The effect of tamoxifen on uterine growth and development

Thesis submitted for the degree of

Doctor of Medicine

January 2012

By

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Abstract

The aim of this research is to examine the abnormal features of uterine growth and development associated with tamoxifen use, to determine if this is due to its failure to replicate all the effects of oestradiol; either inducing or repressing the expression of key paracrine acting regulators of uterine cellular populations or their gene expression profiles, and to examine if such abnormal features could be prevented by the administration of a progestagen-containing levonorgestrel intrauterine device (LNG-IUS) from the onset of tamoxifen treatment for breast cancer.

Postmenopausal women were assessed by hysteroscope and ultrasound scanning for uterine volume, endometrial thickness and the presence of endometrial polyps or submucous fibroids before insertion of the LNG-IUS system and after 1 year. The same was done on a control group that were not treated with LNG-IUS. Benign changes in the uterus were histologically related to, (1) steroid receptor isoform, (2) proliferation and apoptosis markers, (3) mesenchymal marker expression and (4), gene expression and pathway profiles, when compared to the oestrogen induced changes of the proliferative phase uterus and the oestrogen deficient, atrophic, postmenopausal uterus.

The uterotrophic effects and benign changes induced by tamoxifen were confirmed; LNG-IUS prevented both the uterotrophic effects and formation of endometrial polyps. Additionally, ERα, PRA and PRB expression were increased in tamoxifen endometrium similar to that of the proliferative phase endometrium; ERβ expression was also up regulated in the tamoxifen uterus linking it with tamoxifen associated proliferation. Increased Ki67 and Bcl2, but low BAX expression, in the tamoxifen uteri signified increased proliferation and apoptosis failure, coupled with the unique mesenchymal positivity associated with large endometrial cystic glands, suggested a transitional state tissue, undergoing remodelling with significant gene modulation. This was confirmed with microarray analysis where tamoxifen induced not only “oestrogen genes” but also “tamoxifen only genes” and pathways, revealing some of the mechanism(s) whereby tamoxifen causes uterine growth; some of these changes are oestrogen-like and others are brought out through the direct action of tamoxifen itself.

Acknowledgements

I thank my supervisors, Dr Anthony Taylor, Mr Marwan Habiba and Professor Steve Bell for their guidance, support and advice. I also thank Dr Taylor and Mr Habiba for their assistance in the analysis of the microarray data and in their help in data interpretation. The microarray was conducted by scientists at Geneservice Ltd., whilst initial gene expression analysis was performed by Dr Taylor. The confirmatory RT-PCR experiments were kindly and expertly performed by Mrs Muna Abbas to whom I am indebted. The presentation and interpretation of these data are mine and mine alone. I also thank Dr Laurence Brown (Consultant Gynaecology Histopathologist, University Hospitals of Leicester NHS Trust) for the accurate reporting and dating of the numerous endometrial and uterine biopsies and patient instruction on how to identify the histological features identified in these studies.

I would especially like to thank my husband, parents and my sister, Heena for their love and continued support and encouragement throughout my research. Finally, I must thank my four year old daughter, Maya, for her patience, whilst I wrote this thesis.

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Abbreviations

$(NH_4)_2SO_4$	ammonium sulphate
ABC	avidin biotin complex
ACOG	American College of Obstetricians and Gynaecologists
AF-1	activation domain 1
AF-2	activation domain 2
AIB-1	amplified in breast cancer 1
AMV-RT	avian myeloblastosis reverse transcriptase
ANOVA	one-way analysis of variance
AP	anteroposterior uterine diameter
ATAC	arimidex, tamoxifen alone or in combination
BAX	Bcl2 associated X protein
Bcl2	B-cell leukaemia/lymphoma 2 protein
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CA12	carbonic anhydrase 12
Ca-125	cancer antigen 125
CD24	cluster of differentiation 24
cDNA	complimentary deoxyribonucleic acid
cg	cystic glands
CO_2	carbon dioxide
cRNA	complimentary ribonucleic acid
CSS	charcoal stripped serum
CYP2D6	cytochrome P450 2D6
CYP3A4	cytochrome P450 3A4

DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
dH_2O	deionised water
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxyribonucleic acid
E_2	oestradiol
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
ECC1	endometrial cancer cell line
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial to mesenchymal transition
EP	early proliferative
ERα	oestrogen receptor alpha
ERβ	oestrogen receptor beta
EST	expressed sequence tags
ET	endometrial thickness
Fas	tumour necrosis factor receptor superfamily, member 6
FasL	Fas ligand
FBS	fetal bovine serum
FosB	osteosarcoma viral oncogene homolog B
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
ge	glandular epithelial cells
GnRH	gonadotrophin releasing hormone
GPCR	G protein coupled receptor
GRIA2	ionotrophic glutamate receptor 2
GRIP-1	glucocorticoid receptor interacting protein 1

HOX A11	transcription factor homeobox A11
HOX-B13	homeobox protein B13
HSP70	heat shock protein 70
IGF	insulin like growth factor
IGFBP3	insulin-like growth factor binding protein 3
IgG	immunoglobulin G
IL7BR	interleukin-17B receptor
IMS	industrial methylated spirits
K-RAS	Kirsten rat sarcoma viral oncogene homolog
L	longitudinal uterine diameter
LNG-IUS	levonorgestrel intrauterine system
LP	late proliferative
MAPK	mitogen-activated protein kinase
$MgCl_2$	magnesium sulphate
MP	mid proliferative
mRNA	messenger ribonucleic acid
NCoR	nuclear receptor corepressor
ng	normal sized glands
NGF	nerve growth factor
NRS	normal rabbit serum
PBS	phosphate buffered saline
PC3	prostate carcinoma cells
PCR	polymerase chain reaction
PENK	precursor opiod peptide producer
PM	postmenopausal

PR	progesterone receptor
PR-A	progesterone receptor A
PR-B	progesterone receptor B
ps20	prostatic stromal mesenchymal-derives paracrine-acting factor
PTEN	phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SAG	surrounding around endometrial glands
SD	standard deviation
SEM	standard error of the mean
SERM	selective oestrogen receptor modulators
SMF	submucous fibroid
SMRT	silencing mediator of retinoid and thyroid hormone
SRC-1	steroid receptor coactivator 1
st	stromal cells
T	transverse uterine diameter
TAE	tris base, acetic acid and EDTA
TBS	tris-buffered saline
TGF	transforming growth factor
TGF-β1	transforming growth factor beta 1
TIF-2	transcription intermediary factor 2
TMX	tamoxifen
TP53	tumour protein 53

Tris-HCl	tris hydrochloric acid
UtSMC	uterine smooth muscle cells
WAP	whey acidic protein
WFDC1	WAP-type four disulphide core 1
WFDC2	WAP-type four disulphide core 2
WHI	Women's Health Initiative
Wif-1	WNT inhibitory factor 1
WISP2	WNT1 inducible signalling pathway protein 2
WNT1	wingless-type MMTV integration site family, member 1
α-SMA	alpha smooth muscle actin

Publications arising from this thesis

R Panchal, AH Taylor, SC Bell, JC Konje, L Brown & MA Habiba. Mesenchymal markers and extracellular matrix protein expression in the endometrium of women on tamoxifen. *BJOG* 2008; **115(1) :**118.

R Panchal, SC Bell, JC Konje, L Brown & MA Habiba. Uterine effects of levonorgestrel-impregnated intrauterine device (LNG-IUS) inserted at the onset of tamoxifen treatment in post-menopausal women with breast cancer. *BJOG* 2007; **114(8):** 1041

Chapter 1

Introduction

1.1 Tamoxifen and breast cancer

Breast cancer is the second leading cause of death in postmenopausal women and the mostly commonly diagnosed female malignancy, affecting up to 1 in 8 women in the developed world (www.cancer.org). Tamoxifen is a non-steroidal synthetic triphenylethylene oestrogen derivative that has been used extensively in the treatment of all stages of breast cancer since the 1970's in both pre- and post-menopausal women. Tamoxifen belongs to a group of drugs called selective oestrogen receptor modulators (SERMs) that are tissue specific in their action and are target site specific. One factor determining this tissue specificity is the conformational change that occurs when the oestrogen receptor and SERM complex is formed; this results in the varied ability of tamoxifen to activate genes when bound to ERα or ERβ (Paech et al., 1997). Breast tumours that are oestrogen receptor positive benefit the most from tamoxifen therapy due to the anti-oestrogenic effect of tamoxifen that works *via* competitive antagonism at the oestrogen receptor resulting in inhibition of cancer cell growth. It is thought that in the breast epithelial cell, tamoxifen also recruits co-repressors to dimerized oestrogen receptors (Sismondi et al., 2007), these co-repressors bind to the oestrogen receptor resulting in the repression of ERα-target genes, including those involved in cell proliferation, resulting in inhibition of breast cancer cell proliferation.

In the most recent Early Breast Cancer Trialists' Collaborative Group (EBCTCG) review, adjuvant tamoxifen treatment for five years was reported to be associated with a relative reduction in the annual breast cancer mortality by 34% irrespective of patient age,

menopausal status, use of chemotherapy and tumour characteristics (progesterone receptor and lymph node status) ((EBCTCG), 2005), and a relative reduction in the annual recurrence rate by 41% in women with oestrogen receptor positive disease, irrespective of age and menopausal status ((EBCTCG), 2005). In the earlier report, there was a 50% reduction in tumour recurrence rate in women with ER positive tumours compared to 16% in oestrogen receptor negative tumours when treated with tamoxifen for 5 years ((EBCTCG), 1998).

At the time of initiation of this project, there was limited evidence for the use of aromatase inhibitors such as anastrozole, letrozole and exemestane for breast cancer. Breast cancer patients treated with tamoxifen for 2 to 3 years followed by 2 to 3 years of an aromatase inhibitor had an improvement in the disease free survival. Aromatase inhibitors have the additional advantage that they do not stimulate the endometrium (Coombes et al., 2004; Morandi et al., 2004; Patel et al., 2007). However, the use of aromatase inhibitors is associated with an increase rate of arthralgia and fractures due to osteoporosis (ATAC, 2005). Although, the aromatase inhibitors may eventually acquire a more prominent role, tamoxifen is likely to have an important place given the cost differential and different side effect profile (Neven et al., 2006; Rabaglio et al., 2007; Hadji, 2008). Postmenopausal women with high cytochrome P450 2D6 (CYP2D6) metabolism and low homeobox protein B13 (HOX-B13) to interleukin-17B receptor (IL17BR) protein ratios are associated with improved overall survival and disease free period in response to tamoxifen (Goetz et al., 2008; Schroth et al., 2009). Tamoxifen, therefore, still has a role in the treatment of premenopausal women who are diagnosed with oestrogen receptor positive breast cancer (Bao and Davidson, 2007).

1.2 Tamoxifen and adverse uterine effects

Tamoxifen is well recognised as a partial oestrogen agonist in the endometrium (Jordan et al., 1991) as well as in other organs. Its side effects include the production of ovarian cysts, the formation and growth of uterine fibroids, vaginal dryness and the promotion of candidiasis (Cohen et al., 1994b; Varras et al., 2003; Senkus-Konefka et al., 2004). The effect of tamoxifen in the endometrium varies depending on the prevalent levels of oestrogen. In premenopausal women who have higher oestrogen levels, tamoxifen effects are mainly as an antagonist, whilst in postmenopausal women who have lower oestrogen levels, tamoxifen's weak agonist effect become more important (Kedar et al., 1994; Bergman et al., 2000; Mourits et al., 2001). Tamoxifen use by postmenopausal women also promotes cell proliferation inducing changes that include an increase in uterine volume and endometrial thickness and the pattern of growth exhibited by a uterus exposed to tamoxifen is not as coordinated as one that is exposed to its normal oestrogenic environment (Green et al., 2005). Tamoxifen use is associated with several abnormal histological features. These include cystic atrophic glands (Figure 1.1 & 1.2) endometrial hyperplasia, stromal 'fibrosis', disturbance of the normal endometrial-myometrial interface, as exemplified by 'adenomyosis-type' features with endometrium extending into the myometrium, and the formation of 'tamoxifen-associated' endometrial polyps, hyperplasia and endometrial cancer (Ismail, 1994; Kedar et al., 1994; Cohen, 2004). After one year of tamoxifen exposure in postmenopausal women, the incidence of these complications is in the order of 30% (Deligdisch et al., 2000; Cohen, 2004).

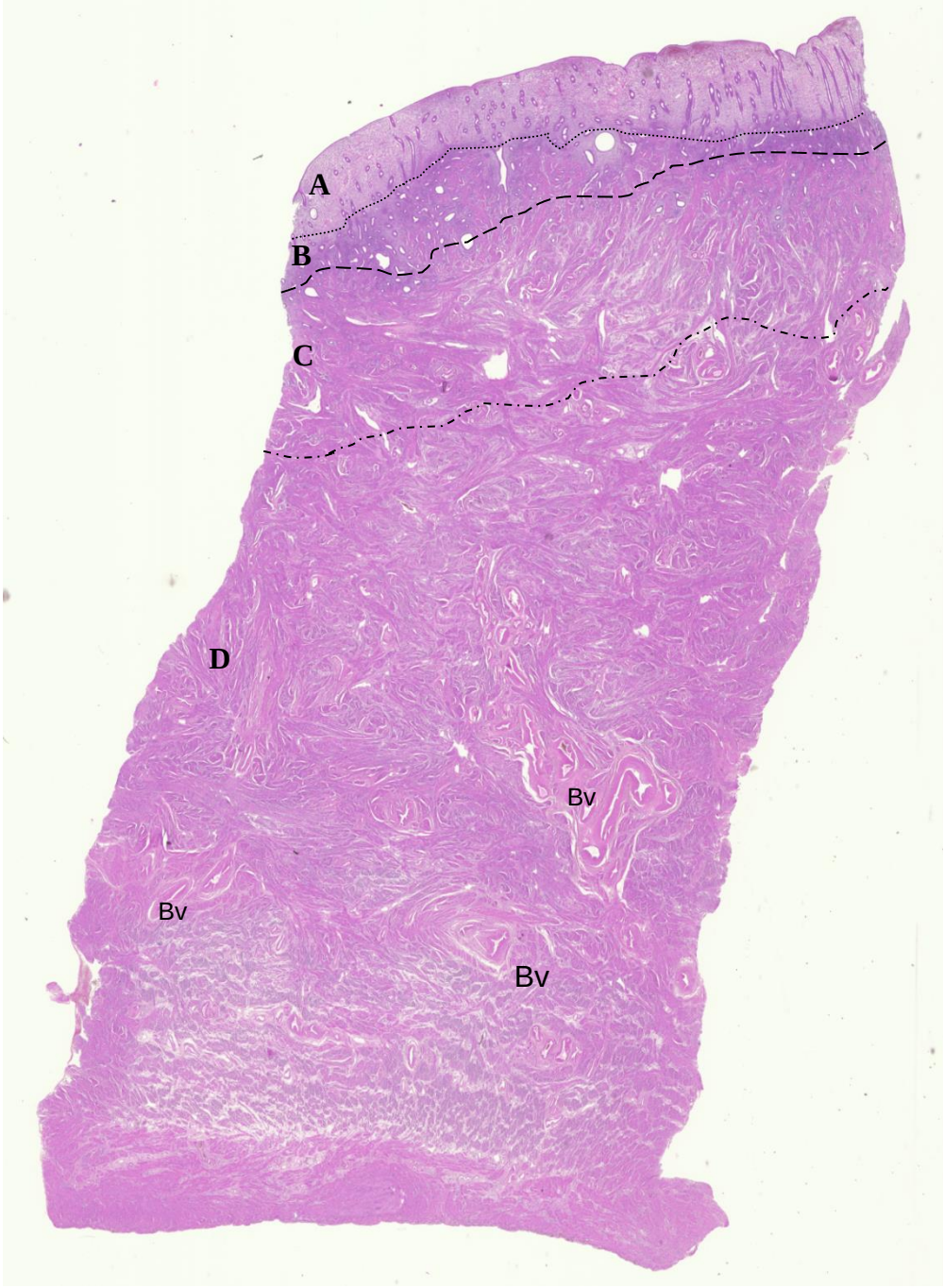


Figure 1.1a Full thickness endometrium and myometrium from an early proliferative phase uterus (routine H&E staining)

Showing the functional (A) and basal (B) endometrium containing normal sized glands. The inner myometrium (junctional zone; C) and the outer myometrium (D) containing blood vessels (Bv).

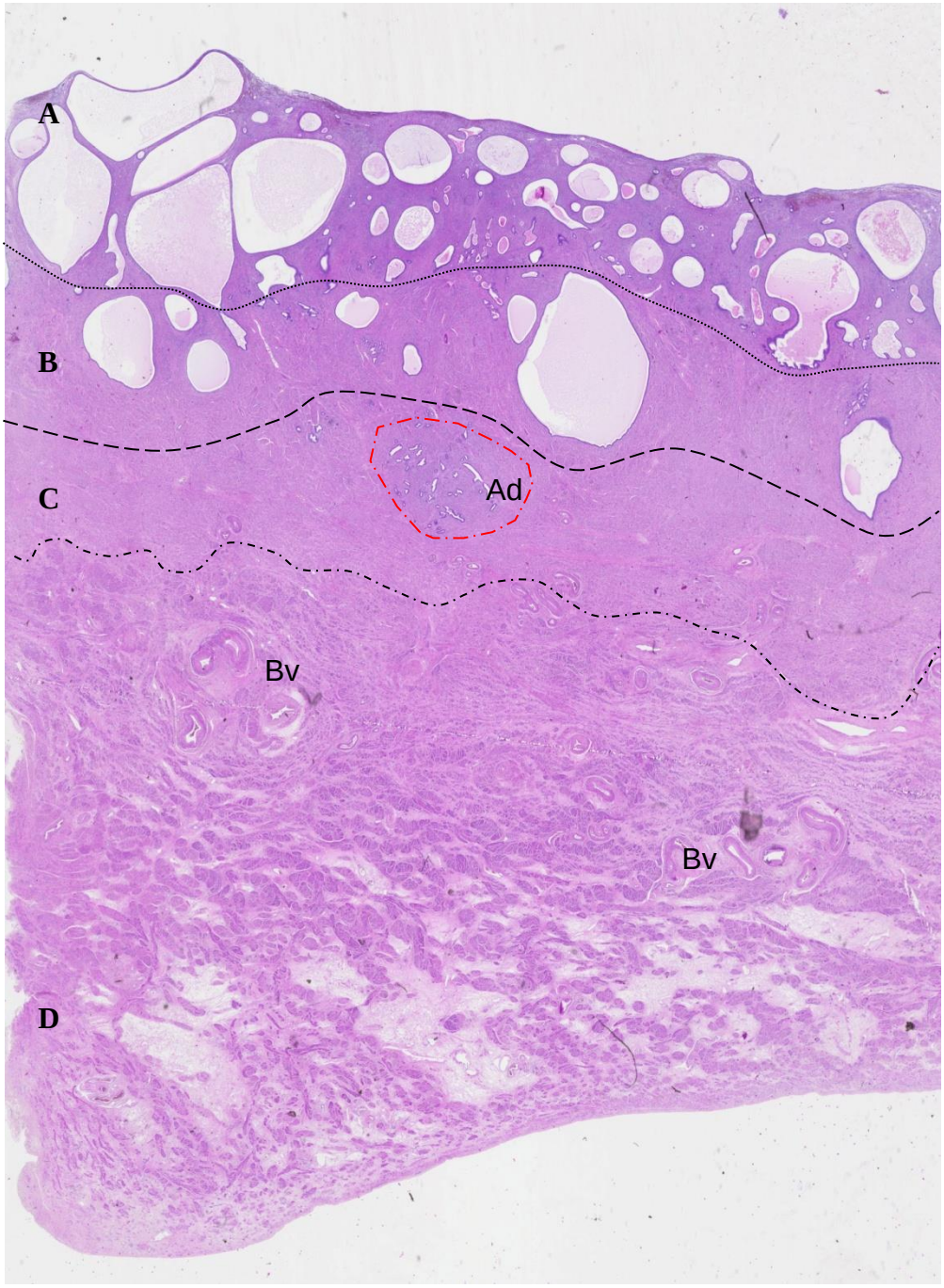


Figure 1.1b Full thickness endometrium and myometrium from a tamoxifen treated uterus (Routine H&E staining)

Showing the functional (A) and basal (B) endometrium containing cystic dilated glands. The inner myometrium (junctional zone (C)) and the outer myometrium (D) containing blood vessels (Bv); this sample also has the inclusion of an adenomyotic lesion (Ad).

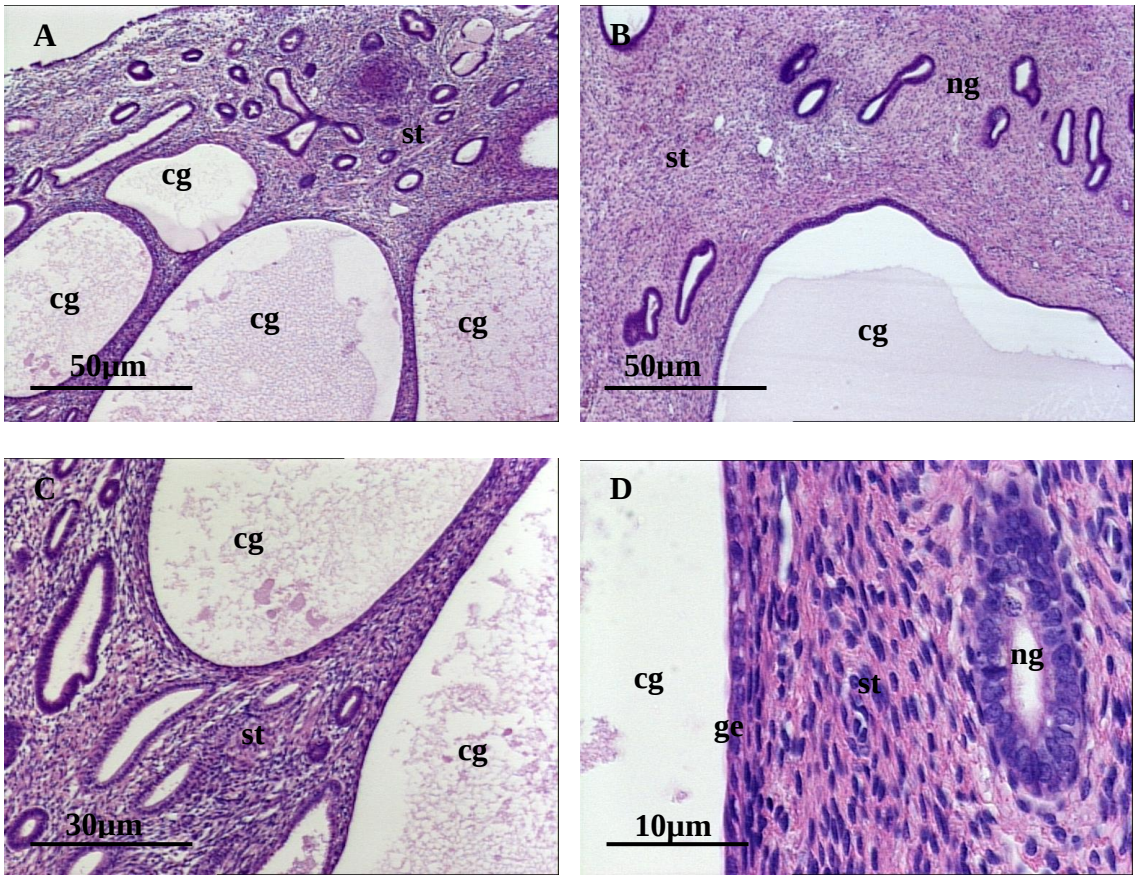


Figure 1.2 Benign histological features of the tamoxifen exposed endometrium

Panel A (x 100), B (x 100) and C (x 200) show histological features associated with tamoxifen treated uteri such as large cystically dilated glands (cg) and densely compacted stromal cells (st) surrounding such glands. The presence of normal sized glands (ng) can also be seen. Panel D shows a higher magnification (x 400) image of the densely compacted stromal cells (st), between a cystic dilated endometrial gland (to the left) and a normal gland (ng) on the right; note the compression of the epithelial cells (ge) in the cystically dilated gland.

