effect (Figure 3.9). Progesterone treatment decreased expression in both cell types. Prostaglandin D_2 (PgD_2) strongly up-regulated *NGF* mRNA expression in both endometrial glands and stroma.

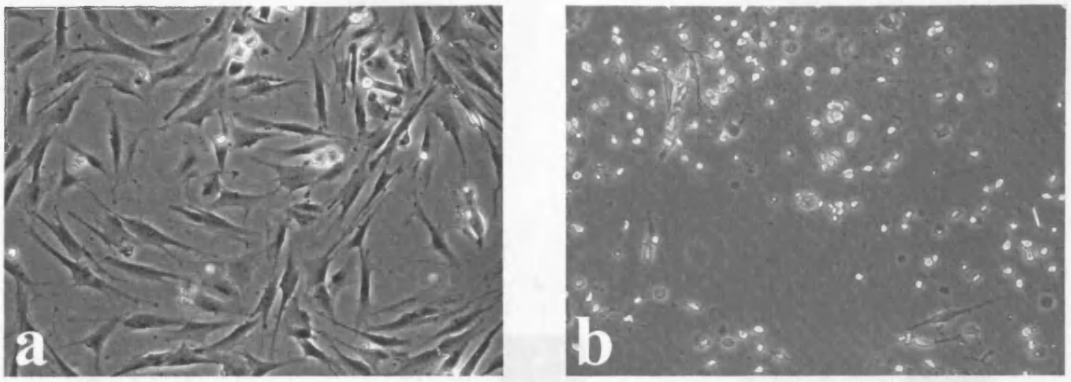


Figure 3.8: Endometrial Stromal Cell 24 Hour Culture.

a. Vehicle only dosed stroma 24 h (control), **b**. PgD_2 dosed stromal cells at 24 h. In cultures dosed with PgD_2, there is a marked difference in the appearance of attached cells. There was no visible difference from controls in cultures dosed with oestradiol, tamoxifen or progesterone. Magnification x 10.

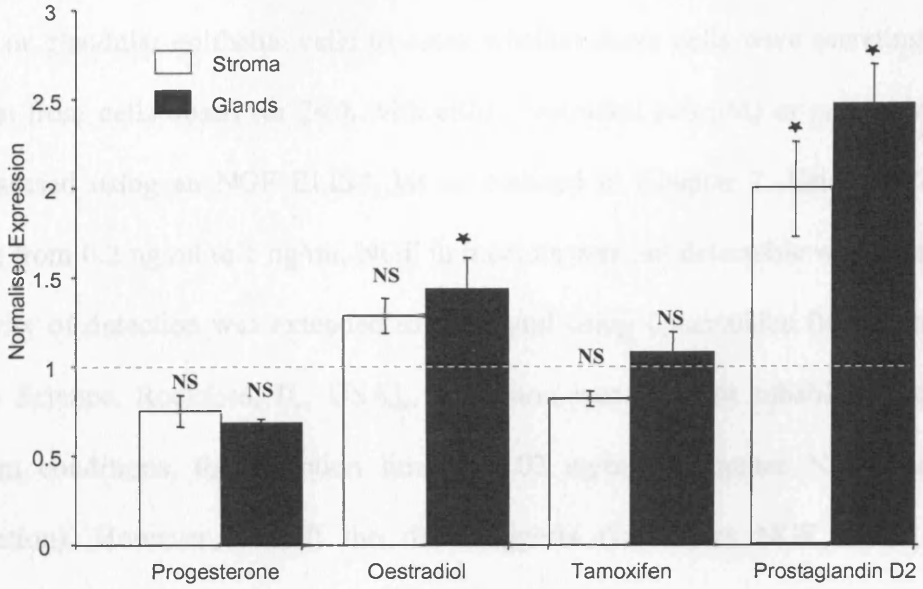


Figure 3.9: *NGF* Expression in Dosed Primary Cell Cultures.

NGF gene expression assessed by RT-PCR is shown relative to control expression. Cells were cultured and then dosed for 24 h with either: vehicle only, oestradiol (10 nM), progesterone (10 nM), tamoxifen (1 μM) or, prostaglandin D_2 (100 nM). Results represent the mean ± SE for experiments. Numbers of experiments from individual patients are: Stroma; control 12, progesterone 9, oestradiol 8, tamoxifen 7 and PgD_2 4, Glands; control 10, progesterone 4, oestradiol 6, tamoxifen 6 and PgD_2 4. Probability of significant difference between treated and controls: ☆ $p < 0.05$, **NS** Not significant.

Attempts were also made to measure NGF protein in the medium of cultured endometrial stroma or glandular epithelial cells to asses whether these cells were secreting this protein. Medium from cells dosed for 24 h with either oestradiol (10 nM) or progesterone (10 nM) was assessed using an NGF ELISA kit as outlined in Chapter 2. Using a standard curve ranging from 0.2 ng/ml to 1 ng/ml, NGF in medium was not detectable within this range. The sensitivity of detection was extended to 0.1 ng/ml using QuantaBlue fluorescence detection [Perbio Science. Rockford, IL, USA], expression was still not reliably detectable. Under optimum conditions, the detection limit is 0.03 ng/ml for human NGF (Roche product information). However, overall the data suggests that either NGF is not secreted by endometrial cells or that the concentrations are below the quantifiable range used in the present study.

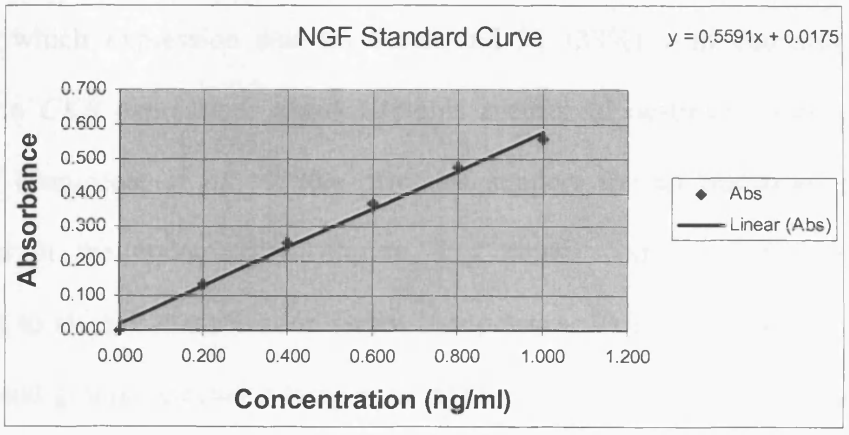


Figure 3.10: NGFβ ELISA Standard Curve.

Figure shows representative NGFβ ELISA standard curve used to asses the concentration of NGF in cell culture media. Recombinant NGF protein was diluted from 1 ng/ml to 0.2 ng/ml and loaded in duplicate.

3.2.4 Gene Expression Changes in the Endometrium of Women Receiving Tamoxifen.

For this study, uterine tissues were obtained from 5 women taking tamoxifen for up to 4 years, and tissues obtained from 6 age matched controls not receiving this drug. The mean age of patients in this study receiving tamoxifen was 56.4 with ages ranging from 42 to 66. Patients were receiving 20 mg per day oral tamoxifen tablets for up to four years for the treatment of breast cancer. The mean age of postmenopausal control patients was 55.8 with ages ranging from 51 to 60. Patients were undergoing a hysterectomy for conditions including menorrhagia, endometrial fibroids and polyps. None of the patients in this study had any visible signs of uterine cancers at the time of surgery. Changes in gene expression were assessed using HGMP oligo arrays and Cy3 / Cy5 labelling (MRC Toxicology Unit protocol).

Table 3.2 shows there were 276 genes in which the Log_2 expression was significantly ($p < 0.05$) changed in the endometrium of women receiving tamoxifen. This included 185 (67%) genes in which expression was increased and 91 (33%) with expression decreased. An increase in *CKB* expression, which contains a classical oestrogen response element in its promoter (Pentecost *et al.,* 1990), provides support for an oestrogen agonist action of tamoxifen in the endometrium. Figure 3.11 shows common gene functions identified according to HGMP classification (www.hgmp.mrc.ac.uk/mircoarray). Gene functions were assigned and groups selected if three or more genes were identified in that group. It should be noted that functions were assigned from HGMP array information files, so the precise gene function may differ from that stated. Arranging genes on their functions, the most common gene function altered was in those involved in developmental processes. Amongst these were *NGFRAP1* and *PAEP* that will be discussed later in this Chapter.

Gene	Log2 Ratio
LOC56757	0.46
20D7-FC4	0.17
ABR	0.45
ACTR10	0.31
AD021	0.98
ADCK2	1.30
ADH5	0.17
ADSS	-0.46
AF010236	1.11
AF052130	0.39
AF052174	0.45
AF070633	-0.45
AF085983	-0.73
AF104914	0.57
AJ012499	-0.28
AJ227869	0.23
AK023059	0.42
AL359334	0.45
ANGPTL1	-1.48
ANP32E	0.40
AP2M1	0.61
APG16L	-1.16
ARC21	1.41
ARF5	0.46
ARHGEF11	0.80
ARRDC4	0.52
ARS	0.58
ATP5A1	0.79
ATP5E	0.97
BAIAP1	0.27
BAT5	0.35
BBS1	0.47
BBX	0.27
BCAT1	0.76
BHLHB3	1.21
BTD	0.23
C10orf117	-0.82
C17orf42	-0.28
C20orf11	0.40
C20orf155	-0.14
C20orf28	-1.49
C20orf67	1.37
C5orf13	1.13
C6orf108	1.36
CAB39L	-0.67
CAG4	1.17
CAPZA2	1.15
CD63	0.90
CDKAL1	-1.40
CDY1	-0.63
CGI-51	0.59
CHI3L1	-0.41
CHM	0.19
CHSY1	0.77
CIDEB	0.40
CKB	2.33
CLECSF2	-1.37
CNN3	0.50
COBRA1	0.68
COG4	0.45
COL27A1	0.71
COMMD8	-0.35
CORO1C	-0.40
CRIP2	0.93
CRK7	0.30
D16914	0.39
D17082	0.66
D17093	0.46
D17152	-0.59
D17184	0.44
D21S2056E	-0.23
D28391	-0.37
D4S234E	1.08
DACH1	1.17
DBN1	1.14
DDX50	0.41
DERPC	0.41
DHX9	0.32
DKFZp547E184	1.52
DKFZp564C2063	-0.86

Gene	Log2 Ratio
DKFZp564D1164	0.31
DKFZp564F133	-1.21
DKFZp564H0616	-0.49
DKFZp586F2323	0.27
DKFZP586O0120	-0.58
DOK1	-0.38
DPYSL3	0.89
E2IG2	-0.26
EDN1	-0.65
EDN3	1.04
EDNRA	0.37
EEF1A1	0.73
EFEMP1	-0.81
EFNB3	0.49
EGR1	-2.07
ELN	0.31
ENPP1	-1.00
F8A1	0.70
FAM19A5	0.50
FGF7	-1.29
FLJ10633	-1.06
FLJ10664	0.57
FLJ11163	0.76
FLJ11177	1.25
FLJ20196	-1.24
FLJ20254	0.36
FLJ20705	-0.16
FLJ21687	-0.68
FLJ22655	-0.86
FLT3LG	-1.40
FMOD	0.23
FNDC3B	-0.86
FRMD4A	0.50
GEM	-1.64
GGCX	-0.38
GLIPR1	0.22
GPRC5B	0.83
H2AFZ	-0.45
HE4	2.45
HMG14	0.53
HMGI-C	0.28
HNF4G	-0.22
HSPA8	-0.36
IGF1	1.49
IL2RB	-0.69
IL6ST	-0.63
INADL	0.30
IPW	0.70
ITPR1	-0.72
ITR	0.65
KCC3	-0.80
KEAP1	0.19
KIAA0280	0.67
KIAA0562	0.86
KIAA0582	-0.59
KIAA0668	-1.39
KIAA0889	-1.29
KLHDC5	0.41
LAMB2	0.65
LAMB3	-0.31
LDB1	-0.89
LEFTY2	-2.51
LMOD1	0.42
LOC51108	0.27
LOC540369	0.77
LOC552891	1.04
LOC57146	-0.34
LRRC19	0.62
LRRFIP1	-0.53
LYRIC	0.21
MAGED2	1.45
MAGED4	0.69
MALAT1	-1.11
MAP2K4	-0.30
MAP3K14	0.32
MARVELD1	0.80
MCLC	-0.57
MEF2B	-0.63
MGC16824	0.19
MGC20460	-0.50

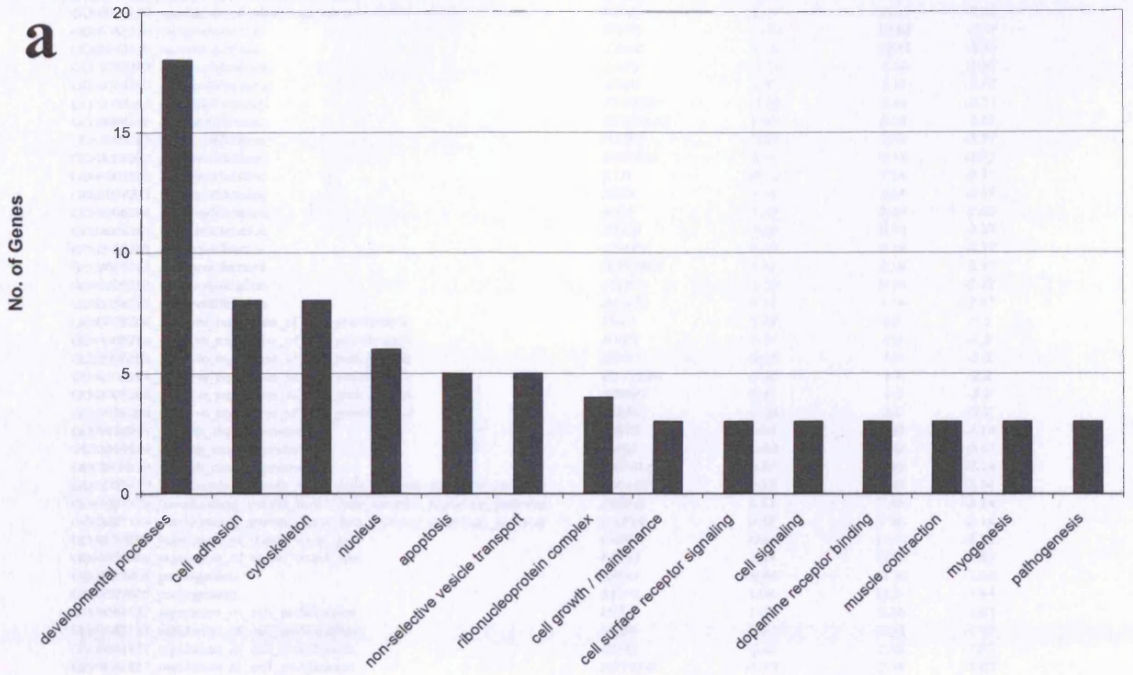
Gene	Log2 Ratio
MGC2603	2.81
MGC3260	0.68
MGC5139	0.36
MGC70863	-0.83
MID1IP1	0.22
MRPL42	-0.15
MSLN	0.52
NCOA3	-0.31
NDRG1	-0.83
NECAP1	0.23
NEU2	0.79
NEURL	0.26
NGFRAP1	0.50
NGLY1	-1.54
NKAP	0.31
ZNF395	0.38
NMU	-0.84
NOSTRIN	0.31
NPY1RL/PP2	1.00
NR2E3	0.60
NR2F6	0.68
NRG1	-0.49
NRP1	0.57
NRXN2	0.69
OTUB1	0.68
PAEP	-3.15
PAWR	0.84
PCP4	-1.64
PDCD6IP	0.73
PDXK	0.83
PENK	2.28
PER2	0.62
PFN2	0.47
PIK3R1	0.75
PKP2	-0.47
PORIMIN	0.59
PPAP2B	0.38
PPIC	0.80
PPP2R2C	1.27
PRKACA	1.15
PRO0529	0.73
PRO1051	1.60
PRO1578	0.26
PRO2007	0.49
PSMB3	0.92
PTD008	0.91
PTPLB	0.38
PTPNS	0.19
PTPRK	0.46
RAF1	-0.54
RAPGEF1	-0.29
RCN3	0.60
RENT1	0.54
RFP	0.38
RGS3	1.88
RN5S1	1.00
RNF5	0.81
RPL19	0.48
RPP20	0.42
RPS12	-0.57
RPS4X	0.52
RYBP	0.36
S100G	-0.69
SCGB3A1	3.00
SDCCAG33	0.17
SELL	0.42
SEMA4A	0.81
SF3A1	0.72
SF3B4	0.58
SFRS14	0.52
SLAP	0.84
SLC40A1	-0.75
SLC41A3	0.47
SLC7A1	-1.14
SMAD7	0.35
SMPDL3A	-0.65
SND1	0.44
SNRP70	-0.36
SOCS7	0.66
SON	0.35

Gene	Log2 Ratio
SPIRE1	-0.73
SRD5A2L	-0.91
SRM300	0.66
SSR3	1.33
STC1	-1.14
STX8	0.82
TACC2	-1.37
TARBP1	0.28
TBK1	0.78
TCEAL4	-0.56
TFPI	-0.65
TIEG	0.86
TIMP1	0.45
TMEM35	-0.71
TMEM9	0.65
TMEPAI	1.55
TOM1L2	0.69
TP53INP2	0.84
TPST1	0.54
TRA1	0.91
TRA1	0.71
TXNRD1	-1.01
U62823	1.18
U88897	-0.93
UBE2H	0.24
USP36	-0.98
Y16704	1.50
Y17175	0.22
Y17180	0.42
YAF2	0.43
Z36782	1.02
Z70709	-1.03
Z70769	0.69
ZFYVE16	-0.97
ZNF304	1.09
ZNF326	-0.91

Table 3.2: Log2 Gene Expression Changes in Uteri from Tamoxifen Treated Women Compared with Those Not Taking this Drug.

All genes in which expression was significantly altered by tamoxifen, $p < 0.05$.

a



b

Gene	Log2 Ratio	Functional Group
ANGPTL1	-1.48	developmental processes
CGI-51	0.59	developmental processes
CHSY1	0.77	developmental processes
CRK7	0.30	developmental processes
DDX50	0.41	developmental processes
DERPC	0.41	developmental processes
DHX9	0.32	developmental processes
KEAP1	0.19	developmental processes
LDB1	-0.89	developmental processes
MCLC	-0.57	developmental processes
NGFRAP1	0.50	developmental processes
PAEP	-3.15	developmental processes
RCN3	0.60	developmental processes
RYBP	0.36	developmental processes
SND1	0.44	developmental processes
TIMP1	0.45	developmental processes
YAF2	0.43	developmental processes
Z36782	1.02	developmental processes

Gene	Log2 Ratio	Functional Group
ANP32E	0.40	cell adhesion
FNDC3B	-0.86	cell adhesion
LRRC19	0.62	cell adhesion
OTUB1	0.68	cell adhesion
PDXK	0.83	cell adhesion
SELL	0.42	cell adhesion
COL27A1	0.71	cell adhesion
PTPNS	0.19	cell adhesion
FRMD4A	0.50	cytoskeletal anchoring
KIAA0582	-0.59	cytoskeletal anchoring
RPP20	0.42	cytoskeletal anchoring
AF104914	0.57	cytoskeleton organization
FLJ10633	-1.06	cytoskeleton organization
NGLY1	-1.54	cytoskeleton organization
ZNF326	-0.91	cytoskeleton organization
CAG4	1.17	cytoskeleton organization

Figure 3.11: Functions of Genes Where Expression was Altered by Tamoxifen.

a. Figure shows functional groups and the number of genes in each group. Gene functions were assigned from HGMP oligo array description files. Genes were sorted on function and groups selected if they contained three or more genes. **b**. Genes identified from the top three groups showing the greatest number of gene expression changes.

GO Category	Gene	Fold change (Log2)	Enrichment	Log10(P)
GO:0019229_regulation_of_vasoconstriction	*EDN1*	-0.65	23.78	-2.57
GO:0019229_regulation_of_vasoconstriction	*EDN3*	1.04	23.78	-2.57
GO:0042310_vasoconstriction	*EDN1*	-0.65	19.82	-2.39
GO:0042310_vasoconstriction	*EDN3*	1.04	19.82	-2.39
GO:0008283_cell_proliferation	*RAF1*	-0.54	2.14	-2.37
GO:0008283_cell_proliferation	*NRP1*	0.57	2.14	-2.37
GO:0008283_cell_proliferation	*FLT3LG*	-1.39	2.14	-2.37
GO:0008283_cell_proliferation	*SCGB3A1*	3.00	2.14	-2.37
GO:0008283_cell_proliferation	*PAWR*	0.84	2.14	-2.37
GO:0008283_cell_proliferation	*EDNRA*	0.36	2.14	-2.37
GO:0008283_cell_proliferation	*ELN*	0.31	2.14	-2.37
GO:0008283_cell_proliferation	*IGF1*	1.49	2.14	-2.37
GO:0008283_cell_proliferation	*RFP*	0.38	2.14	-2.37
GO:0008283_cell_proliferation	*EDN1*	-0.65	2.14	-2.37
GO:0008283_cell_proliferation	*TIMP1*	0.45	2.14	-2.37
GO:0008283_cell_proliferation	*BHLHB3*	1.21	2.14	-2.37
GO:0008283_cell_proliferation	*FGF7*	-1.29	2.14	-2.37
GO:0008283_cell_proliferation	*BCAT1*	0.76	2.14	-2.37
GO:0008284_positive_regulation_of_cell_proliferation	*IGF1*	1.49	3.6	-2.2
GO:0008284_positive_regulation_of_cell_proliferation	*NRP1*	0.57	3.6	-2.2
GO:0008284_positive_regulation_of_cell_proliferation	*EDN1*	-0.65	3.6	-2.2
GO:0008284_positive_regulation_of_cell_proliferation	*FLT3LG*	-1.39	3.6	-2.2
GO:0008284_positive_regulation_of_cell_proliferation	*TIMP1*	0.45	3.6	-2.2
GO:0008284_positive_regulation_of_cell_proliferation	*FGF7*	-1.29	3.6	-2.2
GO:0006939_smooth_muscle_contraction	*CNN3*	0.50	7.43	-2.14
GO:0006939_smooth_muscle_contraction	*NMU*	-0.83	7.43	-2.14
GO:0006939_smooth_muscle_contraction	*EDNRA*	0.37	7.43	-2.14
GO:0007179_transforming_growth_factor_beta_receptor_signaling_pathway	*SMAD7*	0.35	7.43	-2.14
GO:0007179_transforming_growth_factor_beta_receptor_signaling_pathway	*FMOD*	0.23	7.43	-2.14
GO:0007179_transforming_growth_factor_beta_receptor_signaling_pathway	*KLF10*	0.85	7.43	-2.14
GO:0050880_regulation_of_blood_vessel_size	*EDN1*	-0.65	13.21	-2.03
GO:0050880_regulation_of_blood_vessel_size	*EDN3*	1.04	13.21	-2.03
GO:0009405_pathogenesis	*EDN1*	-0.65	11.89	-1.94
GO:0009405_pathogenesis	*EDN3*	1.04	11.89	-1.94
GO:0042127_regulation_of_cell_proliferation	*IGF1*	1.49	2.38	-1.87
GO:0042127_regulation_of_cell_proliferation	*NRP1*	0.57	2.38	-1.87
GO:0042127_regulation_of_cell_proliferation	*EDN1*	-0.65	2.38	-1.87
GO:0042127_regulation_of_cell_proliferation	*FLT3LG*	-1.39	2.38	-1.87
GO:0042127_regulation_of_cell_proliferation	*TIMP1*	0.45	2.38	-1.87
GO:0042127_regulation_of_cell_proliferation	*SCGB3A1*	3.00	2.38	-1.87
GO:0042127_regulation_of_cell_proliferation	*FGF7*	-1.29	2.38	-1.87
GO:0042127_regulation_of_cell_proliferation	*KLF10*	0.86	2.38	-1.87
GO:0042127_regulation_of_cell_proliferation	*PAWR*	0.84	2.38	-1.87
GO:0007165_signal_transduction	*DOK1*	-0.37	1.34	-1.81
GO:0007165_signal_transduction	*STC1*	-1.14	1.34	-1.81
GO:0007165_signal_transduction	*PPP2R2C*	1.27	1.34	-1.81
GO:0007165_signal_transduction	*RGS3*	1.89	1.34	-1.81
GO:0007165_signal_transduction	*NCOA3*	-0.31	1.34	-1.81
GO:0007165_signal_transduction	*NR2E3*	0.60	1.34	-1.81
GO:0007165_signal_transduction	*KLF10*	0.86	1.34	-1.81
GO:0007165_signal_transduction	*BAIAP1*	0.27	1.34	-1.81
GO:0007165_signal_transduction	*ABR*	0.45	1.34	-1.81
GO:0007165_signal_transduction	*PPIC*	0.80	1.34	-1.81
GO:0007165_signal_transduction	*IL6ST*	-0.63	1.34	-1.81
GO:0007165_signal_transduction	*EDN1*	-0.65	1.34	-1.81
GO:0007165_signal_transduction	*PIK3R1*	0.75	1.34	-1.81
GO:0007165_signal_transduction	*TXNRD1*	-1.01	1.34	-1.81
GO:0007165_signal_transduction	*TBK1*	0.77	1.34	-1.81
GO:0007165_signal_transduction	*SMAD7*	0.35	1.34	-1.81
GO:0007165_signal_transduction	*FGF7*	-1.29	1.34	-1.81
GO:0007165_signal_transduction	*NMU*	-0.84	1.34	-1.81
GO:0007165_signal_transduction	*ZFYVE16*	-0.97	1.34	-1.81
GO:0007165_signal_transduction	*SOCS7*	0.66	1.34	-1.81
GO:0007165_signal_transduction	*ITPR1*	-0.72	1.34	-1.81
GO:0007165_signal_transduction	*RAF1*	-0.54	1.34	-1.81
GO:0007165_signal_transduction	*GEM*	-1.64	1.34	-1.81
GO:0007165_signal_transduction	*MAP2K4*	-0.29	1.34	-1.81
GO:0007165_signal_transduction	*NRP1*	0.57	1.34	-1.81
GO:0007165_signal_transduction	*GPRC5B*	0.83	1.34	-1.81
GO:0007165_signal_transduction	*IL2RB*	-0.69	1.34	-1.81
GO:0007165_signal_transduction	*PRKACB*	0.27	1.34	-1.81
GO:0007165_signal_transduction	*FLT3LG*	-1.39	1.34	-1.81
GO:0007165_signal_transduction	*EDN3*	1.04	1.34	-1.81
GO:0007165_signal_transduction	*RAPGEF1*	-0.29	1.34	-1.81
GO:0007165_signal_transduction	*EDNRA*	0.37	1.34	-1.81
GO:0007165_signal_transduction	*ARF5*	0.46	1.34	-1.81
GO:0007165_signal_transduction	*ARHGEF11*	0.80	1.34	-1.81
GO:0007165_signal_transduction	*DPYSL3*	0.89	1.34	-1.81
GO:0007165_signal_transduction	*TMEPAI*	1.55	1.34	-1.81
GO:0007165_signal_transduction	*IGF1*	1.49	1.34	-1.81
GO:0007165_signal_transduction	*PDCD6IP*	0.73	1.34	-1.81
GO:0007165_signal_transduction	*PER2*	0.62	1.34	-1.81
GO:0007165_signal_transduction	*CORO1C*	-0.40	1.34	-1.81
GO:0007165_signal_transduction	*GNG10*	1.03	1.34	-1.81
GO:0007165_signal_transduction	*FMOD*	0.23	1.34	-1.81
GO:0007165_signal_transduction	*NR2F6*	0.68	1.34	-1.81
GO:0007165_signal_transduction	*PENK*	2.28	1.34	-1.81
GO:0007165_signal_transduction	*RAB6A*	0.39	1.34	-1.81

Table 3.3: GoMiner Analysis of the Effects of Tamoxifen on Gene Expression.

In addition to identifying the functions of genes in which expression is altered by tamoxifen based on the HGMP classification, microarray gene expression data was also analysed using GoMiner (http://discover.nci.nih.gov/gominer/GoCommandWebInterface.jsp). Table 3.3 shows some of the most significant categories of gene function to be altered by tamoxifen. This includes cell proliferation itself, positive regulation of cell proliferation and regulation of cell proliferation. *IGF1* was identified as a gene that was potentially involved in all of these cellular functions. Gene expression was increased for a majority of genes for these cellular functions after tamoxifen treatment.