Endometrial polyps are the most common endometrial pathology reported in association with tamoxifen therapy. The incidence of endometrial polyps in women treated with tamoxifen ranges between 8 and 36% (Lahti et al., 1993; Cohen et al., 1994a; Kedar et al., 1994; Cohen et al., 1997a; Deligdisch et al., 2000; Vosse et al., 2002; McGonigle et al., 2006) (Table 1.1), all endometrial polyps were diagnosed at hysteroscopy. Tamoxifen associated polyps are more common in women who present with vaginal bleeding compared to asymptomatic postmenopausal women taking tamoxifen (Cohen et al., 1999b; Gerber et al., 2000). Cohen and colleagues reported a 35.7% (5 of 14 women) presence of endometrial polyps in postmenopausal women presenting with vaginal bleeding on tamoxifen compared to 13.4% (30 of 244 women) in asymptomatic women. It has also been reported that the risk of developing recurrent endometrial polyps was greater in postmenopausal breast cancer patients treated with tamoxifen if there was a history of previous HRT use, lower parity, shorter duration of tamoxifen exposure to diagnosis of endometrial polyp and additional years of tamoxifen therapy (Biron-Shental et al., 2003).

Tamoxifen associated polyps are different both macroscopically and microscopically when compared to the polyps occurring in non treated women. Tamoxifen associated endometrial polyps are larger with a mean diameter of 5 cm (range 1.5 to 16cm) (Ismail, 1994) compared to 0.5 to 3cm in the general population (Polin and Ascher, 2008). Microscopically tamoxifen associated polyps are reported to show proliferative activity in epithelial and stromal cells, focal periglandular stromal condensation and epithelial metaplasias (Ismail, 1994). Tamoxifen associated endometrial polyps in postmenopausal women are also reported to have a high rate of malignant transformation ranging from 3.0 to 10.7% (Cohen et al., 1999a; Deligdisch et al., 2000; Cohen, 2004). Deligdisch and co-workers observed that 15 (5.9%) endometrial carcinomas were found in endometrial

Study	Tamoxifen treated patients (No.=)	Non treated patients (No.=)	Endometrial polyps in tamoxifen treated patients	Endometrial polyps in non treated patients	P value	Sampling method
			No. (%)	No. (%)		
Lahti et al. 1993	51	52	17 (36)	5 (10)	0.004	Prospective case control study
Cohen et al. 1994	93	20	5 (5.38)	0 (0.0)	-	Prospective case control study
Kedar et al. 1994*	61	50	5 (8%)	1 (2%)	-	Randomised controlled trial
Cohen et al. 1997	175	27	14 (8.0)	2 (7.4)	NS	Prospective case control study
Deligdisch et al. 2000	700	-	162 (23.14)	-	-	Retrospective case review
Vosse et al. 2002	256	-	32 (12.5)	-	-	Prospective case control study

Table 1.1 Incidence of endometrial polyps in postmenopausal breast cancer patients receiving tamoxifen.

*Breast cancer prevention trial (healthy women with no personal history of breast cancer but who had at least one first degree relative who had developed breast cancer)

NS = not significant

polyps (Deligdisch et al., 2000). It has therefore been suggested that an endometrial polyp-carcinoma sequence may have a role in the development of endometrial cancer in postmenopausal women on tamoxifen (Ismail, 1994). Therefore, the presence of endometrial polyps in this group of patients should not be overlooked.

The effects of tamoxifen on the uterus are an important consideration with long term treatment. Nevertheless, the benefits of tamoxifen therapy for breast cancer greatly exceeds the potential risk of developing endometrial neoplasia (Jordan and Assikis, 1995). There still remains controversy regarding the most appropriate way of monitoring or protecting the endometrium in women on tamoxifen.

One modality to evaluate the uterus is by using ultrasound scanning. Ultrasound is reported to be sensitive but not specific for assessing the endometrium. The normal postmenopausal endometrium does not exceed above 5mm as a bilayer on transvaginal ultrasound and has the appearance of a single echogenic line (Goldstein et al., 1990; Jordan and Assikis, 1995). The majority of women taking tamoxifen will have a thicker endometrium (9-13mm) compared to that in controls (4.0-5.4mm) (Kedar et al., 1994; Sinawat et al., 2003) (Figure 1.3). The upper limit for normal endometrial thickness in asymptomatic women taking tamoxifen remains controversial and undefined (Cohen I, 1994). The finding of a thickened endometrium on ultrasound scan does not always correlate with endometrial pathology (Gerber et al., 2000; Liedman et al., 2000). Garuti and colleagues suggest that pre-treatment assessment of the endometrium should be performed in all postmenopausal women who are to receive tamoxifen therapy (Garuti et al., 2007), however, there is no uniform consensus as to the maximum endometrial thickness that is acceptable in this patient group to date.

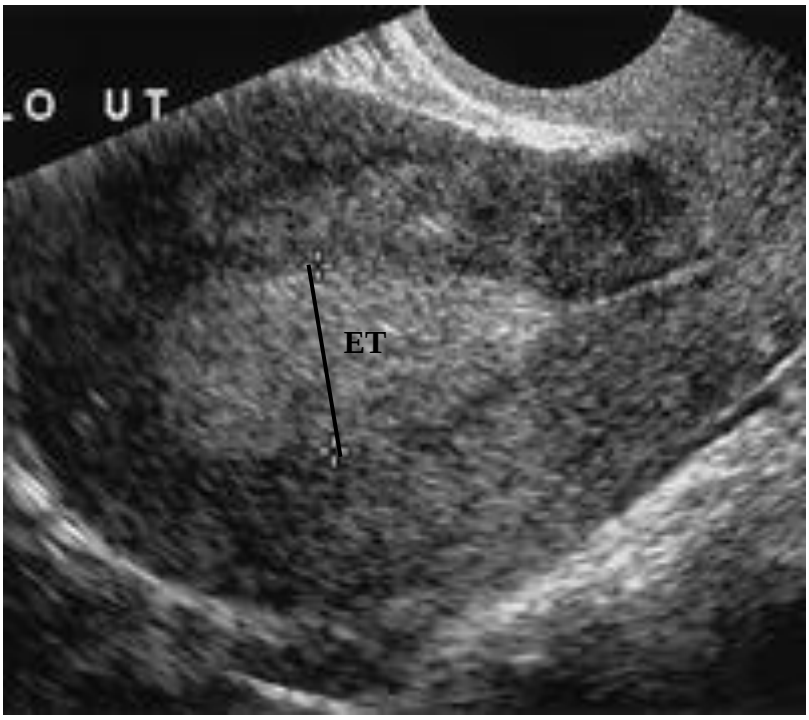


Figure 1.3 Ultrasound scan demonstrating thickened endometrium in a tamoxifen treated uterus. ET = endometrial thickness. The uterine cavity was normal on hysteroscopy.

At the tamoxifen and the uterus consensus meeting in Brussels, it was suggested that asymptomatic postmenopausal women with a normal pre-treatment transvaginal ultrasound scan (i.e. a regular thin endometrium less than 5mm thick) undergo annual screening with transvaginal ultrasonography starting 3 years after commencing tamoxifen therapy and that if at any point the endometrium is irregular or greater than 5mm thick, further assessment of the uterine cavity with hysteroscopy and endometrial biopsy should be performed (Neven and Vergote, 2001). The American College of Obstetricians and Gynaecologists does not recommend screening asymptomatic women taking tamoxifen with transvaginal ultrasound scan or endometrial biopsy (ACOG, 2006). The ACOG have concluded that routine endometrial surveillance is not effective in increasing the early detection of endometrial malignancy in women using tamoxifen. The ACOG do, however, recommend a baseline gynaecological evaluation (pap smear, bimanual and rectovaginal examinations before starting the tamoxifen therapy followed by annual gynaecologic evaluation. All women taking tamoxifen should be carefully counselled about the risks associated with tamoxifen, such as the formation of endometrial polyps, hyperplasia and carcinoma, and be advised to report any symptoms i.e. vaginal bleeding immediately. All women with abnormal vaginal bleeding who are taking tamoxifen should be investigated by means of an endometrial biopsy (ACOG, 2006).

1.3 Mechanisms of tamoxifen action

The exact mechanism whereby tamoxifen exerts its effects remains unclear. Oestradiol acts in the uterus (both the endometrium and myometrium) primarily through the two oestrogen receptor (ER) isoforms, ER-α and ER-β. Both receptor isoforms are transcription factors that regulate the expression of target genes in a ligand dependent manner. The endometrium is a unique tissue, consisting mainly of epithelial cells arranged

in glandular structures surrounded by mesenchymal stromal cells, which in premenopausal women, undergoes monthly cycles of proliferative and secretory changes followed by shedding at menstruation. These phenotypical cyclical changes in the stromal and glandular epithelial cells are regulated and maintained by the interactions of the ovarian steroid hormones, primarily oestradiol and progesterone and their receptors (Fung et al., 1994; Taylor et al., 2005). The follicular phase oestradiol controls endometrial cell proliferation and epithelial hyperplasia, whereas the luteal phase progesterone induces the secretory transformation of the proliferative endometrium in preparation for blastocyst implantation (King and Critchley, 2010). The hormonal receptivity and receptor distribution in the cells change in response to cyclical changes in progesterone and oestrogen concentrations during the menstrual cycle.

After the menopause, the endometrium becomes thinner as it undergoes gradual atrophy (Deligdisch, 2000). There is no cellular proliferation or production of secretory products, the functionalis does not form and the endometrium becomes a very thin layer containing glands lined by flat or cuboidal epithelium (Deligdisch, 2000). The lack of ovulation after the menopause results in the absence of progestagenic stimulation of the endometrium (Deligdisch, 2000). However, a weak oestrogenic stimulation of the endometrium may continue as a result of the conversion of androgens produced by the ovaries and adrenal glands to oestrogen in adipose tissue (Deligdisch, 2000). Higher oestrogen production in the postmenopausal state as a result of extragonadal aromatization in situations such as obesity and diabetes, are associated with endometrial proliferation, hyperplasia and neoplasia (Deligdisch, 2000). Similar changes can occur following tamoxifen treatment.

Tamoxifen is a selective oestrogen receptor modulator which acts as an antagonist in the breast by inducing conformational changes when binding to the oestrogen receptor that block the interaction of the oestrogen receptor with coactivator proteins (Shang, 2006). Conversely, tamoxifen acts as a partial oestrogen agonist in other target organs. This partial oestrogenic action is beneficial in the bones and cardiovascular system of postmenopausal women (Jordan et al., 2001). The oestrogenic effect in the uterus is associated with an increased risk of endometrial cancer (Fornander et al., 1989; Fisher et al., 1994; Mignotte et al., 1998; Bernstein et al., 1999; Bergman et al., 2000; Jordan, 2004). It is the conformational change of the oestrogen receptor as determined by the ligand binding that explains the different biological activity of various oestrogen receptor ligands (McDonnell, 1999). The activation domains allow maximal oestrogen receptor transcriptional activity and have different functional activity. When activation domain 1 (AF-1) is required for transcription, tamoxifen functions as a partial agonist, the converse is true when activation domain 2 (AF-2) is required, tamoxifen functions as an antagonist, as in the breast (McDonnell, 1999). In addition, the differential activity of tamoxifen in different tissues may be as a result of the level of expression of coactivators, such as steroid receptor coactivator 1 (SRC-1), glucocorticoid receptor interacting protein 1 (GRIP-1) (Norris et al., 1998), transcription intermediary factor 2 (TIF-2) or amplified in breast cancer 1 (AIB-1), or co-repressors, such as, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone (SMRT) (McDonnell, 1999). The response of specific cells will depend on the relative levels of corepressors and coactivators and account for the tissue selective activity of SERM's. The concentration of SRC-1 is higher in uterus than in breast, which may explain why some SERMs, such as tamoxifen, are more agonistic in the uterus than in the breast (Shang, 2006).

Tamoxifen itself has some affinity for the oestrogen receptors (Kuiper et al., 1997) and is metabolised in the hepatic microsomal enzyme oxidation system by cytochrome P450 isoforms CYP2D6 and CYP3A4 into the active metabolites 4-hydroxytamoxifen and endoxifen (N-desmethyl-4-hydroxytamoxifen), which have greater affinity for the oestrogen receptor compared to tamoxifen (Desta et al., 2004). Although tamoxifen interacts with both ER-α and ER-β, the precise mechanism by which tamoxifen exerts its oestrogenic affects has yet to be defined (McDonnell, 1999). It is thought that the conformational change of the oestrogen receptor following ligand binding differs between oestrogen and tamoxifen (Norris et al., 1998). It is this conformational change that results in the oestrogen receptor-ligand complex that do not interact with the coactivator and corepressor proteins in an equivalent manner, thereby tamoxifen agonist activity will be restricted to cells in which the oestrogen receptor-tamoxifen complex can find an appropriate coactivator protein (McDonnell, 1999). Shang has suggested it is this conformational change that inhibits the oestrogen receptor from binding to coactivator proteins, by lowering the affinity of oestrogen receptor interaction with the coactivators (Shang, 2006).

Several hypotheses have been proposed whereby promotion of gene expression occurs through binding of the oestrogen receptors to either classical oestrogen response elements (Mosselman et al., 1996) or c-fos and c-jun proteins that bind to AP-1 response elements (Philips et al., 1993). There is differential ligand activation of the oestrogen receptors ERα and ERβ at the AF-1 site (Paech et al., 1997). Paech and co-workers reported that as a ligand of ERβ, tamoxifen, but not oestradiol, acted as a potent transcription activator at the AF-1 site. In the human endometrium, both ERα and ERβ are expressed in luminal epithelial cells and the nuclei of stromal cells (Taylor and Al-Azzawi, 2000), and

tamoxifen-associated polyps are shown to have high levels of proliferation in the glands and ERα and ERβ expression (Hachisuga et al., 2003). Weihua and colleagues demonstrated increased cellular proliferation and an exaggerated response to oestrogen in ERβ knockout mice, suggesting that ERβ has a modulatory role on ERα function in the uterus and is anti-proliferative (Weihua et al., 2000). Therefore, it has been suggested that an imbalance in ERα and ERβ expression may be an important step in the development of oestrogen dependent tumours (Shang, 2006).

1.4 Tamoxifen and endometrial cancer

Many epidemiological, as well as randomized, prospective trials, have shown a moderately increased risk of endometrial cancer induced by tamoxifen (Fornander et al., 1989; Fisher et al., 1994; Fisher et al., 1998; Mignotte et al., 1998; Bernstein et al., 1999; Bergman et al., 2000). This increased risk of endometrial cancer is confined to the postmenopausal population and not to premenopausal women (ACOG, 2006). The increased incidence of endometrial neoplasia is complicated in tamoxifen users by the occurrence of tumours with a poorer prognosis; the majority of tumours are of the endometroid type, but a higher frequency of serous and clear cell carcinomas has been observed in those who take tamoxifen (Mignotte et al., 1998). The relative risk of tamoxifen associated endometrial cancers ranges from 1.5 to 7.5 (Fisher et al., 1998; Mignotte et al., 1998; Bergman et al., 2000) (Table 1.2). This risk of developing endometrial cancer increases with long term use; for example, Mignotte and colleagues observed an increase in the relative risk for endometrial malignancy by 1.5 with each year of tamoxifen use (Mignotte et al., 1998; Fisher et al., 2005). The risk of developing endometrial neoplasia also increases with previous oestrogen replacement therapy, obesity, cumulative tamoxifen dose and pre-existing endometrial pathology (Ismail, 1994; Fisher et al., 1998).

Immunohistochemical studies performed on exposed benign endometrium versus non-exposed endometrium supports the idea that tamoxifen exposure results in increased epithelial proliferation (Mourits et al., 2002b). Thus, it is possible that tamoxifen acts to increase the proliferation of a subset of cells, thereby increasing the likelihood of mutations, or alternatively, promote the growth of cells that have already sustained mutations. As a result, tamoxifen exposure could lead to the production of a spectrum of mutations similar to that of sporadic endometrial cancers and increase the incidence of endometrial carcinoma in this clinical setting (Prasad et al., 2005). It is also possible that tamoxifen exerts a direct neoplastic effect upon the endometrium. The stage and grade of endometrial cancers observed in women who are taking tamoxifen is similar as those in the general population (Fisher et al., 1998).

However, it is considered that the increased risk of endometrial cancer is also linked with the development of polyps in tamoxifen treated women (Ismail, 1994). Additionally malignant transformation has been reported in 2.5-10% of tamoxifen-associated polyps (Cohen et al., 1999a; Deligdisch et al., 2000; Biron-Shental et al., 2003; Cohen, 2004) as compared to only 0.5% in non-tamoxifen associated polyps (Cohen et al., 1999a). These findings suggested that a 'tamoxifen-polyp-carcinoma' sequence plays a role in the development of endometrial malignancy in these patients (Ismail, 1994). This appears to be supported by the observation that mutations in codon 12 of the proto-oncogene, Kirsten ras (K-RAS), have been found in 64% of tamoxifen-associated polyps as compared to only 5-23% incidence in sporadic endometrial hyperplasia (Hachisuga et al., 2003). However, this observation is disputed by the results of a larger study that found similar mutation rates in phophatase and tensin homolog (PTEN), tumour protein 53 (TP53) and K-RAS in

tamoxifen-associated endometrial cancers in women with breast cancer and sporadic cancers (Prasad et al., 2005).

1.5 Uterine growth and paracrine regulators

The molecular basis for the effects of tamoxifen resulting in abnormal uterine features such as the development of adenomyosis and polyps in terms of oestrogenic action is incompletely understood. Indeed, very little is known as to how oestradiol itself acts to ensure the coordinated pattern of endometrial and myometrial growth observed in the normal uterus. Hypothetically, coordinated uterine growth may reflect similar direct proliferative effects of oestrogens upon individual cell types such as the endometrial epithelium, mesenchymal stroma and myometrial components. However, it been suggested that the regulation of certain cell types, such as the epithelium, is achieved *via* the indirect action of stimulatory and inhibitory paracrine-acting regulators. The differences between oestradiol and tamoxifen upon the pattern of uterine development may thus reflect their differential effects, *via* ER-α and ER-β, upon the production of these factors by the various cell types.

Members of the neurotrophin family may represent such oestrogen-induced paracrine factors. In the neonatal mouse, administration of tamoxifen, which is associated with the over expression of factors such as nerve growth factor (NGF) in the endometrial epithelium, subsequently resulted in the uncoordinated development of the uterus to produce ‘adenomyosis-like’ uterine morphology (Green et al., 2003). This may reflect the observation that NGF stimulates myofibroblast growth but inhibited myogenic differentiation (Seidl et al., 1998). Whether NGF functions as a paracrine factor in human myometrial development is unknown, however, other evidence is supportive of a role for

Study	Relative Risk	Duration of tamoxifen treatment (Years)	Tamoxifen dose (mg/day)
Fisher et al. 1998	7.5	5	20
Fornander et al. 1989	6.4	>2	40
Cuzick et al. 2007	1.5	5	20
Bergman et al. 2000	2.0	2-5	10-40
Bergman et al. 2000	6.9	>5	10-40
Bernstein et al. 1999	4.06	>5	20

Table 1.2 Relative risks of endometrial cancer in association with tamoxifen therapy in postmenopausal breast cancer patients.

NGF in oestrogen-induced uterine growth. Treatment of ovariectomised rats with oestradiol increases NGF protein content in uterine stromal/endometrial tissue (Krizsan-Agbas et al., 2003), and treatment of immature mice with NGF increases uterine weight (Akasu et al., 1970). Other members of the neurotrophin family may be involved as oestradiol increases brain-derived neurotrophic factor (BDNF) mRNA and protein levels in the endometrium and myometrium (Krizsan-Agbas et al., 2003). Interestingly, a putative oestrogen response element has been identified in the promoter region of the BDNF gene (Sohrabji et al., 1995). However, little is known as to the expression of neurotrophins in the human uterus, although NGF expression has been reported in the glandular epithelium and stromal cells of both the proliferative and secretory endometrium. Anaf and colleagues showed greater NGF expression in deep adenomyotic nodules, suggesting a role for NGF in hyperalgesia and endometriotic pain (Anaf et al., 2002).

It is also possible that growth-inhibitory paracrine regulatory factors are involved, such that their expression is required to be suppressed by oestradiol to enable normal uterine development. The expression of certain genes may not be fully suppressed with tamoxifen which may contribute to disordered uterine development. One such gene product may be ps20, a 20kDa protein, identified as a prostatic stromal mesenchymal-derived paracrine-acting factor, which regulates epithelial cell proliferation and differentiation (Rowley et al., 1995). Purified ps20 has been shown to inhibit PC-3 prostatic epithelial carcinoma cell proliferation in vitro and promote protein synthesis in a dose dependent and saturable manner (Larsen et al., 1998). The protein ps20 is a member of the whey acidic protein (WAP)-type "Four-Disulphide Core" domain protein family (termed WFDC1), which have

protease inhibitor action. It is this activity that may mediate the variety of growth- and differentiation-regulatory functions.

The expression of ps20 has recently been implicated in the control of growth and the coordinated development of the endometrium and myometrium (Hung, 2005). It has been immunolocalized to both the endometrium and muscle cells of the rat uterus, but appears to originate primarily from the muscle layer (Hung, 2005). The expression of ps20 in the rat is inversely correlated to the level of oestradiol. Tamoxifen was shown to suppress ps20 expression both in vivo and in vitro, but less effectively than oestradiol, suggesting a possible link with abnormal uterine responses to tamoxifen (Hung, 2005). Recently, evidence has emerged that ps20 and another member of the WAP family, WFDC2, are expressed by primary cultures of human uterine smooth muscle cells and that their expression is also suppressed by oestradiol (Taylor & Bell, unpublished observations). It is unknown whether ps20 (WFDC1) and WFDC2 are expressed by other cellular components in the human uterus.

1.6 Tamoxifen and Tissue Remodelling

It has been postulated that tamoxifen associated uterine changes occur through mechanisms involving tissue remodelling, and that tamoxifen-induced adenomyosis and stromal fibrosis are a consequence of disordered regulation of mesenchymal differentiation (Parrott et al., 2001). It is postulated that such changes occur through mechanisms that involve tissue remodelling, during which there is a transition of the mesenchymal cells through an intermediary myofibroblast cell and ends on the production of a fully differentiated myocyte or *vice versa* (Desmoulière et al., 2004).

The key molecules for defining this fibroblast-myofibroblast-smooth muscle cell pathway are, vimentin, an intermediate filament protein only found in mesenchymal cells, α-smooth muscle actin (α-SMA), which is a marker for the smooth muscle cell and can distinguish myocytes from the stromal fibroblast, and tenascin-C, which is an extracellular glycoprotein that is up-regulated in some tissues undergoing tissue remodelling such as those cells that are de-differentiated and proliferating. Examples of this are myofibroblasts in certain types of breast cancer (Pas et al., 2006; Hancox et al., 2009) and cells of the fetal membranes (Castellucci et al., 1991; Bell et al., 1999). Thus, tenascin-C may be considered as a marker of the myofibroblast (Mehasseb et al., 2011).

1.6.1 Vimentin

Vimentin is an intermediate filament protein that is present in the cytoplasm of cells of mesenchymal origin and has a structural role supporting the cell and the nucleus, and maintaining cell shape and integrity (Leader et al., 1987). Vimentin has been shown to be present in the stroma and epithelial glands of the endometrium (Norwitz et al., 1991; Tabibzadeh, 1991). Vimentin expression is thought to be acquired during an epithelial-mesenchymal transition, this results in loss of epithelial differentiation and results in more aggressive and invasive tumours (Dandachi et al., 2001). Vimentin is involved in tissue remodelling through cell motility and migration (Hendrix et al., 1996).

1.6.2 Tenascin-C

Tenascin-C is a large extracellular matrix glycoprotein. It is expressed in the developing embryo during neural, skeletal and vascular morphogenesis and is absent or reduced in fully developed organs, with basal expression present only in tendons and ligament associated tissues (Chiquet-Ehrismann and Chiquet, 2003). However, tenascin-C is up

regulated in situations of tissue remodelling such as carcinogenesis (Natali et al., 1991). In endometrial carcinoma, tenascin C is over expressed in the stroma (Vollmer et al., 1990; Sedele et al., 2002), it is also expressed strongly in the proliferative compared to the secretory endometrium of the menstrual cycle (Harrington et al., 1999). Tenascin-C is classified as having anti-adhesive properties (Chiquet-Ehrismann and Chiquet, 2003), which favours cell motility and growth promotion and together with its increased expression in malignant stroma suggests it is involved in tumour growth, progression and invasion. It has also been shown to be associated with cells that are not differentiated but proliferating (Vollmer et al., 1994).

1.6.3 α-Smooth Muscle Actin

Alpha smooth muscle actin is a cytoskeletal microfilamentous protein that is important for mitosis, cell growth, cell shape change and protein secretion regulation (Christensen et al., 1995). It is absent in endometrial glandular epithelium but present in the stroma and myometrium and involved in the functional changes occurring in the endometrium (Christensen et al., 1995). Alpha-smooth muscle actin is a known marker of myofibroblast differentiation and stromal reaction during tumourigenesis (Chaponnier and Gabbiani, 2004).

During the normal wound healing response there is a stromal reaction, where the stromal cells create a growth promoting environment (Desmoulière et al., 2004). Fibroblasts play a role in the formation of the wound granulation tissue, and myofibroblasts, which express α smooth muscle actin, are responsible for wound contraction facilitating wound healing (Desmoulière et al., 2004). It has been suggested that this stromal or desmoplastic reaction is also induced by cancers in order to aid tumour proliferation, cell survival and invasion

(Dvorak, 1986). Tuxhorn and co-workers demonstrated that normal stroma was altered when compared to that in prostate cancer (Tuxhorn et al., 2002). These reactive stromal cells in the tumour were positively stained for vimentin and α smooth muscle actin, which are markers of myofibroblasts. They also observed positive staining in prostate cancer for tenascin-C, a marker also seen during tissue remodelling situations such as wound healing (Tuxhorn et al., 2002). Reactive stroma has also been described in colon and breast carcinomas (Tuxhorn et al., 2002).

1.7 Approaches to cancer prevention in tamoxifen-treated patients

One strategy to reduce or counter the adverse pro-proliferative actions of tamoxifen upon the uterus has been the local application of a progestagen to oppose the proliferative action and to induce endometrial differentiation. Progestagen treatment for endometrial hyperplasia is widely used (Reed et al., 2009) but may not always work, because one study found that systemic norethisterone does not cause regression of the tamoxifen induced endometrial changes (Powles et al., 1998). In that study, 2.5mg norethisterone was administered daily 21 days out of 28 days for 3 consecutive cycles to 47 healthy postmenopausal women taking tamoxifen in a chemoprevention study (Powles et al., 1998). The inclusion criterion was that the women had an endometrial thickness of greater than 8mm at initial assessment; 26% fulfilled these criteria. Saline hydrosonography revealed polyp in 3%, cysts in 7% and both in 8%, these abnormalities were unaffected by 3 cycles of oral norethisterone. However, 96% of these women had withdrawal bleeding indicating an oestrogen-primed uterus, caused by tamoxifen. It is also important to note that Powles and co-workers used cyclical progestagen and used transvaginal ultrasound measurement of the endometrial thickness to assess reversal of tamoxifen changes. The use of ultrasound does not allow the assessment of endometrial histological changes

including whether norethisterone use has resulted in the development of secretory or decidual changes which will not manifest as thin endometrium on ultrasound. Gardner and colleagues demonstrated that progestagens cause endometrial decidualisation in tamoxifen primed endometrium, and that the decidualised endometrium does not appear thinned on ultrasound (Gardner et al., 2000). This suggests that the type of the progestagen, the route of and duration of administration may be important.

Indeed, the use of the progestagen (levonorgestrel)-impregnated intrauterine device (LNG-IUS) in breast cancer patients being treated with long-term tamoxifen has been demonstrated to induce decidualization of the stroma after 12 months of follow-up in all patients (Gardner et al., 2000). Gardner and colleagues randomized 122 postmenopausal women receiving tamoxifen for a mean duration of 3 years. They observed small but significant increases in the anteroposterior diameter, cavity length and longitudinal diameter of the uterus in women with the levonorgestrel device in situ after 6 and 12 months of follow-up. They attributed this increase to progestagen induced change in the tissue fluid in the inner myometrium. The endometrial histology in the women fitted with the LNG-IUS revealed uniform endometrial decidual response, even in women who withdrew from the study as early as 3 months. Interestingly, no new endometrial polyps were observed in the women fitted with the device, which is an important finding as endometrial polyp is the most common endometrial pathology in tamoxifen treated women (Lahti et al., 1993; Cohen et al., 1994a; Kedar et al., 1994; Cohen et al., 1997a; Deligdisch et al., 2000; Vosse et al., 2002; McGonigle et al., 2006). The prevention of endometrial polyps in this population is also important clinically, since the increased risk of endometrial cancer has been linked with the tamoxifen induced polyp because most tamoxifen associated endometrial cancers are considered to be mainly restricted to the site

of polyps (Ismail, 1994). Whether this treatment will also reduce the incidence of endometrial cancer remains to be determined (Gardner et al., 1998; Gardner et al., 2000; Neven, 2000).

The use of progestagens to oppose the uterine action of tamoxifen and to induce endometrial differentiation can raise concerns with regard to its effect on breast cancer recurrence. It is known that combined oestrogen and progestagen hormone replacement therapy increases the risk of breast cancer (Writing Group for the Women's Health Initiative Investigators, 2002; Beral and Collaborators, 2003). The Women's Health Initiative (WHI) study reported a 26% increase in the relative risk of breast cancer for combined oestrogen and progestagen therapy when compared to placebo (Investigators, 2002). A questionnaire based epidemiological study found no significant increase in the incidence in breast cancer among LNG-IUS users (mean age 35.4 years at time of LNG-IUS insertion) when compared to non-users (Backman et al., 2005). A more recent study by Trinh and co-workers concluded that there was no increased risk of breast cancer associated with LNG-IUS use, but a subgroup analysis revealed borderline significantly higher recurrence rate of breast cancer when breast cancer is diagnosed during the use of the LNG-IUS and where LNG_IUS use continued after cancer diagnosis (Trinh et al., 2008). It must be noted that the control group in their analyses were matched for all the LNG-IUS users, the latter fell into a lower risk for recurrence group who had the LNG-IUS inserted after diagnosis and a higher risk of recurrence group who developed breast cancer whilst using the LNG-IUS. It is inevitable that the high risk group have a poor outcome compared to controls. An in vitro study using the human breast cancer cell lines T47D and MCF-7, showed that levonorgestrel did not stimulate oestrogen activating enzymes in these cells lines and inhibition of oestrogen stimulated proliferation of the breast cancer cells

(Xu et al., 2007). Although, further large scale studies will be needed to confirm the safety of LNG-IUS use in postmenopausal women with breast cancer who are treated with tamoxifen, to date there is no convincing evidence of increased risk of recurrence.

Whether the application of the LNG-IUS at the initiation of tamoxifen treatment would prevent the oestrogenic effects of tamoxifen and the development of tamoxifen associated uterine features from the outset remains to be determined.

1.8 Hypothesis

Tamoxifen is often used as adjuvant treatment of all stages of breast cancer, where it behaves as an oestrogen antagonist. However, tamoxifen affects the endometrium of postmenopausal women and leads to adverse changes resulting in pathologies such as polyps, hyperplasia and more importantly, endometrial cancer.

The key hypothesis being tested is that the abnormal features of uterine growth and development associated with the use of tamoxifen, is due to its failure to replicate all the effects of oestradiol in either inducing or repressing the expression of key paracrine acting regulators of uterine cellular populations or their gene expression profiles.

Secondary to this, the thesis will test the hypothesis that the abnormal features of uterine growth and development associated with the use of tamoxifen can be prevented by the administration of a progestagen-containing levonorgestrel intrauterine device from the onset of tamoxifen treatment.

Chapter 2

The effect of concomitant administration of LNG-IUS and tamoxifen on the uterus of postmenopausal women

2.1 Introduction

As summarised in Chapter 1, tamoxifen is a selective oestrogen receptor modulator that is metabolised to two active metabolites, 4-hydroxy tamoxifen and endoxifen, both of which bind to the oestrogen receptor (Lim et al., 2005). In the breast, tamoxifen acts as an oestrogen antagonist preventing the pro-proliferative action of oestradiol, thereby inhibiting cell proliferation. However, tamoxifen is oestrogenic in the uterus of postmenopausal women, promoting cell proliferation and inducing morphological changes that include an increase in uterine volume and endometrial thickness (Morales et al., 2005). This pattern of growth exhibited by the uterus exposed to tamoxifen suggests that there is a lack of coordinated growth, compared to a uterus exposed to its normal oestrogenic concentrations. This lack of coordination in the pattern of growth results in the development of endometrial polyps, hyperplasia and cancers and histological features such as dilated cystic glands.

In women, tamoxifen use is associated with endometrial abnormalities such as endometrial polyps, hyperplasia and cancer (Ismail, 1994). After one year of exposure in postmenopausal women the incidence of these abnormalities is in the order of 30% (Deligdisch et al., 2000; Cohen, 2004). Many epidemiological, as well as randomised and prospective trials have shown a moderately increased risk of endometrial cancer induced by tamoxifen (Fisher et al., 1994; Fisher et al., 1998; Bernstein et al., 1999; Bergman et al.,

2000). This increased risk of endometrial cancer was also reported in a large randomised trial using tamoxifen for breast cancer prevention (Fisher et al., 1998). Women who present clinically with postmenopausal bleeding all require immediate investigation with ultrasound scan, hysteroscopy and/or endometrial biopsy to rule out pathological changes in the endometrium. However, routine ultrasound surveillance in asymptomatic women taking tamoxifen is not recommended due to the high false positive rate (Fung et al., 2003). However, it has been suggested that all postmenopausal women should have a baseline assessment with a transvaginal ultrasound scan before commencing tamoxifen treatment (Vosse et al., 2002; ACOG, 2006). Those women with a normal endometrium (endometrial thickness less than 8mm) at baseline should undergo annual ultrasound scans after 3 years of tamoxifen treatment, and yearly ultrasound scans should be performed for women with abnormal endometrium (endometrial thickness equalled or measured above 8mm) at baseline after further investigation with hysteroscopy and endometrial biopsy (Vosse et al., 2002).

Progestagen treatment is widely used for endometrial hyperplasia (Reed et al., 2009). Although some progestagens are protective, systemic norethisterone has not been shown to reverse the tamoxifen induced endometrial changes (Powles et al. 1998). This suggests that the nature of the progestagen and its route of administration may be important.

An alternative strategy to reduce the adverse actions of tamoxifen upon the uterus has been the local application of a progestagen. As described in Chapter 1 and above, the application of the progestagen (levonorgestrel)-impregnated intrauterine device (LNG-IUS; Mirena®) in patients treated with long term (minimum 1 year) tamoxifen has been demonstrated to induce pseudo-decidualization of endometrial stroma, and to prevent the

development of endometrial tamoxifen associated polyps at the 12 month follow up following LNG-IUS use (Gardner et al., 2000). Whether this strategy also reduces the incidence of endometrial cancer remains to be determined but would require a large scale study to reach a conclusion.

Endometrial decidualization and the prevention of tamoxifen polyp development are therefore potential mechanisms of endometrial protection in women exposed to tamoxifen. In the study by Gardner and colleagues the LNG-IUS was applied after a minimum 1 year of tamoxifen treatment (mean duration of tamoxifen treatment 3 years). Whether the application of the LNG-IUS at the initiation of tamoxifen treatment would induce similar changes or prevent tamoxifen related changed remains an unanswered question.

I hypothesize that the application of the LNG-IUS at the onset of tamoxifen treatment will prevent the development of tamoxifen effects; namely increased uterine volume, increase endometrial thickness and polyp formation. To test this hypothesis, I undertook a randomised controlled trial in which postmenopausal women with newly diagnosed breast cancer and an intact uterus were recruited when they commenced therapy with tamoxifen. These women were then randomized to receive endometrial surveillance with or without the LNG-IUS device.

2.2 Methods

2.2.1 Design

This was a randomised controlled trial that commenced in March 2004 at a tertiary teaching hospital.

Eligible women who gave informed consent were randomised using a pre-prepared, serially numbered, sealed envelopes method to receive endometrial surveillance with insertion of the LNG-IUS (Mirena group) or endometrial surveillance alone (Control group). The follow-up period was 12 months after inclusion into the study, as described by Gardner and colleagues (Gardner et al., 2000).

The study based its power calculations on the prevention of the uterotrophic effects during tamoxifen therapy. In a pilot study, the mean uterine volume of women commencing tamoxifen therapy was 53.6 cm^3 with a standard deviation of 27.5 (based on 31 patients). Morales and colleagues demonstrated a 36% increase in uterine volume after 6 months of tamoxifen therapy (Morales et al., 2005), and so, power calculations indicated that a minimum of 32 patients were needed per group to demonstrate inhibition of the uterotrophic effect of tamoxifen with 80% power and at the 5% significance level.

2.2.2. Patients

The inclusion criteria were: postmenopausal women (serum oestradiol levels of less than 70 pmol/L) with an intact uterus requiring postoperative adjuvant tamoxifen treatment upon completion of adjuvant radiotherapy and/or chemotherapy for breast cancer; tamoxifen treatment to be commenced; no history of progestagen therapy since diagnosis of breast cancer; no evidence of recurrent disease; healthy pelvic organs as per digital

examination and transvaginal ultrasonography; consent to participate in the study; age less than 80 years.

Women were excluded from the study if they had; suspected pelvic inflammatory disease; active liver disease; a previous history of malignancy; grade III submucous fibroids or refusal of LNG-IUS device insertion.

Patients were identified and approached for inclusion when attending for adjuvant radiotherapy for newly diagnosed breast cancer after initial surgical resection at the Clinical Oncology Department at the University Hospitals of Leicester NHS Trust. All patients were given a patient information sheet (see Appendix 1). Women willing to participate were contacted by the research team and invited to the Obstetrics and Gynaecology Department at the Leicester Royal Infirmary where I undertook all consultations and screening procedures.

2.2.3 The Levonorgestrel Intrauterine System (LNG-IUS)

The levonorgestrel intrauterine system (Mirena®, Schering Health Care, W Sussex, UK) releases 20 μg of levonorgestrel per day, allowing very high local concentrations in the endometrial tissues with low plasma concentrations gained systemically (Nilsson et al., 1982). The low systemic concentrations of levonorgestrel accounts for the low side-effect profile associated with its use. The LNG-IUS is known to effectively prevent endometrial proliferation during postmenopausal oestrogen based hormone replacement treatment (Raudaskoski et al., 2002).

2.2.4 Study Protocol

All women had endometrial surveillance with transvaginal ultrasonography at recruitment and six monthly thereafter. All the women underwent hysteroscopy and endometrial biopsy at entry and at 12 months. Women randomised to the Mirena group had the device inserted at entry and had an additional follow-up at one month for a routine intrauterine device check, in order to check the coil strings were visible at the cervical os.

At transvaginal ultrasonography endometrial thickness was measured, defined as the widest point of the hyper echoic area in a longitudinal section of the uterus (Figure 2.1). Other uterine dimensions were also measured in order to calculate the uterine volume; the anteroposterior diameter (AP) and longitudinal diameter (L) of the uterus in the sagittal plane. A further measurement of the transverse uterine diameter (T) was made in a transverse section of the uterus (Figure 2.1). All measurements were measured in triplicate and were made without reference to measurement taken at previous visits to ensure objectivity.

Outpatient hysteroscopic assessment and endometrial sampling was performed annually with a 3.5mm rigid hysteroscope using saline as the distension media. Large (>20mm) endometrial polyps were removed under general anaesthetic. The same operator performed all the ultrasound and hysteroscopic assessments to prevent interobserver variability. The uterine volume was calculated using the following formula (Morales et al., 2005):

Uterine volume (cm^3) = $(AP.L.T.\pi)/6$

Endometrial samples were obtained using a pipelle endometrial sampler. All specimens were fixed in formalin, embedded in paraffin wax and stained with standard haematoxylin and eosin at the hospital Pathology Department. Sections were examined under a light microscope by an experienced gynaecological histopathologist (Dr Laurence Brown), who assessed and reported all samples blind of randomisation. All those who dropped out of the study, had the device removed and an endometrial biopsy performed at the time of device removal.

2.2.5 Ethical Approval

Ethical approval for the study was granted by the Leicestershire Research Ethics Committee (reference number 6498; see Appendix 3).

2.2.6 Statistical Analyses

Continuous outcome measures, such as uterine volume and endometrial thickness, were reported at entry and at each six month interval. The differences between the groups were assessed using a Student's *t*-test or Mann-Whitney U-test depending on whether the data exhibited Gaussian normal distribution or not, respectively. Examination of the hysteroscopic and histological findings, were analysed using the Fisher's exact test. All statistical analyses were performed using SPSS version 16.0 and a significance level of 5% was considered an indication of statistical significance.

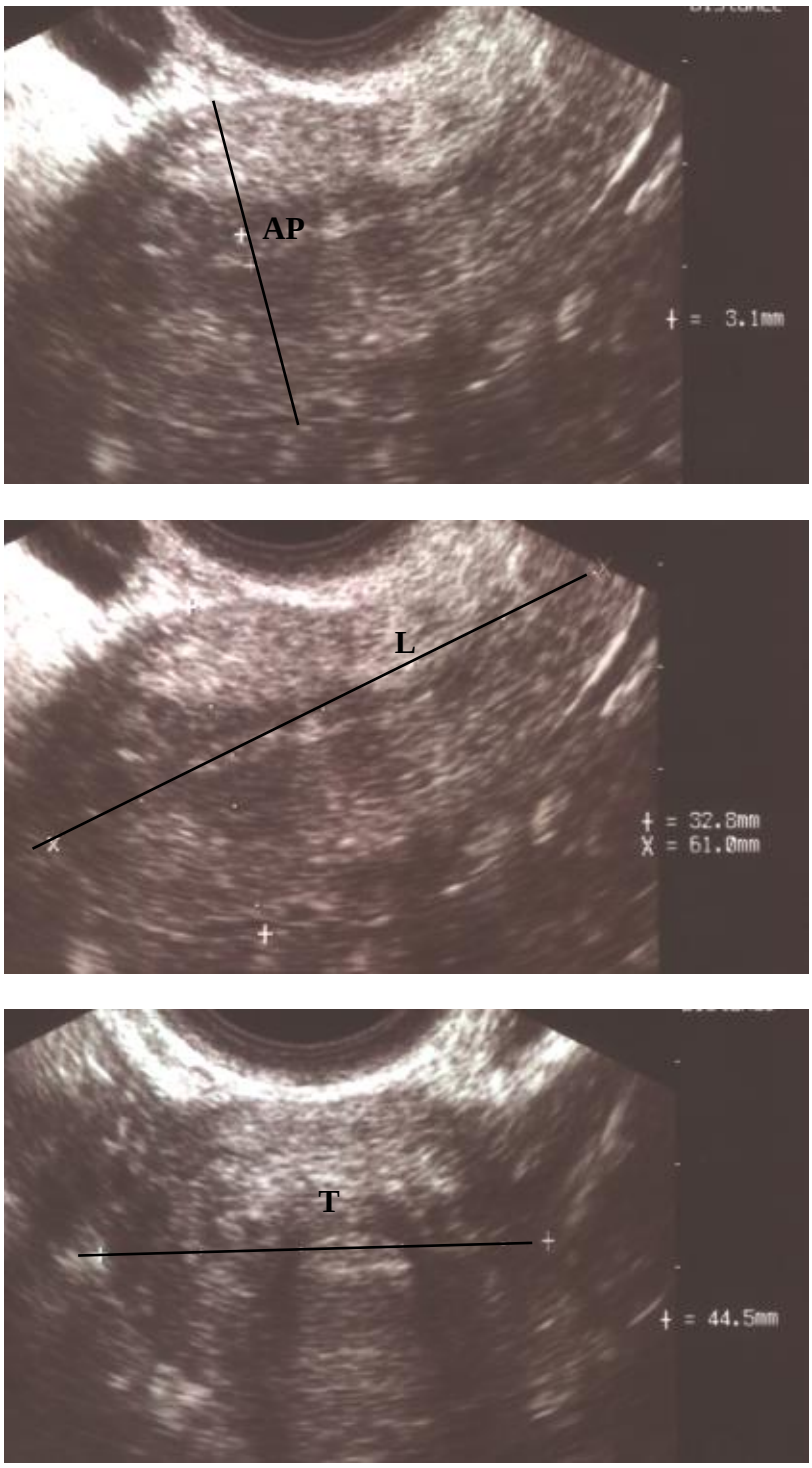
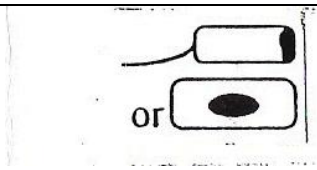
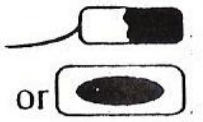
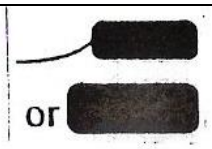


Figure 2.1 Transvaginal ultrasound scan images to demonstrate how the anteroposterior (AP), longitudinal (L) and transverse (T) dimensions were measured.