Large variability can exist between microarray data, due to technical variations or dependent upon the exact sequence printed onto the array. Therefore, expression of three genes identified on the microarrays were assessed using RT-PCR to confirm expression. It was predicted that RT-PCR would confirm both the direction and level of expression change. Log_2 expression of *CKB* was + 5.02 and fold change identified using RT-PCR was 2.74 ± 0.93 in the endometrium of women receiving tamoxifen in comparison to controls. Log_2 expression was + 2.80 for *IGF1* and RT-PCR fold change was 3.79 ± 1.46, and for *PENK* Log_2 expression was 2.28 a compared to 1.75 ± 0.44 for RT-PCR. A change in expression was not identified as significant for *NGFb* on arrays but was 0.64 ± 0.15 in comparison to controls using RT-PCR. Although expression of only four genes was confirmed using RT-PCR, directions and level of expression changes were reasonably consistent with that obtained from microarrays.

3.3 Discussion.

3.3.1 Endometrial Expression of NGF.

Many proteins have been identified that are differentially regulated during the 28-day menstrual cycle. With the introduction of gene array technology, there are now over 100 genes whose expression is known to be changed in this way (Borthwick *et al.,* 2003), although *NGF* was not identified in this group. However an increase in uterine expression of *NGF* in response to tamoxifen in the mouse uterus was recently found (Green *et al.,* 2003; Green *et al.,* 2005). In order to further understand the regulation of NGF expression in the uterus, we examined uterine NGF expression throughout the menstrual cycle and in primary cell cultures dosed with oestradiol or progesterone.

Results presented in this Chapter demonstrate the presence of NGF protein in epithelial and stromal compartments of the normal cycling endometrium of women. Previously, NGF protein expression has only been reported in association with neurites in deep adenomyotic and endometriotic nodules in women (Anaf *et al.,* 2002), or within the endometrial epithelium and diffuse staining through the stroma in the mouse (Bjorling *et al.,* 2002). The presence of NGF protein in the cells suggest continuous synthesis, since in animal models, both NGF mRNA and protein have half-lives of around 2 h and 4 h respectively (Nguyen *et al.,* 2000; Sherer *et al.,* 1998b; Sherer *et al.,* 1998a).

Establishing the function of NGF in non-neuronal tissues has been difficult, complicated by differential NGF receptor expression and interactions. Many functions have been described for NGF. For example, in the human prostate, it has been proposed that NGF regulates cell proliferation mediated through its high affinity receptor TrkA. TrkA and p75, may participate in the paracrine cross-talk between stromal and epithelial cells (Graham *et al.,* 1992). In women, TrkA has only been detected in the sub-peritoneal and peritoneal nerves and not in

glandular epithelium (Anaf *et al.,* 2002). However, p75 protein is expressed in endometrial glands and to a lesser extent in stroma where its expression remains consistent throughout the menstrual cycle (Tabibzadeh *et al.,* 1995). In a mouse model, long-term up-regulation of uterine NGF has been associated with the development of adenomyosis (Parrott *et al.,* 2001; Green *et al.,* 2005). There are some reports to suggest that women taking tamoxifen are more likely to develop adenomyosis (Cohen *et al.,* 1997; Varras *et al.,* 2003).

Neurotrophins clearly play an important role during nerve growth, though this is not thought to be the primary function for NGF in the uterus (Krizsan-Agbas *et al.,* 2003). In our study, human recombinant NGF added to stromal cells in culture did not increase their rate of proliferation as has been described for certain cancer cell lines (Chiarenza *et al.,* 2001; Sortino *et al.,* 2000). It seems most likely that in the uterus NGF has a paracrine / autocrine role in signalling, as has been described in breast, prostate and pancreas (Descamps *et al.,* 2001; Graham *et al.,* 1992; Kanaka-Gantenbein *et al.,* 1995). It remains unclear if such signalling is associated with cell differentiation as seen in premyocytes in culture (Erck *et al.,* 1998) or some other function such as mediating inflammation (Marshall *et al.,* 1999) or inducing apoptosis as seen in hepatocytes (Oakley *et al.,* 2003) although preliminary data (not shown) suggests that there was no increase in apoptosis within the endometrium of patients used in the present study.

The fact that NGF expression was highest during the late secretory phase and in the endometrium of postmenopausal women taking tamoxifen suggests that NGF is not involved in either proliferation or apoptosis within the endometrium. It is possible that elevated levels of NGF observed during the secretory phase, or in the endometrium of postmenopausal women taking tamoxifen, may act to promote polymorphonuclear chemotaxis seen in the

menstrual and proliferative stages. In fact, a role for NGF in endometrial repair has previously been suggested (Bjornstrom *et al.,* 2002). In addition, oestradiol has been shown to inhibit the chemotaxis of these cell types, an effect which was abrogated by pre-treatment with tamoxifen (Ito *et al.,* 1995). Tamoxifen has also been shown to have an effect on immunoregulation in the endometrium, leading to an increase in natural killer cell activity (Garzetti *et al.,* 1994). It would be of interest to examine if levels of polymorphonuclear lymphocytes are elevated in the endometrium of postmenopausal women receiving tamoxifen.

In this Chapter expression of NGF in polymorphonuclear cells in the endometrium was described. The role of NGF in this cell type in the uterus is unknown, although NGF may be involved in the inflammatory response during the menstrual cycle. The presence of neutrophils during the menstrual stage is required to enhance the immune protection within the uterus during menstruation (King *et al.,* 2003a). Previous studies have shown that while antigen-inducible expression of NGF and BDNF may be observed in lymph node cells in humans, neurotrophin expression is confined exclusively to B cells (Edling *et al.,* 2004). T cells, NK1.1 cells and CD11b monocytes and macrophages were found not to express any detectable BDNF or NGF, under any conditions.

In terms of NGF expression in other female reproductive tissues, there has been interest in the expression of NGF in the ovaries. NGF and TrkA are expressed in the ovary and p75 is expressed only in the ovary of foetuses of less than 22 weeks gestation (Abir *et al.,* 2005), where the presence of NGF is thought to act as a proliferative signal involved in early follicular development (Dissen *et al.,* 2001).

3.3.2 Primary Endometrial Cell Culture Model.

Oestradiol and tamoxifen have been shown to stimulate proliferation of both stromal and epithelial cell cultures (Pole *et al.,* 2005), though they induced expression of only a few classical replication associated genes. In addition oestradiol has been shown to stimulate proliferation of endometrial cells, an effect inhibited by tamoxifen (Zhang *et al.,* 2003), although no attempt was made to separate cell types, thus allowing essential interactions between different cell types.

Communication between endometrial stromal and glandular cells is likely to be vital in the proliferative response of epithelial cells *in vivo* (Bigsby, 2002) and *in vitro* (Astrahantseff *et al.,* 1994). It is thought oestradiol acts through stromal ERα, which then initiates a paracrine cell communication system resulting in proliferation of epithelial cells (Bigsby *et al.,* 2003). However, stromal cell proliferation itself may also be independent of tissue ERα expression. Using uterine explants, stroma from ERKO mice did not proliferate in response to oestradiol when implanted into an ERKO mouse but did when in a wild type mouse. This suggests that proliferation of stromal cells may not require direct expression of ERα. It may be possible therefore, that oestradiol does not directly act on stroma to induce proliferation (Bigsby, 2002). It is likely that oestradiol induced proliferation is mediated through a locally produced growth factor such as EGF or TGFα (Komatsu *et al.,* 2003).

3.3.3 *NGF* Expression in Primary Cell Cultures.

A primary cell culture model was established to study the regulation of *NGF* expression. Cell purity of epithelial and stromal cultures was similar to that previously described (Pole *et al.,* 2005). We show here that oestradiol and prostaglandin D_2 (PgD_2) are able to induce expression of *NGF* in primary endometrial and stromal cells in culture. Progesterone

decreased expression minimally and tamoxifen was without effect. Data presented here using oestradiol are supported by findings that oestradiol may induce *Ngf* expression in the uteri of rodents (Bjorling *et al.*, 2002; Krizsan-Agbas *et al.*, 2003).

In the present study, PgD_2 was used as it is a strong inducer of NGF protein expression in astrocytes (Toyomoto *et al.*, 2004), where PgD_2 induced NGF secretion is thought to have a neuroprotective effect. In our study we found that endometrial cells dosed with PgD_2 exhibited altered morphology compared with vehicle dosed controls. PgD_2 may be produced by both the endometrium and myometrium, with production in the endometrium peaking during the menstrual and mid-luteal phase (Rees *et al.*, 1986). In terms of PgD_2 function in the uterus, experimentally PgD_2 is involved in T-cell recruitment (Michimata *et al.*, 2002) and in myometrial contractility (Cao *et al.*, 2002). Considering that NGF was strongly up-regulated by PgD_2 and that polymorphonuclear cells stain for NGF, it may be possible that NGF is involved in the endometrial inflammatory response during the menstrual cycle.

An elevated expression of NGF protein in the late secretory phase suggests a regulatory role for both progesterone and oestradiol in its expression, although isolated cell studies show it is primarily oestradiol that up-regulates gene expression. In cell cultures, *NGF* gene expression was similar in gland and stromal cells while in women, protein expression was highest in gland cells. For cell culture, cells were obtained from pre-menopausal women who were within the proliferative stage of the menstrual cycle where we show NGF protein levels to be low or absent.

Recent genome-wide identification did not show an oestrogen response element (ERE) in the human NGF promoter region (Bourdeau et al., 2004). However, the NGF promoter does

contain an AP-1 site (Zheng et al., 1988), from which the ligand bound oestrogen receptor may activate transcription (Paech et al., 1997; Jones et al., 1999). In rats, uterine NGF protein and mRNA expression is increased by oestradiol treatment (Krizsan-Agbas *et al.,* 2003) while in ovariectomised mice, both oestradiol and progesterone up-regulate this protein in the uterus (Bjorling *et al.,* 2002). In the present study *NGF* mRNA was increased by oestradiol but decreased by progesterone in human primary cell cultures. The fact that expression of NGF protein was high in the postmenopausal endometrium of women receiving tamoxifen, yet tamoxifen did not directly increase expression of *NGF* mRNA, suggests that tamoxifen may induce NGF protein expression by an indirect mechanism.

3.3.4 Gene Expression Changes in the Endometrium after Tamoxifen Treatment.

Identifying genes and their functions that are altered by tamoxifen will hopefully aid understanding of the mechanisms of tamoxifen action in the uterus. Clinically, tamoxifen treatment has variable effects on the endometrium, which may range from endometrial atrophy (Ugwumadu *et al.,* 1998) to endometrial hyperplasia of which glandular epithelial atrophy is a factor (Neis *et al.,* 2000). Overall, however, the weight of evidence suggests that in the endometrium, tamoxifen has an oestrogen agonist action and this contributes to a thickening of the endometrium and occasionally the generation of adenocarcinomas (IARC Monographs, 1996). Analysis of gene expression microarray data using GoMiner shows some of the most significant categories of gene function to be cell proliferation where expression of a majority of genes was increased. Further evidence to support the role of tamoxifen in cell proliferation comes from studies that show tamoxifen increase expression of cell proliferation markers. This effect that was associated with enhanced growth as confirmed by increased expression of oestrogen receptors and Ki-67, in addition to a high incidence of glandular hyperplasia (Mourits *et al.,* 2002; Tregon *et al.,* 2003). It is also of interest that GoMiner

analysis showed high significance of the TGFβ1 signalling pathway (GO:007179). This supports an earlier observation of endometrial dysplasia and glandular hyperplasia, in addition to significant elevation in gland-associated TGFβ1 protein (Carmichael *et al.,* 2000). The following discussion outlines a few additional genes involved in developmental processes, cell adhesion and the cytoskeleton that were thought to be relevant to endometrial changes after tamoxifen treatment.

Expression of the developmental gene that encodes the progestagen-associated endometrial protein, *PAEP*, was decreased in the endometrium of women receiving tamoxifen. This protein is secreted by glandular epithelium during the secretory phase of the menstrual cycle, and is thought to effect epithelial differentiation (Seppala *et al.,* 2001). Decreased expression of *PAEP* and *IGF1* (discussed later in this Section) have recently been shown to discriminate between normal and malignant endometrium (Mutter *et al.,* 2001). Another progesterone responsive gene identified using GoMiner was proenkephalin (*PENK*), in which expression decreases during the secretory phase (Ace *et al.,* 2004). In the present study, expression was strongly increased. Considering the changes in expression of *PENK* and *PAEP* seen in this study, it appears as though tamoxifen may have an anti-progestin effect on gene expression in the endometrium. Furthermore in terms of clinical symptoms, locally administered progestins in the uterus have been shown to protect against the oestrogen agonist effects in the endometrium of postmenopausal tamoxifen users (Gardner *et al.,* 2000). Whether alteration of the normal endometrial response to progestins is involved in tamoxifen pathogenicity would provide interesting further research.

Additionally, in the developmental group of genes, expression of the Nerve Growth Factor Receptor Associated Protein 1, *NGFRAP1* (*NADE*) was increased. This gene is involved in

p75 mediated NGF induced cell growth arrest and apoptosis. *NGF* expression increased in the uteri of adult CD-1 mice dosed with tamoxifen on days 2 to 5 after birth (Green *et al.*, 2003) and was suggested to be involved in adenomyosis. In this Chapter it was shown that oestradiol (but not tamoxifen) was able to increase expression of *NGF* mRNA in primary human endometrial stromal cell cultures. No change in *NGF* expression itself was identified in the present study using endometrial specimens from women receiving tamoxifen. This does however provide further support that increased NGF protein expression in the endometrium after tamoxifen is induced through an indirect mechanism.

It was of interest to note that there was no change after tamoxifen treatment in expression of classical tumour suppressor genes such as *PTEN* (Mutter *et al.*, 2001) or tumour growth regulators. In addition, the small number of oestrogen responsive genes identified in this study supports evidence that a purely oestrogen agonist action is unlikely to be primarily responsible for the effects of tamoxifen in the uterus (Carthew *et al.*, 1999).

There was, however, an increase in *IGF1* expression. Peptide growth factors, such as IGF1, are thought to be important mediators of estrogenic promotion of tumour growth. Receptors for this growth factor are expressed in stromal and epithelial cells (Tang *et al.*, 1994). Both oestradiol and tamoxifen increase *IGF1* expression in both mouse and human uterus (Elkas *et al.*, 1998; Moyano *et al.*, 2004; Stygar *et al.*, 2003). In addition, oestradiol and tamoxifen but not raloxifene increase expression in the Ishikawa endometrial carcinoma cell line (Shang *et al.*, 2002). Considering that raloxifene does not have oestrogen agonist action in the uterus (Wakeling ,2000), *IGF1* could be involved in the oestrogen agonist action of tamoxifen in the uterus. Also, supporting the role for progestins as described above, locally administered

progestins have been shown to increase expression of endometrial IGF1 binding proteins which modulate IGF1's action in the uterus (Pekonen *et al.*, 1992).

Recently, gene expression changes have been identified in isolated-cultured primary stromal and epithelial cells in response to treatment with tamoxifen and oestradiol for 24 h using Affymetix gene arrays (Pole *et al.*, 2005). In this study, tamoxifen led to most changes in gene expression in the glandular epithelium. A majority of genes in which expression was altered by tamoxifen in this study have a role in regulation of gene transcription, the cell cycle or signal transduction. The largest functional groups identified in the present study also included regulation of transcription and signal transduction but also apoptosis and cell adhesion.

In addition, Pole *et al.*, (2004) used short-term cultures of uterine epithelial cells to identify genes associated with tamoxifen treatment. In this system eight genes showed major transcriptional changes associated specifically with tamoxifen treatment. These were: RNA polymerase II, *POLR2J*; hydroxymethylbilame synthase, *HMBS*; Checkpoint suppressor 1, *CHES1*; Nuclear Factor I/X, *NFIX*; Aryl Hydrocarbon Receptor Interacting Protein-Like 1, *A1PL1*; 5,10-Methylenetetrahydrofolate Reductase, *MTHFR*; Stabilin 1, *STAB 1* and Myotubularin-Related Protein 2, *MTMR2*. Expression of none of these genes were significantly altered either in uterine tissues of women taking tamoxifen or in the tamoxifen treated neonatal mouse model (Chapter 5). The results of Pole *et al.*, (2004) were obtained using primary epithelial cell cultures treated for 24 h with tamoxifen. The results in the present study were from whole endometrial tissue taken from women receiving long term tamoxifen treatment, which may explain the differences in gene expression identified between these two studies.

Considering gene expression data presented here and also in published literature, there is evidence that tamoxifen affects expression of genes which are likely to be involved in the development of uterine pathologies. It is likely that there is variability between individual patients receiving tamoxifen in terms of uterine effects. In the patients used in this study no cancers were present, though polyps or fibroids were a common feature of the endometrium. The lack of expression of classical tumour promoting genes suggests that in these patients, tamoxifen may not be promoting endometrial cancers at the time of operation. However, the tamoxifen-induced gene expression changes presented here could contribute to the phenotypic changes observed in the postmenopausal tamoxifen endometrium.

In the present study whole endometrium samples were used, and it is recognised that the cell-specific response is likely to vary considerably. Further studies utilising laser capture microdissection may aid identify the cell specific response to tamoxifen. Additionally using this method, gene expression changes in endometrial pathologies after tamoxifen treatment could be normalised to areas of 'normal' endometrium from the same patient to minimise patient variability effects.

In conclusion, in this Chapter it was shown that NGF protein expression follows a cyclical pattern throughout the menstrual cycle, peaking during the secretory phase. mRNA expression increased in response to oestradiol and decreased in response to progesterone though, protein levels were undetectable. NGF levels were shown to increase in the endometrium of women receiving tamoxifen, although this drug was without effect on mRNA expression in either endometrial tissue or primary cell cultures. NGF expression in polymorphonuclear cells was described for the first time in the endometrium. It was suggested that NGF may play a role in the inflammatory response in the uterus.

Chapter 4.

The Uterotrophic Activity of SERMs in the Immature CD-1 Mouse.

4.1 Introduction.

4.1.1 Introduction to the Uterotrophic Assay.

Concern over the potential of certain chemicals to disrupt the normal endocrine system has intensified over recent years following reports of adverse effects on reproductive health in human and wildlife after exposure to endocrine disrupters (Baker, 2001). It is likely that that the oestrogenic activity of tamoxifen could contribute in its uterine toxicity. There is evidence that this effect is primarily mediated through ERα as tamoxifen has no oestrogen agonist action in the ERKO mouse uterus (Korach *et al.,* 1996). ER-independent pathways are also likely to be important in the oestrogenic response in the uterus as shown by involvement of WNT signalling (Hou *et al.,* 2004) and the induction of oestrogen responsive gene expression in the ERKO mouse uterus by 4-hydroxyoestradiol (Das *et al.,* 1997). There is a real need to screen compounds for oestrogenic or endocrine disrupting potential. The most commonly used short term *in vivo* screening models for the study of endocrine disrupters are the rodent uterotrophic assay in females and Hershberger assay in males (Kennel *et al.,* 2004).

The rodent uterotrophic assay is a successful model used in the study of oestrogen agonists or oestrogen antagonists in females (Gelbke *et al.,* 2004). The most commonly used end point is uterine weight, which increases in response to oestrogen agonists. Protocols used for the uterotrophic assay often vary although this is of fundamental importance (Odum *et al.,* 1997). A range of protocols have been used including rats or mice, and either immature or ovariectomised to avoid interference from endogenous oestrogens (Odum *et al.,* 1997). Rats have traditionally been used in toxicological studies, though the immature mouse is a comparable model to the rat in the uterotrophic assay (Padilla-Banks *et al.,* 2001). Additionally either oral or subcutaneous routes of administration have been used, with

subcutaneous being the most common. The effects of the route of administration may play an important role in the physiological response due to potential differences in metabolism.

The uterotrophic assay has been used in the study of a variety of endocrine disrupters. 17β-Oestradiol is often used as a reference compound and has been studied extensively including oral and subcutaneous dosing (Odum *et al.,* 1997). The uterotrophic responses of tamoxifen (Carthew *et al.,* 1999; Newbold *et al.,* 2001b) and 4-hydroxyestradiol (Barnea *et al.,* 1983; Franks *et al.,* 1982) have been studied though the protocols used vary widely between individual studies making it difficult to compare data.

4.1.2 Alternative Endpoints for the Uterotrophic Assay.

The classical end point in the uterotrophic assay is an increase in uterine weight. However additional endpoints have been used to enhance the sensitivity of the uterotrophic assay (Ashby, 2001; Newbold *et al.,* 2001b). Other physiological responses include the time to vaginal opening (Odum *et al.,* 1997), uterine epithelial cell height and gland number, (Jefferson *et al.,* 2002) or cell proliferation, as assessed by Proliferating Cell Nuclear Antigen (PCNA) or Ki-67 expression (Stygar *et al.,* 2003). These additional endpoints provide a greater understanding into the mechanisms of action of endocrine disrupters (Newbold *et al.,* 2001b). Gene and protein expression has more recently been used as a uterotrophic marker in the immature mouse model. These include induction of oestrogen responsive genes and proteins such as lactotransferrin and complement C3 (Padilla-Banks *et al.,* 2001), uterine heat shock proteins in ovariectomised adult mice (Papaconstantinou *et al.,* 2002) shown to be oestrogen responsive (Shyamala *et al.,* 1989), and transcriptional activation using the luciferase reporter assay (Jefferson *et al.,* 2002). More recently cDNA microarrays have been

used to further understand the transcriptional profile of the uterotrophic response (Moggs *et al.*, 2004b).

4.1.3 Chapter Aims.

The primary aim of the work described in this Chapter was to establish a maximal uterotrophic dose of oestradiol, 4-hydroxyoestradiol and tamoxifen using the same oral dosing protocol. As most previously published work has been done using subcutaneous dosing, we also wanted to compare our oral dosing model with other published literature where subcutaneous dosing is generally used. Assays employed to study the oestrogenic response were:

- Uterotrophic assay,
- Cell proliferation within the endometrial epithelium,
- Gene expression studies.

Data obtained using the above assays were used to decide a maximal oral uterotrophic dose of each compound which was then used in Chapter 5 for neonatal dosing studies.

4.2 Results.

4.2.1 Immature CD-1 Mouse Uterotrophic Assays.

a. Oral Dosing.

To determine the maximal uterotrophic doses of oestradiol, 4-hydroxyoestradiol and tamoxifen, a standard uterotrophic assay in 14 day old CD-1 mice was used (Newbold *et al.*, 2001b; Shelby *et al.*, 1996) using oral dosing. Mice were dosed each day on days 14, 15 and 16 after birth then they were killed and uteri weighed on day 17, 24 h after the last dose. There were at least four mice per dosed group, larger numbers of animals were used in some groups to obtain tissues for other experiments. Control mice received vehicle only, and control data shown are pooled from all control animals in similar experiments.

Results for this Section are shown in Figures 4.1, 4.2, and 4.3 and summarised in Table 4.1. Oestradiol, 4-hydroxyoestradiol and tamoxifen all had a positive uterotrophic effect. Uterotrophic assays show that at the maximal uterotrophic dose, 4-hydroxyoestradiol has the greatest potential to increase uterine weight out of the compounds tested. The rank order of uterotrophic potentials was 4-hydoxyoestradiol > oestradiol > tamoxifen. For oral dosing the following dose levels gave the maximal uterotrophic response:

- Oestradiol 100 μg/kg
- 4-Hydroxyoestradiol 386 μg/kg
- Tamoxifen 250 μg/kg

Oral dosing generally did not have any statistically significant effect on body weight in comparison to control animals. The exceptions to this was at 10 μg/kg oestradiol where body weight was increased and, 193 and 386 μg/kg 4-hydroxyoestradiol leading to a decrease in weight. Though statistically significant ($p < 0.05$), for oestradiol at least, this effect was not repeatable in a similar study (Section 4.2.3).

	Compound	Maximal Uterotrophic Dose		n =	Uterine Weight at Max. Uterotrophic Dose (mg ± SE)
		µg/kg/day	µmoles/kg/day		
Oral	Control	0	0	50	12.46 ± 0.76
	Oestradiol	100	0.26	24	40.72 ± 2.90 *
	4-Hydroxyoestradiol	386	1.34	11	97.29 ± 10.11 *
	Tamoxifen	250	0.67	12	39.95 ± 2.07 *
SC	Control	0	0	4	15.42 ± 1.07
	Oestradiol	100	0.26	4	54.62 ± 4.25 *
	Tamoxifen	250	0.67	4	37.9 ± 2.41 *

Table 4.1: Summary of Maximal Uterotrophic Doses.

SC refers to subcutaneous dosing. n = refers to the number of animals used. * Uterine weights significantly different from controls ($p < 0.05$) using ANOVA and Dunnetts comparison with a control. Control animals received vehicle only.

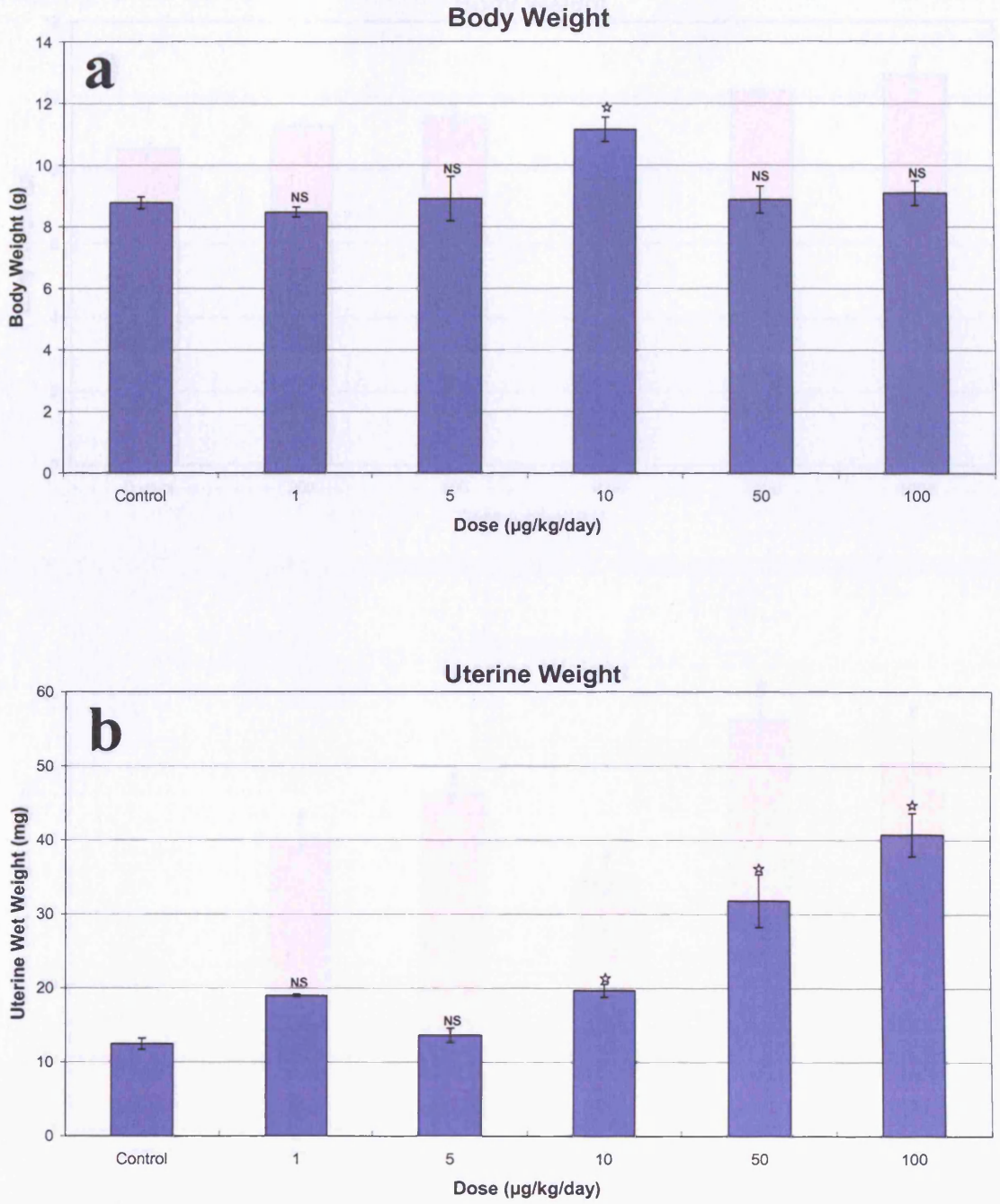


Figure 4.1: Oral Oestradiol Uterotrophic Assay.

a. Body weights (g) ± SE. Control (n = 50), 1 µg/kg (n = 4), 5 µg/kg (n = 4), 10 µg/kg (n = 4), 50 µg/kg (n = 4) and 100 µg/kg (n = 24). **b**. Uterine wet weights (mg ± SE) following oral oestradiol at 1, 5, 10, 50 and 100 µg/kg. Probability of significant difference from control: $p < 0.05$ ☆, **NS** not significant.

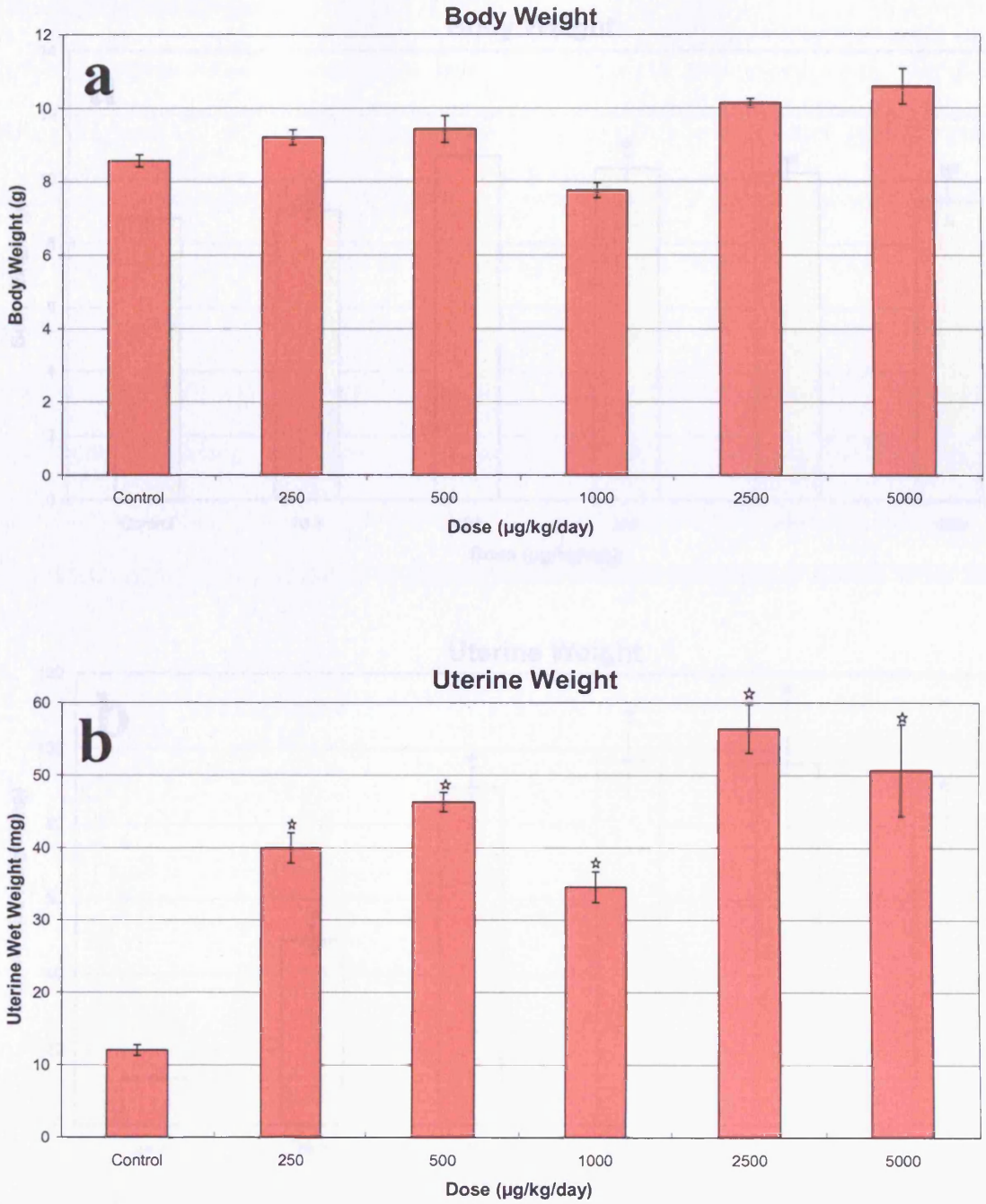


Figure 4.2: Oral Tamoxifen Uterotrophic Assay.

a. Body weights (g) ± SE. Control (n = 50), 250 µg/kg (n = 12), 500 µg/kg (n = 4), 1000 µg/kg (n = 4), 2500 µg/kg (n = 4) and 5000 µg/kg (n = 4). **b.** Uterine weights (mg) ± SE following oral tamoxifen at 250, 500, 1000, 2500 and 5000 µg/kg. Probability of significant difference from control: ☆ $p < 0.05$, **NS** not significant.

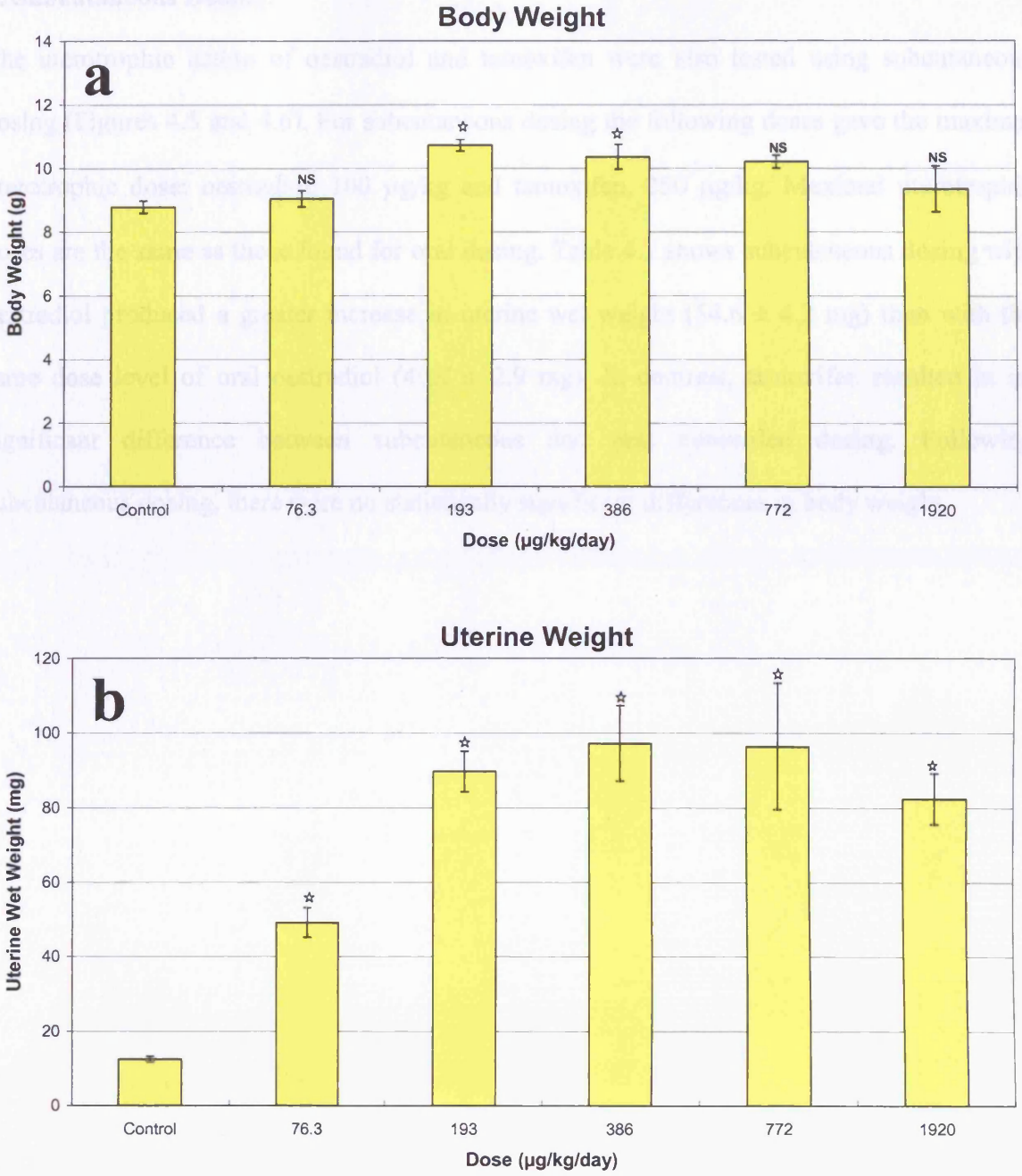


Figure 4.3: Oral 4-Hydroxyoestradiol Uterotrophic Assay.

a. Body weights (g) ± SE. Control (n = 50), 76.3 µg/kg (n = 4), 193 µg/kg (n = 3), 386 µg/kg (n = 11), 772 µg/kg (n = 4) and 1920 µg/kg (n = 4). **b**. Uterine weights (mg) ± SE following oral 4-hydroxyoestradiol at 76.3, 193, 386, 772 and 1920 µg/kg. Probability of significant difference from control: ☆$p < 0.05$, **NS** not significant.

b. Subcutaneous Dosing.

The uterotrophic action of oestradiol and tamoxifen were also tested using subcutaneous dosing (Figures 4.5 and 4.6). For subcutaneous dosing the following doses gave the maximal uterotrophic dose: oestradiol, 100 μg/kg and tamoxifen, 250 μg/kg. Maximal uterotrophic doses are the same as those found for oral dosing. Table 4.1 shows subcutaneous dosing with oestradiol produced a greater increase in uterine wet weight (54.6 ± 4.2 mg) than with the same dose level of oral oestradiol (40.7 ± 2.9 mg). In contrast, tamoxifen resulted in no significant difference between subcutaneous and oral tamoxifen dosing. Following subcutaneous dosing, there were no statistically significant differences in body weight.

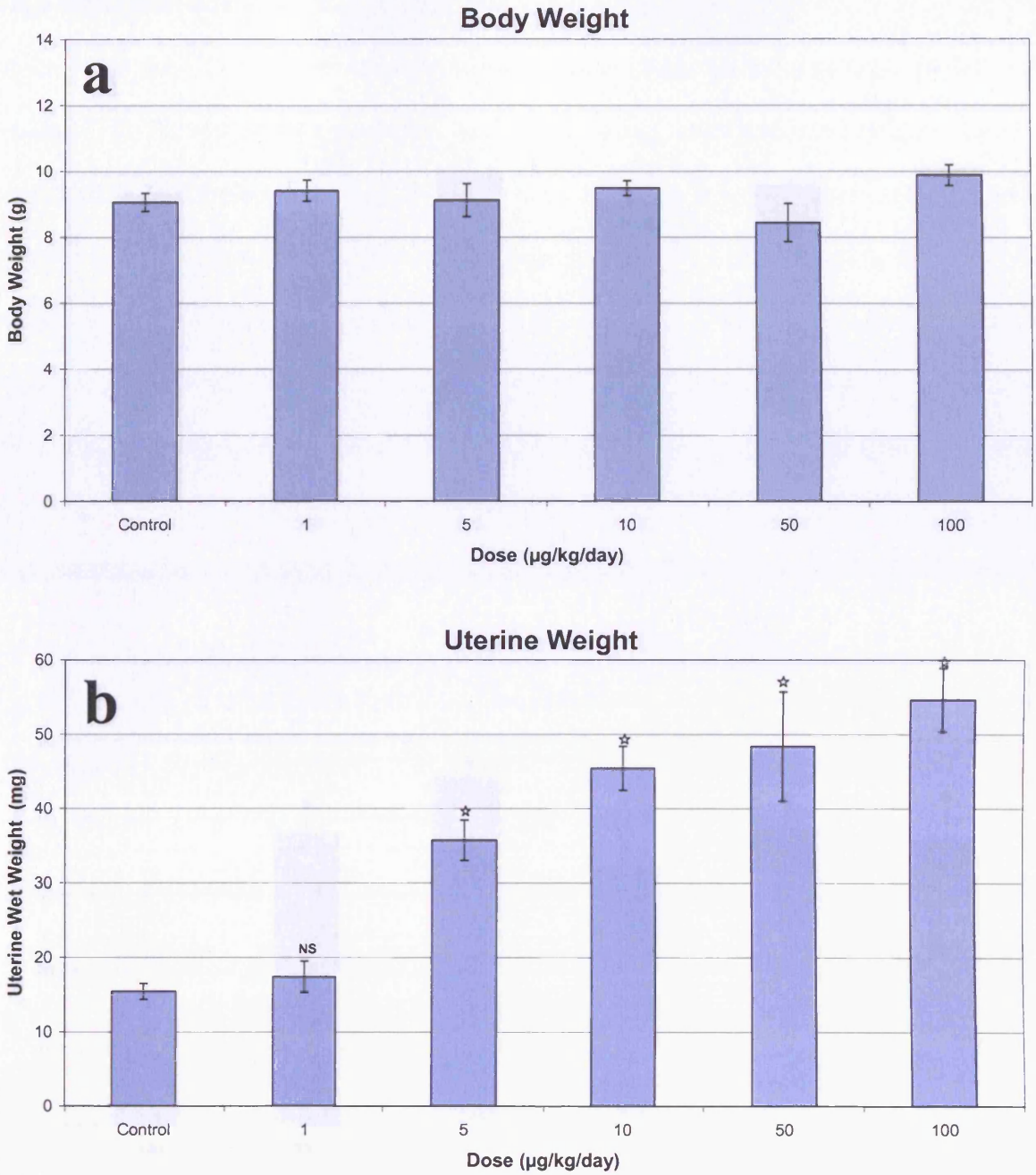


Figure 4.5: Subcutaneous Oestradiol Uterotrophic Assay.

a. Body weights (g) ± SE. Control (n = 8), 1 µg/kg (n = 4), 5 µg/kg (n = 4), 10 µg/kg (n = 4), 50 µg/kg (n = 4) and 100 µg/kg (n = 4). **b**. Uterine weights (mg) ± SE following subcutaneous oestradiol at 1, 5, 10, 50 and 100 µg/kg. Probability of significant difference from control: $p < 0.05$ ☆, **NS** not significant.

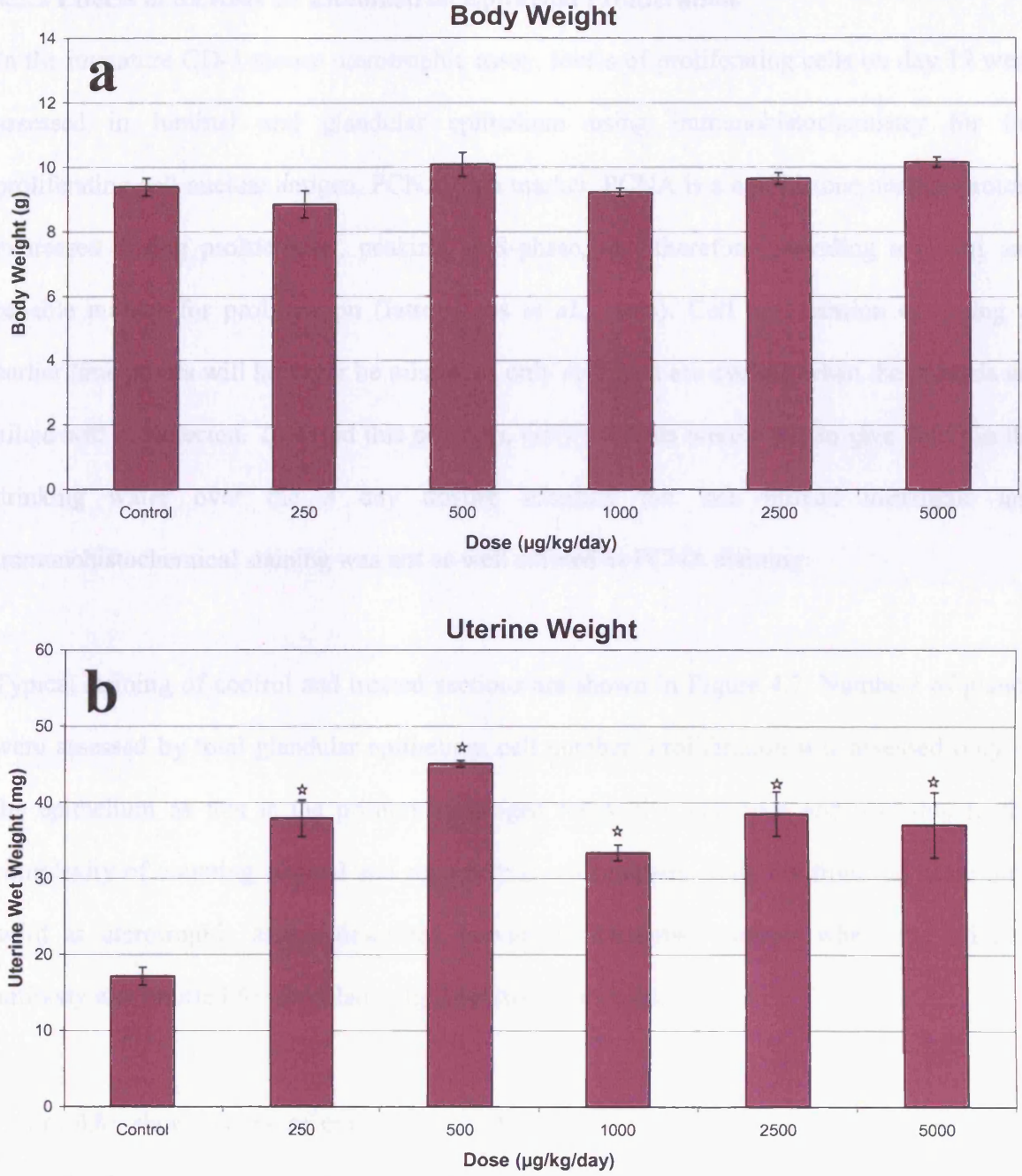


Figure 4.6: Subcutaneous Tamoxifen Uterotrophic Assay.

a. Body weights (g) ± SE. Control (n = 8), 250 µg/kg (n = 4), 500 µg/kg (n = 3), 1000 µg/kg (n = 4), 5200 µg/kg (n = 4) and 5000 µg/kg (n = 4). **b**. Uterine weights (mg) ± SE following subcutaneous tamoxifen at 250, 500, 1000, 2500 and 5000 µg/kg. Probability of significant difference from control: ☆ $p < 0.05$, **NS** not significant.

4.2.2 Effects of SERMs on Endometrial Epithelial Proliferation.

In the immature CD-1 mouse uterotrophic assay, levels of proliferating cells on day 17 were assessed in luminal and glandular epithelium using immunohistochemistry for the proliferating cell nuclear antigen, PCNA, as a marker. PCNA is a non-histone nuclear protein expressed during proliferation, peaking at S-phase, and therefore providing a useful and reliable marker for proliferation (Iatropoulos *et al.,* 1996). Cell proliferation occurring at earlier time points will however be missed as only cells that are cycling when the animals are killed will be detected. To avoid this problem, early attempts were made to give BrdU in the drinking water over the 3 day dosing schedule but this proved unreliable and immunohistochemical staining was not as well defined as PCNA staining.

Typical staining of control and treated sections are shown in Figure 4.7. Numbers of glands were assessed by total glandular epithelium cell number. Proliferation was assessed only in the epithelium as this is the primary oestrogen responsive cell type and also due to the complexity of counting stromal and myometrial cell numbers. Data are from the same time point as uterotrophic assays described previously. Negative controls where the primary antibody was omitted for the relative IgG control were negative.

Figure 4.8a shows doses of oral oestradiol between 5 and 100 μg/kg. Proliferation in the luminal epithelium decreased relative to controls, but this dosing had no significant effect on total cell number. All doses of oestradiol caused a significant decrease in both total and proliferating glandular epithelium cell number relative to controls (Figure 4.8b). This suggests that oral oestradiol inhibits proliferation of glandular epithelium in neonatal animals, assessed on day 4 after dosing.

In the case of mice dosed orally with tamoxifen, the highest dose of oral tamoxifen (5000 μg/kg) caused a significant reduction on both total cell number and proliferation in the luminal epithelium relative to controls (Figure 4.9a). Both low doses of tamoxifen (250 to 500 μg/kg) caused an increase in total glandular epithelium cell number, although this was only significant in mice receiving 500 μg/kg. However, no effect on proliferation was seen in terms of PCNA staining (Figure 4.9b).

Figure 4.10a shows that in mice receiving 4-hydroxyoestradiol, four out of five doses lead to a significant increase in proliferation within the luminal epithelium, but had no effect on total cell number. 4-Hydroxyoestradiol treatment does not result in any statistically significant change in total glandular epithelial cell number at any dose level (Figure 4.10b).

Results suggest that increase in uterine weight assessed during the uterotrophic assay is not due to increase in cell number or proliferation, as determined on day 4 after treatment. It is possible that cell proliferation may occur at an earlier time point although time course studies were not carried out to establish this.

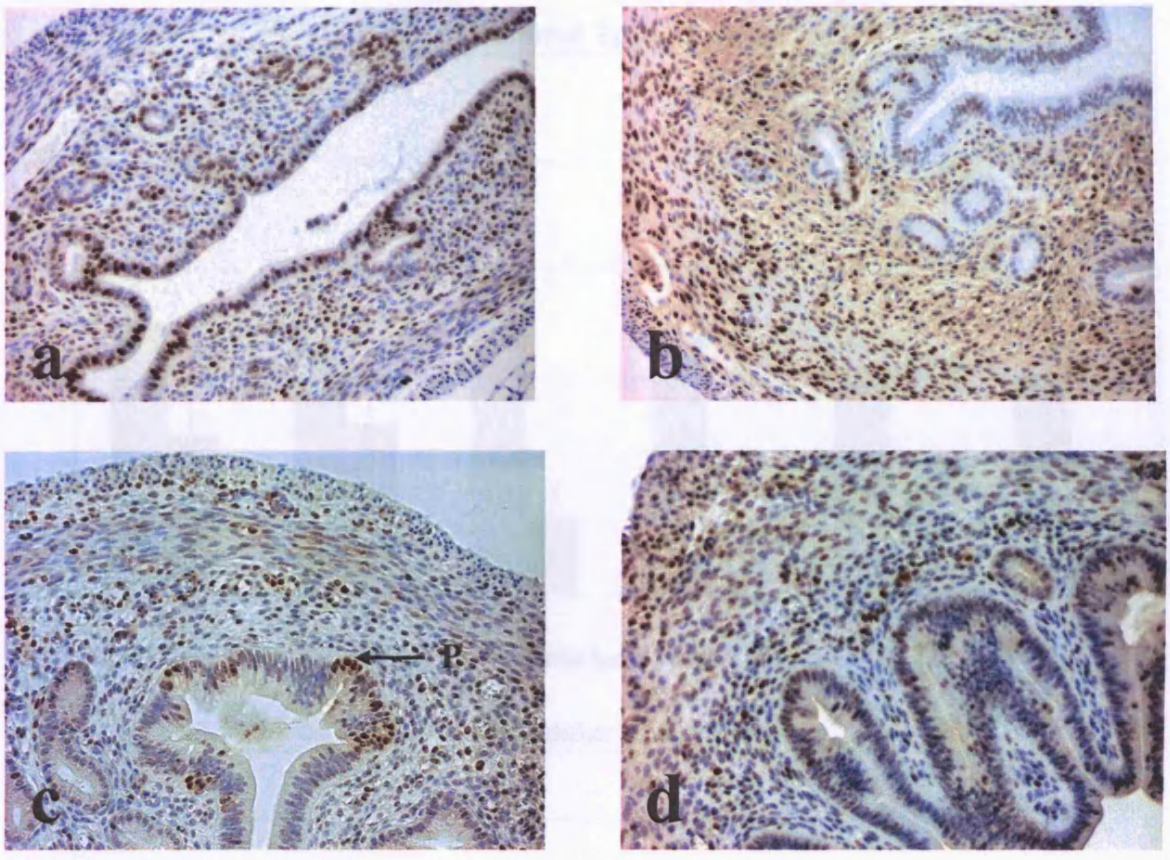


Figure 4.7: PCNA Immunohistochemistry as Uterotrophic Endpoint.

Representative images used for assessment of cell proliferation in luminal and glandular epithelium by PCNA immunohistochemistry. **a**. Vehicle only control; **b**. 100 μg/kg oestradiol; **c**. 250 μg/kg tamoxifen showing PCNA stained cells (**P**); **d**. 386 μg/kg 4-hydroxyoestradiol. All pictures original magnification x 10.

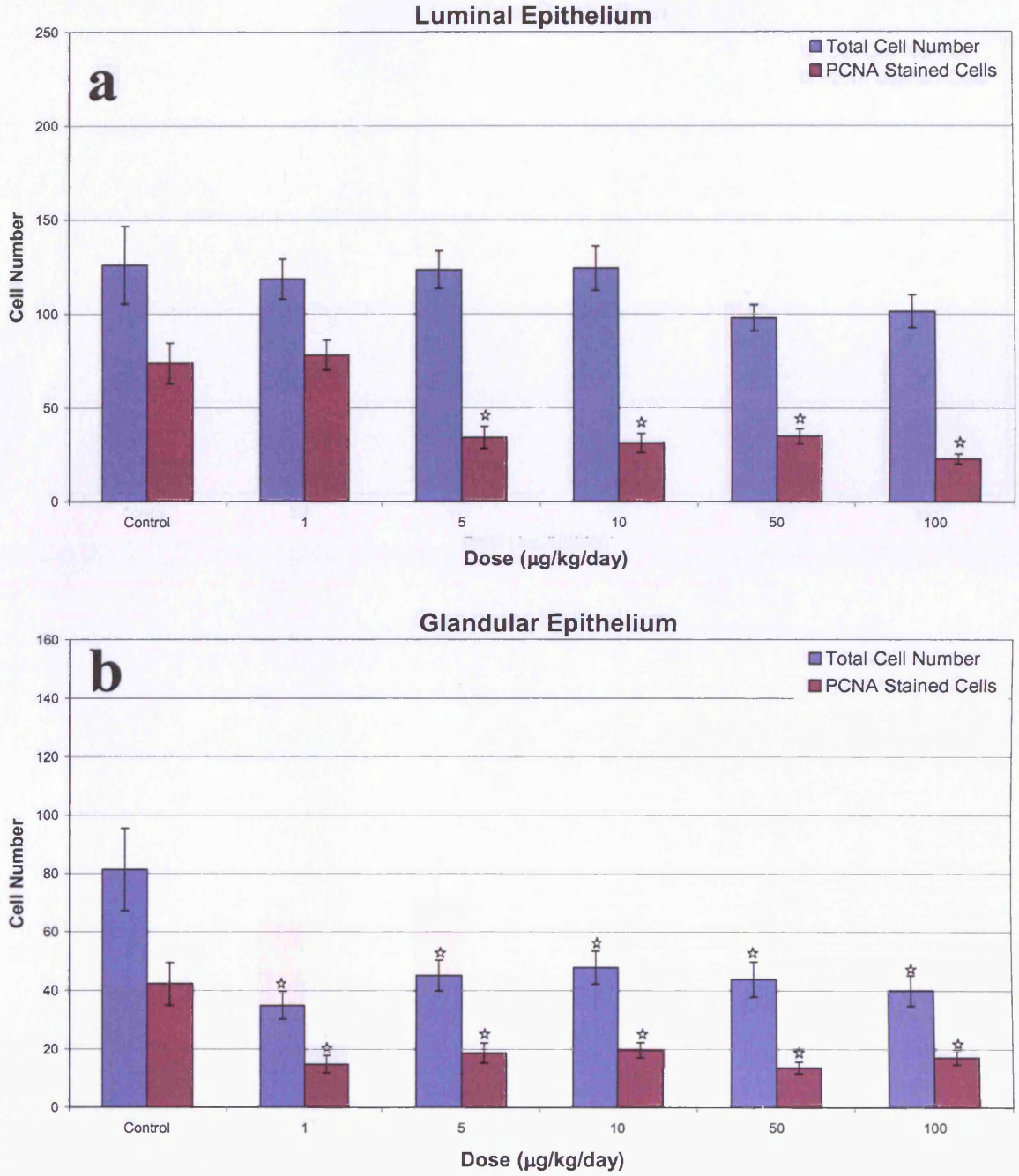


Figure 4.8: Oral Oestradiol PCNA Labelling in Endometrial Epithelium.

a. PCNA Staining within the luminal epithelium (± SE), and, **b**. glandular epithelium (± SE) in comparison to total number of epithelial cells counted per field. Each data point is the mean count from 10 individual fields from 4 animals per dose. ☆ Significant difference from control ($p < 0.05$).