All women were given diaries to record daily blood loss. The amount of bleeding in any 24-hour period was scored as follows with the aid of the following pictorial chart (Gardner et al., 2000):

Table 2.1 Bleeding diary definitions of maximum amount of bleeding in 24 hour period

Score	Amount	Definition	
1	Spotting	Fresh blood on wiping, not requiring sanitary protection	
2	Light		Small amount of bleeding on any sanitary pad/tampon that day
3	Moderate		Moderate amount of bleeding on any sanitary pad/tampon that day
4	Heavy		Soaking any sanitary pad/tampon on that day

The maximum amount of daily bleeding was assessed in eight 90-day intervals over the year to give number of bleeding episodes, total number of days of bleeding and total bleeding scores per 90-day interval;

Total bleeding score = sum of maximum daily bleeding scores

2.3 Results

A total of 75 women were randomised into the study. Of these, 42 were randomly assigned to the control group (surveillance alone), and 33 were assigned to the Mirena group (LNG-IUS and endometrial surveillance).

There was incomplete follow up data at 6 months for 5 women in the control group and for 7 women in the Mirena group due to withdrawals. At 12 months there were a further 14 and 2 withdrawals in the control and Mirena groups, respectively. No women had stated that unwanted vaginal bleeding was a reason for their withdrawal.

Figure 2.2 details the trial profile and details of the patients who withdrew from the trial.

2.3.1 Demographic Details

The baseline characteristics of the women on entry into the study are shown in Table 2.1. All the parameters were similar in both the control and Mirena group. There were no statistically significant differences in the demographic details of women in the control and Mirena groups.

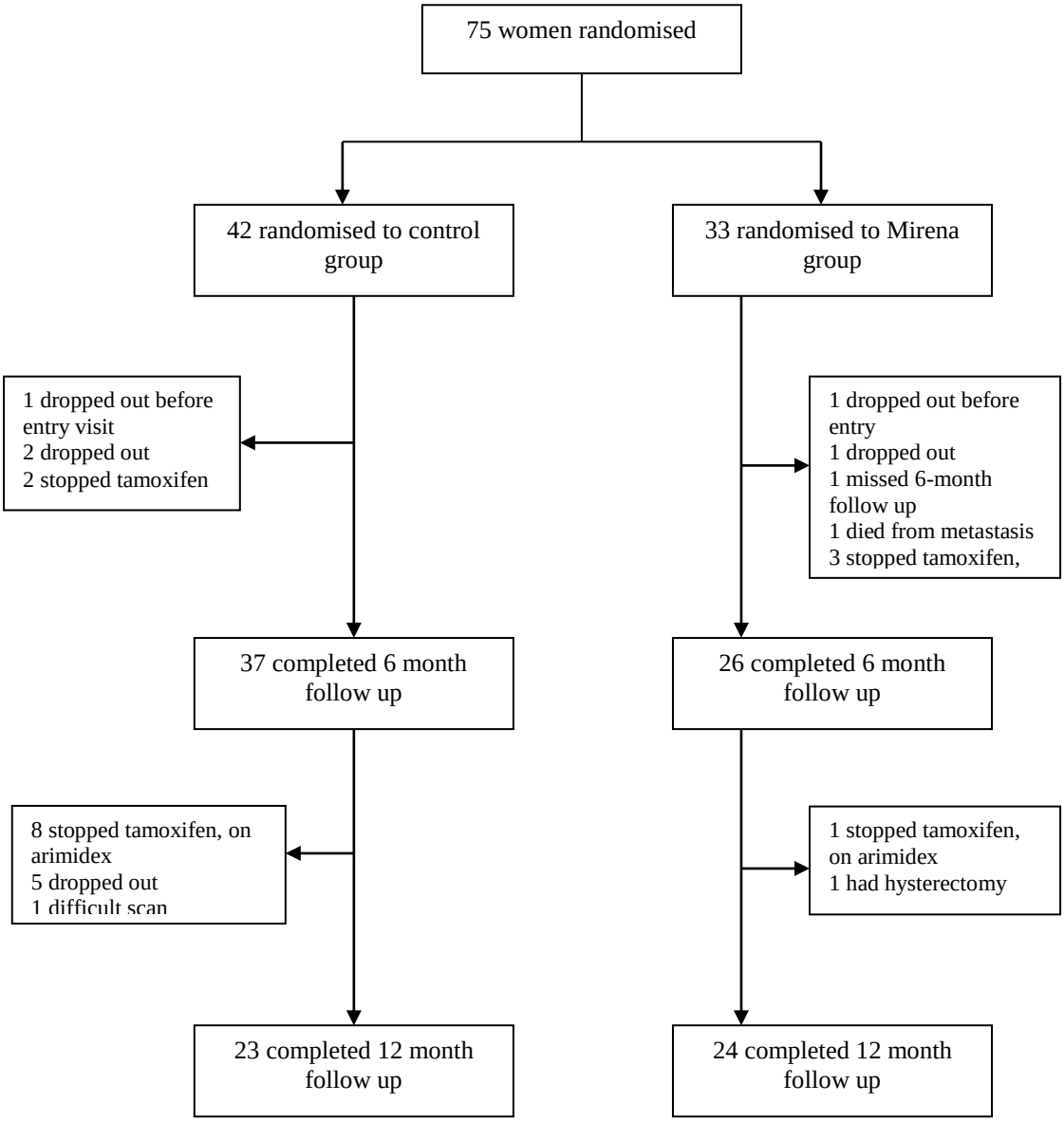


Figure 2.2 Trial profile

Characteristic	Control group (n=42)	Mirena group (n=33)
Age at entry to study (years)	60.26 (6.58)	59.32 (6.06)
Age at diagnosis of breast cancer (years)	60.07 (6.63)	58.84 (6.06)
Age at menopause (years)	50.88 (4.62)	51.58 (3.84)
Body Mass Index (kg/m^2)	26.79 (7.04)	26.61 (4.40)
Duration of tamoxifen treatment (days)	39.36 (20.32)	33.06 (23.31)
ER positive tumour	40 (95%)	31 (94%)
PR positive tumour	35 (83%)	25 (76%)
Lymph nodes positive	9 (21%)	11 (33%)
Chemotherapy	9 (21%)	11 (33%)
Radiotherapy	42 (100%)	31 (94%)

Table 2.2 Baseline characteristics of the women who entered the study.

Data are shown as the mean number and (percentage); there were no statically significant differences between the two groups.

2.3.2 Endometrial Thickness

The mean endometrial thickness (ET) in the control and Mirena groups at entry was 3.87mm and 3.40mm, respectively. At the 6 and 12 month follow up, the ET in the control group increased to 3.98mm and 4.32mm, respectively. In the Mirena group, ET decreased to 2.66mm at 6 months and then increased to 3.11mm at 12 months. However, the ET showed no statistically significant difference after 6 and 12 months between the two groups (Table 2.2). It appeared that the levonorgestrel intrauterine system had no significant effect on endometrial thickness, however, its presence can interfere with the measurement of endometrial thickness. This may have affected the accuracy and significance of the endometrial thickness in the Mirena group.

2.3.3 Anteroposterior, transverse and longitudinal dimensions on ultrasound scan

Assessment with transvaginal sonography showed that there was no significant difference in anteroposterior (AP), longitudinal (L) or transverse (T) uterine dimensions at baseline between the control and Mirena groups (Table 2.2).

In the control group, all 3 parameters increased slightly at 6 and 12 months compared to baseline. However, in the Mirena group the converse was observed, all 3 dimensions decreased slightly at 6 and 12 months from the baseline measurements (Table 2.2).

The changes in the uterine dimensions between the control group and the Mirena group were statistically significant at 12 months in the AP ($p=0.0216$) and T ($p=0.0093$) dimensions, but not at the 6 month assessment (Table 2.2).

There was no significant difference in the longitudinal dimension between the groups.

	Control group	Mirena group	Comparison	
			Mean Difference (95% CI)	P value*
Endometrial Thickness (mm)				
Baseline	3.87 (3.10)	3.40 (3.34)		0.6219
6 months	3.98 (3.23)	2.66 (0.96)		0.3457
12 months	4.32 (3.89)	3.11 (1.56)		0.3326
Anteroposterior diameter (mm)				
Baseline	33.88 (8.14)	35.75 (5.88)	1.88 (-1.55 to 5.29)	0.2779
6 months	35.60 (7.57)	35.18 (4.82)	-0.42 (-3.79 to 2.96)	0.8048
12 months	39.20 (8.20)	34.66 (4.40)	-4.54 (-8.39 to 0.69)	**0.0216**
Longitudinal diameter (mm)				
Baseline	59.21 (10.25)	59.56 (9.55)	0.36 (-4.35 to 5.07)	0.8799
6 months	60.53 (9.94)	59.19 (7.41)	-1.35 (-5.93 to 3.27)	0.5625
12 months	61.80 (9.83)	56.84 (9.47)	-4.96 (10.63 to 0.72)	0.0851
Transverse diameter (mm)				
Baseline	44.92 (10.92)	46.84 (6.59)	1.92 (-2.46 to 6.31)	0.3836
6 months	47.40 (9.21)	44.23 (6.63)	-3.16 (-7.38 to 1.06)	0.1393
12 months	50.01 (9.44)	43.61 (6.48)	-6.40 (-11.14to -1.66)	**0.0093**
Uterine volume (cm^3)				
Baseline	51.14 (30.56)	55.25 (21.63)	4.11 (8.66 to 16.88)	0.5223
6 months	57.57 (33.11)	50.07 (18.87)	-7.50 (-21.95 to 6.95)	0.3033
12 months	69.21 (40.30)	47.87 (17.91)	21.35 (-39.55 to -3.14)	**0.0225**

Table 2.3 Ultrasound assessment of endometrial thickness, anteroposterior, longitudinal and transverse dimensions, and uterine volume at baseline, 6 and 12 month visits.

Values are shown as mean (SD). Endometrial thickness analysed using non parametric Mann Whitney U-test (non equal variance). Anteroposterior, longitudinal, transverse diameters and uterine volume analysed using Student's *t*-test. *Statistical differences are shown in bold typeface.

2.3.4 Uterine Volume

The uterine volume in the control group was 51.14cm^3 at baseline, and this increased to 57.57cm^3 at 6 months and 69.21 cm^3 at 12 months. In the Mirena group, the baseline uterine volume at 55.25cm^3 was not statistically different to that in the control group (p=0.5223). At 6 months the uterine volume in the Mirena group decreased to 50.07cm^3 and further still to 47.87cm^3 at 12 months (Table 2.2). The changes in the uterine volume between the control group and the Mirena group were statistically significant at 12 months (p=0.0225).

2.3.5 Hysteroscopy and histology findings

Histological assessment of endometrial samples obtained at baseline demonstrated that the majority of endometrium, but not all of the endometrium was sufficient for analysis; 68.3% in the control and 62.5% in the Mirena group. Table 2.3 demonstrates that the endometrial histology in both groups was comparable at study entry. There were 2 cases (4.9%) of weak proliferative endometrium in the control group. There were 4 cases in which endometrium samples were not obtained, these were in the control group where the introduction of the hysteroscope was not possible due to cervical stenosis. There were no cases of endometrial hyperplasia or carcinoma at study baseline.

Outcome	Control group		Mirena Group	
	Baseline (%) (n=41)	12 months (%) (n=23)	Baseline (%) (n=32)	12 months (%) (n=24)
Benign polyp*	5 (12.2)[a]	6 (26.1)[b]	7 (21.9)	1 (4.2)
SMF**	7 (17.1)	4 (17.4)	9 (28.1)	0(0.0)
Insufficient for diagnosis	9 (21.9)	11 (47.8)	12 (37.5)	1 (4.2)
Atrophic or Inactive	26 (63.4)	11 (47.8)	20 (62.5)	0 (0.0)
Weak proliferative or secretory	2 (4.9)	(0.0)	0(0.0)	1 (4.2)
Endometritis	0(0.0)	(0.0)	0(0.0)	1 (4.2)
Decidualised endometrium	0(0.0)	(0.0)	0(0.0)	21 (87.5)
No hysteroscopy	4 (9.8)[c]	1 (4.3)[d]	0(0.0)	0 (0.0)

Table 2.4 Hysteroscopic and histological findings at entry and at 12 months for the control and Mirena groups.

[a] 1 patient dropped out at 6 months, 2 stopped tamoxifen and then dropped out

[b] 4 patients with new polyps, 1 with the same number of polyps as at baseline, 1 developed multiple polyps at 12 months

[c] Unable to introduce hysteroscope

[d] Declined hysteroscopy

Histological assessment of the endometrial samples at 12 month follow up showed benign changes in both groups (Table 2.3 and Figure 2.3). In the control group 11 (47.8%) specimens were insufficient for analysis, 11 (47.8%) exhibited atrophic or inactive endometrium and the remaining 1 (4.3%) was missing because the woman declined hysteroscopy so there was no sample to analyze. One (4.2%) sample in the Mirena group was insufficient for analysis, 1 (4.2%) demonstrated weak proliferative endometrium and 1 (4.2%) demonstrated endometritis. The remaining 21 (87.5%) endometrial samples from the Mirena group had undergone pseudo-decidual changes (Table 2.3). Before the 6 month follow up 7 women in the Mirena group dropped out, there was histology taken from 4 of these women when the coil was removed, of the remaining three, one died from metastases, one missed the follow up appointment and the third dropped out before coil insertion. A further two women dropped out after the 6 months follow up, one had stopped tamoxifen and the other had a hysterectomy. All six endometrial samples taken when the six dropped out had a pseudo-decidual response, and of these samples two samples were taken only 27 and 50 days after the LNG-IUS was inserted.

At the start of the study there were 12 women with endometrial polyps (Figure 2.5); 5 (12.2%) in the control and 7 (21.9%) in the Mirena group. Of the seven women with endometrial polyps in the Mirena group, two had polyps larger than 2cm, only one patient had a polypectomy, the second patient declined intervention. At the 12 month follow up, the control group had 6 women (26.1%) and the Mirena group had 1 woman (4.2%) with an endometrial polyp. Only 2 of the 5 women with endometrial polyps in the control group remained in the study at 12 months, one had no change in the number of polyps and the other had multiple endometrial polyps. Therefore, at the 12 month hysteroscopic assessment, 4 women developed new polyps in the control group. By contrast, in the

Mirena group the 7 women with polyps at the start of the study had no polyps at 12 months follow up (one of these was surgically removed); the 1 polyp at 12 month was a newly developed polyp. The difference between the two groups was statistically significant (p=0.0044, Fisher's exact) (Table 2.3).

There were 16 sub-mucous fibroids observed on hysteroscopy at baseline, 7 (17.1%) in the control group, and 9 (28.1%) in the Mirena group. At 12 months, there were a total of 4 submucous fibroids and these were all in the control group (9.8%; Figure 2.5). There were 2 women from baseline with unchanged submucous fibroids, 2 *de novo* submucous fibroids and 3 women whose submucous fibroids had regressed at 12 months. The difference between the two groups was statistically different (p=0.0006, Fisher's exact test). This suggests that the LNG-IUS causes the SMF to regress.

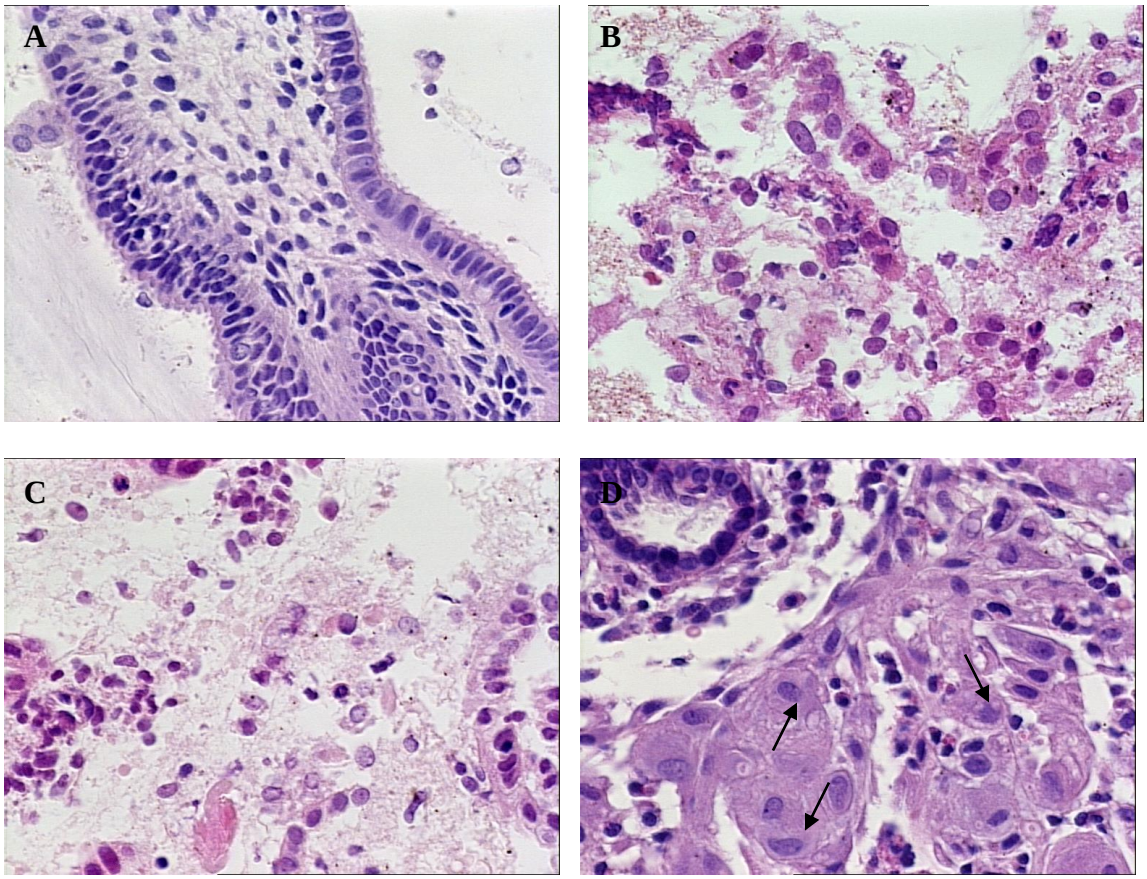


Figure 2.3 Histology of the endometrium from women in the control and Mirena groups at entry and then at 12 months follow up. Magnification x400

The photomicrographs show representative images of inactive atrophic postmenopausal endometrium in the control (A) and Mirena (B) group at entry, and the control group (C) at 12 month follow-up. The biopsies obtained from the Mirena group at 12 month follow-up show diffuse decidualization of the stroma (D). Arrows indicate the decidualized stromal cells.

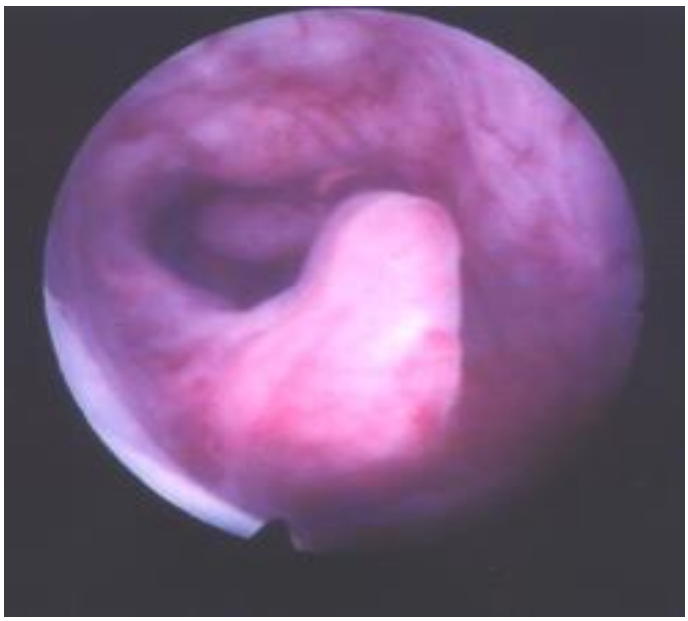


Figure 2.4 Hysteroscopic image of an endometrial polyp.