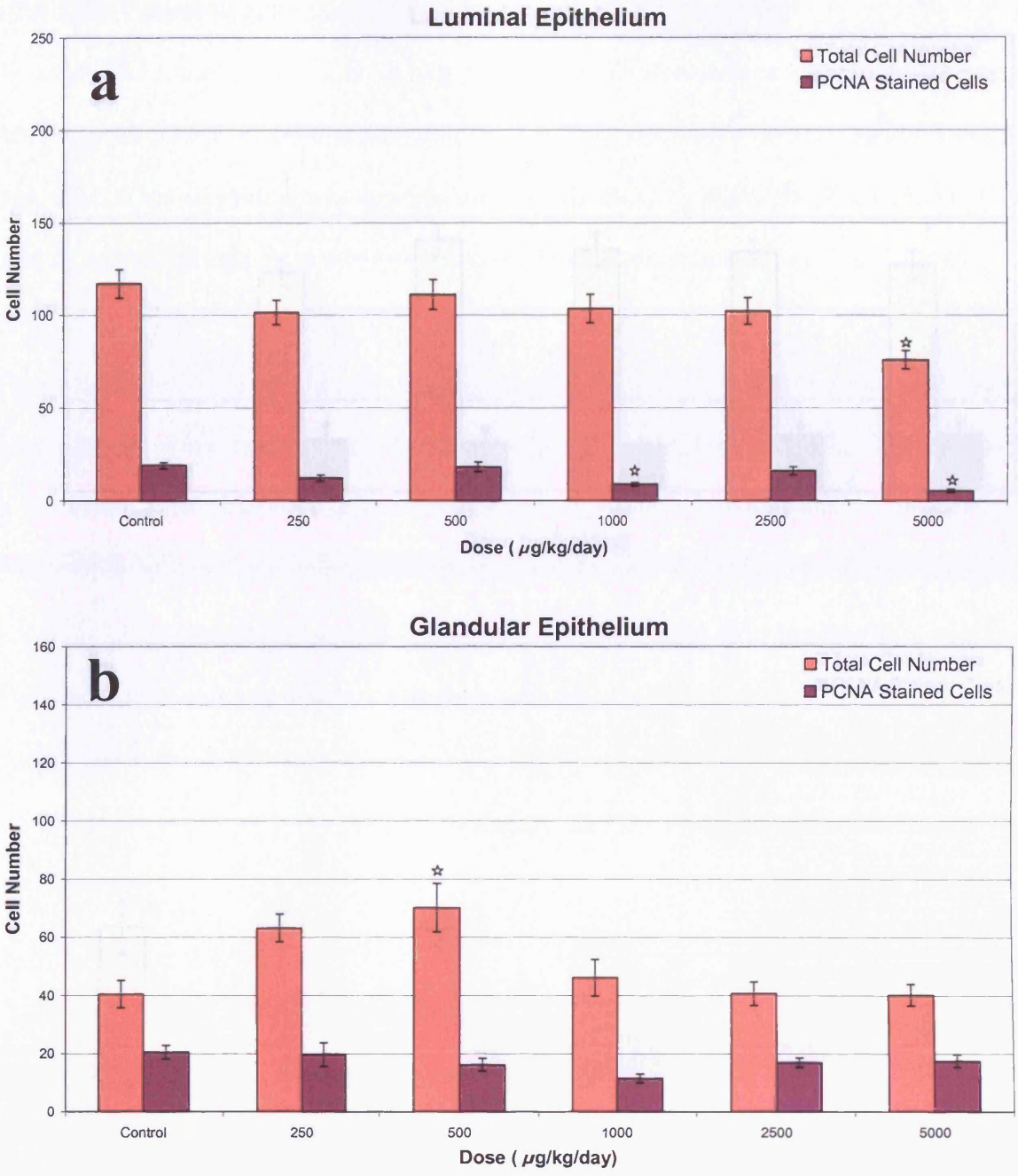


Figure 4.9: Oral Tamoxifen PCNA Labelling in Endometrial Epithelium.

a. PCNA Staining within the luminal epithelium (± SE), and, **b**. glandular epithelium (± SE) in comparison to total number of epithelial cells counted per field. Each data point is the mean count from 10 individual fields from 4 animals per dose. ☆Significant difference from control ($p < 0.05$).

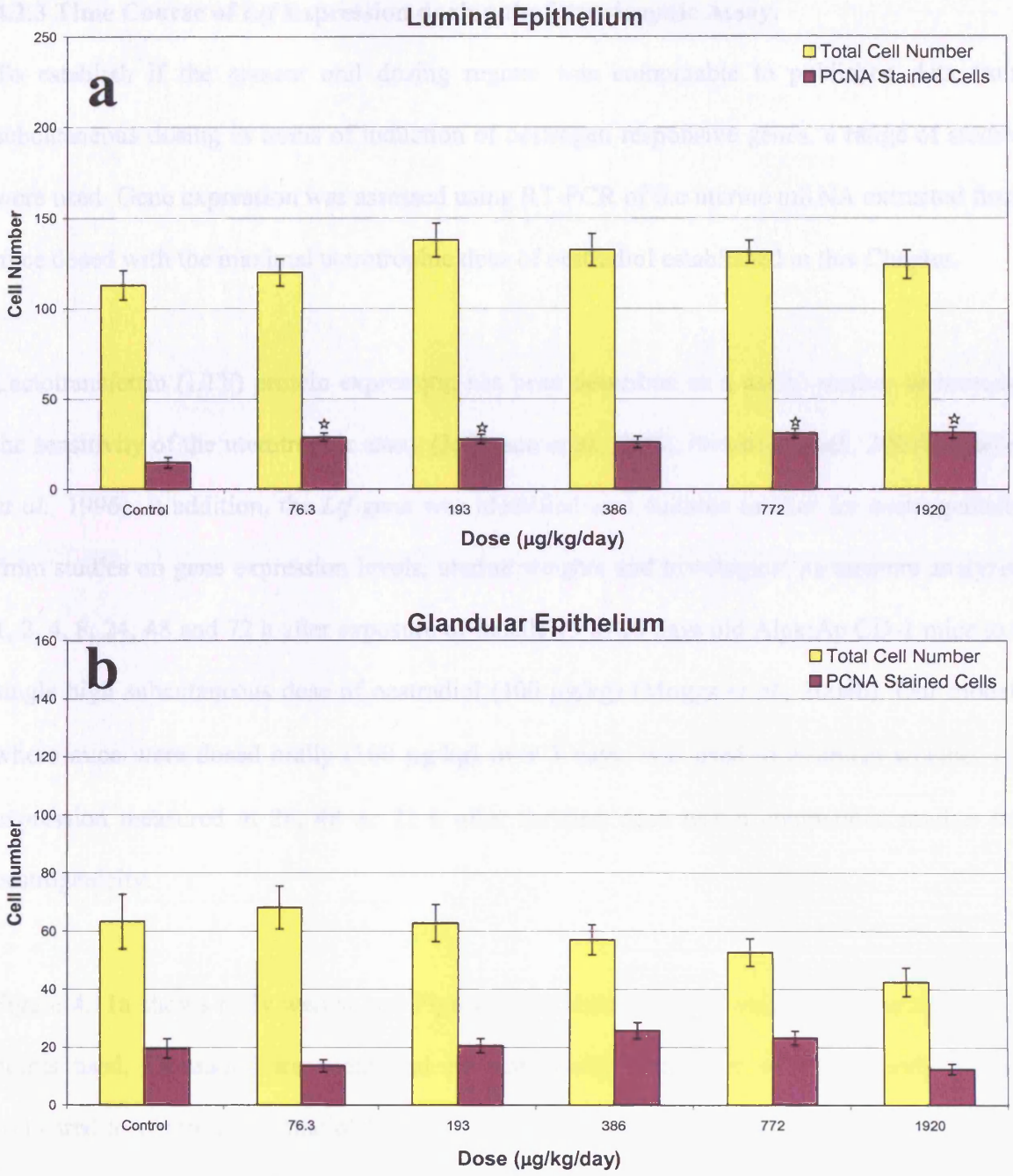


Figure 4.10: Oral 4-Hydroxyoestradiol PCNA Labelling in Endometrial Epithelium.

a. PCNA Staining within the luminal epithelium (± SE), and, **b**. glandular epithelium (± SE) in comparison to total number of epithelial cells counted per field. Each data point is the mean count from 10 individual fields from 4 animals per dose group. ☆ Significant difference from control ($p < 0.05$).

4.2.3 Time Course of *Ltf* Expression during the Uterotrophic Assay.

To establish if the present oral dosing regime was comparable to published data from subcutaneous dosing in terms of induction of oestrogen responsive genes, a range of studies were used. Gene expression was assessed using RT-PCR of the uterine mRNA extracted from mice dosed with the maximal uterotrophic dose of oestradiol established in this Chapter.

Lactotransferrin (LTF) protein expression has been described as a useful marker to increase the sensitivity of the uterotrophic assay (Jefferson *et al.,* 2002; Newbold *et al.,* 2001b; Shelby *et al.,* 1996). It addition, the *Ltf* gene was identified as a suitable marker for oestrogenicity from studies on gene expression levels, uterine weights and histological parameters analyzed 1, 2, 4, 8, 24, 48 and 72 h after exposure of female 19 to 20 days old Alpk:Ap$_f$CD-1 mice to a single high subcutaneous dose of oestradiol (400 μg/kg) (Moggs *et al.,* 2004b). Our model, where mice were dosed orally (100 μg/kg) over 3 days, was used to establish whether *Ltf* expression measured at 24, 48 or 72 h after the first dose was a comparable marker for oestrogenicity.

Figure 4.11a shows body weight and Figure 4.11b shows uterine weight over the three time points used. Oestradiol treatment had no statistically significant effect on body weight compared to controls. Oestradiol led to a significant increase in uterine weight compared to time matched controls. Maximal increase in uterine weight was achieved by 72 h, consistent with previous findings in this Chapter. Figure 4.12b shows induction of *Ltf* was significantly greater than that of control animals at each time point. Expression increased significantly on each day compared to the previous day. Out of the time points used in the present study, both uterine weight and *Ltf* expression was highest 24 hours after the last dose (72 h after the first dose).

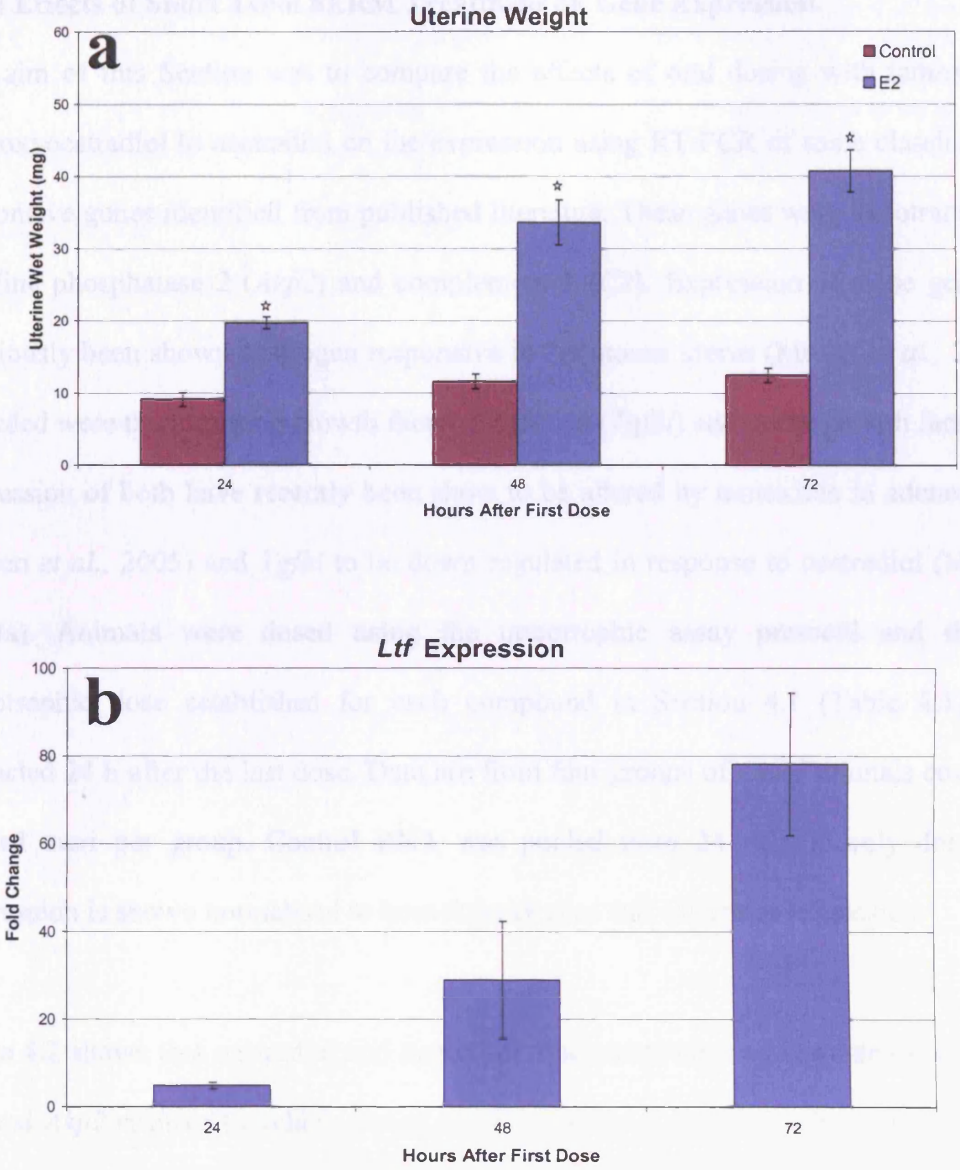


Figure 4.11: Oral Oestradiol Uterotrophic Weight and *Ltf* Expression.

Mice received 100 µg/kg oral oestradiol on days 14, 15 and 16 after birth. **a**. Uterine weights (mg) ± SE. Numbers of mice used were: 24 h n = 4; 48 h n = 4; 72 h controls n = 50 and dosed n = 24. ☆ Significant difference from control ($p<0.05$). **b**. Real time PCR analysis of *Ltf* expression relative to time-matched vehicle controls and normalised to housekeeper (*Gapdh*) gene expression in the endometrium. Each data point is the mean fold change (± SE).

4.2.4 Effects of Short Term SERM Treatment on Gene Expression.

The aim of this Section was to compare the effects of oral dosing with tamoxifen and 4-hydroxyoestradiol to oestradiol on the expression using RT-PCR of some classical oestrogen responsive genes identified from published literature. These genes were lactotransferrin (*Ltf*), alkaline phosphatase 2 (*Akp2*) and complement 3 (*C3*). Expression of these genes have all previously been shown oestrogen responsive in the mouse uterus (Moggs *et al.*, 2004a). Also included were transforming growth factor-β induced (*Tgfbi*) and nerve growth factor-β (*Ngfb*), expression of both have recently been show to be altered by tamoxifen in adenomyotic uteri (Green *et al.*, 2005) and *Tgfbi* to be down regulated in response to oestradiol (Moggs *et al.*, 2004a). Animals were dosed using the uterotrophic assay protocol and the maximal uterotrophic dose established for each compound in Section 4.1 (Table 4.1) and RNA extracted 24 h after the last dose. Data are from four groups of dosed animals containing two pooled uteri per group. Control RNA was pooled from 24 vehicle only dosed animals. Expression is shown normalised to housekeeper gene and calibrator expression.

Table 4.2 shows that oestradiol and tamoxifen treatments both up regulate expression of *Ltf*, *C3* and *Akp2* relative to vehicle dosed controls, with *Ltf* expression being the most strongly induced. 4-hydroxyoestradiol increased expression of *Ltf* and *Akp2*, but was without effect on *C3* expression at this time point. Oestradiol, 4-hydroxyoestradiol and tamoxifen treatments led to the down regulation of expression of *Tgfbi* and *Ngfb* with oestradiol having the greatest effect. These data show that in terms of a selection of oestrogen responsive genes, all three compounds tested have a similar direct effect on gene transcription in the uterus with differences only in intensity of expression.

Gene	Treatment			
	Control	**Oestradiol**	**Tamoxifen**	**4-Hydroxyoestradiol**
Ltf	0.05 ± 0.005	3.47 ± 0.72	1.30 ± 0.06	8.22 ± 1.88
C3	0.09 ± 0.03	0.20 ± 0.07	1.88 ± 0.31	Not detectable
Akp2	0.29 ± 0.15	1.90 ± 0.33	1.50 ± 0.18	1.71 ± 0.3
Tgfbi	9.56 ± 5.18	1.08 ± 0.02	2.20 ± 0.2	1.36 ± 0.33
Ngfb	3.50 ± 1.14	1.09 ± 0.21	1.23 ± 0.2	2.52 ± 0.27

Table 4.2: Changes in Gene Expression in the Mouse Uterus.

Gene expression changes were measured 24 h after 3 daily doses of SERMs. Doses are: oestradiol 100 μg/kg/day, 4-hydroxyoestradiol 386 μg/kg/day and tamoxifen 250 μg/kg/day. Gene expression was determined by RT-PCR normalized to *Gapdh* housekeeping gene and calibrator. Data are the mean ± SE (n = 4) , with PCR repeated in at least duplicate.

4.3 Discussion.

4.3.1 Uterotrophic Assays.

A maximal uterotrophic dose of 100 μg/kg oral oestradiol was identified where uterine weights were 40 mg. The maximal uterine weight induced by 4-hydroxyoestradiol was *circa* 100 mg. It is probable that oestradiol if tested at higher doses than the top dose used in this study (100 μg/kg) would induce uterine weights to a similar level as 4-hydroxyoestradiol. Therefore 100 μg/kg oestradiol dose not represent a true maximal uterotrophic dose as was identified for tamoxifen and 4-hydroxyoestradiol.

The finding that 4-hydroxyoestradiol gave the highest uterotrophic response was consistent with previously published data (Newbold *et al.,* 2000), in 1 day old mice dosed with 2 μg/kg of oestradiol or 4-hydroxyoestradiol. In the present study the maximal uterotrophic dose of oral 4-hydroxyoestradiol was *circa* three fold higher than that of oral oestradiol (386 μg/kg and 100 μg/kg, respectively). Even at the lowest dose of 4-hydroxyoestradiol (76.3 μg/kg), uterine weight was still slightly higher than that at obtained with maximal dose of 100 μg/kg oestradiol. These doses of oestradiol and 4-hydroxyoestradiol were equimolar (0.26 μmoles/kg/day).

4-hydroxyoestradiol has been shown to associate with ERα in MCF-7 cells with 1.5 fold greater relative affinity than oestradiol (Van Aswegen *et al.,* 1989) although they both have similar dissociation rates from ERα (Wawrzak *et al.,* 1988), suggesting that they form an equally stable complex. Thus altered receptor binding seems unlikely to solely explain the strong oestrogen agonist action of 4-hydroxyoestradiol. However, 4-hydroxyoestradiol but not oestradiol, has been shown to increase uterine weight and induce oestrogen responsive gene expression in ERKO mice (Das *et al.,* 1997). This indicates that 4-hydroxyoestradiol

functions via both oestrogenic and non-oestrogenic pathways, which may account for the increased uterotrophic response seen by this compound.

The uterotrophic activity of tamoxifen in rodents has been well documented, using subcutaneous dosing and a variety of protocols including ovariectomised mice (Carthew *et al.,* 1999) and rat (Carthew *et al.,* 2000; Stygar *et al.,* 2003), and in immature mice (Carthew *et al.,* 2000; Newbold *et al.,* 2001b; Papaconstantinou *et al.,* 2002). Often this involves only a single dose level of tamoxifen in contrast to the range of doses used in our study. For tamoxifen, 250 μg/kg was selected as the maximum uterotrophic oral dose as assessed by uterine weights. Using the same strain of immature mice as used in the present study, 1 mg/kg was found to be the maximal uterotrophic dose using subcutaneous dosing (Newbold *et al.,* 2001b), and 400 μg/kg to be the maximal uterotrophic dose using subcutaneous dosing in ovariectomised mice (Carthew *et al.,* 1999).

Though studies using subcutaneous dosing provide insight into the actions of tamoxifen, women take tamoxifen orally and there are likely to be differences in metabolism of the drug by the two routes. In the present study there were only minor differences in the uterotrophic response to tamoxifen after oral or subcutaneous dosing. However, subcutaneous oestradiol treatment produced a considerably higher uterotrophic response than oral oestradiol. It is well established that oestradiol is well absorbed orally but undergoes extensive first-pass metabolism in the liver resulting in the production of less potent metabolites such as oestrone and oestrone sulphate (Grow, 2002). It is likely that oestradiol undergoes more extensive metabolism effects via the oral route than subcutaneous. To our knowledge, this is the first study where the uterotrophic effects of oral and subcutaneous tamoxifen and oestradiol have been directly compared in the immature CD-1 mouse.

4.3.2 Proliferation Assays.

Primarily it was found that proliferation or cell number in the endometrial epithelium did not correlate with uterine weight assessed over the same time frame. This supports the findings of Carthew *et al.,* (1999) who found that stromal BrdU labelling did not predict the uterotrophic effects of tamoxifen. In contrast, Newbold *et al.,* (2001) observed that oestradiol and tamoxifen led to increases in both PCNA and BrdU markers 18 h after a single subcutaneous dose in 17 day old CD-1 mice.

The present data show that oestradiol leads to a decrease in proliferation and cell number in the endometrial glandular epithelium and at higher doses in the luminal epithelium. Administration of subcutaneous oestrogens on postnatal days 10 to 14 in rats is known to delay the onset of gland genesis (Branham *et al.,* 1985), with a similar effect being apparent in the present study after oral administration on postnatal days 14, 15 and 16 in CD-1 mice.

Tamoxifen also lead to a decrease in proliferation in the luminal epithelium at 1000 and 5000 μg/kg, and an increase in glandular epithelium cell number at 500 μg/kg. As there is an increase in cell number without PCNA labelling, it is likely that proliferation has already occurred leading to a higher cell number. Recently published work has identified that the peak of proliferation in the luminal epithelium occurs at 24 to 36 h after a single dose of tamoxifen (Zhang *et al.,* 2005), suggesting that in our study tamoxifen induced proliferation in the luminal epithelium occurred at an earlier time point.

To our knowledge this is the first study of proliferation in the endometrium in response to 4-hydroxyoestradiol treatment. As mentioned above, this compound was the most effective compound of those tested in terms of increasing uterine weight over the 3-day period.

However, this compound caused no significant effects within the glandular epithelium. Four out of five doses increased proliferation in the luminal epithelium though total cell number was not affected. These results suggest that the large increases in uterine weight after 4-hydroxyoestradiol are more likely due to hypertrophy than hyperplasia. Taking both these results and the large increase in uterine weight observed, the results suggest that 4-hydroxyoestradiol has a more powerful uterotrophic effect than oestradiol. This is supported by the finding that 4-hydroxyoestradiol increased luminal epithelial PCNA staining or had no effect (glandular epithelium) whereas oestradiol treatment generally decreased luminal and glandular epithelial PCNA staining. Alternatively, the potential chemical reactivity of 4-hydroxyoestradiol (Devanesan *et al.,* 2001), may enhance the ability of this compounds to bind oestrogen responsive sites. It would be interesting to establish if 4-hydroxytamoxifen, the putative reactive metabolite of tamoxifen (White, 2003), has a greater effect on the mouse uterus than tamoxifen.

4.3.3 Gene Expression Studies.

Recently, it has been shown that the oestrogenic compounds oestradiol, diethylstilboestrol and genistein induce a comparable transcriptional response in the immature mouse up to 72 h after a single subcutaneous dose (Moggs *et al.,* 2004a). In this Thesis, oral dosing was used, which is arguably more clinically relevant. Due to differences in the metabolism of a compound between oral and subcutaneous dosing, it is not unreasonable to expect a difference in physiological effect observed, as shown in this Chapter by the difference in oestrogen agonist activity for oral and subcutaneous oestradiol in the uterotrophic assay. An aim of this Chapter was to asses if oral dosing produced a response comparable to Moggs *et al.,* (2004a) between oestradiol, 4-hydroxyoestradiol and tamoxifen.

It was shown that oestradiol induced an increase in *Ltf* expression that was accompanied by increases in uterine weight. In addition, *Ltf* expression was a good indicator of the level of response, as expression was highest in mice dosed with 4-hydroxyoestradiol (Table 4.3), which also led to the greatest increase in uterine weight. In this study, *Ltf* expression increased in a comparable pattern to that observed by Moggs *et al.*, (2004b) where *Ltf* gene expression peaked 48 to 72 h after a single subcutaneous dose, showing that in terms of a classical oestrogen responsive gene, the timing of the expression response is not affected by the route of dosing. Other oestrogen responsive genes such as *c-fos*, would be less likely to correlate with uterine weight as expression peaks 1 to 6 h after oestradiol administration (Bigsby *et al.*, 1994; Moggs *et al.*, 2004b).

Both ERα and ERβ are expressed at similar levels in the immature mouse uterus (Weihua *et al.*, 2000) although ERα is the predominant mediator of the oestrogenic response. Non-ERα mediated pathways may be also play a vital role in the oestrogenic response, as shown in the ERKO uterus after treatment with 4-hydroxoestradiol (Das *et al.*, 1997). In this Chapter we show that oestradiol, 4-hydroxyoestradiol and tamoxifen have similar effects on expression of a selection of oestrogen responsive genes. The only exception was the lack of *C3* expression in response to 4-hydroxyoestradiol. 4-hydroxyoestradiol was shown in this Chapter to lead to the greatest increase in uterine weight and strongly increases expression of *Ltf* and *Akp2*. The lack if response observed with *C3* suggests that regulation of *C3* expression may involve complex mechanisms. It was also shown that *Ngfb* and *Tgfbi* are down regulated in response to SERMs. In 3 month old mice treated with tamoxifen, expression of *Ngfb* has shown to be increased (Green *et al.*, 2003; Moggs *et al.*, 2004b). Our results for *Tgfbi* support the findings of Moggs *et al.*, (2004a), who also showed that expression of this gene was decreased in response to a single subcutaneous dose of oestradiol (400 μg/kg). It seems unlikely that there

are differences in the uterotrophic response in terms of gene expression between the three compounds tested, supporting work by Moggs *et al.*, (2004a).

In summary, maximal uterotrophic doses of oral oestradiol, 4-hydroxyoestradiol and tamoxifen have been established and increases in uterine weight did not correlate with cell proliferation. Differences in uterotrophic response were seen between oral and subcutaneous oestradiol but not tamoxifen. All three compounds were shown to have a direct effect on gene expression in the uterus shown using a selection of oestrogen responsive genes. Dosing orally over 3 days used in this study was shown to have comparable effects on gene expression with studies using a single subcutaneous dose.

Chapter 5.

Neonatal Mouse Model to Study the Long Term Effects of SERMs.

5.1 Introduction.

It is now well established that tamoxifen leads to uterine pathologies in both women and mice. Classically, in mice it is thought that tamoxifen acts as an oestrogen agonist in the uterus, leading to endometrial epithelial hyperplasia and promotes neoplasia in initiated cells (Carthew *et al.,* 2000). However, endometrial cancers have been shown to develop in the absence of hyperplasia (Carthew *et al.,* 2000), suggesting that an oestrogen agonist action in the uterus is not the only mechanism involved in tamoxifen toxicity. This idea is further supported by neonatal dosing studies, where short term neonatal exposure to the oestrogen agonist effect of tamoxifen leads to long term uterine changes (Carthew *et al.,* 2000; Parrott *et al.,* 2001). In addition, tamoxifen does not just lead to uterine cancers and may affect normal uterine development in both the endometrial and myometrial compartments leading to adenomyosis (Parrott *et al.,* 2001). Previous research has shown adenomyosis developed by three months of age in mice that have been given oral tamoxifen on neonatal days 2 to 5 (Carthew *et al.,* 2000; Parrott *et al.,* 2001). Adenomyosis is a fairly frequent disorder of the uterus of women characterised by the haphazard location of endometrial glands and stroma deep within the myometrial layer. It is most commonly found in premenopausal women between the ages of 40 and 50 years (Ferenczy, 1998) and may occur more frequently in those taking tamoxifen (Cohen *et al.,* 1997). It is clear that complex mechanisms are likely to be involved in uterine abnormalities in response to tamoxifen which may include actions directed through the oestrogen receptor, but also activation of non-genomic pathways and possible formation of DNA adducts, outlined in Chapter 1.

Many compounds with oestrogenic activity have been shown to induce uterine histopathological abnormalities after neonatal treatment in rodents. This includes tamoxifen and toremifene (Newbold *et al.,* 1997; Parrott *et al.,* 2001), genistein and diethylstilbestrol (Newbold *et al.,* 2001a), and the oestradiol metabolite 4-hydroxyoestradiol (Newbold *et al.,* 2000), though oestradiol itself does not lead to uterine cancers (Newbold *et al.,* 2000).

To what extent the oestrogen receptor is involved in uterine pathology is still unclear, though the development of the ERKO and βERKO mouse models have gone some way to aid the study of this phenomenon. For example, ERα is thought to mediate the toxic effects of diethylstilbestrol in the uterus as activating of gene expression in the ERKO uterus is abolished (Couse *et al.,* 2004). However, 4-hydroxyoestradiol is capable of activating gene transcription in this model, an effect not seen in response to oestradiol (Das *et al.,* 1997). It is likely that there is some other signalling pathway involved in the response to 4-hydroxyoestradiol but not oestradiol, which may contribute to its detrimental effects. Tamoxifen is unable to induce an oestrogenic response in the ERKO mouse uterus (Korach *et al.,* 1996), though the long term effects of tamoxifen in this model are unknown.

In mice, after neonatal dosing, it has been suggested that tamoxifen acts through the oestrogen receptor of endometrial stroma and epithelium resulting in abnormalities in other tissue compartments (Newbold *et al.,* 1997). This may account for the disruption of the myometrium seen in adenomyosis. However, it is clear that other mechanisms must occur for the long term changes seen after neonatal dosing. Alteration in the methylation pattern of a gene promoter region by tamoxifen has been suggested as one mechanism (Carthew *et al.,* 2000; Newbold *et al.,* 2004). In six day old mice, expression studies show that tamoxifen is capable of altering gene expression patterns (Carthew *et al.,* 2000; Green *et al.,* 2003; Parrott *et al.,* 2001). It is

thought that long term gene expression changes, including changes in *Ngfa* and *Pref-1* altered at this stage may contribute to the development of adenomyosis (Parrott *et al.*, 2001).

It has been shown that tamoxifen may activate many pathways that potentially mediate its toxic effects in the uterus. This includes alteration of expression of certain growth factors including TGF, EGF and IGF1 (Stygar *et al.*, 2003; Ugwumadu *et al.*, 1998). Many studies investigating the effects of tamoxifen on gene and protein expression have looked at only a few candidates in isolation. We wanted to examine a global gene expression profile in the uterus after tamoxifen and relate theses changes to phenotypic changes observed.

In this section, CD-1 mice were dosed orally on days 2 to 5 after birth with either the maximal uterotrophic dose of SERM, described in Chapter 5. A dose that was 4 to 8 fold higher than maximal uterotrophic dose was also used to establish if any phenotypic changes could be linked to uterotrophic oestrogen agonist activity. Gene expression was examined at three months of age to correlate long term changes with phenotypic changes present in the uterus. We also wanted to investigate to what extent genotoxicity might be involved in changing gene expression. To do this we studied the effects of 4-hydroxyoestradiol, a metabolite of oestradiol known to be capable of forming DNA adducts (Devanesan *et al.*, 2001) and also activate pathways other than those activated by oestradiol itself (Das *et al.*, 1997). To further understand the mechanism of tamoxifen toxicity in the uterus, we also employed the ERKO mouse model to study the role of ERα in the development uterine pathologies

5.2 Results.

5.2.1 Phenotypic Changes in the Uteri of Mice after Neonatal Dosing with SERMs.

In this Chapter, all mice were dosed orally on days 2 to 5 after birth and then culled at three months of age. Control mice were dosed with peanut oil / lecithin / milk vehicle only. There were no significant differences in body weight in any groups receiving oestradiol or tamoxifen at the dose level tested (Table 5.1). In mice receiving 4-hydroxyoestradiol at both dose levels (386 and 1920 μg/kg), body weights were significantly higher than controls. There was only one significant change in uterine weight (Table 5.1), where weight was decreased in mice receiving 1000 μg/kg tamoxifen. There were however, non-significant decreases in uterine weight in mice receiving 250 μg/kg tamoxifen.

Pathology was assed in H&E stained sections from three month old mice (Figure 5.1). The uteri from vehicle only dosed control mice showed normal cycling uterine endometrium (Figure 5.1a). The ovaries and oviducts appeared normal and ovaries all contained abundant developing follicles and corpora lutea (Figure 5.2a). None of the uteri from mice dose with 100 μg oestradiol contained any significant pathological change in comparison to controls (Figure 5.1c and d). The endometrium was normally cycling in these animals and there was no evidence of either endometrial hyperplasia or adenomyosis. The ovaries and oviducts were all also within normal limits.

All mice treated with either 250 or 1000 μg/kg tamoxifen showed adenomyosis of the uterus (Figure 5.1b) similar to that previously reported (Parrott *et al.,* 2001). The endometrium showed evidence of normal cycling and ovaries appeared within normal limits and contained abundant corpora lutea (Figure 5.2b). Adenomyosis was slightly more prominent in some mice receiving 1000 μg/kg group.

All mice treated with either 386 or 1920 μg/kg 4-hydroxyoestradiol showed hyperplasia of the endometrial glands (Figure 5.1e). Hyperplasia was graded as minimal although it appeared slightly more marked in the uteri of mice treated with 1920 μg/kg. This is interesting as in Chapter 4, a dose of 386 μg/kg was shown to induce a greater uterotrophic response than 1920 μg/kg. In addition, endometrial glands were often arranged in an abnormal cribriform manner and this was associated with a reduction in the thickness of the uterine stroma and myometrium. There was little histological evidence of normal cycling in the endometrium. The ovaries in this group contained developing follicles but showed a complete absence of corpora lutea (Figure 5.2c).

	n =	Body Weight (g)	Uterine Weight (mg)
Control	52	38 ± 0.52	193 ± 18.39
E2 (100 μg)	4	41 ± 3.52	231 ± 49.10
4OHE2 (386 μg)	9	44 ± 1.93*	164 ± 6.26
4OHE2 (1920 μg)	9	49 ± 3.01*	106 ± 7.88
Tamoxifen (250 μg)	9	37 ± 1.69	143 ± 11.85
Tamoxifen (1000 μg)	9	35 ± 1.46	92 ± 8.17*

Table 5.1: Body and Uterine Weights of Three Month Old CD-1 Mice.

Neonatal CD-1 mice were dosed orally on days 2 to 5 after birth with the compounds indicated and culled 3 months later. Weights are body weight (g ± SE) and uterine wet weights (mg ± SE). Star (*) denotes significant difference from control ($p < 0.05$) using ANOVA and Dunnetts post-hoc comparison with a control.

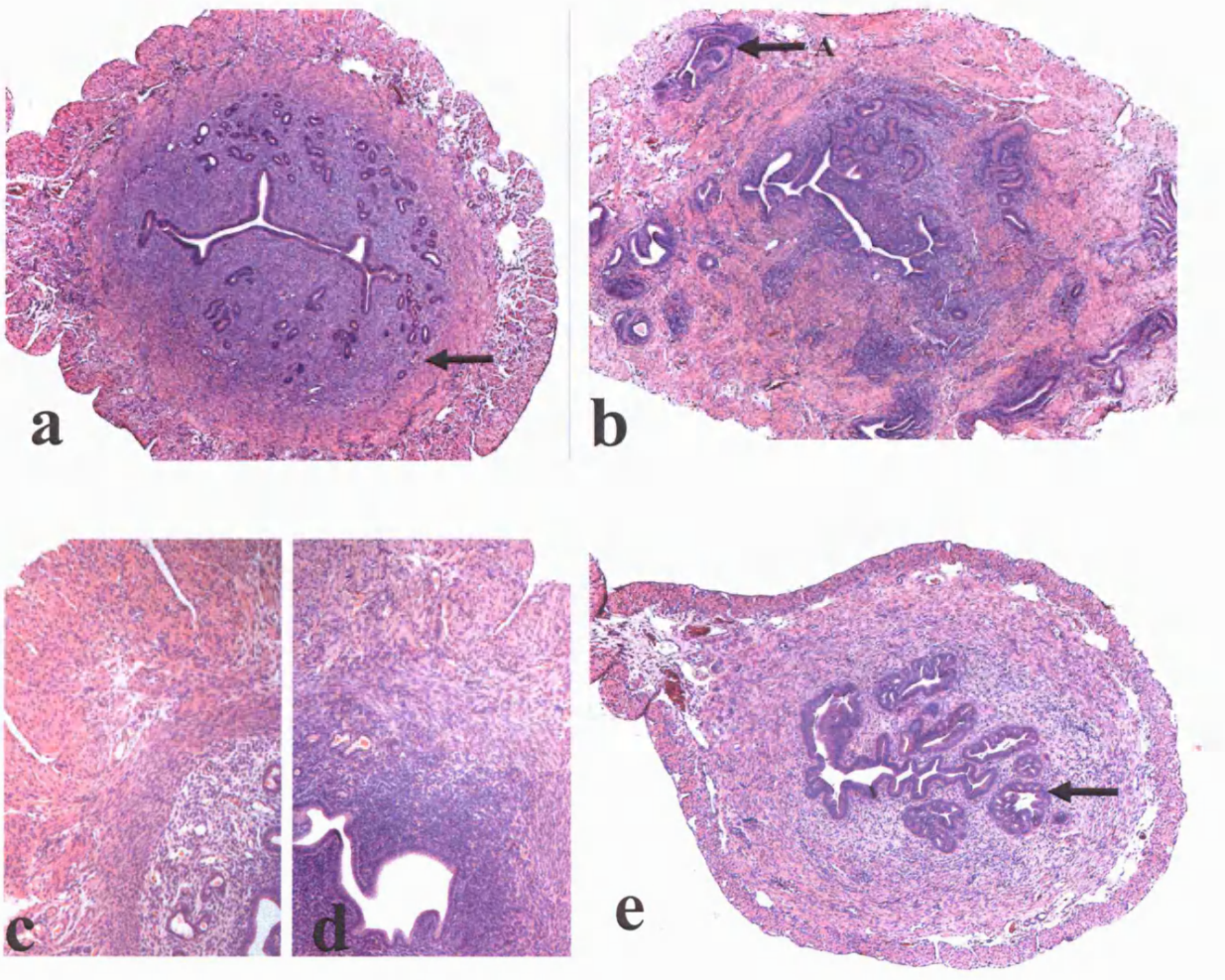


Figure 5.1: Representative Uterus Section from Three Month Old CD-1 Mice.

H&E sections show uteri from three month old CD-1 mice after neonatal dosing with SERMs **a**. Vehicle only dosed control uterus, showing luminal and glandular epithelium in the endometrium surrounded by a well differentiated myometrial / endometrial junction (arrowed) (magnification x 4). **b**. Uterus from tamoxifen (1000 μg/kg) dosed mouse (magnification x 4). Adenomyosis presents as glands and stroma deep within the myometrial layer (**A**). The myometrial layer is poorly defined. **c**. control uterus (magnification x 10) **d**. uterus from oestradiol (100 μg/kg) dosed mouse (magnification x 10) **e**. Uterus from 4-hydroxyoestradiol (1920 μg/kg) dosed mouse, showing epithelial hyperplasia (arrowed, magnification x 4).

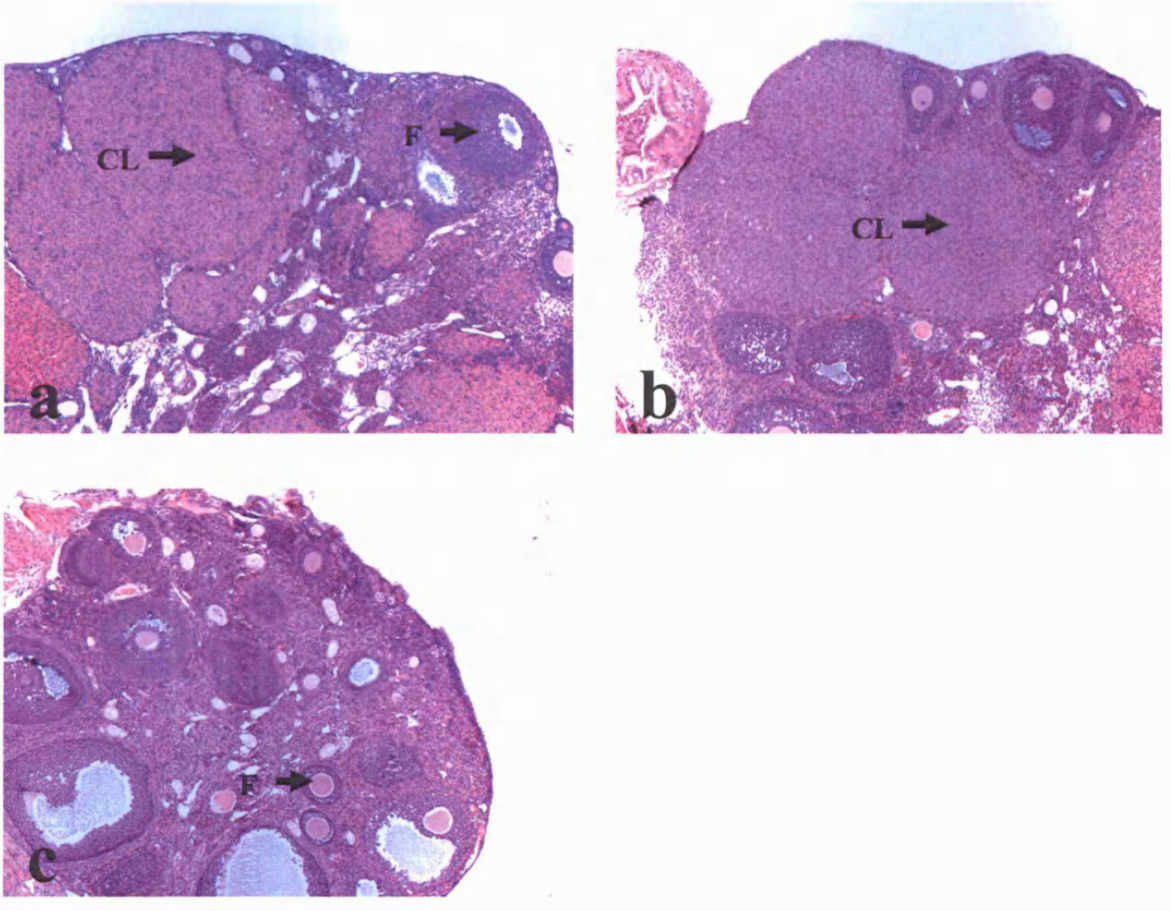


Figure 5.2: Representative Ovary Section from Three Month Old CD-1 Mice.

H&E sections show ovaries from three month old CD-1 mice after neonatal dosing with SERMs **a**. Vehicle only dosed control ovary, showing developing follicles (**F**) and mature corpora lutea (**CL**) (magnification x 6). **b**. Ovary from tamoxifen (1000 μg/kg) dosed mouse (magnification x 6), containing mature corpora lutea comparable with controls. **c**. Ovary from 4-hydroxyoestradiol (1920 μg/kg) dosed mouse, developing follicles (**F**) but no mature corpora lutea.

5.2.2 Expression of ERα in the Three Month Old CD-1 Mouse Uterus.

In order to determine expression of ERα and ERβ in three month old CD-1 mice, protein levels were assessed using Western blotting of whole uterine protein. In addition oestrogen receptor expression was measured in the five day old mouse uterus, to determine if ERα and ERβ were expressed at the time of dosing. Figure 5.3 shows that ERα was expressed in representative mice from all dosing groups. Expression of ERα appears to be higher in 5 day old mice than in three month old mice. Expression of ERβ was too low to be quantified accurately.

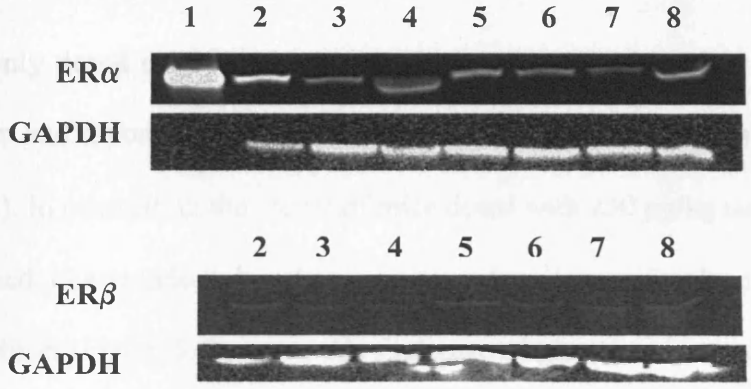


Figure 5.3: Representative ER Expression in CD-1 Mice .

Representative Western blot of ERα and ERβ using 70 μg total uterine protein (10 and 20 min image acquisition respectively). Samples are: Lane 1, human ERα recombinant protein (10 ng); Lane 2, 5 day old mouse uterus; Lane 3, 3 month control; Lane 4, 3 month oestradiol; Lane 5, 3 month tamoxifen (1000 μg/kg); Lane 6, 3 month tamoxifen (250 μg/kg); Lane 7, 3 month 4-hydroxyoestradiol (1920 μg/kg); Lane 8, 3 month 4-hydroxyoestradiol (386 μg/kg).

5.2.3 Smooth Muscle α-Actin Staining in the Adenomyotic Uterus.

Adenomyosis is characterised by the presence of glands deep within the myometrial layer. The myometrial layer may also appear undifferentiated. In order to further define the complex phenotypic changes that occur in adenomyosis, paraffin sections of uterus from 3-month old mice were immunostained with an antibody for smooth muscle α-actin (Figure 5.4). This marker is specific for differentiated muscle cells and myofibroblasts. For negative controls, primary antibody was replaced with relevant IgG at the same dilution as the primary antibody (Figures 5.4e). Overall, Figure 5.4 shows that smooth muscle α-actin is a good marker for myometrial disruption in adenomyosis.

In vehicle only dosed control animals, staining shows there is a clear junction between the endometrium and myometrium, and concentric and longitudinal layers of muscle are visible (Figure 5.4a). In contrast, in the uterus of mice dosed with 250 μg/kg tamoxifen, adenomyosis is visible and characterised by the presence of endometrial glands and stroma in the myometrial layer (Figure 5.4b, arrow A). Endometrial glands and stroma are often completely surrounded by smooth muscle bundles (Figure 5.4c), although this may simply be a consequence of sectioning.

In the uterus of mice treated with 4-hydroxyoestradiol (Figure 5.4d), the distinction between concentric and longitudinal myometrium and endometrium is more clear than in mice dosed with tamoxifen although the myometrium is much thinner.

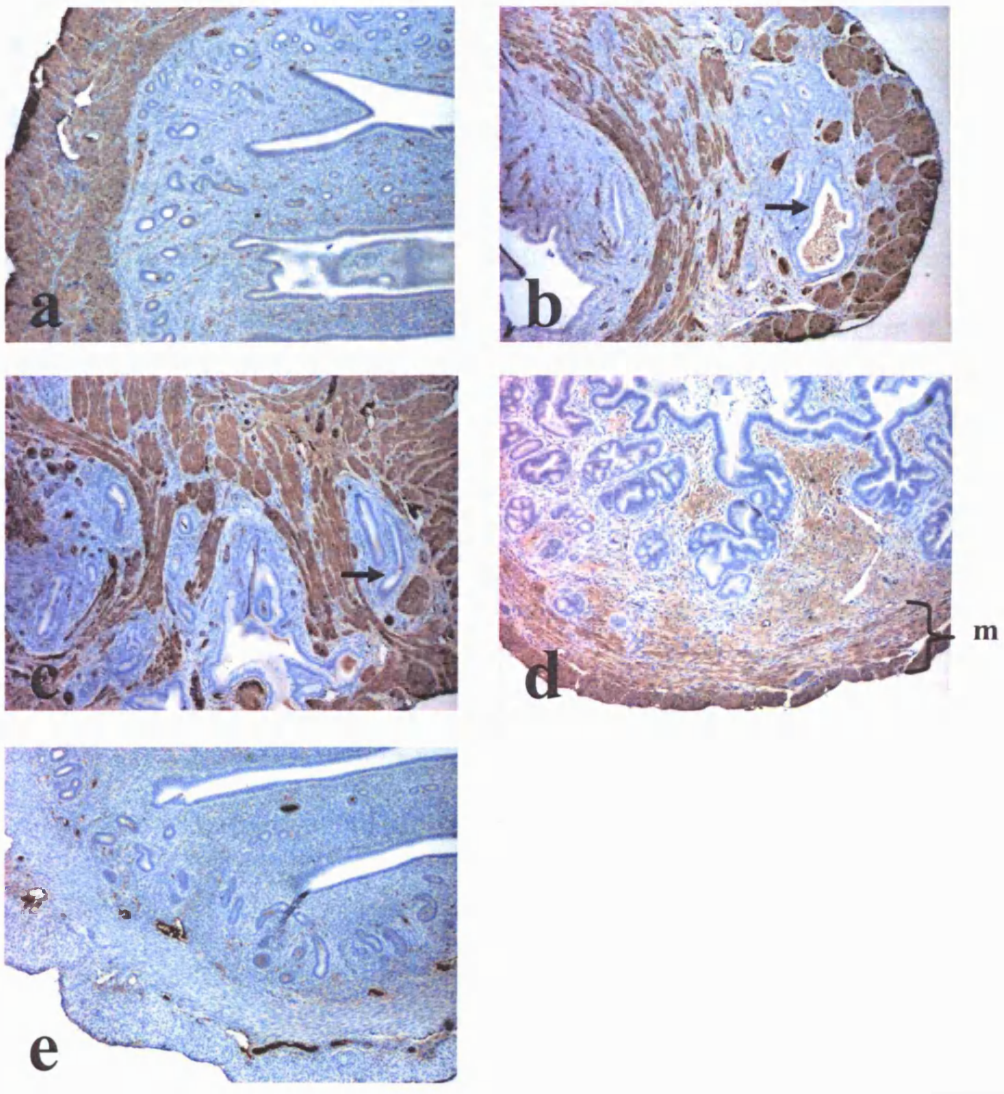


Figure 5.4: Smooth Muscle α-Actin Staining in the CD-1 Mouse Uterus.

Smooth muscle α-actin in the 3 month old mouse uterus after neonatal treatment with tamoxifen or 4-hydroxoestradiol: **a**. Vehicle only dosed control (x 10); **b**. tamoxifen (250 μg/kg, x 10); **c**. tamoxifen (1000 μg/kg) showing adenomyosis (**A**) (x 10); **d**. 4-hydroxyoestradiol (386 μg/kg, x 100) (myometrial layer is illustrated to distinguish from background staining, **m**); **e**. vehicle only dosed control IgG control (no primary antibody, x 10). In figures b and c, arrows show endometrial glands and stroma in the myometrial layer.

5.2.4 Effects of Time of Dosing on the Development of Adenomyosis.

To establish if there was a specific time window during which neonatal exposure to tamoxifen could induce the onset of adenomyosis, groups of newborn mice were dosed with 1000 mg/kg tamoxifen on different days after birth for four days. These time windows were postnatal days 2 to 5, 6 to 9, 10 to 13 or 14 to 17. The only histological changes seen in this study were in mice dosed with tamoxifen on days 2 to 5 after birth where adenomyosis was present. Adenomyosis was not present in the concurrent control group, or within any other of the control or tamoxifen treated groups. There was evidence of normal endometrial cycling and corpora lutea in all groups, both tamoxifen treated and vehicle controls.

5.2.5 Effects of Tamoxifen Induced Adenomyosis on Fertility.

To establish whether tamoxifen induced adenomyosis could affect fertility, female CD-1 mice were dosed on days 2 to 5 after birth with 1000 μg/kg oral tamoxifen. At three months mice were mated and then kept until they gave birth at which point the litter size and sex of offspring were determined (Table 5.2). All vehicle control mice successfully gave birth with an average litter size of 13.66 ± 0.33 (SE) with 55% of the litter being males. In contrast, tamoxifen severely affected the fertility of mice with one out of six mice giving birth. The litter size was markedly smaller than controls. Tamoxifen dosed animals numbers 1, 2 and 5 did not get pregnant. Animal number 3 was culled due to difficult birth, after which it was found that the mouse was carrying one large pup and an ectopic pregnancy. The litter was lost from animal number 4.

	Animal No.	Litter Size	No. Males	No. Females
Control	1	14	9	5
	2	14	8	6
	3	15	5	10
	4	13	8	5
	5	13	8	5
	6	13	7	6
Tamoxifen	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
	6	5	3	2

Table 5.2: Litter Size of Mated Mice Treated with Tamoxifen Neonatally.

Female CD-1 mice were dosed on days 2 to 5 after birth with 1000 μg/kg oral tamoxifen. At three months mice were mated and then kept until they gave birth at which point the litter size and sex of offspring were determined.

5.2.6 Gene Expression Changes in the Uteri of Mice after Neonatal Dosing with SERMs.

Long term gene expression changes were investigated in three month old mice after neonatal dosing with oestradiol (100 μg/kg), tamoxifen (250 μg/kg) or 4-hydroxyoestradiol (1920 μg/kg). At these doses, adenomyosis was present in the uteri of all mice receiving tamoxifen. In mice receiving 1920 μg/kg 4-hydroxyoestradiol, no adenomyosis was present although there was a reduction in thickness of the myometrium. Adenomyosis was not present in control mice or those receiving oestradiol. Previous studies using microarray data have often omitted genes where fold expression falls outside a cut-off point, usually 2-fold. However, using these criteria genes may be excluded in which there is a very small but highly significant change in expression, for example signalling cascades where only a small increase in gene expression is involved. In our study, we decided to select genes where the change in expression was significant at $p < 0.05$.

All gene expression data have been deposited on the GeoDatabase (Appendix 1). Figure 5.5. shows a Venn diagram representing the numbers of genes changed for each compound and also compares the number of genes changed in common (also listed in Appendix 1). There were only 12 genes in common where expression was altered by oestradiol, 4-hydroxyoestradiol and tamoxifen (Table 5.3). The direction of expression of 9 genes was similar between all compounds, and 3 genes were different. Relative to control expression, oestradiol lead to a significant change in gene expression in the greatest number of genes (n = 833), where expression of 568 genes (68%) was increased. 4-Hydroxyoestradiol and tamoxifen had a weaker effect on gene expression in the uterus (totals = 464 and 286, respectively). After neonatal tamoxifen treatment, expression of 78% of genes was decreased relative to controls. In contrast, expression of a majority of genes was increased after oestradiol (68%) or 4-hydroxyoestradiol (62%) treatment.

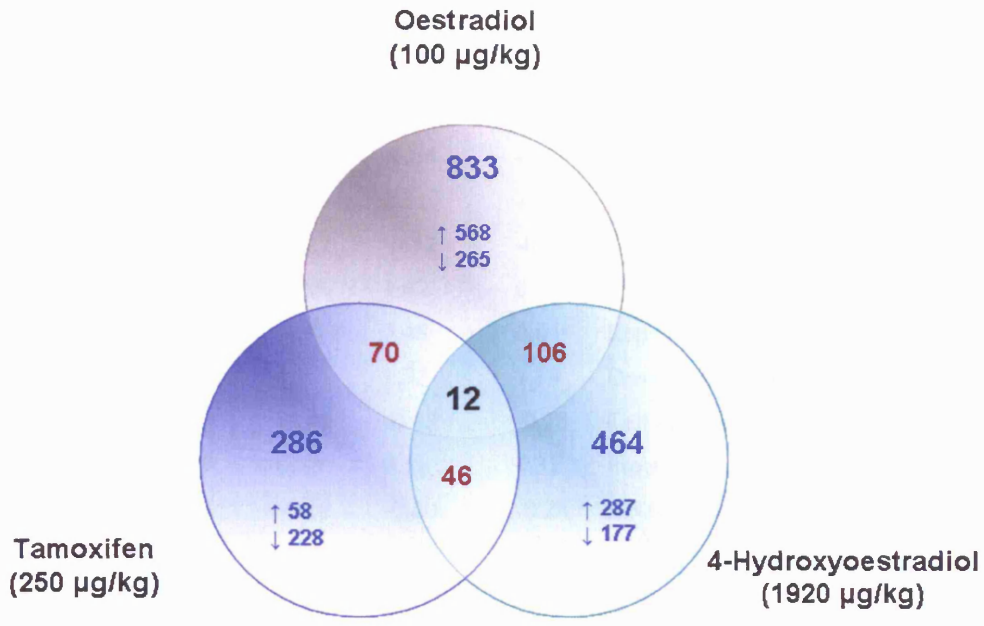


Figure 5.5: Venn Diagram of Mouse Uterus Arrays after Neonatal SERM Treatment.

Diagram shows numbers of genes in which Log_2 expression was significantly changed ($p < 0.05$) by either oestradiol (100 µg/kg), tamoxifen (250 µg/kg) or 4-hydroxyoestradiol (1920 µg/kg). Blue text represent the total number of genes changed for each compound, red text show numbers of genes that were significantly changed in a pair of compounds. There were only 12 genes that were significantly changed by all three compounds (Table 5.3). Further comparisons of gene expression are given in Tables 5.3 to 5.5.

Gene Name	Gene ID	Compound			Gene Function
		E2	4OHE2	Tam	
Similar					
1810014L12Rik	X81718	-0.71	-0.64	-1.12	Unknown
Cenpb	NM_007682	2.49	2.81	1.58	Chromosome organization and biogenesis
E2f6	AF032131	-0.27	-0.12	-0.39	Cell cycle
Fkbp8	NM_010223	1.25	1.42	0.78	Intracellular signalling cascade
Hdac7a	NM_019572	0.70	0.48	0.64	Repression of transcription
Kail	NM_007656	1.73	1.93	1.39	Developmental processes
Lrrn2	D49375	-0.47	-0.63	-0.25	Cell adhesion
Rps6ka1	NM_009097	0.43	0.51	0.31	Protein phosphorylation
Ruvbl2	NM_011304	-0.28	-0.20	-0.24	DNA repair
Different					
Cyp2e1	NM_021282	1.03	-2.05	-0.89	Electron transport
Eng	NM_007932	1.13	1.22	-0.67	Cell adhesion
Gnl2	U69600	-0.13	0.16	0.48	Intracellular protein traffic

Table 5.3 Gene Expression Significantly Changed in Response to all SERMs Tested.

Table shows Log_2 ratio of gene expression relative to control. Doses were 100 μg/kg oestradiol, 386 μg/kg 4-hydroxyoestradiol or 250 μg/kg tamoxifen. Using the MRC Toxicology Unit protocol, 25 μg total uterine RNA was reverse transcribed and hybridised onto HGMP oligo microarrays. Data is from four dosed CD-1 mice uteri individually challenged against a pooled control of four vehicle only dosed mice. Microarrays were repeated using reverse labelling and data filtered to give genes in which expression was significant ($p < 0.05$) across all microarrays of similarly dosed uteri.