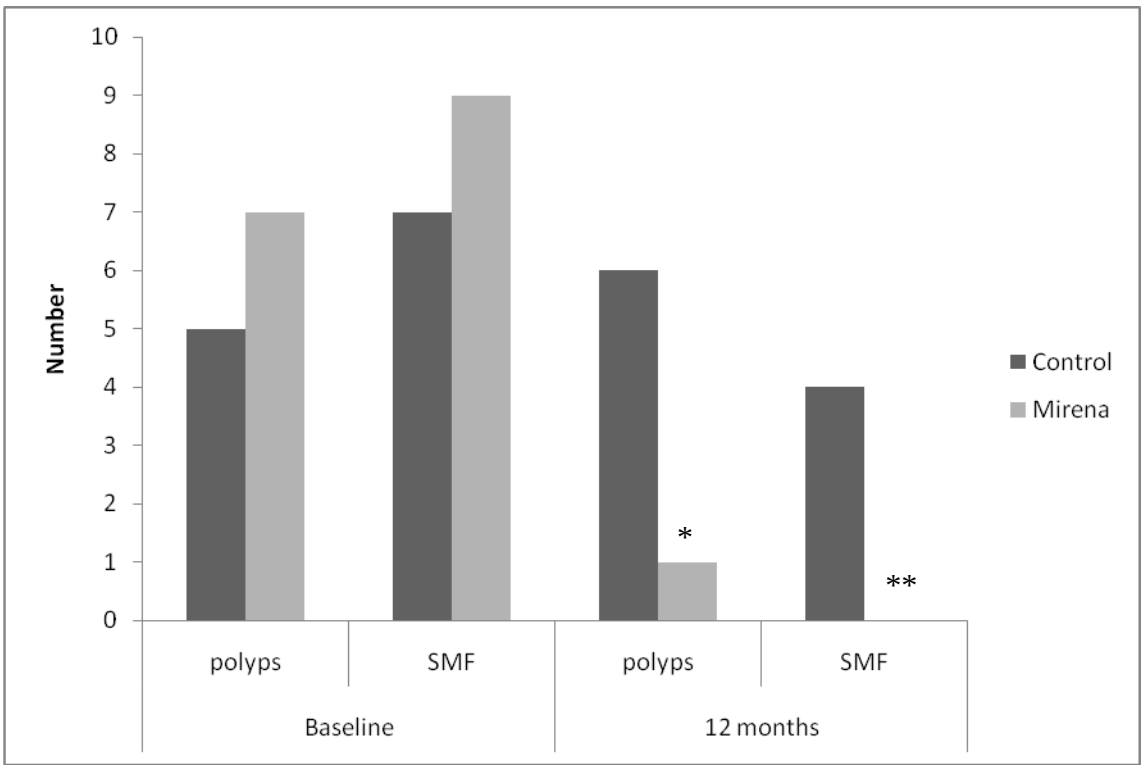


Figure 2.5 Number of endometrial polyps and submucous fibroids (SMF) at baseline and 12 months in the control and Mirena groups.

*Fisher's exact test p=0.0044 (comparing polyps in the control and Mirena group at 12 months); **Fisher's exact test p=0.0006 (comparing SMF in the control and Mirena group at 12 months); SMF = submucous fibroids.

2.3.6 Bleeding patterns

All women in the study were given diaries to record their vaginal bleeding patterns over the course of the twelve months. Not all of the women under follow up during the intervals returned their diaries. In the control group 82.9%, 78%, 70.3% and 100% of women returned the bleeding diaries in the first, second, third and fourth 90 day interval, respectively. In the Mirena group, the compliance to recording and returning the diaries was slightly better at 87.5%, 87.5%, 96.1% and 100% in the first, second, third and fourth 90 day interval, respectively.

There were more bleeding episodes and days of bleeding in both groups in the first interval of the study than in subsequent intervals (Table 2.4). The bleeding scores were higher in the Mirena group in the first two intervals (6 months) due to an increase in both the number of episodes of bleeding and their duration. In the control group, the bleeding episodes, number of days of bleeding and the bleeding scores, all reduced after the first interval to a steady baseline thereafter. In the Mirena group, the bleeding episodes, duration of bleeding and scores decreased through the duration of the study with each interval, but this was at a slower rate compared to the control group (Figure 2.6). However, after the first, second and third interval (9 months) the bleeding scores in the Mirena group were similar to the control group (Table 2.4).

There were no other adverse effects of the levonorgestrel intrauterine device reported by participants.

Interval measure (90 days)	r/n* (N)	Control	r/n*	Mirena	p value**
1st Interval					
Episodes	21/34 (41)	0.68 (0.59)	26/28 (32)	2.14 (2.09)	<0.0001
Days		1.59 (3.12)		21.32 (26.9)	<0.0001
Score		1.74 (3.17)		38.07 (55.6)	<0.0001
2nd Interval					
Episodes	1/32 (41)	0.031 (0.18)	13/28 (32)	1.68 (3.08)	<0.0001
Days		0.094 (0.53)		8.5 (11.96)	<0.0001
Score		0.190 (1.06)		12.64 (18.28)	<0.0001
3rd Interval					
Episodes	2/26 (37)	0.12 (0.43)	7/25 (26)	0.56 (1.29)	0.063
Days		0.27 (0.96)		2.76 (7.09)	0.0556
Score		0.31 (1.09)		4.44 (11.96)	0.0487
4th Interval					
Episodes	3/23 (23)	0.17 (0.48)	4/24 (24)	0.46 (1.22)	0.6143
Days		0.17 (0.48)		1.83 (6.36)	0.6025
Score		0.25 (0.85)		2.13 (0.69)	0.6262

Table 2.5 Vaginal bleeding patterns at different intervals during the 12 month study period.

Values are shown as mean (SD). *r/n=number of women experiencing bleeding/number of women who returned bleeding diaries (n=number of women under follow up in that group); ** Mann Whitney U-test.

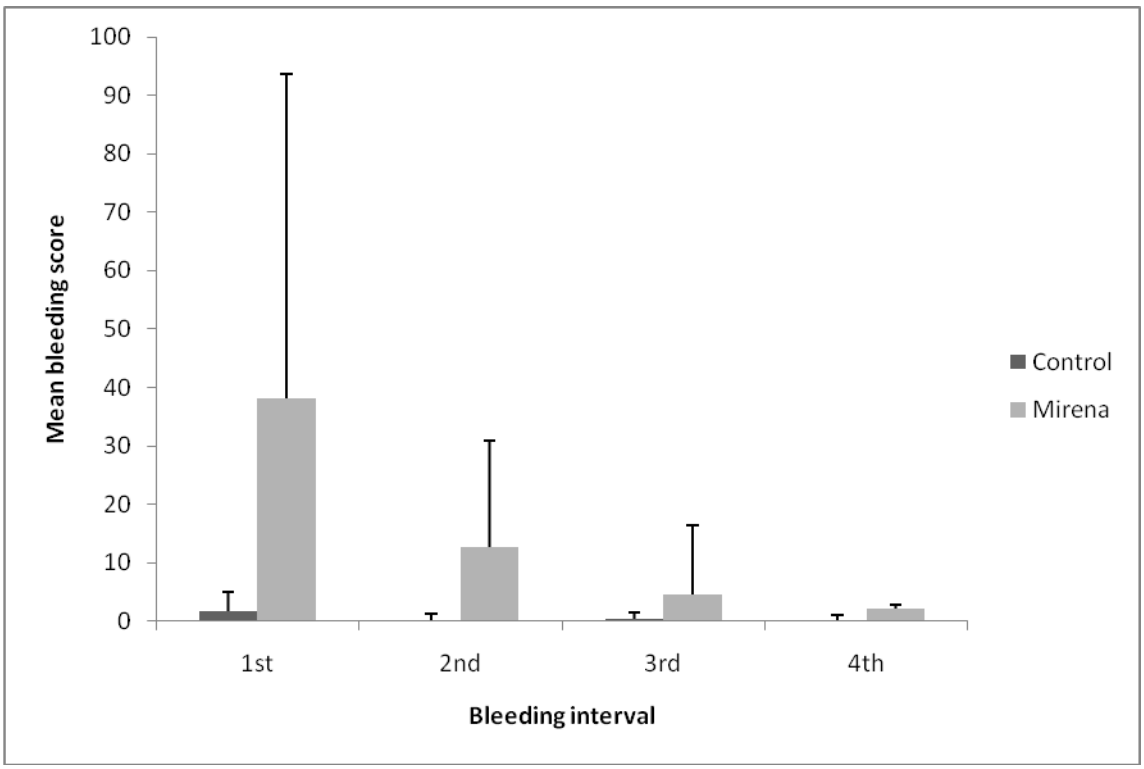


Figure 2.6 Mean bleeding score during each 90 day interval in the control and Mirena groups.

Total bleeding score = sum of maximum daily bleeding. Values are shown as mean ± SD.

2.4 Discussion

The use of tamoxifen has been the mainstay of adjuvant hormonal therapy in oestrogen receptor positive breast cancer, since the 1970's. Tamoxifen has improved both the prognosis and survival following breast cancer diagnosis ((EBCTCG), 2005). In recent years, there has been a move towards increased use of aromatase inhibitors, either following two to three years of tamoxifen therapy or from the outset. This shift to using adjuvant aromatase inhibitors has been associated with improved disease-free survival (Baum et al., 2002; Coombes et al., 2004). Another advantage of the aromatase inhibitors is that they do not stimulate the endometrium, thereby reducing the incidence of endometrial abnormalities including the increased risk of endometrial carcinoma associated with tamoxifen use. This reduces the need for endometrial assessment (Baum et al., 2002; Duffy et al., 2005; Gerber et al., 2006; Morales et al., 2006; Patel et al., 2007). However, tamoxifen continues to play an important role in the treatment of breast cancer in premenopausal women and low risk women with oestrogen responsive tumours (Patel et al., 2007).

This study confirmed the uterotrophic effects of tamoxifen and importantly, that this effect is prevented by the LNG-IUS used over 12 months. The increase in uterine volume observed here has also been reported by Morales and colleagues in 2005, where they showed a 38% increase in the uterine volume after 3 months of tamoxifen therapy. This uterotrophic effect did not occur when women were treated with aromatase inhibitors, and there was a reduction in the uterine volume when women switched from tamoxifen to aromatase inhibitor therapy (Morales et al., 2005). There was no change in uterine volume in the women in the tamoxifen with the LNG-IUS group, where the LNG-IUS was inserted after long-term tamoxifen therapy (minimum one year) (Gardner et al., 2009), this is

probably because prior tamoxifen induced uterotrophic effects cannot be reversed by progesterone. These data suggest that the histological features described in the control group at 12 months are consistent with the use of tamoxifen (Ismail, 1994). There were no cases of endometrial hyperplasia or carcinoma in either group throughout the duration of the study.

At hysteroscopic assessment, five women in the control group had endometrial polyps at baseline and 6 women had endometrial polyps at 12 months. However, three of the five women with polyps at baseline dropped out and of the remaining two, one had developed multiple polyps and the other had the same number as at baseline. Therefore, at the 12 month follow up hysteroscopy there were four women who had developed new polyps and one had developed multiple polyps. In the Mirena group, at baseline there were seven women with endometrial polyps, and at 12 months there was only one, which was a *de novo* polyp. The reduction in new polyp formation was statistically significant (p=0.0044), and was consistent with previous studies where the LNG-IUS was inserted either after long term tamoxifen treatment (Gardner et al., 2000; Chan et al., 2007) or at the initiation of tamoxifen therapy (Chan et al., 2007; Kesim et al., 2008).

In the Mirena group, the majority of women (87.5%) developed a uniform pseudo-decidual response in the endometrium at the 12 month endometrial sampling. There were eight patients in the Mirena group who left the study (one died, one dropped out after having only one scan and the remaining six dropped out when tamoxifen therapy was stopped). All six endometrial samples taken when the six dropped out (when tamoxifen therapy was stopped) had a decidual response, and of these samples two samples were taken only 27 and 50 days after the LNG-IUS was inserted. This decidual reaction is consistent with

previous studies were the LNG-IUS was inserted following tamoxifen treatment for a mean duration of 3 years, therefore the endometrium had been primed with tamoxifen, whereas in this study the Mirena was inserted into a postmenopausal oestrogen deficient environment (Gardner et al., 2000; Gardner et al., 2009). However, in contrast to the study by Chan and co-workers, who reported 43.6% atrophic or inactive, 27.8% proliferative or secretory endometrium at 12 months following the LNG-IUS insertion (Chan et al., 2007), none of the endometrial samples in the present study were atrophic or inactive, and only 4.2% (i.e. one patient) had a weak proliferative endometrium, but this patient had her coil removed 8 weeks prior at which point the endometrium was pseudo-decidualised. There are no obvious differences between the patients in the two studies other than Chan and colleagues studied a mixture of pre- and post-menopausal women and the ethnic differences; Chan's group studied Asian women, I studied mainly Caucasian women. However, the differences may be related to reporting differences between pathologists. To my knowledge, there are no data to indicate ethnic differences in the responses to the combination of tamoxifen and levonorgestrel.

Vaginal bleeding was the only adverse effect reported by women in the Mirena group. Both control and Mirena groups reported vaginal bleeding after the first visit. However, there were more bleeding episodes and duration of bleeding in the Mirena group compared to the control group ($p<0.0001$), this bleeding occurred for up to and more than 6 months.

After 6 months, the bleeding score reduced in the women who had the LNG-IUS, but the difference when compared to the control group was still significant ($p=0.0487$). However, after the third interval, the bleeding patterns of the women in the Mirena group were similar to that in the control group. The bleeding patterns in this study concur with that in

the literature (Gardner et al., 2000; Chan et al., 2007). None of the women in the Mirena group reported the vaginal bleeding as unacceptable, requiring removal of the device.

The data presented in this chapter show that tamoxifen induces uterotrophic effects in women commencing tamoxifen for adjuvant breast cancer treatment, as reported in the literature (Morales et al., 2005). This uterotrophic effect was prevented by the LNG-IUS (Mirena®). The uterotrophic effects of tamoxifen cannot be accounted for as purely endometrial as this would not account for the almost 40% increase in uterine volume in the control group after 12 months. It has been shown that tamoxifen administration to rats causes stromal and myometrial oedema causing an increase in the uterine wet weight (Kwekel et al., 2009). The expression of both oestrogen (ER) and progesterone (PR) receptor is higher in the benign endometrium of breast cancer patients using tamoxifen compared to non users (Elkas et al., 2000). This may indicate that the uterotrophic effects of tamoxifen are as a result of increased ER signalling, and up-regulation of the PR, a known oestrogen-responsive gene. The observation of the prevention of the tamoxifen induced uterotrophic effects with the LNG-IUS strengthens this hypothesis. The LNG-IUS also prevented the formation of endometrial polyps and induced a pseudo-decidual response in the endometrium. This suggests tamoxifen is oestrogenic and must be inducing the progesterone receptor, as has been shown in benign endometrium of breast cancer patients using tamoxifen when compared to atrophic controls (Elkas et al., 2000) and directly within a post-menopausal tamoxifen user (Cohen et al., 1994). The prevention of polyp formation suggests antagonism of tamoxifen effects by the progestagen, levonorgestrel. This progestogenic antagonism must be acting on both the endometrium and myometrium as suggested by the prevention of uterotrophy in the Mirena group.

Gardner and colleagues inserted the LNG-IUS in women taking tamoxifen for an average of 3 years, they observed that the endometrium was decidualised but there was no reduction in uterine volume (Gardner et al., 2000). Powles and colleagues also reported that the ultrasound changes were not reversed by progestagen treatment in tamoxifen users (Powles et al., 1998). The data in this chapter also demonstrated endometrial decidualisation and the prevention of the uterotrophic effects of tamoxifen when the LNG-IUS was inserted at the onset of tamoxifen therapy. Based on these various sources of data it is now becoming clear that there is a difference in response to levonorgestrel in the endometrium and myometrium depending on whether there has been tamoxifen priming or not. Whether the difference in response could be related to a difference in receptor expression between the endometrium and myometrium is unknown.

I hypothesized that the application of the LNG-IUS at the onset of treatment would prevent the development of increased uterine volume. The data presented in this chapter tend to support this hypothesis. The data also show that LNG-IUS causes a reduction in endometrial polyp formation, possibly through mechanism involving pseudo-decidualisation. Although these observations are clinically useful, how the abnormal histological features associated with tamoxifen use are caused, and how tamoxifen therapy results in an increase in uterine volume and later endometrial pathologies, is going to be the focus of the remainder of my thesis. As stated above, one change that could occur during tamoxifen therapy is a change in the expression patterns of steroid receptors (ERα, ERβ, PR-A and PR-B). This is examined in the following chapter.

Chapter 3

Steroid receptor expression in the uterine endometrium and myometrium of postmenopausal women following tamoxifen treatment

3.1 Introduction

The effects of tamoxifen on the endometrium have been shown to be associated with alterations in the expression of oestrogen and progesterone receptors (Schwartz et al., 1997; Kommoss et al., 1998; Hachisuga et al., 1999; Elkas et al., 2000; Mourits et al., 2002b; Tregon et al., 2003; Leslie et al., 2007). The majority of studies demonstrated up regulation of the oestrogen receptor in the endometrial glandular epithelium and stromal cells (Hachisuga et al., 1999; Mourits et al., 2002b; Tregon et al., 2003), however, Schwartz and co-workers observed a decrease in the oestrogen receptor expression in postmenopausal women on tamoxifen for breast cancer (Schwartz et al., 1997).

The effect of tamoxifen on progesterone receptor expression have also been variably reported with most studies reporting up regulation of progesterone receptor in the glands and stroma (Kommoss et al., 1998; Mourits et al., 2002b; Tregon et al., 2003), but one study reported a decrease in stromal PR-A in tamoxifen treated women (Hachisuga et al., 1999).

One study reported to examine the expression in the basalis is that of Wang et al., who examined ERα and ERβ in the basal and functional endometrium through the menstrual cycle in macaques (Wang et al., 2002). They observed an increase in ERα expression in the basalis and functionalis and minimal ERβ expression (Wang et al., 2002) in ovariectomized macaques treated with tamoxifen.

Although several studies have studied oestrogen and progesterone receptor expression they have been limited because the antibodies used did not define which oestrogen and progesterone receptor isoform was being studied in their immunohistochemical analysis (Schwartz et al., 1997; Kommoss et al., 1998; Elkas et al., 2000; Mourits et al., 2002b). One group examined ERα and PR-A isoform expression (Hachisuga et al., 1999), and another the distribution of PR-B (Leslie et al., 2007) and yet another used a combined PR-A and PR-B antibody to examine total progesterone receptor isoform (Tregon et al., 2003), but none to date have examined all four receptor isoforms, ERα, ERβ, PR-A and PR-B, in the normal human uterus. Additionally, there is minimal literature on studying these four receptors isoforms in the endometrium or myometrium of postmenopausal women treated with tamoxifen.

The aim of this chapter is therefore to describe and quantify the distribution and amount of the oestrogen and progesterone receptor isoforms in the endometrium and myometrium of women receiving tamoxifen for breast cancer and compare that to the proliferative phase of the menstrual cycle and that of untreated postmenopausal women. To do that, I have used semi-quantitative analysis of the staining patterns in the endometrial functionalis and basalis, and the inner (junctional) zone and the outer layers of the myometrium.

3.2 Methods

3.2.1 Tissue collection

Uteri were collected from women attending at the Leicester Royal Infirmary Women's Hospital, University Hospitals of Leicester NHS Trust, according to Leicestershire Health Authority Ethics Committee (Appendix 1). All patients provided written consent indicating that they had been informed of the use of their tissue sample for research purposes (Appendix 2).

3.2.2 Patient groups

Full thickness uterine tissue was obtained from women undergoing hysterectomy. These were women formed one of three groups:

3.2.2.1 Menstrual cycle control group

Women undergoing total abdominal hysterectomy with or without bilateral salpingo-oophorectomy for dysfunctional uterine bleeding with or without dysmenorrhoea were included. Samples were collected from each phase of the menstrual cycle; early-, mid- and late-proliferative, early-, mid- and late-secretory phases. A total of 8 samples for each of the sub-divisions of proliferative and secretory phases were collected making 48 samples in total.

3.2.2.2 Postmenopausal control group

These were women who had their last menstrual period at least one year prior to surgery and were undergoing total abdominal for benign adnexal pathology or vaginal hysterectomy for prolapse. Eight samples were collected from this group.

3.2.2.3 Tamoxifen treated group

These women, who had been taking 20mg tamoxifen for a minimum of six months for the treatment of breast cancer, underwent hysterectomy for benign pathology. A total of 18 uteri were collected from this group.

3.2.3 Sampling technique

In all cases, uterine samples were obtained immediately after the uterus had been removed. An incision was made through the anterior surface of the uterus in the midline until the uterine cavity was reached; two further fundal incisions were made to create a Y-shaped incision on the anterior surface of the uterus.

Uterine tissue from the serosal to mucosal surfaces and containing the entire endometrium and myometrium, measuring approximately 50 x 20 x 5 mm was obtained. Further dissection of the biopsy was made so that a "full thickness" uterine sample measuring approximately 20mm x 20mm could be placed into a tissue cassette and fixed in 4% formal saline at room temperature from 2 to 5 days, before being embedded in paraffin wax by the Histopathology Department at Leicester Royal Infirmary.

3.2.4 Paraffin-embedded tissue sections

Paraffin embedded sections (5µm) were cut using a microtome and floated onto silane-coated slides; prepared by dipping the glass slide in 3-aminopropyltriethoxy-saline (3% vol/vol in methanol) for 2 minutes and rinsing in deionised water twice for 10 seconds followed by drying overnight at 37°C. The cut sections were racked and allowed to air dry in an oven at 37°C for 4 days.

3.2.5 Histological examination

Haematoxylin and eosin stained tissue sections were prepared using standard methods, and examined under a light microscope to date menstrual phase uteri into cycle phases and to confirm normal histological features and the absence of pathology. This was done by an experienced gynaecological histopathologist (Dr Laurence Brown), according to the criteria laid down by Noyes and colleagues (Noyes et al.,1950). Dr Brown also confirmed the atrophic nature of the postmenopausal samples and noted the presence of any endometrial pathologies in the tamoxifen treated uteri.

3.2.6 Single labelling immunohistochemistry

ERα, ERβ, PR-A and PR-B immunoreactivity was visualised using an avidin-biotin complex amplification methodology with 3, 3' diaminobenzidine as the chromogen. In all the cases the procedure was as follows:

Paraffin embedded tissue sections were dewaxed in xylene three times for 3 minutes each and dehydrated in 99%, 99% and 95% industrial methylated spirits (IMS) for 3 minutes each. Slides were then rehydrated in deionised water for 3 minutes, followed by microwave antigen retrieval. For this, slides were placed in 10mM citric acid buffer (pH 6.0) and placed in a microwave oven for 30 minutes at 800 Watts, with the citrate buffer being topped up at 10-minute intervals. The slides were then allowed to cool for 20 minutes in the citrate buffer and then washed in tap water for 5 minutes.

Endogenous peroxidases were then suppressed by incubating the sections in 6% hydrogen peroxide (Fisher Scientific, Loughborough, UK) for 10 minutes. The slides were washed in running tap water for 5 minutes, followed by a 5-minute wash in TBS-0.1% BSA buffer

with stirring. Subsequently, each tissue sections was blocked with 100µl normal rabbit serum (NRS, Dako Ltd.), diluted to 10% in TBS-0.1% BSA and incubated at room temperature for 20 minutes. Tissue sections were then incubated with 100µl primary antibody diluted in TBS-0.1% BSA at the specified dilution indicated in Table 3.1, and incubated overnight in a humidified chamber at 4°C. The sections were washed in TBS-0.1% BSA for 20 minutes and then incubated with biotinylated rabbit anti-mouse Fab fragment Ig secondary antibody (Dako Ltd.), diluted 1 in 400 in TBS-0.1% BSA at room temperature for 20 minutes. Tissue sections were washed in TBS-0.1% BSA for 20 minutes with stirring and then incubated in Vectastain ABC Elite (Vector Laboratories) for 30 minutes at room temperature. The tissue sections were then washed in 1 x PBS twice for 15 minutes each and then incubated with 3, 3'-diaminobenzidine substrate (DAB, Vector Laboratories) for 5 minutes. After rinsing in deionised water for 5 minutes, the tissue sections were counterstained in Mayer's haematoxylin (Sigma) for 30 seconds, washed in running tap water for 5 minutes, dehydrated through increasing concentrations of industrial methylated spirits, and cleared in xylene three times for 3 minutes each and mounted using DPX mountant medium, covered with a cover slip and allowed to set.

All experiments included a negative control where the primary antibody was replaced with an equivalent concentration of mouse IgG (Figures 3.1, 3.6, 3.11 and 3.16). A positive control tissue for each experiment was also determined and included.

Antibody	Clone name	Antibody type	Working concentration (µg/mL)	Manufacturer
ERα	6F11	Mouse monoclonal IgG1	1.5	Novocastra
ERβ	14C8	Mouse monoclonal IgG2b	5.0	Abcam
PR-A	16	Mouse monoclonal IgG1	1.8	Novocastra
PR-B	SAN27	Mouse monoclonal	0.167	Novocastra

Table 3.1 Sources of antibodies used. The table shows the monoclonal name, the type of antibody (where known) and the final working concentration.

3.2.7 Image Analysis

The pattern of distribution of the positively stained cells was determined by examining the tissue sections and semiquantitative analysis was done by image capture of 10 randomly selected fields per slide in each of the functional endometrium, the basal endometrium, the junctional zone of the myometrium and the outer myometrium.

All images were captured at 400 x magnification, in the presence of daylight and light grey neutral density filters with standardised illumination set at just under maximum exposure. Images were captured using an Axioplan microscope (Carl Zeiss, Herts. UK) and a Sony DXC-151P colour camera and AxioVision version 4 software.

For the histomorphometric analyses, the proportion of positively stained stromal and glandular cells per field were assessed by counting the number of positive (brown) and negative (blue) cells. Each specimen provided an independent mean score for the endometrial functionalis, the endometrial basalis, and the inner and outer layer of the myometrium. The average and standard error of the mean was calculated for each phase of the menstrual cycle, for the postmenopausal uteri and for the tamoxifen treated specimens. Because the staining patterns for all antigens in the dilated cystic and normal sized glands in the endometria of tamoxifen treated uteri were similar, these data were combined. Endothelial cells in the stromal and myometrial compartment were omitted from the count.

3.2.8 Statistical analysis

Data are presented as mean ± standard error of the mean and analysed using one-way analysis of variance (ANOVA) with Tukey's Honestly Significance Difference post-test, with $p<0.05$ accepted as being statistically significant.

3.3 Results

Immunohistochemistry showed that ERα, ERβ, PR-A and PR-B were all present in the nuclei of cells, in all compartments of the endometrium and myometrium (Figure 3.2). The nuclear immunoreactivity was mainly observed in stromal fibroblast, the glandular epithelium of the endometrium, and in the myometrial smooth muscle cells. The distribution of these antigens will now be described in turn.

3.3.1 ERα distribution

Staining for ERα was observed in the stromal cell, and glandular epithelial cell of the endometrium, both within the functionalis and the basalis, and myometrial smooth muscle, both within the junctional zone myocytes and the outer myometrium cells.

3.3.1.1 Functionalis

A *Menstrual Cycle*

Histomorphometric analysis of the ERα staining in the glandular epithelium and stromal cells showed cyclical changes (Table 3.2). Both glandular epithelium and stromal cells showed greatest ERα immunoreactivity in the proliferative phases (Table 3.2 and Figure 3.3).

The greatest glandular ERα staining was seen in the early-, mid- and late-proliferative phases, with 97.1%, 97.7% and 95.9% of the cells staining, respectively (Figure 3.2).

In the stromal cells, the highest ERα immunostaining was in the early- and mid-proliferative phases, with 85.4% and 84.9% immunopositivity, respectively. There was a small decline to 80.1% in the late proliferative phase.

B *Tamoxifen treatment group*

In the tamoxifen functional endometrium, ERα immunoreactivity was present at high levels at 88.1% in the stromal and 98.8% in the glandular cell nuclei (Table 3.2 and Figure 3.3). Both the glandular epithelial and stromal cell staining was similar to that seen in the proliferative phases of the menstrual cycle (Figure 3.2). A similar trend was seen in the glandular cells, there was no difference between ERα staining in tamoxifen uteri compared to the proliferative phase.

These data indicate that ERα expression was up-regulated by tamoxifen in the functionalis and similar to that of the proliferative phase of the normal menstrual cycle (Figure 3.3).

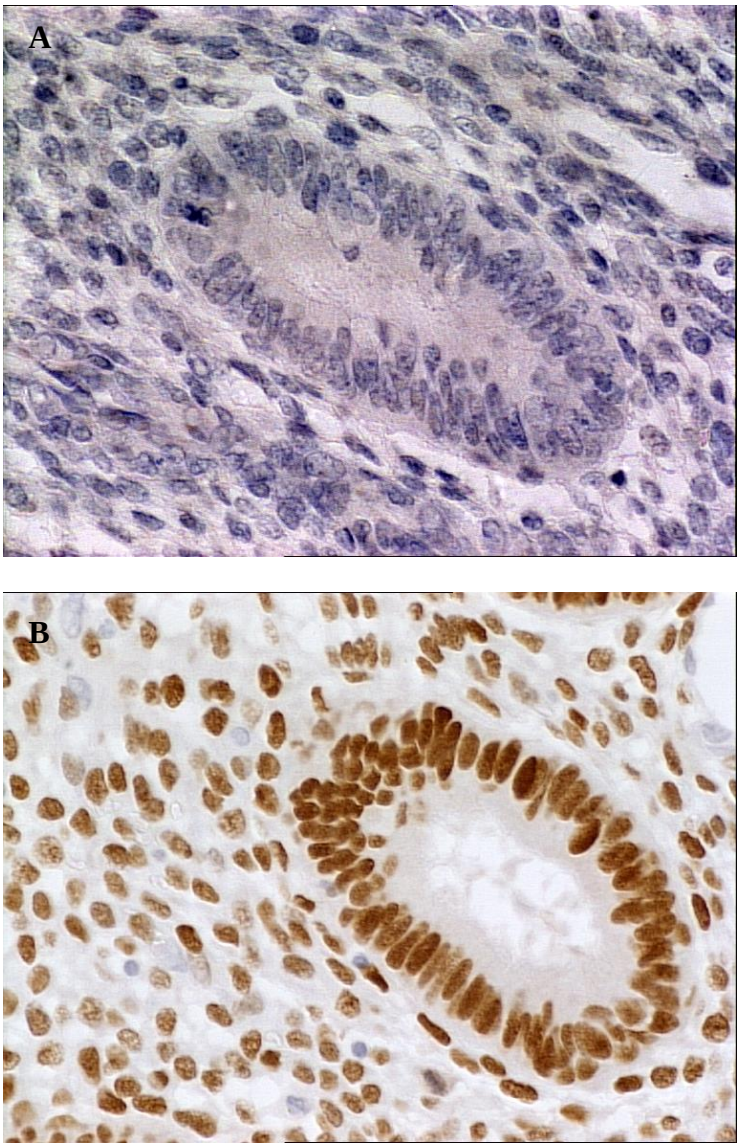


Figure 3.1 Demonstration of ERα antibody specificity.

The tissue sample is endometrium from the mid proliferative phase of the menstrual cycle. Panel A shows the IgG control (1.5µg/ml) and Panel B shows ERα (1.5µg/ml). Both images were taken at x400 magnification.

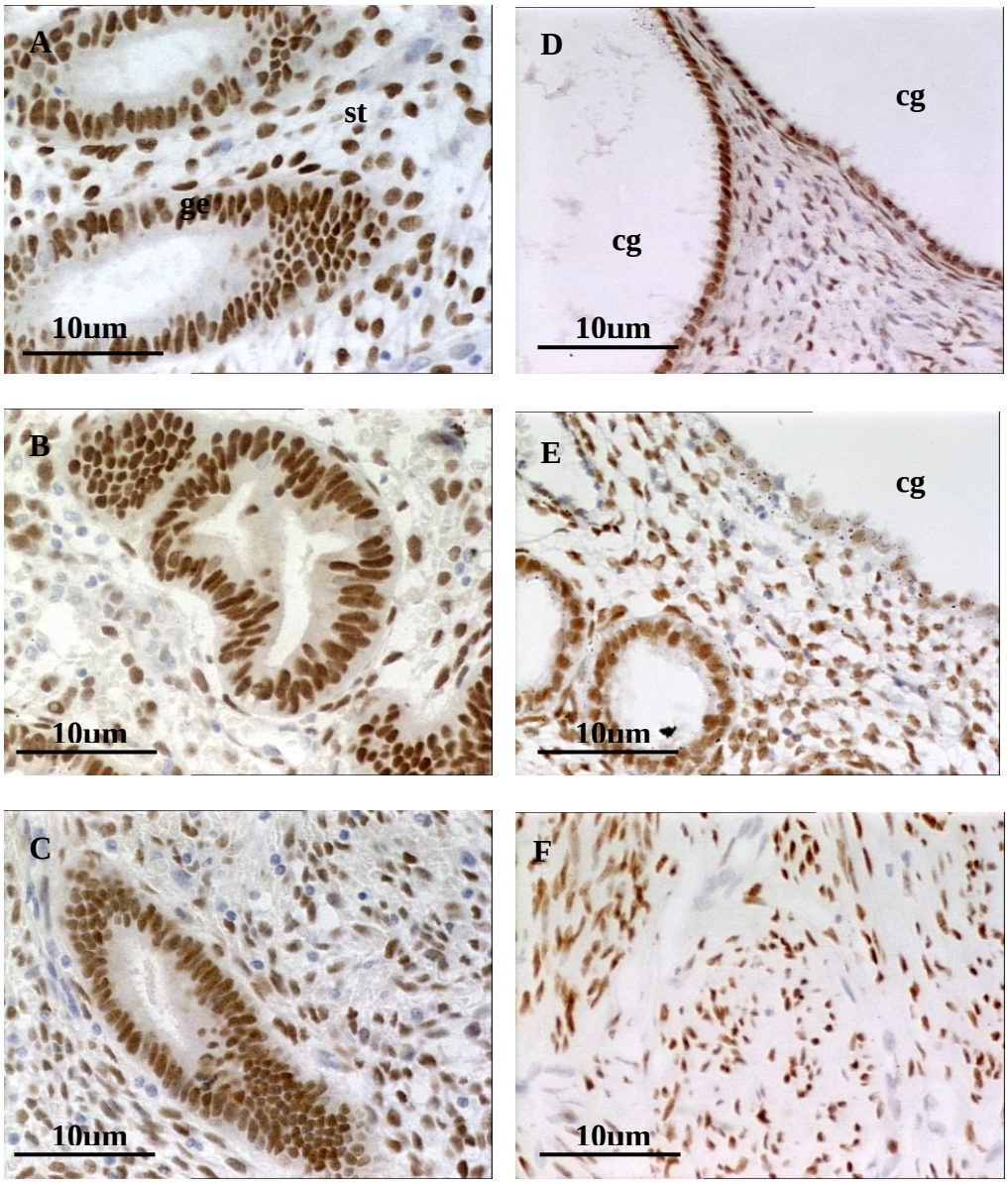


Figure 3.2 Comparison of ERα immunohistochemical staining in the functionalis of the early-, mid- and late-proliferative phase endometrium with that of tamoxifen treated endometrium and inner myometrium.

Dense nuclear staining was observed in the both the glandular epithelium (ge) and stromal (st) cells of the early- (panel A), mid- (panel B) and late-proliferative (panel C) phase endometrium and some cells in the tamoxifen treated myometrium (panel F). The expression of ERα in the tamoxifen treated endometrium (panel D & E) was similar to that observed in the proliferative endometrium both within the stroma and the epithelial cells

lining the cystic dilated (cg) glands. The tamoxifen treated inner myometrium (panel F) demonstrated ERα staining, primarily in the myocytes.

Category (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Inner	Outer
EP (8)	85.4 (2.3)	97.1 (0.6)	90.7 (0.7)	99.2 (0.1)	88.3 (1.6)	81.6 (7.2)
MP (8)	84.9 (2.1)	97.7 (0.5)	88.1 (1.4)	99.4 (0.1)	84.5 (2.2)	80.8 (2.9)
LP (8)	80.1 (2.9)	95.9 (1.0)	73.6 (12.7)	97.8 (1.2)	66.8 (8.6)	54.8* (15.0)
PM (8)	tissue not present	tissue not present	89.2 (2.5)	96.4 (3.3)	84.8 (2.86)	62.9 (6.31)
TMX (18)	88.1 (1.5)	98.8 (0.2)	82.9 (5.1)	99.7 (0.2)	89.3 (1.4)	73.5 (5.2)

Table 3.2. ERα expression in the glandular and stromal cells in the functionalis, basalis and myometrium.

Data are presented as percentage positive cells (standard error of the mean, SEM).

Numbers of samples assessed (N) in each category are shown in parentheses.

Statistical significance was determined using one way ANOVA analysis with Tukey's honestly significance difference post test comparing menstrual cycle and postmenopausal samples to the tamoxifen treated specimens, * $p<0.05$.

EP = early proliferative, MP = mid proliferative, LP = late proliferative, PM = postmenopausal, TMX = tamoxifen.

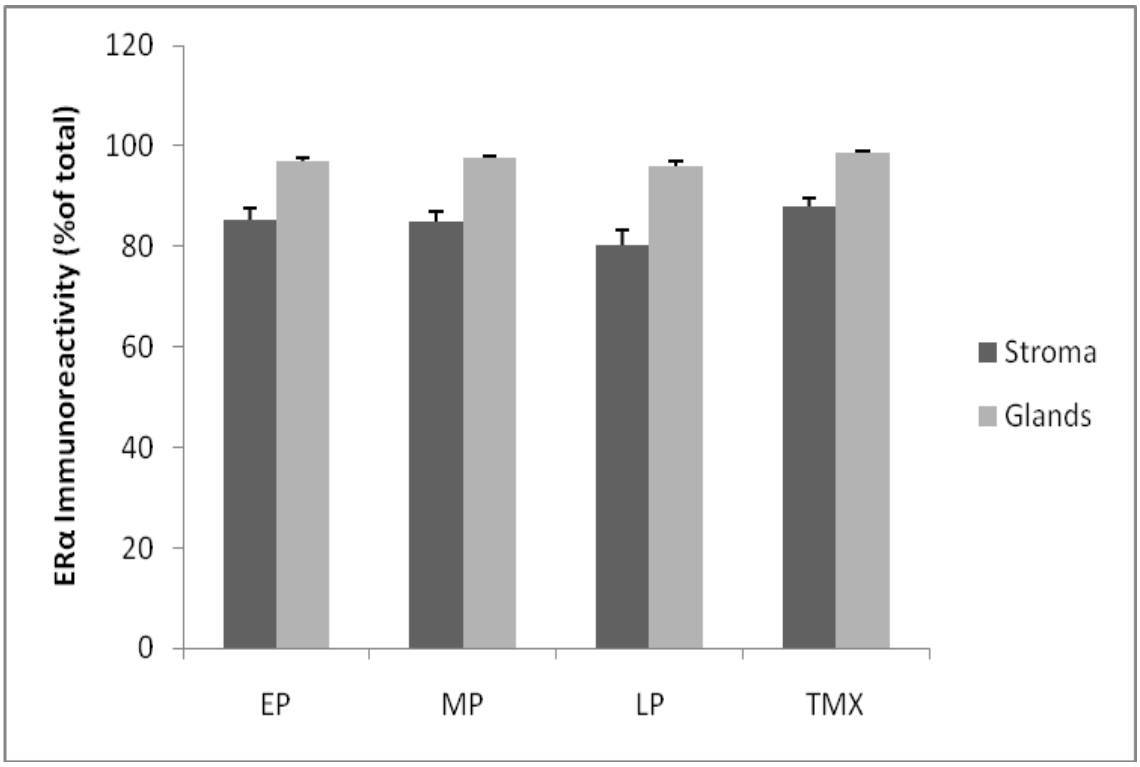


Figure 3.3 Histomorphometric analysis of ERα expression in the stroma and glandular epithelial cells in the functionalis in the proliferative phases of the menstrual cycle and tamoxifen treated women.

Data are presented as mean percentage ± SEM of cells expressing ERα. Statistical significance was determined using one way ANOVA with Tukey's honestly significance difference post test. The data were not significantly different.

3.3.1.2 Basalis

A *Menstrual Cycle*

Histomorphometric analysis of the ERα staining in the stromal and glandular epithelial cells of the basalis did not show any clear cyclical trend (Table 3.2). The glandular epithelial cell ERα immunopositivity in the proliferative phase ranged from 97.8% to 99.4%, with the lowest levels of staining found in the late proliferative phase and the highest levels found in the early- and mid-proliferative phases, but these data were not significantly different.

ERα staining in the stromal cells in the proliferative phases ranged from 73.6% to 90.7% (Table 3.2) and these also were not significantly different.

B *Postmenopausal group*

ERα immunostaining in the postmenopausal stroma was 89.2% and 96.4% in the glandular epithelium. This level of staining was similar to that seen in the menstrual cycle (Figure 3.4).

C *Tamoxifen treatment group*

In the tamoxifen basal endometrium, ERα was present in the stromal and glandular cell nuclei, at 82.9% and 99.7%, respectively (Table 3.2). ERα immunoreactivity in both the glandular epithelial and stromal cells was similar to that found in other phases of the menstrual cycle and in postmenopausal uteri (Figure 3.4).

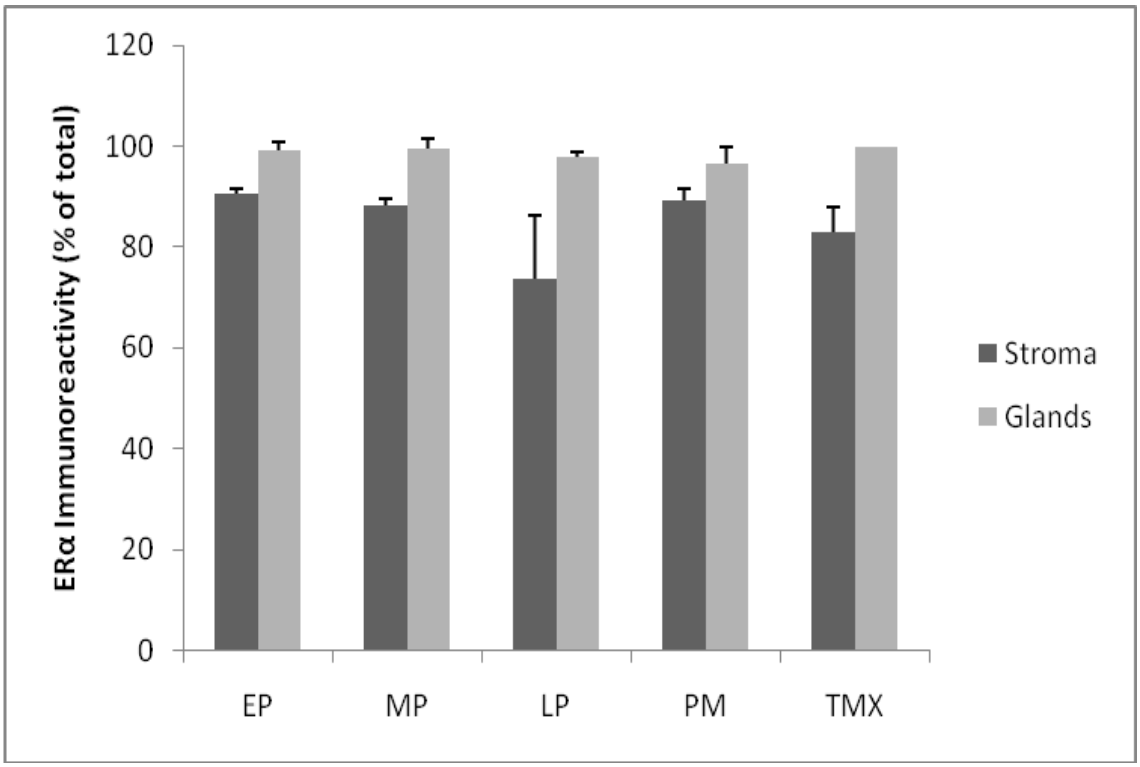


Figure 3.4 Histomorphometric analysis of ERα expression in the stroma and glandular epithelial cells in the basalis in the proliferative phases of the menstrual cycle, in postmenopausal and tamoxifen treated uteri.

Data are presented as mean percentage ± SEM of cells expressing ERα. Statistical significance was determined using one way ANOVA with Tukey's honestly significance difference post test. The data were not significantly different.

3.3.1.3 Myometrium

A *Menstrual cycle*

The inner myometrium was immunopositive for ERα with 66.8 to 88.3% of the cells in the proliferative phase of the menstrual cycle, showing ERα immunoreactivity (Table 3.2).

The outer myometrium was 81.6% and 80.8% positive in the early- and mid-proliferative phases, respectively, and staining was significantly decreased to 54.8% in the late-proliferative phase (Table 3.2).

B *Postmenopausal group*

In the inner myometrium, 84.8% of the cells were positive for ERα. This was similar to the levels seen in the early- and mid-proliferative phases of the menstrual cycle (Figure 3.5). The outer myometrium had significantly lower ERα staining compared to the inner myometrium at 62.9% (Table 3.2).

C *Tamoxifen treatment group*

The inner junctional zone myometrium demonstrated ERα immunostaining in 89.3% of the cells examined. This was not different compared to either the menstrual cycle or the postmenopausal myometrium (Figure 3.5). By contrast, in the outer myometrium, 73.5% of the smooth muscle cells were immunopositive for ERα, which was significantly higher compared to the levels observed in the late proliferative phase ($p<0.05$) but was statistically insignificant when compared to all other phases of the menstrual cycle or the postmenopausal myometrium (Figure 3.5).