As tamoxifen and 4-hydroxyoestradiol but not oestradiol lead to histological changes in the uterus, it was of interest to identify genes that were similarly affected by tamoxifen and 4-hydroxyoestradiol but not by oestradiol. Of the 45 genes in this category (Table 5.3), expression of 33 genes are similarly increased or decreased. It was therefore assumed that some of this sub-set of 33 genes may be involved in adenomyosis. However, these genes may not necessarily be involved in the initiation or the early progression of adenomyosis.

In the tamoxifen and 4-hydroxyoestradiol arrays there were several genes involved in muscle development (*Tpm1*), cell cycle (*Cdc2a*, *E2fb*, *Pdgfra*), cell adhesion and motility (*Col4a2*, *Aif1*) and cell communication (*Gja1*). These may be associated with the phenotypic changes that occur in the adenomyotic uterus.

Considering that tamoxifen but not oestradiol lead to the development of adenomyosis, Table 5.4 identifies 38 genes in which the direction of gene expression is different after tamoxifen or oestradiol treatments. There are only two genes in which expression is decreased by oestradiol and increased by tamoxifen. Expression of 36 genes was increased by oestradiol and decreased by tamoxifen. This suggests that suppression and not induction of gene expression is involved in the development of adenomyosis. To further illustrate the functional effects of gene suppression, microarray data from all three compounds was analysed using GenMapp 2 (Figures 5.6 – 5.7). Table 5.5 identifies genes in which the direction of gene expression was the same, it is seems unlikely that theses genes are involved in adenomyosis.

Microarray data were interrogated for changes in gene expression in specific cell mechanisms, e.g. apoptosis. Three of these pathways were identified that were consistent with gene functions already identified as possibly being involved in adenomyosis. Within these

pathways there were differences in gene regulation by oestradiol and tamoxifen, with tamoxifen generally suppressing pathway activity. These pathways were apoptosis (Figure 5.6), actin cytoskeleton and integrin mediated cell adhesion (Figure 5.7).

From the gene profiles we chose a number of genes to confirm by real time PCR that we thought of interest (Table 5.6), in particular candidate genes that are known to be regulated by oestrogens (*Akp2*, *Ltf*, *C3*) or have been previously associated with the development of adenomyosis (*Ngfb* and *Tgfbi*). In addition, six genes were chosen after searching published literature to establish a potential role in disruption of uterine structure or development. These genes were Collagen IV (*Col4a2*), platelet derived growth factor receptor-α (*Pdgfra*), thrombospondin (*Thbs1*), gap junction a1 (*Gja1*) and tropomyosin (*Tpm1*) and their functions will be discussed later.

	Gene Symbol	Accession No.	Log_2 Ratio Tam	4OHE2	Gene Function
Similar	*Stra6*	NM_009291	-1.56	-1.02	Neurogenesis
	Trpc5	NM_009428	-1.38	-0.88	Neurogenesis
	Slc9a3	AF139194	-1.21	-0.77	Sodium transport
	1810014L12Rik	X81718	-1.12	-0.64	Unknown
	Sult1d1	NM_016771	-1.11	-4.65	Steroid metabolism
	Thbs1	NM_011580	-1.10	-0.24	Cell adhesion
	Ptger3	NM_011196	-1.09	-1.05	G-protein receptor signalling
	Cdc2a	NM_007659	-0.97	-0.59	Cell cycle
	Tcf23	AF142405	-0.97	-1.27	Actin polymerization
	Cyp2e1	NM_021282	-0.89	-2.05	Electron transport
	Tpm1	M22479	-0.85	-0.69	Muscle development
	Kdelc1	AJ404004	-0.81	-0.56	Unknown
	Gpx3	NM_008161	-0.71	-1.87	Peroxidase reaction
	Gja1	NM_010288	-0.59	-0.60	Cell communication
	Psmb5	NM_011186	-0.48	-0.40	Protein degradation
	E2f6	AF032131	-0.39	-0.12	Cell cycle
	Pdgfra	NM_011058	-0.31	-1.05	Cell proliferation
	Lrrn2	D49375	-0.25	-0.63	Cell adhesion
	Ruvbl2	NM_011304	-0.24	-0.20	DNA repair
	Col4a2	J04695	-0.14	-0.67	Cell adhesion
	ORF5	NM_016924	0.21	0.16	Holocytochrome c synthase
	Rps6ka1	NM_009097	0.31	0.51	Protein phosphorylation
	Bcl10	NM_009740	0.37	0.63	Induction of apoptosis
	Gnl2	U69600	0.48	0.16	Intracellular protein traffic
	Mpeg1	L20315	0.49	0.65	Steroid metabolism
	5830458K16Rik	AJ251364	0.62	0.49	Unknown
	Hdac7a	NM_019572	0.64	0.48	Repression of transcription
	AF291821	AF291821	0.66	2.02	Transcription regulation
	Psmb8	NM_010724	0.72	1.20	Protein degradation
	Fkbp8	NM_010223	0.78	1.42	Intracellular signalling
	Aif1	NM_019467	1.07	0.74	Cell motility
	Nucb1	NM_008749	1.29	2.43	Calcium ion homeostasis
	Kai1	NM_007656	1.39	1.93	Developmental processes
	Cenpb	NM_007682	1.58	2.81	Chromosome organization
Different	*Apobec2*	NM_009694	-1.32	2.23	mRNA editing
	Pdlim1	NM_016861	-1.07	0.60	Oxidative stress response
	Padi1	NM_011059	-0.75	0.73	Protein modification
	Eng	NM_007932	-0.67	1.22	Cell adhesion
	Ppp1r1b	AF281662	-0.64	1.29	Female receptivity
	5031400M07Rik	NM_020586	-0.61	0.31	Intracellular protein traffic
	Dkk2	NM_020265	-0.49	1.21	Cell adhesion
	Krt2-8	M21836	-0.44	0.40	Cytoskeleton organization
	Kcnj10	NM_020269	-0.44	0.52	Potassium transport
	Parg	NM_011960	-0.18	0.48	Actin polymerization
	Ptprj	D45212	-0.51	0.67	Transmembrane RTK signalling

Table 5.3: Differential and Similar Gene Expression Changes Common between Tamoxifen and 4-Hydroxyoestradiol.

Gene Symbol	Accession No.	Log2 Ratio E2	Log2 Ratio Tam	Gene Function
4921504N20Rik	AB010374	0.80	-0.47	Transcription regulation
Adam10	NM_007399	0.53	-1.27	Cell-cell signalling
Adnp	NM_009628	0.45	-0.57	Neuropeptide signalling pathway
Ak3	AB020203	0.91	-0.26	Nucleic acid metabolism
App	NM_007471	0.08	-0.42	Signal transduction
Asb2	AF155353	0.51	-0.26	Signal transduction
Atf2	M77167	0.22	-1.02	Transcription regulation
Cacna1h	NM_021415	0.21	-0.46	Calcium ion transport
Cacnb4	AF068899	0.47	-0.96	Calcium ion transport
Cd164	NM_016898	0.67	-0.89	Cell adhesion
Cox6a2	NM_009943	0.24	-0.28	Energy pathways
Cyfip1	NM_011370	0.58	-0.87	DNA replication
Cyp2e1	NM_021282	1.03	-0.89	Electron transport
Drg2	NM_021354	0.29	-0.31	DNA recombination
Eng	NM_007932	1.13	-0.67	Cell adhesion
Galc	NM_008079	0.64	-0.43	Carbohydrate metabolism
Gdi1	U07950	0.48	-0.57	Protein-nucleus import
Gnl2	U69600	-0.13	0.48	Intracellular protein traffic
Grb2	NM_008163	0.21	-0.09	RAS protein signal transduction
Gstm1	NM_010358	0.38	-0.59	Protein synthesis
Ivns1abp	AL355706	1.17	-0.92	Transcription regulation
Kcnk1	NM_008430	0.46	-0.89	Potassium transport
Mapk6	NM_015806	0.60	-0.46	Protein phosphorylation
Matr3	NM_010771	0.56	-1.03	Cell growth and maintenance
Mxi1	NM_010847	0.43	-0.22	Transcription regulation
Nf2	NM_010898	0.96	-0.44	Oncogenesis
Nptx1	NM_008730	0.95	-1.31	Acute-phase response
Pnck	NM_012040	0.49	-0.36	Signal transduction
Ppp1cb	M27073	0.25	-1.01	Cell cycle
Prdx3	NM_007452	-0.36	0.44	Peroxidase reaction
Pum2	AF315590	0.34	-0.90	Cell growth
Qk	NM_021881	0.59	-0.60	Nerve ensheathment
Rnf25	NM_021313	0.44	-0.37	Ubiquitin cycle
Sfrs5	NM_009159	0.27	-0.64	mRNA splice site selection
Sts	NM_009293	0.65	-0.72	Steroid metabolism
Sumo3	NM_019929	0.59	-0.41	Small ubiquitin-related protein 1 conjugation
Tcea1	NM_011541	0.25	-0.66	Transcription regulation
Tln1	NM_011602	1.18	-0.30	Signal transduction

Table 5.4: Differential Gene Expression between Tamoxifen and Oestradiol.

Gene Symbol	Accession No.	Log_2 Ratio E2	Log_2 Ratio Tam	Gene Function
1810014L12Rik	X81718	-1.12	-0.71	Unknown
2010004O20Rik	AB030183	-0.27	-0.43	Actin polymerization
Arl6ip6	AF133913	-0.52	-0.64	Unknown
Arntl	NM_007489	-0.76	-0.16	Circadian rhythm
Batl a	NM_019693	-0.78	-0.35	Developmental processes
Birc5	NM_009689	-1.02	-1.17	Apoptosis
C3arl	NM_009779	1.00	0.70	Chemotaxis
Cenpb	NM_007682	1.58	2.49	Chromosome organization
Chn2	AJ279014	0.43	0.86	Cell growth
Corolc	NM_011779	0.47	1.46	Actin cortical patch assembly
E130016I23Rik	NM_021434	-0.37	-0.19	Unknown
E2f6	AF032131	-0.39	-0.27	Cell cycle
Eef2k	NM_007908	0.37	0.84	Cell cycle
Fkbp8	NM_010223	0.78	1.25	Intracellular signalling
Gasl	NM_008086	-0.55	-0.22	Cell cycle arrest
Gmnn	NM_020567	-0.38	-0.79	Protein modification
Gtpbp2	NM_019581	0.15	0.28	Protein biosynthesis
Hdac7a	NM_019572	0.64	0.70	Repression of transcription
Inmt	NM_009349	0.89	1.30	Catecholamine metabolism
Kail	NM_007656	1.39	1.73	Developmental processes
Lrrn2	D49375	-0.25	-0.47	Cell adhesion
Noc4	NM_010926	-0.46	-0.16	Energy pathways
Npml	NM_008722	-0.36	-0.33	Transcription regulation
Pcdh7	NM_018764	-0.57	-0.35	Cell adhesion
Rps6kal	NM_009097	0.31	0.43	Protein phosphorylation
Ruvbl2	NM_011304	-0.24	-0.28	DNA repair
Sh3dl9	NM_012059	0.33	1.39	Synaptic vesicle endocytosis
Sqle	NM_009270	-0.40	-0.57	Ergosterol biosynthesis
Strap	NM_011499	-0.03	-0.45	Invasive growth
Timm8a	NM_013898	-0.29	-0.69	Neurogenesis
Trf	M23016	0.37	0.62	Iron transport
Ywhae	NM_009536	-0.25	-0.23	Signal transduction

Table 5.5: Similar Gene Expression between Tamoxifen and Oestradiol.

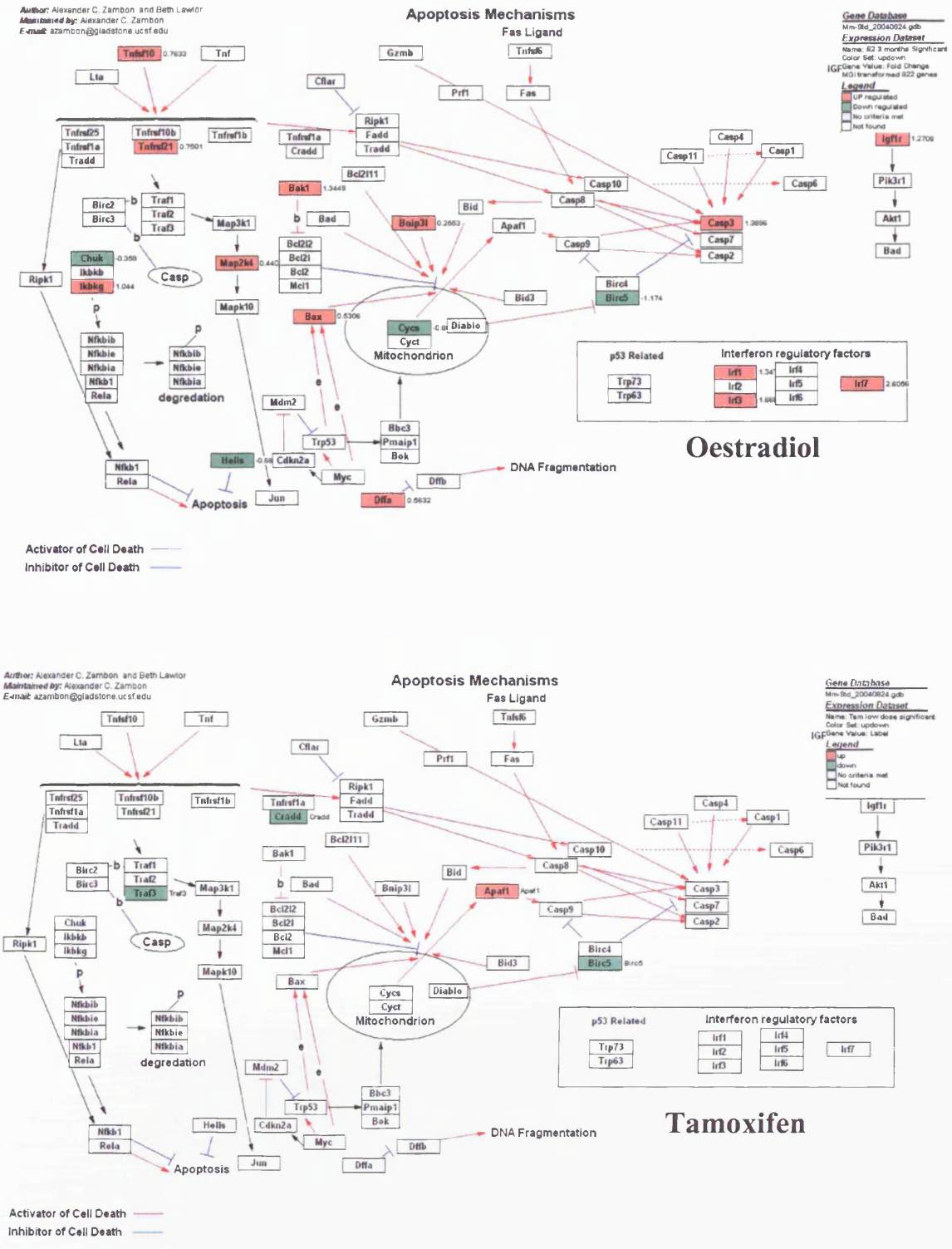
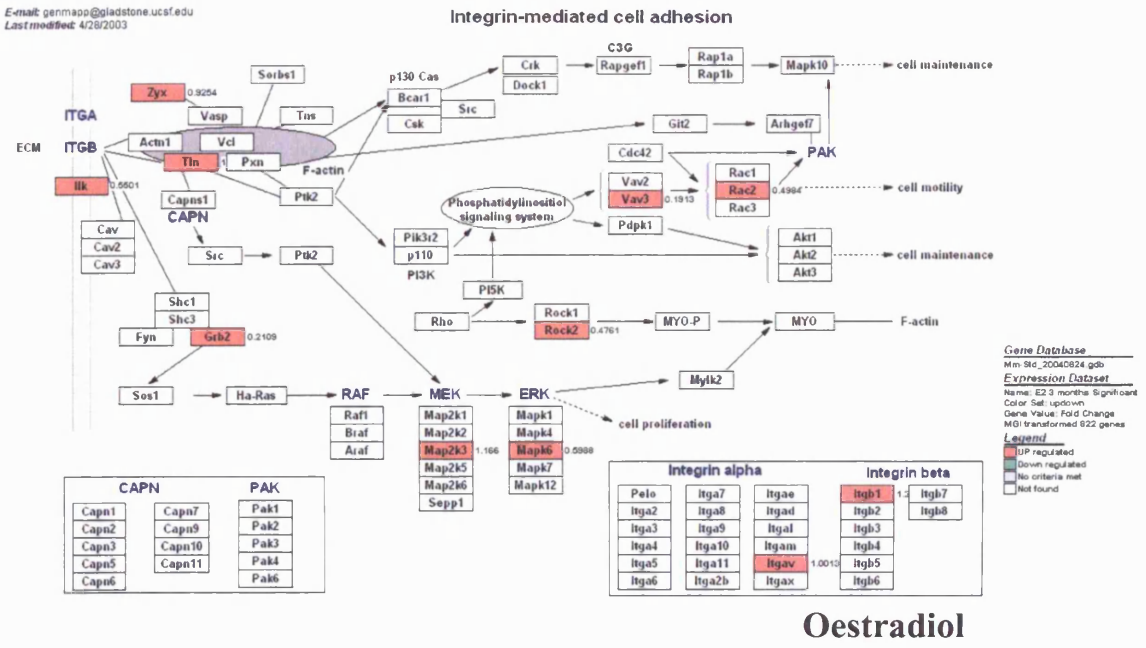
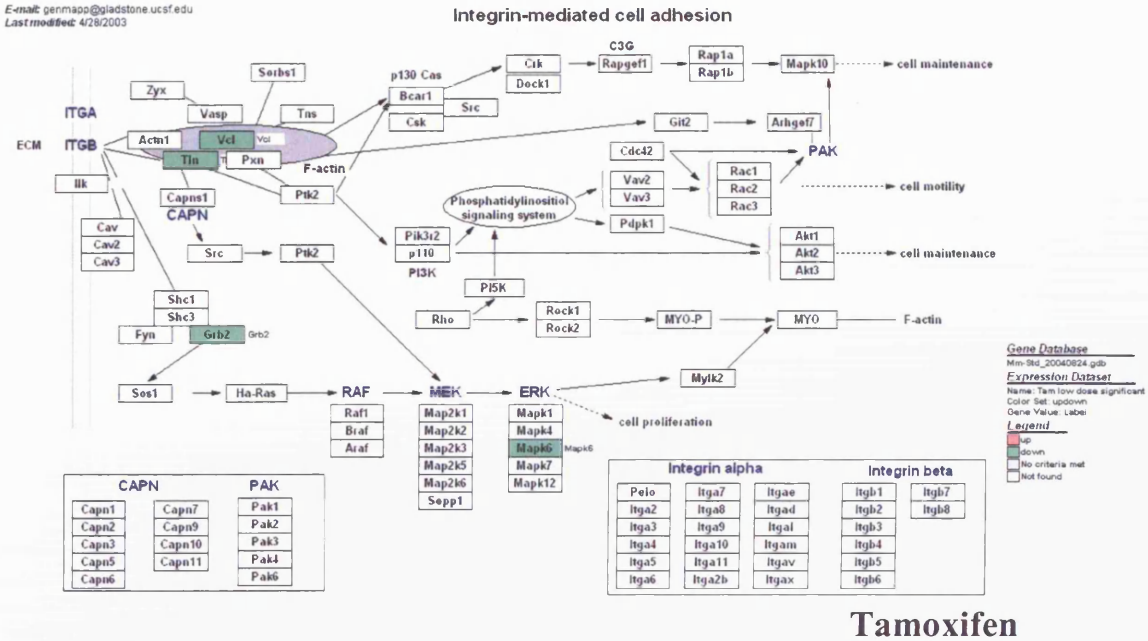


Figure 5.6: Apoptosis Mechanisms Regulated by Oestradiol and Tamoxifen.

Figure shows genes involved in apoptosis in which expression was altered by neonatal treatment with either oestradiol of tamoxifen.



Oestradiol



Tamoxifen

Figure 5.7: Regulation of Integrin Mediated Cell Adhesion Mechanisms by Oestradiol or Tamoxifen.

Gene	Promoter	E2		4OHE2		Tam	
		PCR	Array	PCR	Array	PCR	Array
Cyp2e1		3.3 ± 0.64	2.04	↓0.58 ± 0.24	↓4.14	↓0.70 ± 0.24	↓1.85
Pdgfra		1.2 ± 0.08	NS	↓0.62 ± 0.59	↓2.07	↓0.65 ± 0.19	↓1.30
Col4a2	ERE	↓0.62 ± 0.1	NS	↓0.93 ± 0.1	↓1.59	↓0.80 ± 0.13	↓1.10
Tpm1		2.43 ± 0.29	NS	1.99 ± 0.34	↓1.61	↓0.69 ± 0.16	↓1.80
Gja1		1 ± 0.04	NS	↓0.93 ± 0.06	↓1.50	↓0.90 ± 0.16	↓1.50
Thbs1	ERE	1.31 ± 0.54	NS	1.19 ± 0.27	↓1.18	1.82 ± 0.50	↓2.14
Tgfbi		↓0.74 ± 0.06	NS	↓0.52 ± 0.07	↓2.17	↓0.82 ± 0.08	NS
Ckb	ERE/Sp 1	↓0.98 ± 0.06	NS	1.01 ± 0.12	NS	↓0.89 ± 0.08	NS
C3	ERE	1.47 ± 0.39	NS	1.84 ± 0.22	NS	1.12 ± 0.30	NS
Akp2		1.34 ± 0.3	NS	1.55 ± 0.14	↓1.54	↓0.53 ±0.13	NS
Ltf	ERE/AP-1	3.45 ± 1.35	NS	5.94 ± 1.13	NS	1.85 ± 0.66	NS
Ngfb	AP-1	1.34 ± 0.23	NS	↓0.74 ± 0.09	NS	↓0.77 ±0.10	NS

Table 5.6: Real Time PCR of Selected Genes from Microarrays.

Table shows real time PCR confirmation of selected genes identified from microarrays. Microarray data represent fold change of expression relative to control expression for each compound using EST arrays (HGMP) and Cy3, Cy5 labelling protocol (MRC Toxicology Unit). All microarray data expressed are significant at $p < 0.05$. NS not significant. PCR data represents expression normalised to control expression. PCR data are the mean of four groups of similarly dose animals normalised to *Gapdh* expression, the calibrator and controls. Blue text identifies a decrease in expression relative to controls. For certain genes such as *Ltf*, there were large changes in expression as assessed by PCR but no significant changes using microarrays. The raw microarray data for *Ltf* showed that there was an increase in expression. However variance was large and thus was not classed as significant using our microarray statistical analysis software.

5.2.7 Effects of Neonatal Dosing with Tamoxifen in the ERKO Mouse Uterus.

As outlined in Chapter 1, the oestrogenic effects of SERMs in the uterus are primarily mediated by ERα signalling through both genomic and non-genomic pathways. It has been established that tamoxifen has a uterotrophic effect on the uterus, and may disrupt gene expression and uterine development. To further investigate whether the development of adenomyosis after neonatal tamoxifen treatment is mediated through ERα, ERKO mice were dosed on days 2 to 5 after birth with 1000 μg/kg/day tamoxifen. This high dose was used because we found adenomyosis was more marked in CD-1 mice receiving this dose than in animals that received the maximal uterotrophic dose.

Figure 5.8 shows uterine weights for wild type (WT) and ERKO mice either dosed with 1000 μg/kg tamoxifen or vehicle only. At three months after dosing, uterine wet weights for WT control mice were around 15 to 20 mg higher than any of the other groups, though this difference was not significant. There was no significant difference in body weight between groups (data not shown).

Figure 5.9 shows expression of ERα in the ERKO and WT uterus of vehicle only dosed controls. Analysis of protein molecular weight by SDS-gel electrophoresis showed ERα in WT mice to be 69.8 kDa. Human recombinant ERα (66 kDa actual molecular weight) was shown to be 69.3 kDa. A protein recognised as ERα in ERKO mice was 63.3 kDa. In WT mice ERα has been reported to be 66 kDa while an isoform of ERα in the ERKO mouse uterus has a molecular weight of 61 kDa (Kos *et al.*, 2002) The antibody used to detect ERα protein was the same in both studies [SantaCruz Biotechnology. Santa Cruz, CA, USA. Cat No. SC7207]. This isoform appears to be expressed at lower levels than WT ERα in WT animals (Figure 5.9b).

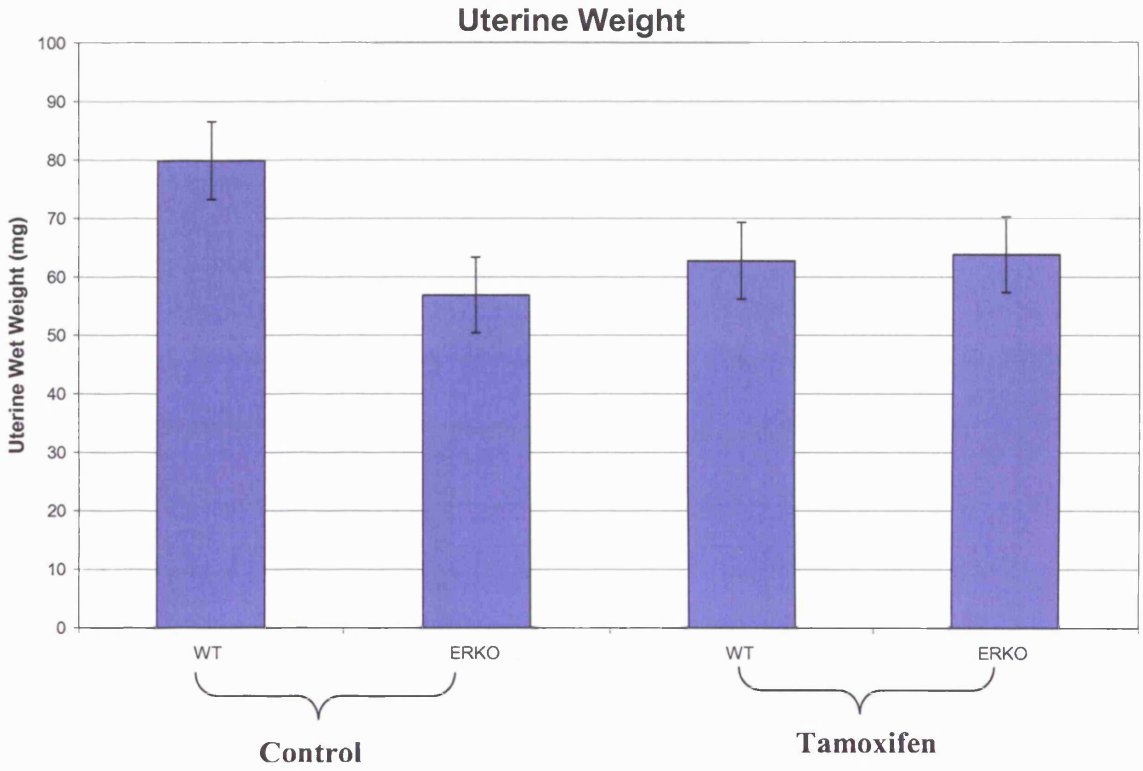


Figure 5.8: ERKO Mice Uterine Wet Weights at Three Months.

Uterine wet weights (± SE) for WT and ERKO mice dosed neonatally with either vehicle only (controls) or 1000 µg/kg tamoxifen. Numbers of animals in each group are: WT controls n = 14; ERKO control n = 9; WT tamoxifen n = 21; ERKO tamoxifen n = 7. There were no significant difference in either uterine or body weights ($p > 0.05$). Body weights were: controls WT 23.56 ± 0.37; controls ERKO 25.59 ± 0.64; tamoxifen WT 23.69 ± 0.61 and; tamoxifen ERKO 24.69 ± 0.53.