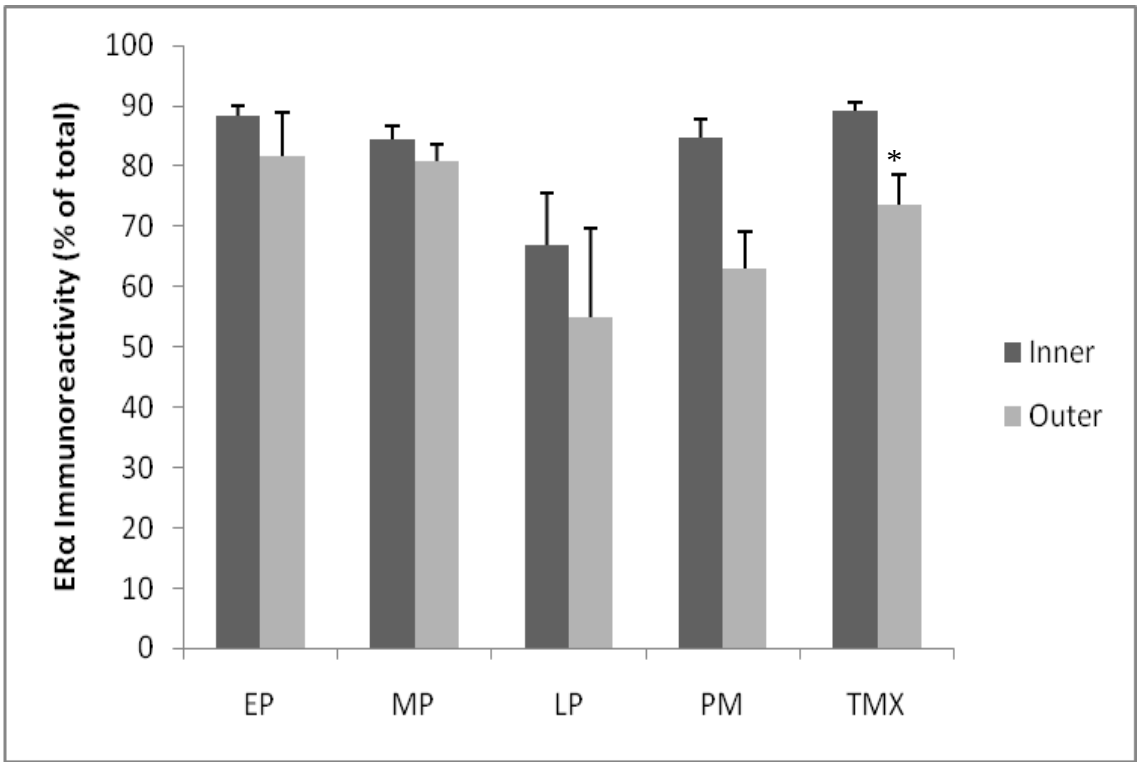


Figure 3.5 Histomorphometric analysis of the ERα expression in the inner and outer myometrium in the proliferative phases of the menstrual cycle, untreated postmenopausal uteri and tamoxifen treated uteri.

There was very little difference in the ERα expression levels in the myometrium, with only the outer myometrium in the late-proliferative phase showing significantly lower levels of ERα immunoreactivity when compared to the tamoxifen treated uteri.

Data are presented as mean percentage ± SEM cells expressing ERα.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test; *p<0.05 when LP outer myometrium was compared to tamoxifen outer myometrium. All other comparisons were not significantly different.

3.3.2 ERβ distribution

ERβ immunoreactivity was primarily confined to the nuclei of cells in all compartments of the endometrium and myometrium (Figure 3.7). This included the stromal cells and glandular epithelial cells of the endometrium (both in the functionalis and the basalis) and in the myometrial smooth muscle cells both within the inner zone and the outer myometrium.

3.3.2.1 Functionalis

A Menstrual cycle

Histomorphometric analysis of the ERβ immunoreactivity showed greater staining in the glandular epithelium compared to the stromal cells (Table 3.3).

The glandular ERβ staining was consistently seen in the early-, mid- and late-proliferative phases, at 66.9%, 68.5% and 67.7% of cells, respectively (Figure 3.7).

In the stromal cells, the lowest ERβ expression was observed in the early proliferative phase at 11.2%. There was an increase to 27.6% in the mid proliferative phase, followed by a further decrease to 13.9% in the late proliferative phase.

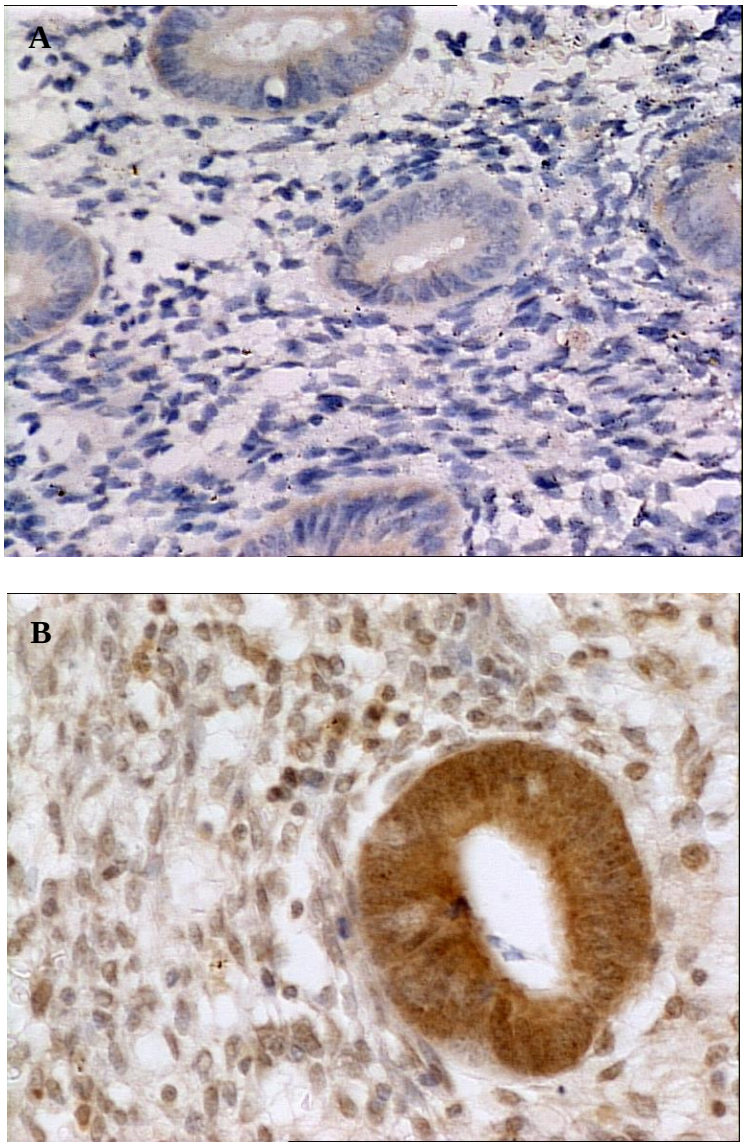


Figure 3.6 Demonstration of ERβ antibody specificity.

The tissue sample used is a mid-proliferative phase endometrium of the menstrual cycle. Panel A shows the IgG control (5.0µg/ml) and Panel B shows ERβ (5.0µg/ml). Both panels were captured at x400 magnification.

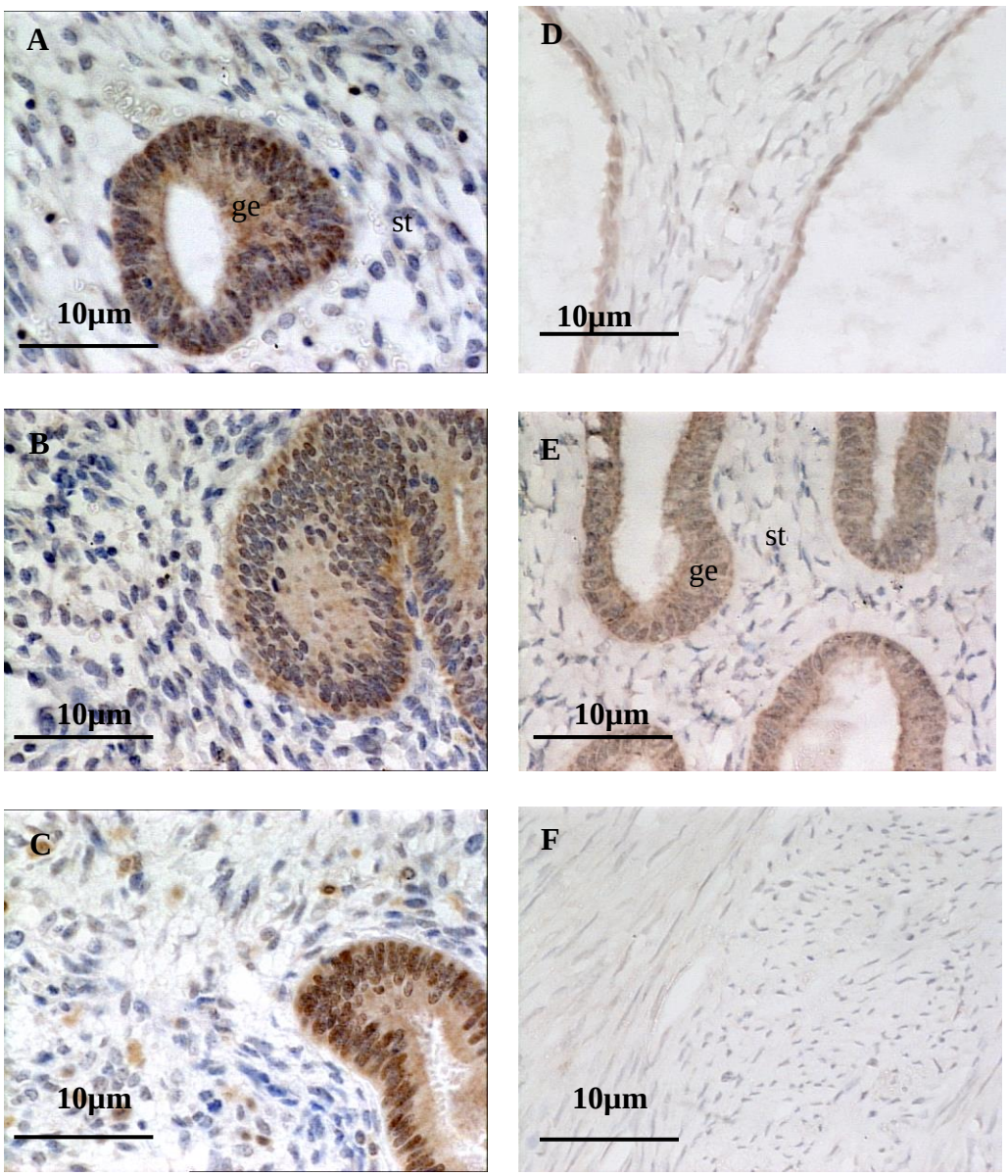


Figure 3.7 Comparison of the ERβ immunohistochemical staining in the functionalis of the early-, mid- and late-proliferative phase endometrium. ERβ was present in greater amounts in the glandular epithelium (ge) than the stromal (st) cells of the early- (panel A), mid- (panel B) and late-proliferative (panel C) phase endometrium. The expression of ERβ in the tamoxifen treated endometrium (panels D & E) was greater than that observed in the proliferative endometrium in the glandular epithelium but similar to that observed in the stroma. The tamoxifen treated inner myometrium (panel F) demonstrated ERβ staining, but this was 'weaker' than that found in the functional endometrium. All panels magnification x400.

Cycle phase (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Inner	Outer
EP (8)	11.2 (4.1)	66.9 (4.8)*	1.8 (1.0)***	42.9 (10.1)***	0.6 (0.1)***	0.2 (0.1)***
MP (8)	27.6 (7.7)	68.5 (10.4)*	3.5 (1.4)***	76.9 (12.7)***	1.4 (0.4)***	0.2 (0.1)***
LP (8)	13.9 (9.2)	67.7 (8.0)*	3.3 (2.3)***	85.1 (9.1)	7.7 (7.1)***	6.8 (6.1)*
PM (8)	tissue not present	tissue not present	31.1 (3.4)***	88.4 (5.8)	44.0 (3.3)	34.21 (3.3)*
TMX (18)	21.3 (1.7)	98.3 (0.4)	19.79 (1.3)	97.32 (0.8)	31.59 (1.7)	21.0 (2.9)

Table 3.3 ERβ expression in the glandular and stromal cells in the functional and basal endometrium and myometrium.

Data are presented as percentage positive cells (standard error of the mean).

Numbers of samples assessed in each category are shown in parentheses.

Significance was determined by one way ANOVA analysis with Tukey's post test comparing tamoxifen treated to menstrual cycle and postmenopausal samples,*p<0.05; **p<0.01; ***p<0.001.

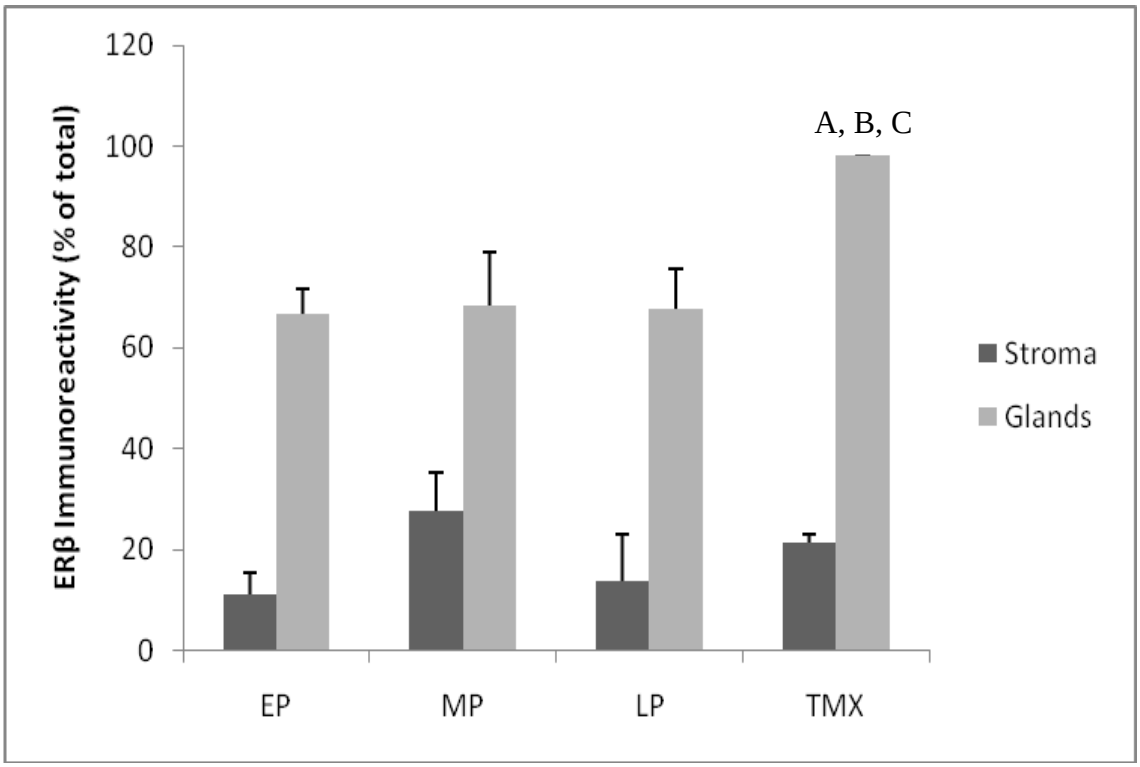


Figure 3.8 Histomorphometric analysis of ERβ expression in the stromal and glandular epithelium in the functionalis in the proliferative phases of the menstrual cycle and in tamoxifen treated uteri.

ERβ staining was suppressed in the proliferative stroma when compared to the tamoxifen treated stroma, whereas there was no difference in glandular epithelial staining.

Data are presented as the mean ± SEM of percent of ERβ positive cells.

Statistical significance was determined using one way ANOVA analysis with Tukey's honestly significant difference test; A, B and C denotes $p<0.05$ for tamoxifen treated gland staining compared to glandular staining in EP (A), MP (B) and LP (C) samples of the menstrual cycle.

B Tamoxifen treated uteri

In the functionalis of tamoxifen treated uteri, ERβ immunoreactivity was present at high levels in the glandular epithelial cells (98.3%) (Figure 3.8). By contrast, ERβ staining in the stromal cells was only observed in 21.3% (Table 3.3). The stromal cell staining was similar to that seen in the functionalis in the menstrual cycle, and there was no statistically significant difference between ERβ staining in tamoxifen treated uteri when compared to the proliferative phase of the menstrual cycle. By contrast, in glandular epithelial cells there were significant differences between ERβ staining in the tamoxifen treated uteri compared to the early- (66.9%, $p<0.05$), mid- (68.5%, $p<0.005$) and late- (67.7%, $p<0.05$) proliferative phases of the menstrual cycle (Table 3.3).

These data indicate that ERβ expression in the stromal cells was similar to that seen in the menstrual cycle, however, in the glandular epithelial cells the ERβ staining in the functionalis of tamoxifen treated uteri was greater than that seen in the proliferative phase.

3.3.2.2 Basalis

A Menstrual Cycle

Histomorphometric analysis of the ERβ staining showed cyclical changes in both the stromal and glandular epithelial cells (Table 3.3). Both the glandular epithelial and stromal cells showed high levels of ERβ immunoreactivity in the proliferative phases (Table 3.3). The glandular ERβ staining was 42.9% in the early proliferative phase, and increased in the mid- and late-proliferative phases to 76.9% and 85.1%, respectively (Figure 3.9).

In the stroma, only 1.8% of the cells were ERβ positive in the early proliferative phase. The ERβ expression increased to its highest levels in the mid- and late- proliferative phases to 3.5% and 3.3%, respectively (Table 3.3).

B Postmenopausal group

ERβ immunostaining in the postmenopausal stroma was 31.1% and 88.4% in the glandular epithelium. This level of staining was greater than that seen in all phases of the menstrual cycle (Figure 3.9).

C Tamoxifen treated uteri

In the tamoxifen basal endometrium, ERβ was present in the stromal and glandular cell nuclei, at 19.8% and 97.3%, respectively (Table 3.3). ERβ immunoreactivity in the stromal cells was significantly greater compared to all phases of the menstrual cycle and that in the postmenopausal uteri (Figure 3.9). In the glandular epithelial cells, ERβ immunopositivity was significantly greater when compared to the early- and mid-proliferative phases of the menstrual cycle.

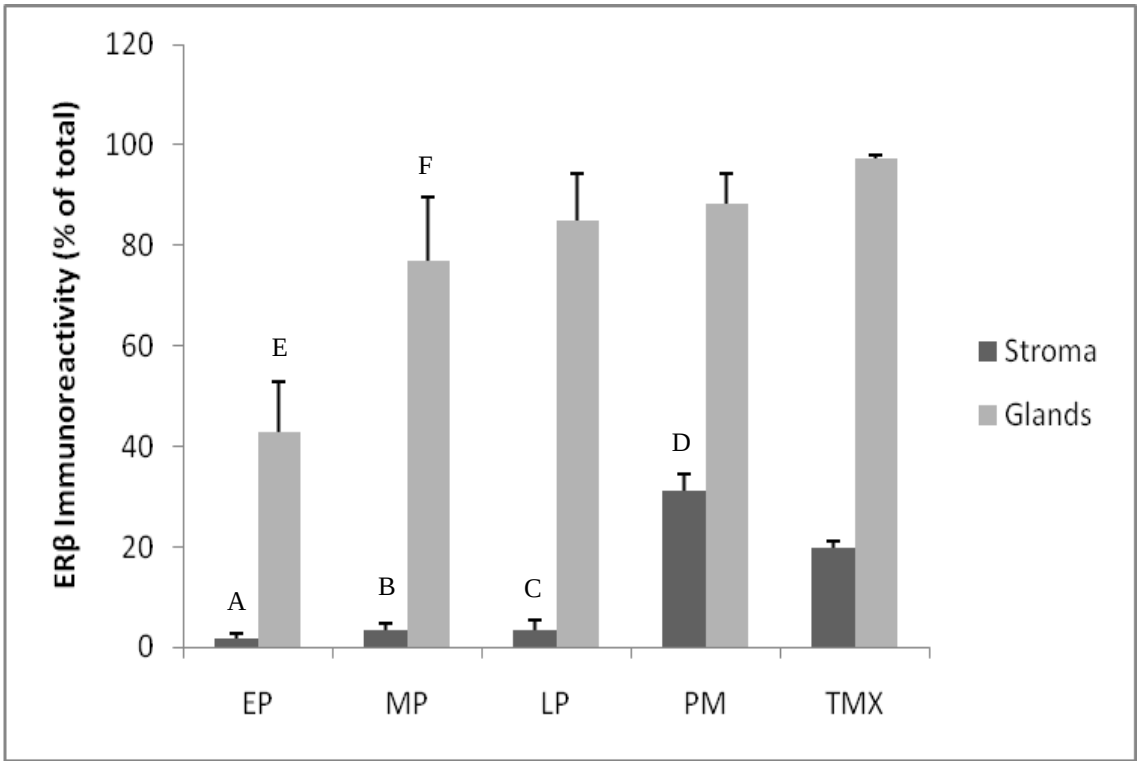


Figure 3.9 Histomorphometric analysis of ERβ expression in the stroma and glandular epithelial in the basalis in the proliferative phases of the menstrual cycle, untreated postmenopausal and tamoxifen treated uteri.

ERβ staining was significantly lower in the proliferative stroma and significantly higher in the postmenopausal stroma when compared to the tamoxifen treated uteri, whereas glandular staining was only reduced in the early-and mid-proliferative glands.

Data are presented as mean percentage ± SEM of cells expressing ERβ.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test; A to F $p<0.001$ when using the menstrual cycle samples as the control, with the tamoxifen treated sample being the test.

Stroma in the tamoxifen treated samples when compared to EP (A), MP (B), LP (C) and PM (D), $p<0.001$. Glands in the tamoxifen treated samples compared to EP (E) and MP (F), $p<0.001$.

3.3.2.3 Myometrium

A Menstrual cycle

ERβ immunoreactivity in the smooth muscle cells of the inner myometrium was between 0.6% and 7.7% in the proliferative phase of the menstrual cycle (Table 3.3). In the outer myometrium, ERβ levels were equally low with 0.2% of cells in the early- and mid-proliferative phases being immunopositive. There was an increase in immunopositive cells to 6.8% in the late proliferative phase (Table 3.3).