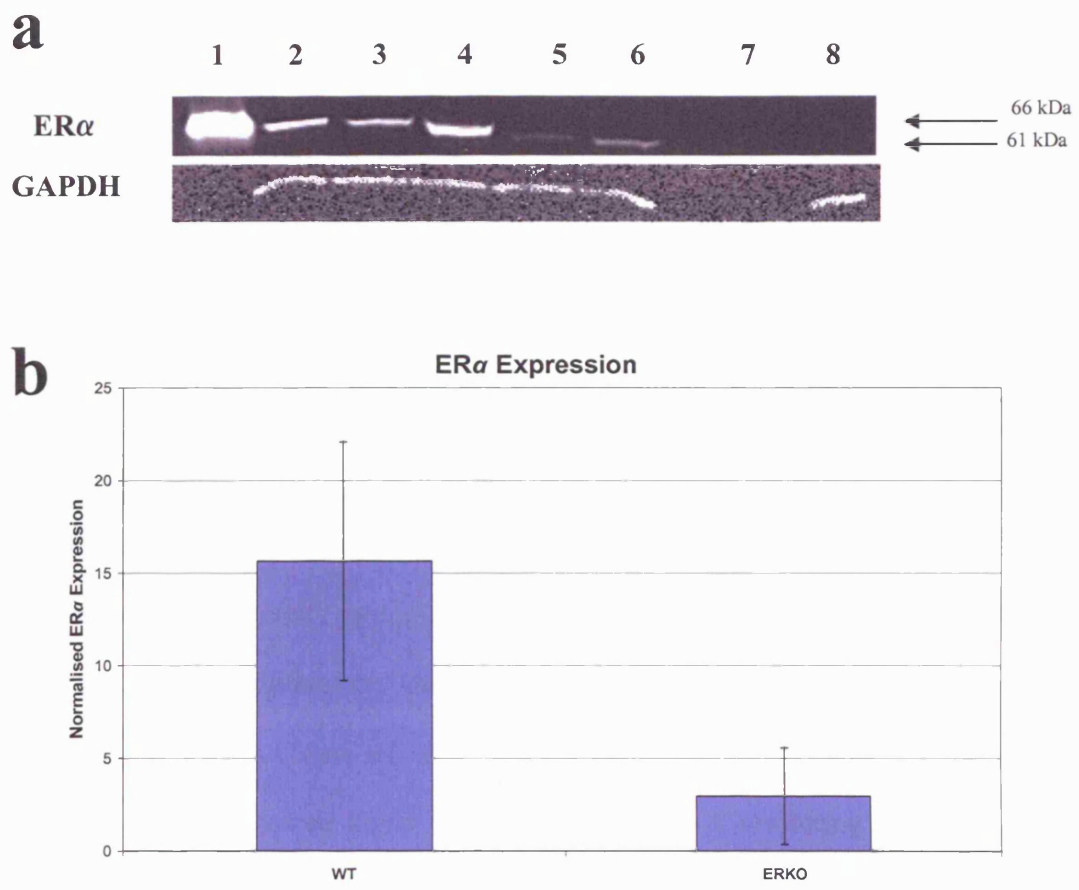


Figure 5.9: Representative Western Blot for ERα in the ERKO Mouse Uterus.

a. Lane **1**. ERα human recombinant protein 66 kDa (10 ng protein); Lanes **2**, **3** and **4**, WT ERα (45 μg protein); Lanes **5** and **6**, ERKO ERα (45 μg protein); Lane **7**, No primary antibody control with ERα human recombinant (10 ng protein), and; Lane **8**, WT ERα (45 μg protein). 20 min Image acquisition. Molecular weights of 66 kDa and 61 kDa are indicated. GAPDH expression is shown as a re-probe of ERα blot (2 min image acquisition). **b**. ERα Expression in WT and ERKO mouse uterus normalised to GAPDH expression and total protein loaded (45 μg). Data are from 3 WT and 2 ERKO (due to limited availability) control dosed mice uteri with western blots repeated 3 times. Statistics have not been applied due to the limited availability of ERKO uteri.

a. Pathology.

ERKO and WT mice were dosed on days 2 to 5 after birth with tamoxifen or peanut oil / lecithin / milk vehicle (controls) and killed at three month of age. Formalin fixed, paraffin embedded H&E stained 5μm sections were reviewed for phenotypic changes.

In ERKO controls (Figure 5.10a), some uteri showed signs of normal cycling. Others had haemorrhagic ovarian follicles which were associated with atrophic and non-proliferative endometrium. There were no consistent differences between the uteri of ERKO controls and ERKO mice treated with tamoxifen. In tamoxifen treated ERKO mice (Figure 5.10c), the endometrium was generally minimally to moderately proliferative, although occasionally showed no signs of proliferation. Other abnormalities included endometrial atrophy and one focus of adenomyosis. Uteri of WT mice dosed with tamoxifen were all moderately proliferative though showed signs of stromal sclerosis. Considering that the presence of stromal sclerosis was the only difference between WT and ERKO mice treated with tamoxifen, it is possible that this effect is mediated through ERα. There were no treatment related effects in the liver, lung, kidney or spleens of tamoxifen dosed mice or controls.

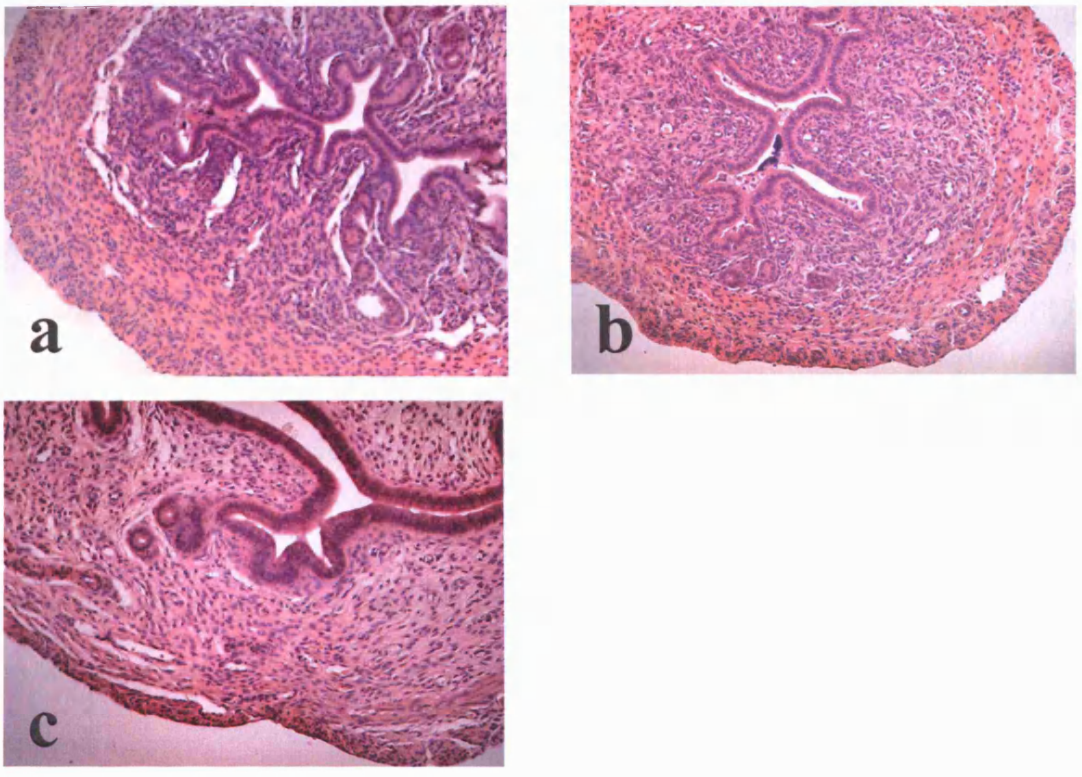


Figure 5.10: Representative Uterus Section from Three Month Old ERKO Mice.

H&E sections show uteri from three month old ERKO mice after neonatal dosing with SERMs **a**. Vehicle only control dosed ERKO mouse uterus section. **b**. 1000 µg/kg dosed WT mouse uterus section. **c**. 1000 µg/kg dosed ERKO mouse uterus section. There were no clear consistent histopathological changes in the ERKO mouse uterus after dosing with tamoxifen in comparison with controls. (original magnification x 16)

b. Gene Expression Studies.

To examine the role ERα plays in mediating pathology and gene expression changes in response to tamoxifen, long term gene expression changes in the ERKO mouse uterus were analysed using EST (cDNA) microarrays and Cy3, Cy5 labelling. Mice were dosed on days 2 to 5 after birth and culled at three months of age, using the same protocol used for CD-1 mice described previously in this Chapter. Gene expression was compared for tamoxifen (1000 μg/kg) dosed ERKO mice individually challenged against a pooled control of four vehicle only dosed ERKO mice. Microarrays were analysed to identify genes significantly changed across all microarrays at $p < 0.05$. Due to limitation in time, microarrays were not carried out for WT controls Vs WT tamoxifen dosed mice. All gene expression data are available (Appendix 1).

205 genes were significantly ($p < 0.05$) changed in neonatally dosed ERKO mice treated with tamoxifen in comparison to vehicle dosed controls. Unlike CD-1 mice, tamoxifen treatment resulted in expression of a majority of genes being increased. Expression of 108 (53%) genes was increased and 97 (47%) in which expression was decreased. Table 5.7 shows all genes in which expression was significantly changed relative to controls in both CD-1 and ERKO tamoxifen dosed animals. Expression of only nuclear RNA export factor 1 homolog, *Nxf1*, was changed differentially in the two mice strains, where expression is increased by tamoxifen in ERKO mice and decreased in CD-1 mice. In ERKO mice, *Ltf* expression was increased 4.6 fold relative to controls and was the most strongly induced by tamoxifen in the present microarray data. Although *Ltf* has an oestrogen responsive element in its promoter region, and prolonged expression has been shown in response to diethylstilboestrol in neonatal CD-1 mice (Li *et al.,* 1997b), data presented here suggest that there are complex mechanisms involved in regulation of gene expression that may be ERα independent.

Gene ID	Gene Name	Fold Change		Gene Function
		CD-1	ERKO	
AJ251364	*5830458K16Rik*	1.54	1.84	Integral to membrane
NM_011338	*Ccl9*	1.23	1.46	Chemotaxis
NM_010926	*Noc4*	-1.37	-1.20	Energy pathways
NM_008749	*Nucb1*	2.43	1.06	Calcium ion homeostasis
NM_016813	*Nxf1*	-1.04	1.22	Nucleocytoplasmic transport
NM_013585	*Psmb9*	1.30	1.20	Ubiquitin-dependent protein degradation
M23016	*Trf*	1.29	1.20	Iron transport

Table 5.7: All Genes in which Expression is Significantly Changed by Tamoxifen in both CD-1 and ERKO Mice.

None of these genes were identified in a recent gene wide identification of high affinity oestrogen response elements (Bourdeau *et al.*, 2004). CD-1 mice received 250 μg/kg/day where as ERKO mice received 1000 μg/kg/day oral tamoxifen. 25 μg total RNA was labelled using Cy3 / Cy5 (MRC Toxicology Unit protocol) and hybridised to HGMP Oligo microarrays (CD-1) or EST cDNA microarrays (ERKO).

5.3 Discussion.

5.3.1 Development of Adenomyosis in CD-1 Mice.

In this Chapter it is shown that neonatal exposure to oral tamoxifen and 4-hydroxyoestradiol, leads to long term phenotypic changes in the uterus by three months of age. Adenomyosis develops after tamoxifen treatment specifically on days 2 to 5 after birth. However, in mice dosed with 4-hydroxyoestradiol, there was endometrial epithelial hyperplasia and a reduction in the thickness of the endometrial stroma and myometrium and not adenomyosis. Also, in contrast to tamoxifen, there was a complete lack of mature corpora lutea in the ovaries with an absence of cycling within the endometrium in mice treated with 4-hydroxyoestradiol. Adenomyosis did not develop after oestradiol treatment or in control mice, implying that a purely oestrogen antagonist action is not responsible for this effect. Although adenomyosis develops in mice at 6 to 12 months of age even in control animals (Green *et al.,* 2005), it is uncommon at the three month period. Tamoxifen induced adenomyosis was further shown in the present study to affect the fertility of mice.

The effects of tamoxifen in the uterus have mainly been studied in the endometrium due to the increased incidence of endometrial cancers. However, tamoxifen clearly affects the myometrial layer of the uterus, as illustrated in the present study. Other research has shown, the myometrium to be underdeveloped in mice dosed subcutaneously with tamoxifen on days 1 to 5 after birth (Newbold *et al.,* 1997). In addition, oral treatment with tamoxifen in adult rats has a long term antagonist effect in the myometrium (Nephew *et al.,* 2000), suggesting that these antagonist effects occur not only in the developing myometrium. Although regarded as an oestrogen agonist in the uterus, it has been suggested that tamoxifen has an oestrogen antagonist effect at high doses (Newbold *et al.,* 1997). In our model, it is unlikely that the

effects of tamoxifen on the myometrium are due to a simple oestrogen antagonist effect in the myometrium as all doses had oestrogen agonist action (Chapter 4).

Adenomyosis that develops after tamoxifen treatment was shown in this Chapter to affect fertility in mice, where treated mice had a lower successful birth rate than control animals. Recently, using magnetic resonance imaging, the prevalence of adenomyosis was shown to be around 90% in infertile women presenting with endometriosis and who had fertile partners (Kunz *et al.*, 2005).

In the uteri of mice treated with 4-hydroxyoestradiol but not oestradiol or tamoxifen, there was an apparent absence of cycling in the endometrium. In addition, the ovaries of mice receiving 4-hydroxyoestradiol did not contain mature corpora lutea. As hormonal secretion by the corpora lutea plays a salient role in the endometrial cycle, this may account for the lack of cycling seen in the endometrium. It is known that 4-hydroxyoestradiol has a particularly high binding affinity in the ovary in comparison to other tissues (Philips *et al.*, 2004), which may play a role in the differential effects seen in this tissue between tamoxifen and 4-hydroxyoestradiol.

In the present study, the use of oral dosing of tamoxifen was considered to be the most clinically relevant route of administration. Comparing our results obtained here with published literature where subcutaneous dosing has been used, there are clear differences in the effects on the uterus of tamoxifen via the different dosing routes. Using subcutaneous dosing of neonatal CD-1 mice (from a breeding colony maintained at the NIEHS) on days 1-5 after birth, uterine cancers developed in up to 50% of animals dosed with tamoxifen (10 μg/pup/day) and in 66% of mice dosed with 4-hydroxyoestradiol (2 μg/pup/day) by 17

months of age (Newbold *et al.*, 1997; Newbold *et al.*, 2000). Power calculations to detect 40% tumours at the 5% significance level in treated mice show that 19 mice should have been used per group in this thesis. Although only four mice were assessed for histopathological changes per dosed group, no evidence of uterine cancers or pre-neoplastic changes were found in the in 3 month old CD-1 mice after oral dosing with either tamoxifen or 4-hydroxyoestradiol on days 2-5 after birth. Supporting this finding, no atypical cytological changes suggestive of premalignant change were found by 12 months of age in CD-1 mice treated with 1000 μg/kg/day oral tamoxifen on days 2-5 after birth (Green *et al.*, 2005). This evidence suggests that uterine tumours are not a common feature after oral dosing as is found after subcutaneous dosing.

Why subcutaneous dosing of tamoxifen should lead to cancers and oral dosing lead to adenomyosis is unclear. It is known for oestradiol at least, that oral administration leads to high serum concentrations of oestrone, whereas subcutaneous administration generally leads to higher serum concentrations of oestradiol (O'Connell, 1995), most likely as a result of first pass metabolism effects. It is likely that differential metabolism of the compounds plays a salient contribution to differential effects on the uterus.

5.3.2 Genotoxicity of Tamoxifen and 4-Hydroxyoestradiol.

As outlined in Chapter 1, it is still unclear whether metabolism of tamoxifen to a DNA reactive intermediate is involved in tamoxifen induced uterine histopathological changes. Generally, it is accepted that though the tamoxifen metabolite α-hydroxytamoxifen or its sulphate ester may form DNA adducts (White ,2003), 4-hydroxyoestradiol also has the potential to form DNA adducts (Devanesan *et al.*, 2001; Newbold *et al.*, 2000). There is no evidence that with either tamoxifen or 4-hydroxyoestradiol such DNA adducts are formed in

the uterus of treated neonatal mice. Even in adult mice given tamoxifen, no DNA adducts could be detected in uterine tissues (Martin *et al.,* 1997).

Recently gene expression profiling has been used to identify 20 genes that discriminate between genotoxic and non-genotoxic carcinogens in human HepG2 cells (van Delft *et al.,* 2004). None of the genes identified was significantly changed in expression in any of our arrays. Assuming that markers of genotoxicity are similar in HepG2 cell and the uterus, then it is reasonable to suggest that phenotypic changes in the uterus in response to tamoxifen or 4-hydroxyoestradiol are not due to genotoxic activity. It is possible that expression of genes associated with genotoxicity may have been changed transiently in the uteri of treated mice at the time of dosing and that these were not involved in the subsequent evolution of adenomyosis.

There were obvious differences in the overall morphology of the uteri and ovaries of mice receiving tamoxifen and 4-hydroxyoestradiol. Due to differences in pathology and gene expression caused by the two compounds, it is suggested that there are likely to be other mechanisms which play a more salient role than a genotoxic effect of tamoxifen in the uterus.

5.3.3 Understanding the Development of Adenomyosis.

Adenomyosis has been reported in 60% of postmenopausal women on tamoxifen (Cohen *et al.,* 1997). Adenomyosis may occur in conjunction with endometriosis with a prevalence of up to 79% (Kunz *et al.,* 2005). Although, current evidence suggests that adenomyosis and endometriosis are merely clinical variations of the same disease process (Leyendecker *et al.,* 1998). The precise aetiology of adenomyosis is not clearly understood, and may result from primary disruption of either the endometrium or myometrium.

Adenomyosis is defined as 'the presence of endometrial glands and stroma located haphazardly and deep within the myometrium', though the precise mechanisms of how adenomyosis develops are still unclear (Ferenczy ,1998). Because continuity between basal endometrium and adenomyotic lesions is often seen, it has been suggested that adenomyosis is the consequence of down growth and invagination of the uterine endometrium into the myometrium. Also, endometriotic stromal cells are highly invasive compared to normal endometrial stroma (Gaetje *et al.,* 1995), supporting the notion that adenomyosis is an 'invasion' of the myometrium by the endometrium. However it has also been proposed that adenomyosis may be the consequence of disorganised myometrium differentiation from stroma (Parrott *et al.,* 2001) and magnetic resonance imaging has identified that the primary defect in adenomyosis may be myometrial dysfunction (Brosens *et al.,* 1998; Kunz *et al.,* 2000).

In the new born rat, only the luminal epithelium is differentiated with differentiation of the myometrium and glandular epithelium occurring postnatally (Branham *et al.,* 1985). The two layers of the myometrium become distinguishable by day 10 postpartum (Brody *et al.,* 1989). Myometrial differentiation from the mesenchyme is known to be dependent upon interaction with uterine epithelium (Cunha *et al.,* 1989), which is thought to be mediated through interactions with the extracellular matrix. In this Thesis it was shown that in the tamoxifen induced adenomyotic mouse uterus and endometrium from women receiving tamoxifen, expression of genes involved in tissue structure and cytoarchitecture were down regulated which could alter the normal interactions needed for the differentiation or maintenance of myometrium.

5.3.4 Long Term Gene Expression Changes.

There is increasing evidence that long term changes in gene expression following neonatal dosing may be an important mechanism involved in tamoxifen toxicity in the uterus. The mechanism whereby long-term gene expression changes occur is not fully understood. It is known that lactotransferrin is strongly up-regulated in mice treated neonatally with diethylstilbestrol. Alteration in DNA de-methylation in five CpG sites immediately upstream from the oestrogen response element promoter of the *Ltf* gene can be detected 17 to 30 days later (Li *et al.,* 1997b). This effect has also been shown for the *c-Fos* gene (Li *et al.,* 2003). It was shown in this Chapter that *Ltf* expression was greatly increased in ERKO mice dosed with tamoxifen, and therefore if alteration of promoter methylation is responsible, suggests that this effect may occur independents of ERα.

However, this mechanism may not be universal. Uterine *Hoxa-10* and *Hoxa-11* expression, potently repressed by perinatal diethylstilbestrol exposure show no alteration in proximal promoter CpG methylation patterns, these being highly un-methylated in both control and diethylstilbestrol-dosed mice from postnatal days 5 to 30 (Li *et al.,* 2001). In the present study, gene microarray data showed a significant decrease in *Hoxa-11* expression (-0.35) but not *Hoxa-10* at 3 months after dosing with tamoxifen in CD-1 mice. Despite this, alteration of promoter methylation is likely to be an important factor to be considered for the long term changes in gene expression seen in this Chapter. Some genes identified in this study are discussed later in this Section.

It is known that in rats, exposure to tamoxifen leads to long term decrease in uterine oestrogen receptor and that exposure must occur before postnatal day 4 for this to occur (Csaba *et al.,* 1998; Csaba *et al.,* 2000; Csaba *et al.,* 1998). In this Thesis it was shown that exposure to

tamoxifen must occur before postnatal day 4 for adenomyosis to occur. This is likely to be a combined effect of the sensitivity of the developing uterus and ability for long term gene expression to be changed during this time frame.

Six genes that were thought to be involved in the development of adenomyosis were identified from our microarray data and their expression confirmed using real time PCR. Platelet derived growth factor (PDGF) has been shown be a myometrial cell mitogen (Chegini *et al.,* 1992; Rossi *et al.,* 1992), which potentially plays an important role in myometrial organisation. A tyrosine kinase receptor for PDGF (*Pdgfra*), previously shown to be expressed in smooth muscle cells (Hu *et al.,* 1998), was up regulated by oestradiol but down regulated by tamoxifen and 4-hydroxyoestradiol in our study. Recent genome wide analysis identified 50 genes which discriminate normal from malignant endometrium in women (Mutter *et al.,* 2001). In that study, expression of *Pdgfra* was strongly decreased in malignant endometrium. However, expression of this gene was found to be increase in isolated cell cultures treated with tamoxifen (Pole *et al.,* 2005), though as previously stated cell interactions play an important role in the response to oestrogen agonists in the endometrium. Although adenomyosis is generally considered a benign condition, decreased expression of this gene may be a common mechanism in the development of adenomyosis and endometrial cancers.

Microarray data show that expression of the thrombospondin gene, *Thbs1* (or *Tsp-1*) was decreased after tamoxifen and 4-hydroxyoestradiol treatment. Thrombospondin is an extracellular protein that interacts with other extracellular proteins and surface receptors. Expression has been identified in the myometrium (Morimoto *et al.,* 1998) and is responsive to TGFβ and PDGF (discussed above). *Thbs1* expression has also been shown to be

permissive for PDGF mediated smooth muscle cell proliferation and migration (Isenberg *et al.,* 2005) and is thought to be involved in tissue remodelling. It is possible that decreased expression of *Thbs1* after tamoxifen and 4-hydroxoestradiol may lead to disruption of normal myometrial organisation. Analysis of gene expression profiles using Ingenuity Software [http://www.ingenuity.com], showed that *Thbs1* expression was linked in a pathway involving cellular function and maintenance.

Collagen IV (*Col4a2*) was up-regulated by all three compounds tested. Collagen IV is a basement membrane protein and is involved in tissue remodelling. Expression has been shown to be higher in endometriotic lesions (Konno *et al.,* 2003) and myometrial fibroids (Weston *et al.,* 2003). This suggests that neonatal exposure to oestrogen agonists disrupts long term expression of a fundamental gene involved in tissue remodelling. However, it is unlikely that this gene is involved in the onset of uterine disease due to the lack of phenotypic change in oestradiol treated mice.

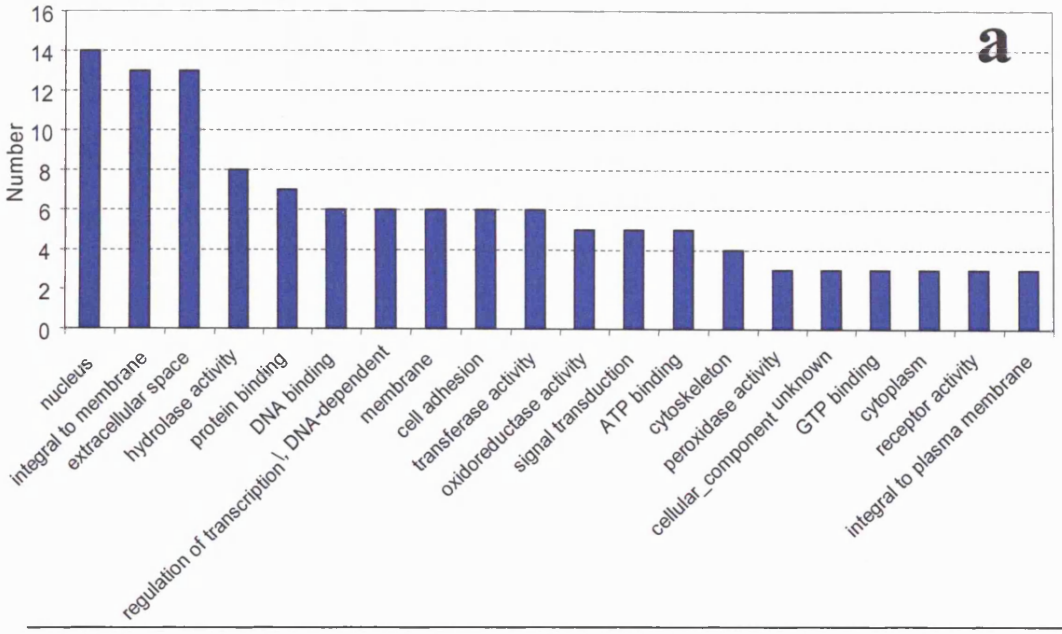
Gap junction A1 (*Gja1*), also know as connexin 43 (*Cx43*), is the major gap junction protein in the myometrium (Loch-Caruso *et al.,* 2004; Brosens *et al.,* 1998; Loch-Caruso *et al.,* 1992). Throughout the menstrual cycle different patterns of myometrial contractions occur in order to maintain functional polarity in the myometrium. This is most probably mediated through connexion 43 (Brosens *et al.,* 1998). Aberrant expression of CX43 has been reported in the epithelium of nearly all cases of endometriosis (Regidor *et al.,* 1997). In our study expression was not changed by oestradiol, though there was a small but significant decrease caused by tamoxifen and 4-hydroxyoestradiol. Oestradiol has been shown to increase expression of *Gja1* mediated through a conserved zinc-finger (Oltra *et al.,* 2003), most likely mediated through an AP-1 site regulated by oestradiol (Hu *et al.,* 2003; Webb *et al.,* 1999).

Decreased expression of *Gja1* in response to tamoxifen and 4-hydroxyoestradiol may lead to altered non-gravid myometrial contractions potentially involved in maintenance of myometrial structure, disruption of which may lead to adenomyosis.

In our arrays *Cyp2e1* was the only gene to be expressed on all our arrays where expression was altered in the opposite direction after oestradiol (increased) than to 4-hydroxyoestradiol and tamoxifen (decreased), although expression of *Pdgfra* showed this same pattern as assessed by RT-PCR. This supports previous findings that oestradiol can increase expression of *Cyp2e1* in the endometrium (Hukkanen *et al.,* 1998; Kikkawa *et al.,* 1994). Literature suggests that the CYP2E1 enzyme does not metabolise oestradiol (Brosens *et al.,* 1998; Lee *et al.,* 2003), so it is unlikely that CYP2E1 mediated metabolism is involved in pathogenesis in the uterus. Although CYP2E1 may mediate metabolism of other oestrogenic compounds such as genistein (Helsby *et al.,* 1998; Hu *et al.,* 2003), it is not suggested that this gene is involved in the development of adenomyosis.

Further into this project, the microarray data analysis programme GenMAP was used to analyse microarray data. Genes that were significant at $p < 0.01$ were grouped according to their 'GO' function (Figure 5.11a) and then groups identified that were involved in certain cellular processes, these were: regulation of transcription, cell adhesion and cytoskeleton. These groups were identified as in the adenomyotic uterus where there is an obvious change in tissue structure which may be normally regulated by gene in these groups. Genes from the groups that were identified in our study are listed in Figure 5.11b and further details about their function provided in Table 5.8. This analysis provides further evidence that tamoxifen may suppress gene transcription as expression of 15 out of 16 genes identified was decreased after tamoxifen treatment. In contrast three out of four genes were up regulated by oestradiol.

Gene ontology of tamoxifen induced uterine gene expression changes (P<0.01) (GenMAPP 2, http://www.GenMAPP.org/)



Expression of genes that may be involved in uterine morphogenesis 3 months after neonatal treatment with tamoxifen, 4-hydroxyoestradiol or oestradiol **b**

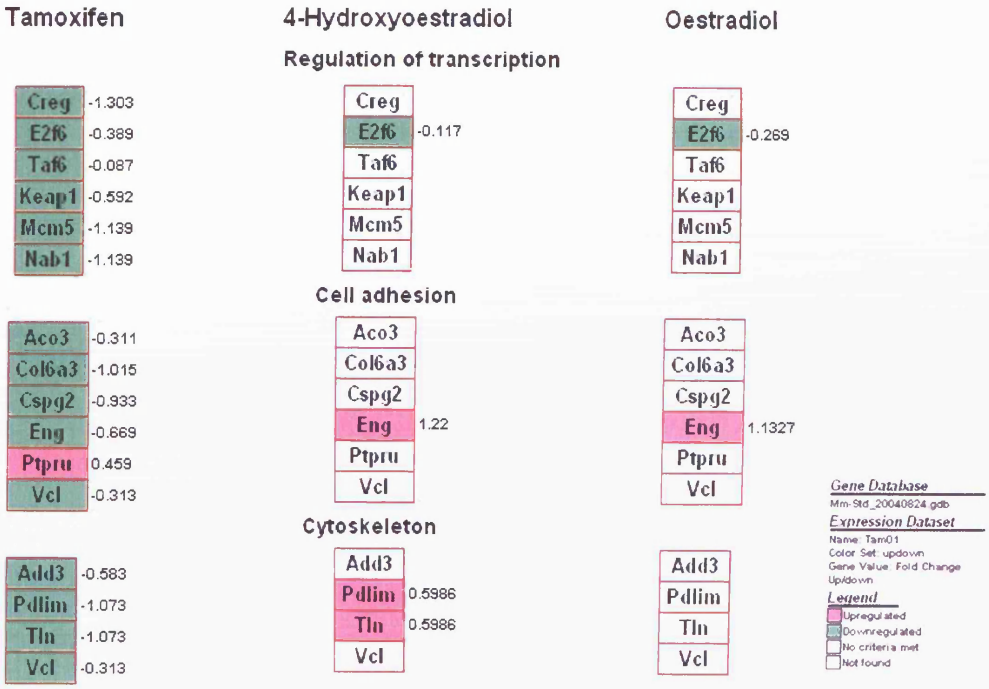


Figure 5.11: GenMap Analysis of Three Month Gene Expression Functions.

Gene	Fold change	Function
1.Regulation of transcription, DNA dependent		
Creg	-1.30	Cellular repressor of E1A-stimulated genes. May inhibit cell growth or promote differentiation (Veal *et al.*, 1998; Veal *et al.*, 2000)
E2f6	-0.39	Inhibitor of a subset of E2F-dependent genes Regulates cellular proliferation (Kherrouche *et al.*, 2004; Pohlers *et al.*, 2005)
Taf6	-0.09	Transcription initiation factor that plays a role in mediating promoter responses to various activators and repressors (Kotova *et al.*, 2001)
Gna14	-0.55	Guanine nucleotide-binding protein, alpha-14 subunit. involved as modulators or transducers in various transmembrane signalling systems (Strathmann *et al.*, 1989).
Keap1	-0.59	Kelch-like ECH-associated protein 1. Regulates *Nfe2l2/Nrf2* acting as a negative regulator of Nrf2 and as a sensor of xenobiotic and oxidative stresses (Velichkova *et al.*, 2005; Motohashi *et al.*, 2004)
Mcm5	-1.14	Minichromosome maintenance deficient 5, cell division cycle 46. Required to initiate eukaryotic DNA replication. Over expression has been associated with dysplasia and malignancy (Guida *et al.*, 2005)
Nab1	-0.31	Ngfi-A binding protein 1. Acts as a transcriptional repressor for EGR transcription factors (Buitrago *et al.*, 2005)
2. Cell adhesion		
Aco3	-0.31	Cell adhesion protein that participate in lymphocyte recirculation by mediating the binding of lymphocytes to peripheral lymph node vascular endothelial cells (Stolen *et al.*, 2005b)
Col6a3	-1.01	Extracellular matrix structural constituent conferring tensile strength. Regulated by TGF-beta (Verrecchia *et al.*, 2001)
Cspg2	-0.93	Encoding the core protein of the cartilage chondroitin sulphate proteoglycan, versican. May play a role in intercellular signalling and cell adhesion Binds hyaluronic acid (Stolen *et al.*, 2005a)
Eng	-0.67	Endoglin precursor. TGF-beta binding protein of endothelial and stromal cells (Ouellette *et al.*, 1999; St Jacques *et al.*, 1994)
Ptpru	+0.46	Transmembrane receptor protein tyrosine phosphatase signalling (Taniguchi *et al.*, 1999)
Vcl	-0.31	Vinculin. May be involved in the attachment of the actin-based microfilaments to the plasma membrane and in myogenic differentiation (Zhang *et al.*, 2004b; Tomczak *et al.*, 2004)
3. Cytoskeleton		
Add3	-0.69	Gamma adducing: Membrane-cytoskeleton-associated protein that promotes assembly of the spectrin-actin network. Binds to calmodulin (Suriyapperuma *et al.*, 2000; Gilligan *et al.*, 1999)
Pdlim1	-1.08	Programmed cell death 1 ligand 1mouse. Cytoskeletal protein that may act as an adapter that brings other proteins to the cytoskeleton (Kioussi *et al.*, 2002)
Tln	-0.30	Talin. Involved in connections of major cytoskeletal structures to the plasma membrane (Jiang *et al.*, 2003)
Vcl	-0.31	As above

Table 5.8: Genes that may be involved in uterine morphogenesis where expression is significantly changed (P<0.01) by neonatal tamoxifen treatment.

(GenMAPP2 analysis http://www.genmapp.org)

A recent review has highlighted the role of repression of gene expression by oestrogen as a mediator of oestrogen action (Zubairy *et al.,* 2005). The classical model of transcription involves oestrogen binding to the ER leading to repositioning of helix 12. This allows recruitment of transcriptional machinery and thus inducing transcription. However, as highlighted in our study, 32% of genes changed in response to oestradiol were decreased in expression. This was even more for tamoxifen, with expression of 78% of genes being repressed by tamoxifen. The precise mechanisms or functions of oestrogen agonist induced gene repression remains unclear. Mechanism could include recruitment of corepressor instead of coactivators, the involvement of nonclassical ERα pathways and / or involvement of ERβ in oestrogen-mediated gene repression (Zubairy *et al.,* 2005).

It is interesting to note that oestradiol led to a large number of gene expression changes yet led to no phenotypic change. It was shown in this Chapter that, where tamoxifen generally led to a decrease in expression of genes (78%) which could contribute to adenomyosis, oestradiol led to an increase in expression (68%). It is possible that oestrogen has a protective effect against the development of adenomyosis. Oestrogen is thought to promote myometrial differentiation from mesenchyme at the junction zone between stroma and the inner myometrium (Brosens *et al.,* 1998), an interface that is less well defined in adenomyosis. Adenomyosis often develops by 12 months in CD-1 mice dosed with vehicle only on days two to five after birth (Green *et al.,* 2005), it would be interesting to examine if neonatal dosing with oestradiol prevents this.

5.3.5 Effects of Tamoxifen in the ERKO Mouse Uterus.

It was shown that there was a non-significant decrease in uterine weight in both control and tamoxifen dosed ERKO mice and also WT tamoxifen dosed mice. As shown in this Chapter, tamoxifen at 1000 μg/kg given to neonatal CD-1 mice leads to a significant decrease in uterine weight at three months of age. If this effect is largely mediated via ERα, uterine weight changes in ERKO mice caused by tamoxifen would be expected to be less that in the ERα expressing CD-1 strain.

At three months after dosing, there were no consistent histopathological changes observed between the uteri of ERKO controls and those treated with tamoxifen. Although there was one focus of adenomyosis in one tamoxifen treated mouse, this was not interpreted as being significant. In addition, adenomyosis did not develop in WT mice treated with tamoxifen. The WT mouse is based on the C57BL/6 mouse, which may be less susceptible to develop adenomyosis than CD-1 mice.

Using gene microarrays, it was shown that there are only six genes in which expression is similarly changed by tamoxifen in comparison to controls in both CD-1 and ERKO mice. It is likely that altered expression of these genes after tamoxifen treatment is independent of ERα. However, considering tamoxifen led to adenomyosis in only the CD-1 mouse and not in ERKO or C57BL/6 WT mice, it is unlikely that these effects are mediated by the six genes identified in the present study. Changes in gene expression between WT controls and tamoxifen treated mice were not assessed in this study, though this would make an interesting comparison to establish genes in which expression was mediated though ERα using a comparable strain of mouse.

Previous research with the ERKO mouse has revealed a distinct oestrogen signalling pathway independent of ERα (Das *et al.,* 1997), where 4-hydroxyoestradiol but not oestradiol was able to strongly induce *Ltf* expression. It has been proposed that this may be a result of isoforms of ERα, such as the 61kDa ERα (Kos *et al.,* 2002), or cross talk between growth factors signalling pathways or due to a novel oestrogen-type receptor (Philips *et al.,* 2004). It is known that the *Ltf* promoter contains a full palindromic ERE and also a functional half ERE site (O'Lone *et al.,* 2004). The truncated ERα protein (61 kDa) lacks transcriptional activity through the AF-1 domain (Kos *et al.*, 2002). However, at 3 months after dosing, all tamoxifen is likely to have been cleared from the body and increase in expression is likely to have occurred through epigenetic mechanisms such as promoter hypomethylation (Li *et al.*, 1997).

Gene expression studies described in this Chapter have used RNA isolated from whole uterus. It is clear that the response will differ between both the endometrium and myometrium, but also the separate cell types. For example, it has been shown that oestrogen induces proliferation of ERα negative glandular epithelium if the underlying stroma expresses ERα (Bigsby, 2002). Recently, gene expression has been analysed in isolated cell cultures (Pole *et al.,* 2005), although this removes cell interaction that may be critical in the gene expression response to tamoxifen. Future work utilising Laser Capture Microdissection will help identify the contribution of each tissue in the detrimental effects of tamoxifen in the uterus.

It is also of note that the CD-1 mouse strain used in theses studies is an out-bred strain. It is likely therefore that there is a large influence of biological variability between mice. It is possible that if an inbred mouse strain were to be used, such as the C57BL/6J, on which the ERKO mice are based, the variability in the gene expression response to SERMs would be minimised.

The effects of endogenous oestrogens or hormonal cycling was avoided in our long term studies treating mice with SERMs on days 1 to 4 after birth. However, in these long term studies gene expression was assessed in three month old mice in which hormonal cycling was well established with the exception of mice dosed with 4-hydroxyoestradiol. No assessment of oestrous stage was made in this study, though in retrospect this would have been appropriate. In addition neonate mice dosed with SERMs were housed with mothers until weaning. It is recognised that these neonates will have also been exposed to oestrogens in mothers milk that may have had an effect on the neonate uterus. However, any potential effect from this should have been controlled for since vehicle only dosed mice also had access to mother's milk.

5.3.6 Summery of Chapter Findings.

In this Chapter it was shown that an oestrogen agonist action does not predict long term histopathological changes in the uterus as tamoxifen but not 4-hydroxyoestradiol or oestradiol led to adenomyosis. However, all compounds tested may lead to long term gene expression changes after neonatal exposure. Expression of a majority of genes was increased after oral oestradiol but decreased after tamoxifen. It is suggested that down regulation of gene expression by tamoxifen, for example in *Pdgfra* and *Gja1*, may alter normal uterine structure and lead to adenomyosis. It was difficult to interpret findings from studies in ERKO mice in relation to the role of ERα in the development of adenomyosis. However gene expression studies in this model show that ERα is not required to effect long term expression of genes.

Chapter 6.

General Discussion.

Tamoxifen is successfully used for the treatment of breast cancer, although long term use is associated with an increase in endometrial pathologies including carcinomas and adenomyosis (Cohen *et al.,* 1997; Fisher *et al.,* 1994; Rutqvist *et al.,* 1995). These adverse actions in the uterus may be mediated through several mechanisms including: i, a purely oestrogen agonist action; ii, differential gene expression between oestradiol and tamoxifen, such as signalling through AP-1 pathways (Webb *et al.,* 1999); or iii, the formation of DNA adducts (Shibutani *et al.,* 2000). The affect of oestradiol, 4-hydroxyoestradiol, or tamoxifen in the neonate mouse model has not previously been studied in detail at the molecular level. The main aims of the work described in this Thesis were:

1. To determine the maximum uterotrophic dose of these compounds in a standard uterotrophic assay using 14 day old immature mice.
2. Use these doses to assess if the development of adenomyosis in the neonate mouse model was directly related to oestrogen agonist activity.
3. To establish if gene expression changes could be correlated with alterations in uterine pathologies associated with adenomyosis.
4. Determine if changes in uterine gene expression at 3 months after neonatal dosing with the different oestrogen agonists were all similar, as suggested by Moggs *et al.*, (2004*a*), using data from immature mice.
5. Investigate if neonatal treatment of mice with SERMs leads to adverse affects on the reproductive success.
6. To see if the neonate mouse could be used to predict the effects of tamoxifen in uterine tissues of women taking tamoxifen therapeutically.

The overall goal of the work was not to assess the risks to neonate mice of taking SERMs but to establish if the model might be a plausible one for women taking this class of drug.

6.1 Uterotrophic Effects of SERMs in the Immature Mouse Uterus.

The gravimetric uterotrophic assay is currently the best established, short-term rodent test for oestrogenicity. Other end-points include morphometric (epithelial cell height, lamina propria area), luminal and glandular epithelial labelling index, time to vaginal opening or lactotransferrin expression (Ashby, 2001). In Chapter 4 it was shown that oestradiol, 4-hydroxyoestradiol and tamoxifen were all able to induce increases in uterine weight and oestrogen responsive gene expression. In the present study, the maximal uterotrophic dose for oestradiol (100 μg/kg) and tamoxifen (250 μg/kg) broadly agree with published data for immature CD-1 mice: 60 μg/kg for subcutaneously administered oestradiol (Moggs *et al.,* 2004a); and 1 mg/kg for tamoxifen (Newbold *et al.,* 2001). It is interesting that in the latter study, a dose of 10 mg/kg of 4-hydroxytamoxifen was needed to give a maximal uterotrophic dose, in spite of 4-hydroxytamoxifen being generally considered more oestrogenic than tamoxifen (Lyman *et al.,* 1985). In the case of 4-hydroxyoestradiol, information on its uterine effects are more limited. In C57BL/6J mice, two injections (10 μg/kg) at 6 h intervals of oestradiol or 4-hydroxyoestradiol resulted in similar up regulation of the lactotransferrin gene (Das *et al.,* 1997). In newborn CD-1 mice, 4-hydroxyoestradiol given subcutaneously at a dose of 2 μg/pup on days 1 to 4 of neonatal life resulted in an increase in uterine weight of 213% on day 5, considerably greater than the 133% increase caused by 17β-oestradiol (Newbold *et al.*, 2000). To our knowledge, a uterotrophic assay for 4-hydroxyoestradiol on immature mice has not previously been carried out.

In terms of changes in gene expression, Moggs *et al.*, (2004a) showed the transcriptional response for 179 genes in the intact immature mouse uterus using the standard three day assay protocol in the uterus to be similar between oestradiol, genistein and diethylstilboestrol. To establish that gene expression changes occurring in the uteri of mice in our study were

behaving similarly to the above study, we confirmed by quantitative PCR that three of these genes: lactotransferrin (*Ltf*); alkaline phosphatase (*Akp2)* and Complement 3 (*C3)* were similarly upregulated by oestradiol (Chapter 4, Table 4.2). For all of the SERMs tested, we also measured luminal cell proliferation, using immunostaining for PCNA. However, this did not give as reliable an indication of oestrogen agonist effect as uterine weight increases. Therefore, the increase in uterine wet weight was used as the primary index of oestrogen agonist action.

It is appreciated that a uterine response to estrogens involves a highly complex sequence of events initially involving, for example, binding of the SERM to the oestrogen receptors, water imbibition, DNA synthesis and cell proliferation, eventually leading to an increased uterine weight. Oestrogenicity determined in an immature 14-day old mouse need not necessarily be the same in a neonate mouse on days 2 to 5 days after birth. However, these three oestrogen agonists were investigated at their maximal uterotrophic doses with respect to longer-term pathological changes related to the development of adenomyosis.

6.2 Relationship Between the Development of Adenomyosis in the Neonate Mouse Model and Oestrogen Agonist Activity.

The present results show unambiguously that oestrogenicity *per se* is not the only factor that leads to the development of adenomyosis at three months following neonatal treatment of CD-1 mice. Results demonstrate that treatment with tamoxifen but not 4-hydroxyoestradiol or oestradiol leads to the development of this condition. The only other SERM know to have similar effects resulting in adenomyosis is the closely related triphenylethylene, toremifene (Parrott *et al.,* 2001). Treatment of neonatal mice with raloxifene which is a much weaker oestrogen agonist in the uterus, at an equimolar dose, resulted in minimal adenomyosis

(Green *et al.,* 2005). In terms of mechanisms, it would be interesting to see if other SERMs such as the triphenylethylene clomiphene used clinically in women for the induction of ovulation or the naturally occurring genistein found in soy milk were able to cause adenomyosis in this model.

Metabolites of both 4-hydroxyoestradiol and tamoxifen may be bioactivated to form DNA reactive intermediates (Dasaradhi *et al.,* 1997; Devanesan *et al.,* 2001; White *et al.,* 1992). For tamoxifen, the current model of hepatic genotoxicity involves α-hydroxylation of tamoxifen by CYP3A (White, 1999). Concern arises from the DNA reactive potentials of 4-hydroxyoestradiol and tamoxifen as both these compounds have been shown to cause uterine cancers (Newbold *et al.,* 1997; Newbold *et al.,* 2000). However, the capacity of the liver of newborn mice to carry out drug metabolism is generally much lower than in adults. Additionally, in mice tamoxifen is very rapidly detoxified by pathways such as *N*-oxidation and 4-hydroxylation rather than α-hydroxylation (Lim *et al.,* 1997). Even in adult animals only low levels of DNA adducts are detected in the liver and none in the uterus after tamoxifen treatment (Martin *et al.,* 1997). It is not thought therefore that the formation of DNA adducts play a role in the development of adenomyosis. Epigenetic mechanisms such as hypomethylation of gene promoters, which have been described for lactotransferrin after diethylstilbestrol treatment of neonatal mice (Li *et al.,* 1997b) may be responsible.

6.3 Gene Expression Changes Associated with Adenomyosis.

It has been suggested that high oestrogen levels are necessary for development and maintenance of adenomyosis (Yamamoto *et al.,* 1993). Data presented in this Thesis contradicts this finding as neonatal exposure to oestradiol did not lead to this condition (Chapter 5). Key genes that are suggested as predicting adenomyosis are *Pdgfra* and *Gja1*,

where a decrease in expression in both is associated with adenomyosis since both are thought to be involved in normal myometrial functioning (Chapter 5). Investigating the mechanisms involved in long term alteration of gene expression, for example, altered promoter methylation, protein expression and the precise roles of these in the development of adenomyosis provides grounds for future research. *Ngf*, previously identified (Green *et al.*, 2004) as a key gene that may be involved the development of this condition, was not up-regulated (*see below*). The reason for this is unclear. In the present study, a dose of tamoxifen of 250 μg/kg was used compared to 1 mg/kg previously. However, in both instances, adenomyosis developed in the uterus.

6.4 Changes in Uterine Gene Expression between the Different Oestrogen Agonists.

Results shown in Chapter 5 categorically demonstrate that with oestradiol, 4-hydroxyoestradiol and tamoxifen, all oestrogens are not similar with respect to long-term changes in uterine gene expression following neonatal treatment. Oestradiol leads to predominantly long term increases in expression where as tamoxifen leads to long term decreases. There were practically no similarities between genes changed in expression during the uterotrophic response (Moggs *et al.*, 2004a) and those altered three months after neonatal treatment with oestradiol. Table 6.1 shows of the 10,000 genes on the HGMP array, only six were changed in common and only three: protective protein for beta-galactosidase (*Ppgb*); desmin (*Des*); and ring finger protein 10 (*Rnf10*), were mutually up-regulated. This suggests that neonatal exposure to an oestrogen agonist may affect long-term expression of genes involved in the initial uterotrophic response, leading to a prolonged oestrogen agonist effect.

The presence of non-ERα mediated pathways has been shown. In the ERKO mouse model, 4-hydroxyoestradiol and tamoxifen (Chapter 5) are able to induce expression of an 'oestrogen responsive' gene, *Ltf*, while oestradiol itself had no effect (Das *et al.*, 1997). In the latter study, an injection of 4-hydroxoestradiol led to an increase in *Ltf* expression after six hours suggesting that short term mechanisms are involved. However, in the present study, *Ltf* expression was increased three months after four daily neonatal doses with tamoxifen in the ERKO mouse, which may occur through hypomethylation of the promoter as previously reported for diethylstilbestrol (Li *et al.*, 1997b).

RefSeq	**Gene Symbol**	**Uterotrophic Response in Immature Mice at 17 Days**	**Changes at 3 Months Following Neonatal Dosing**
		Fold change (Log_2)	
NM_008906	*Ppgb*	1.5	1.44
NM_010043	*Des*	1.2	1.49
NM_016698	*Rnf10*	0.6	0.29
NM_016809	*Rbm3*	1.1	-0.53
NM_019639	*Ubc*	0.7	-0.15
NM_010106	*Eef1a1*	-0.6	0.95

Table 6.1: Expression of Genes Altered by Oestradiol in both the Uterotrophic Response and at 3 Months after Neonatal Treatment.

6.5 Neonatal Treatment of Mice with Tamoxifen Leads to Adverse Affects on the Reproductive Success.

We have shown that neonatal dosing with oestradiol, 4-hydroxyoestradiol or tamoxifen in neonate mice leads to large long-term changes in gene expression in the uterus. One consequence of this, following tamoxifen treatment, is the early development of adenomyosis where the myometrial layers are disorganised and weakened. Additionally, for this effect, exposure must occur no later than days 2 to 5 after birth. In Chapter 5 it was demonstrated that this resulted in a dramatic reduction in the number of pups produced by the mated dams. Time did not allow an investigation to establish if this was a failure of blastocyst implantation or to other causes. A recent publication showed a reduction in the number of pups in mice treated with genistein. Here animals were fertile, as determined by implantation sites, but the pregnancy was not maintained (Jefferson *et al.*, 2005). No pathology was presented so it is not known if these animals had developed adenomyosis. Our studies show neonatal treatment of mice with 4-hydroxyoestradiol leads to loss of cycling of the uterus (Chapter 5), so it might be expected that these animals would be largely infertile. After oestradiol treatment, there are no obvious pathological changes and the animals cycle normally. Since female mice are normally exposed to low levels of oestradiol it would be interesting to see if the gene changes observed in the absence of adenomyosis affected fertility. In women there is an association between endometriosis, adenomyosis and infertility that was attributed to impaired uterine sperm motility (Kunz *et al.*, 2000). Adenomyosis has been reported in 60% of postmenopausal women on tamoxifen (Cohen *et al.*, 1997) and may occur in conjunction with endometriosis with a prevalence of up to 79% (Kunz *et al.*, 2005). A number of normal births have been reported in breast cancer patients taking tamoxifen even though this drug is contraindicated in pregnancy (Barthelmes *et al.*, 2004). Further work need to be carried out on adenomyosis and fertility in women.

6.6 Neonate Mouse as a Predictor of the Effects of Tamoxifen in Uterine Tissues of Women.

Comparing the results of Chapters 3 and 5 shows there is no correlation between changes in gene expression in women continuously treated for up to 4 years with tamoxifen and mice treated neonatally on days 2 to 5 with this drug. This may be related to both a species difference and an effect of different exposure. In terms of dose received, women receiving tamoxifen commonly take 20 mg per day, (0.28 mg/kg/day) and CD-1 mice in our studies received 0.25 mg/kg/day. This raises the question as to whether studies in rodents produce data applicable to a clinical situation. However, this model was initially developed to study the development of endometrial adenocarcinomas in mice (Newbold *et al.,* 1997). Although it was shown that the pathology of the uterine tumours was similar to that seen in women taking tamoxifen, there have been no reports comparing changes in gene expression known to occur in human uterine tumours to those in treated mice.

Gene expression studies in the normal endometrium of women treated therapeutically with tamoxifen show up-regulation of classical oestrogen responsive genes such as creatine kinase B (*CKB)* and insulin like growth factor 1 *IGF-1.* Although not detected on the arrays, real time PCR showed down-regulation of nerve growth factor (*NGFb*), even though protein expression was upregulated (Chapter 6). This is a further example of species differences since in the mouse NGF protein and *Ngfb* genes are upregulated by neonatal tamoxifen treatment (Green *et al.,* 2003).

6.7 Conclusions.

Results of this Thesis show that in the neonatal mouse model oestradiol, 4-hydroxyoestradiol and tamoxifen lead to long term changes in gene expression but major differences exist between the compounds. Changes in gene expression play an important role in the different uterine response between oestradiol and tamoxifen, where tamoxifen leads to adenomyosis but the mechanisms involved have not yet been positively identified. Hormonal imbalance that leads to adenomyosis is dependent on oestrogenic agonist action but as yet, unresolved additional factors require further study. Adenomyosis occurs naturally in normal mice 6 to 12 months of age, suggesting non-physiological drugs such as tamoxifen are not essential for the development of this condition.

Appendix. Individual Microarray Details and Geo References.

Species	Treatment	Group/ Sample	Control Labelling	Platform	Geo Reference
Mm CD-1	Oestradiol	1	Cy3	HGMP Oligo	GSM83169
Mm CD-1	Oestradiol	2	Cy3	HGMP Oligo	GSM83170
Mm CD-1	Oestradiol	3	Cy3	HGMP Oligo	GSM83171
Mm CD-1	Oestradiol	4	Cy3	HGMP Oligo	GSM83172
Mm CD-1	Oestradiol	1	Cy5	HGMP Oligo	GSM83173
Mm CD-1	Oestradiol	2	Cy5	HGMP Oligo	GSM83174
Mm CD-1	Oestradiol	3	Cy5	HGMP Oligo	GSM83175
Mm CD-1	Oestradiol	4	Cy5	HGMP Oligo	GSM83176
Mm CD-1	4-Hydroxyoestradiol	1	Cy3	HGMP Oligo	GSM83161
Mm CD-1	4-Hydroxyoestradiol	2	Cy3	HGMP Oligo	GSM83162
Mm CD-1	4-Hydroxyoestradiol	3	Cy3	HGMP Oligo	GSM83163
Mm CD-1	4-Hydroxyoestradiol	4	Cy3	HGMP Oligo	GSM83164
Mm CD-1	4-Hydroxyoestradiol	1	Cy5	HGMP Oligo	GSM83165
Mm CD-1	4-Hydroxyoestradiol	2	Cy5	HGMP Oligo	GSM83166
Mm CD-1	4-Hydroxyoestradiol	3	Cy5	HGMP Oligo	GSM83167
Mm CD-1	4-Hydroxyoestradiol	4	Cy5	HGMP Oligo	GSM83168
Mm CD-1	Tamoxifen	1	Cy3	HGMP Oligo	GSM83177
Mm CD-1	Tamoxifen	2	Cy3	HGMP Oligo	GSM83178
Mm CD-1	Tamoxifen	3	Cy3	HGMP Oligo	GSM83179
Mm CD-1	Tamoxifen	4	Cy3	HGMP Oligo	GSM83180
Mm CD-1	Tamoxifen	1	Cy5	HGMP Oligo	GSM83181
Mm CD-1	Tamoxifen	2	Cy5	HGMP Oligo	GSM83182
Mm CD-1	Tamoxifen	3	Cy5	HGMP Oligo	GSM83183
Mm CD-1	Tamoxifen	4	Cy5	HGMP Oligo	GSM83184
Mm ERKO	Tamoxifen	1	Cy3	MRC cDNA	GSM83187
Mm ERKO	Tamoxifen	2	Cy3	MRC cDNA	GSM83188
Mm ERKO	Tamoxifen	3	Cy3	MRC cDNA	GSM83189
Mm ERKO	Tamoxifen	1	Cy5	MRC cDNA	GSM83190
Mm ERKO	Tamoxifen	2	Cy5	MRC cDNA	GSM83191
Mm ERKO	Tamoxifen	3	Cy5	MRC cDNA	GSM83192
Hs	Tamoxifen	1	Cy3	HGMP Oligo Slide A	GSM83840
Hs	Tamoxifen	2	Cy3	HGMP Oligo Slide A	GSM83841
Hs	Tamoxifen	3	Cy3	HGMP Oligo Slide A	GSM83842
Hs	Tamoxifen	4	Cy3	HGMP Oligo Slide A	GSM83843
Hs	Tamoxifen	1	Cy5	HGMP Oligo Slide A	GSM83844
Hs	Tamoxifen	2	Cy5	HGMP Oligo Slide A	GSM83845
Hs	Tamoxifen	3	Cy5	HGMP Oligo Slide A	GSM83846
Hs	Tamoxifen	4	Cy5	HGMP Oligo Slide A	GSM83847
Hs	Tamoxifen	1	Cy3	HGMP Oligo Slide B	GSM83848
Hs	Tamoxifen	2	Cy3	HGMP Oligo Slide B	GSM83849
Hs	Tamoxifen	3	Cy3	HGMP Oligo Slide B	GSM83850
Hs	Tamoxifen	4	Cy3	HGMP Oligo Slide B	GSM83851
Hs	Tamoxifen	1	Cy5	HGMP Oligo Slide B	GSM83852
Hs	Tamoxifen	2	Cy5	HGMP Oligo Slide B	GSM83853
Hs	Tamoxifen	3	Cy5	HGMP Oligo Slide B	GSM83854
Hs	Tamoxifen	4	Cy5	HGMP Oligo Slide B	GSM83855

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