B Postmenopausal group

In the inner myometrium, 44.0% of the myometrial cells were positive for ERβ. This value was significantly higher compared to that observed in any of the menstrual cycle phases (Figure 3.10). The ERβ staining in the outer myometrium at 34.2% was low compared to staining observed in the inner zone myometrium (Table 3.3).

C Tamoxifen treatment group

In the inner junctional zone myometrium, 31.6% of cells examined were ERβ positive, a level that was statistically significantly higher when compared to all phases of the menstrual cycle (Table 3.3). By contrast, the inner zone myometrium was not statistically different compared to the postmenopausal group. The outer myometrium, 21.0% of the smooth muscle cells were immunopositive for ERβ, which was significantly higher when compared to the levels observed in all the phases of the menstrual cycle and postmenopausal uteri (Table 3.3 and Figure 3.10).

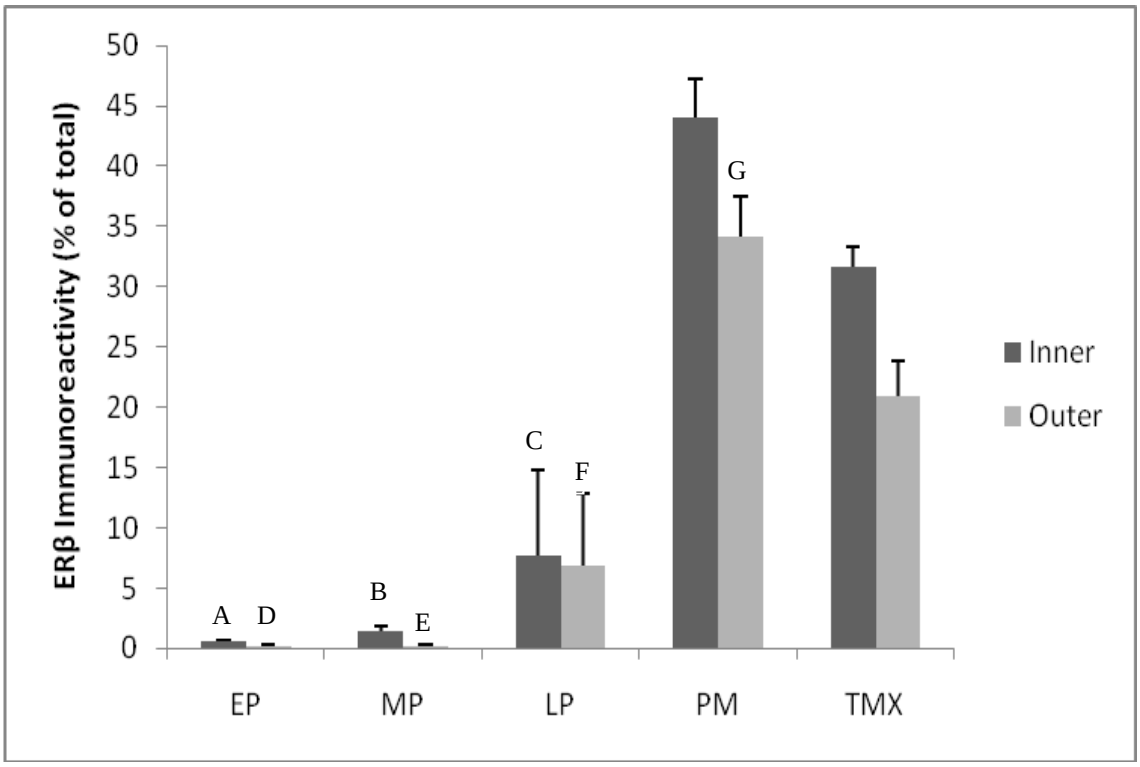


Figure 3.10 Histomorphometric analysis of the ERβ expression in the inner and outer myometrium in the proliferative phases of the menstrual cycle, untreated postmenopausal and tamoxifen treated uteri.

ERβ staining was significantly lower in the proliferative myometrium when compared the tamoxifen treated myometrium, whereas ERβ staining was significantly higher in the outer postmenopausal myometrium.

Data are presented as mean percentage ± SEM of cells expressing ERβ.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test; F and G $p<0.05$; A to E $p<0.001$ when using the menstrual cycle samples as the control and the tamoxifen treated sample being the test.

Inner myometrium in the tamoxifen treated samples when compared to EP (A), MP (B) and LP (C), $p<0.001$. Outer myometrium in the tamoxifen treated samples compared to EP (D), MP (E), $p<0.001$ and LP (F) and PM (G), $p<0.005$.

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3.3.3 PR-A distribution

3.3.3.1 Functionalis

PR-A immunoreactivity was primarily confined to the nuclei of cells in all compartments of the endometrium and myometrium. This included the stromal and glandular epithelial cells of the basal and functional endometrium and the myometrial smooth muscle cells, both within the inner zone and in the outer myometrium (Figure 3.12).

A Menstrual Cycle

Histomorphometric analysis of the PR-A staining showed cyclical changes in the glandular epithelial cells but not the stromal cells (Table 3.4). The glandular epithelium showed high PR-A immunostaining in the proliferative phases (Table 3.4). The greatest glandular epithelial staining was observed in the early- (96.7%), mid- (98.3%), late- (97.5%) proliferative phases (Figure 3.12; Table 3.4).

In the stromal cells there was no apparent cyclical change in PR-A expression. In the proliferative phases PR-A immunoreactivity ranged from 86.0% to 89.6%.

B Tamoxifen treatment group

In the tamoxifen treated endometrium, PR-A immunoreactivity was present at very high levels in both the stromal and glandular cell nuclei, at 92.5% and 98.9%, respectively (Table 3.4). Both the glandular epithelium and stromal cell staining was similar to that observed in the proliferative phases of the menstrual cycle (Figure 3.12 and Table 3.4). A similar trend was observed in the stromal cells. Although, there were no statistically significant differences between PR-A immunoreactivity in the tamoxifen treated

functionalis when compared to the levels observed in the proliferative phases (Figure 3.13).

These data indicate that PR-A expression was up-regulated by tamoxifen in the stromal and glandular functionalis and similar to that of the proliferative phases of the menstrual cycle.

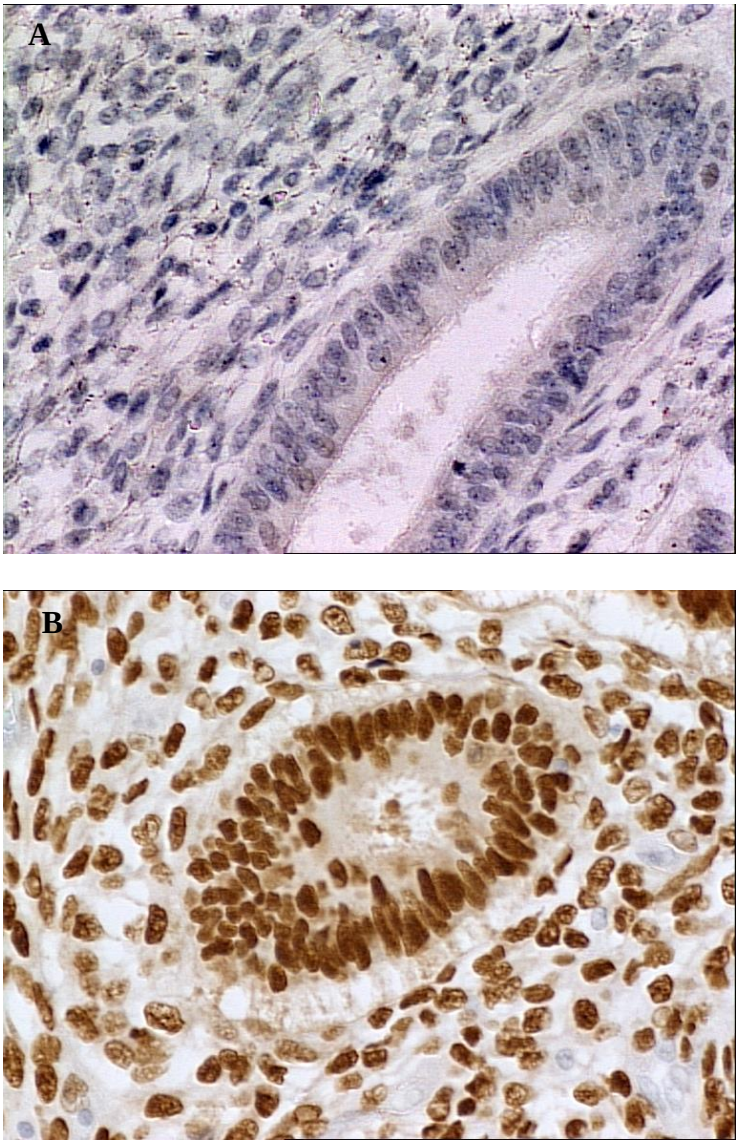


Figure 3.11 Demonstration of PR-A antibody specificity.

The tissue sample is an endometrial sample taken from the late proliferative phase of the menstrual cycle.

Panel A shows the IgG control (1.8µg/ml) and Panel B shows PR-A (1.8µg/ml). Both images were taken at x400 magnification.

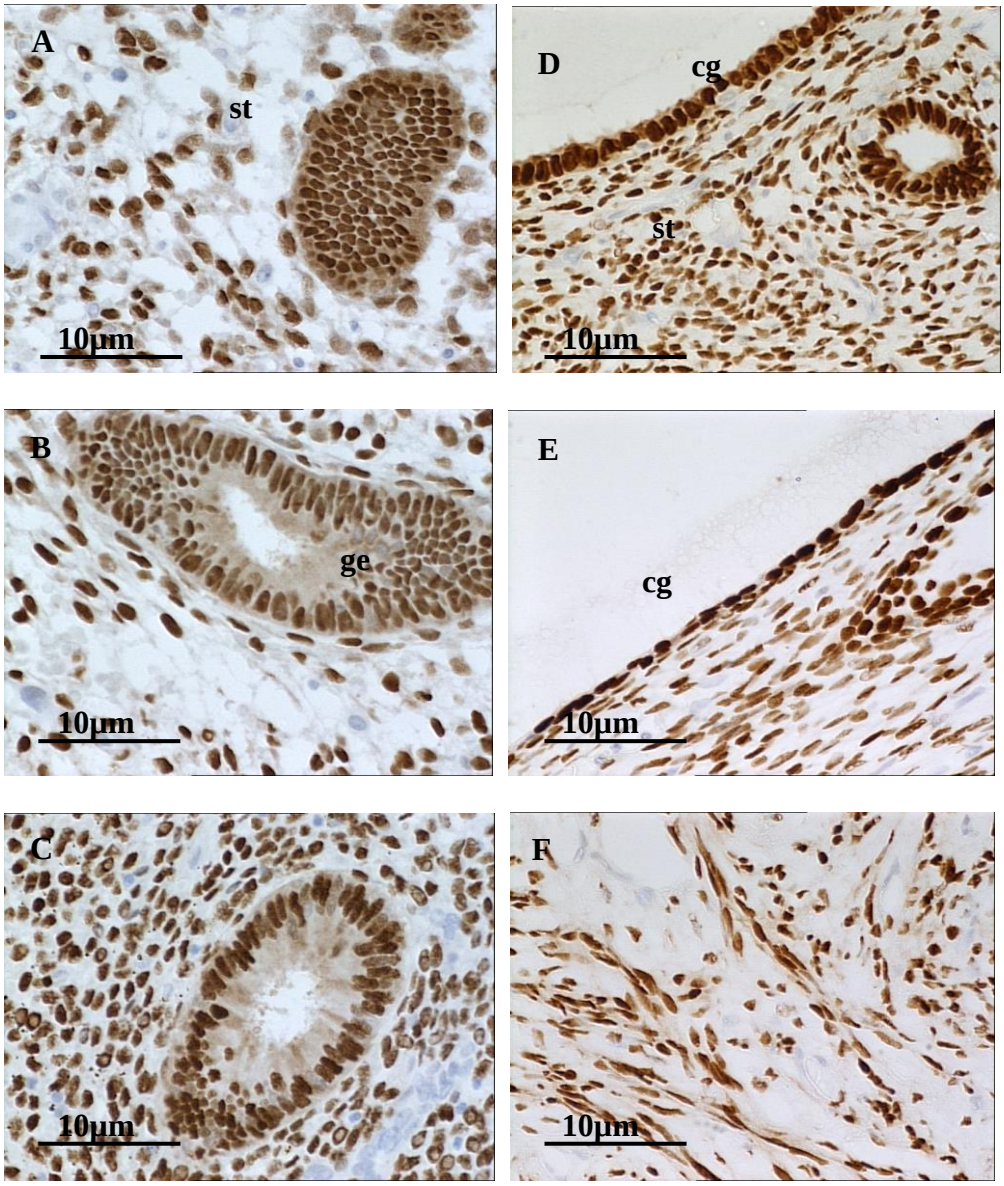


Figure 3.12 PR-A immunoreactivity in the functionalis of the early-, mid- and late-proliferative phase endometrium.

PR-A staining was greater in the glandular epithelium (ge) than the stromal (st) cells in the functionalis of the early (panel A), mid (panel B) and late (panel C) proliferative phase endometrium, with some cyttoplasmic staining that was not present in the stromal cells. The expression of PR-A in the tamoxifen treated endometrium (panels D & E) was similar to that observed in the proliferative endometrium in the stromal and in the epithelial cells

lining cystic dilated (cg) glands. The tamoxifen treated inner myometrium (panel F) demonstrated PR-A staining in the myocytes. All panels; magnification is x 400.

Category (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Inner	Outer
EP (8)	86.0 (1.0)	96.7 (0.6)	91.7 (1.5)	99.5 (0.1)	91.8 (1.2)	89.1 (3.3)
MP (8)	89.6 (1.3)	98.3 (0.4)	92.5 (1.2)	99.6 (0.1)	90.0 (1.1)	88.4 (1.6)
LP (8)	88.6 (1.4)	97.5 (0.6)	94.2 (0.7)	99.8 (0.1)	90.0 (1.5)	84.0 (3.2)
PM (8)	tissue not present	tissue not present	84.0 (3.9)	99.8 (0.1)	85.7 (2.0)***	57.7 (5.0)***
TMX (23)	92.5 (1.1)	98.9 (0.3)	87.1 (3.7)	98.9 (0.3)	96.3 (0.4)	90.5 (1.1)

Table 3.4. PR-A expression in the glandular and stromal cells of the functional and basal endometrium and of the myometrium.

Data are presented as percentage positive cells (standard error of the mean, SEM).

Numbers of samples assessed in each category are shown in parentheses.

Statistical significance was determined using one way ANOVA analysis with Tukey's honestly significance difference post test,*$p<0.05$; **$p<0.01$; ***$p<0.001$.

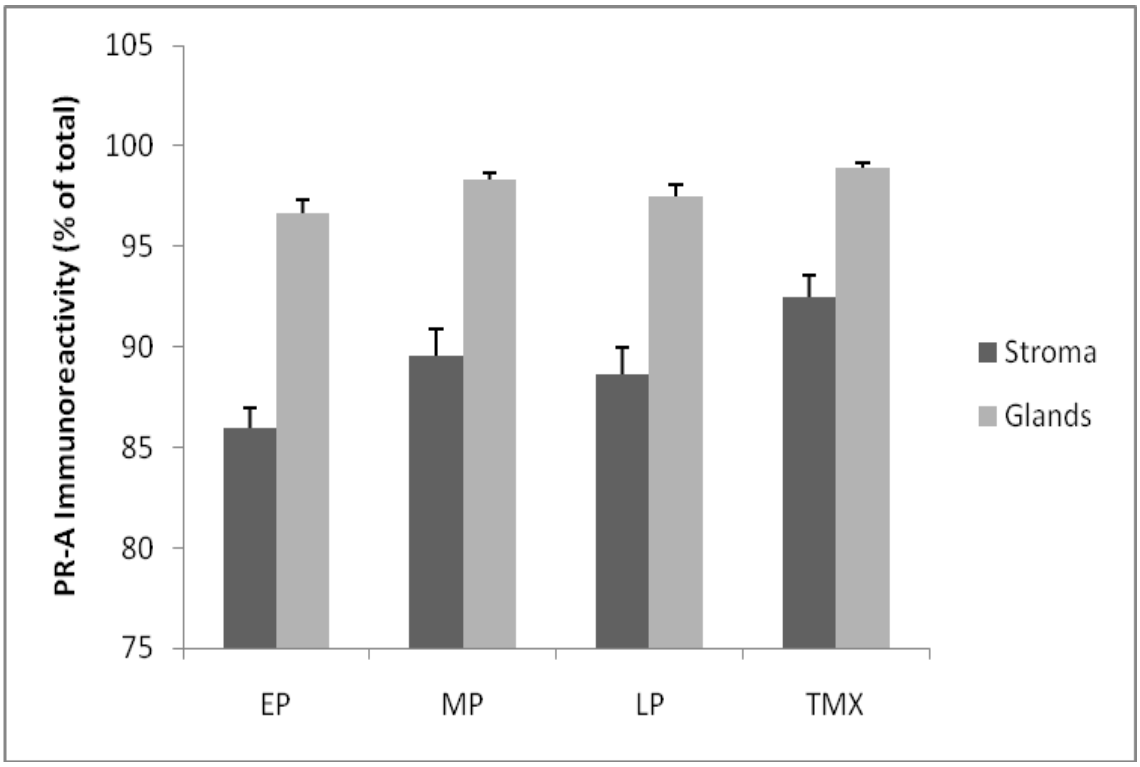


Figure 3.13 Histomorphometric analysis of PR-A expression in the stroma and glandular epithelial in the functionalis in the proliferative phases of the menstrual cycle and in tamoxifen treated uteri.

Data are presented as mean percentage and SEM of cells expressing PR-A.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test, comparing tamoxifen treated samples to the menstrual cycle samples. There were no statistically significant differences.

3.3.3.2 Basalis

A Menstrual Cycle

Histomorphometric analysis of the PR-A staining in the stromal cells of the basalis did not show any clear cyclical trend, however, a cyclical pattern was observed in the glandular epithelial cells of the basalis (Table 3.4).

The stromal cell PR-A immunoreactivity ranged from 91.7% to 94.2%, with the highest level found in the late proliferative phase, but these data were not statistically different.

The PR-A immunostaining in the glandular epithelial cells was greater in the proliferative (Table 3.4). The highest glandular epithelial PR-A staining was seen in the early- (99.5%), mid- (99.6%), late- (99.8%) proliferative phases (Figure 3.14).

B Postmenopausal group

PR-A immunoreactivity in the postmenopausal stromal cells was 84.0% and 99.8% in the glandular epithelial cells. This level of staining was similar to that observed in the glands and stroma of the normal menstrual cycle (Table 3.4).

C Tamoxifen treatment group

In the tamoxifen basal endometrium, PR-A was present in the stromal and glandular epithelial cells, at 87.1% and 98.9%, respectively (Table 3.4). PR-A immunoreactivity in

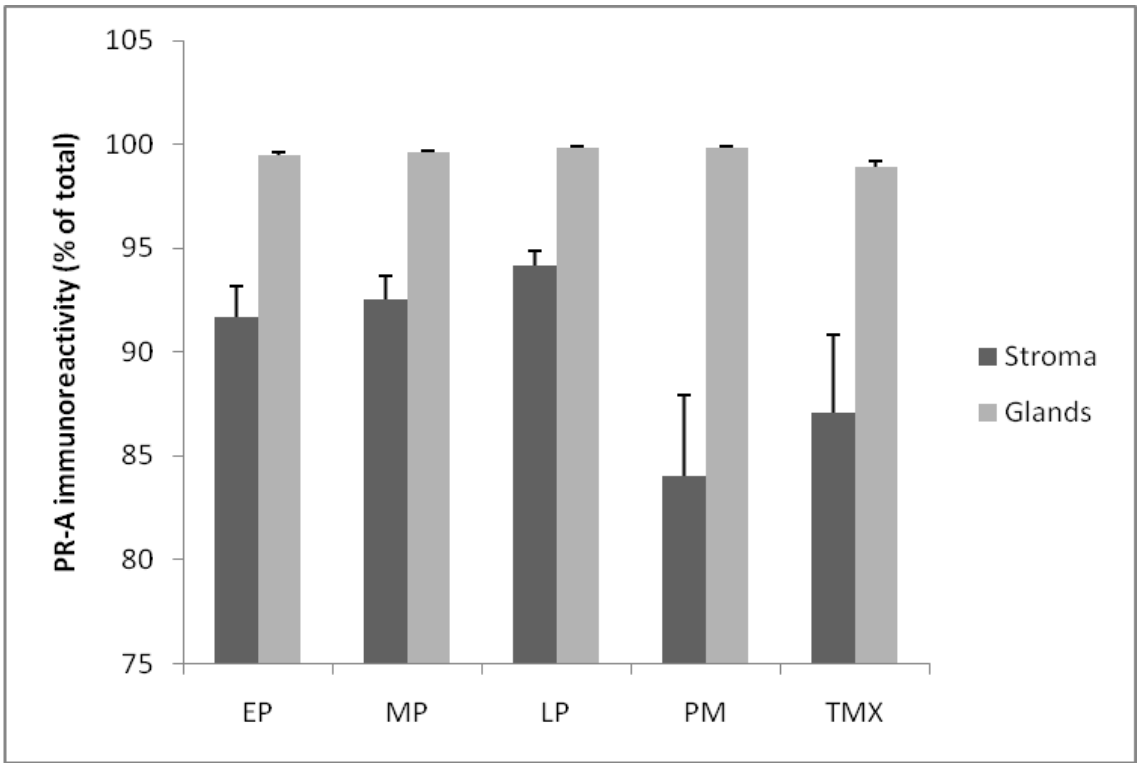


Figure 3.14 Histomorphometric analysis of PR-A expression in the stroma and glandular epithelial in the basalis in the proliferative phases of the menstrual cycle, untreated postmenopausal and tamoxifen treated uteri.

PR-A staining in the stroma was less than in the corresponding glands, regardless of tissue. Although the percentage of PR-A positive cells was lower in the postmenopausal stroma when compared to that observed in the tamoxifen stroma, the difference was not statistically significant.

Data are presented as mean percentage ± SEM of cells expressing PR-A.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test, comparing the tamoxifen treated samples to menstrual cycle and postmenopausal samples.

the stromal cells were similar to that found in the proliferative phases of the menstrual cycle and in postmenopausal uteri (Figure 3.14). PR-A immunostaining in the glandular epithelium was also similar to that observed in the proliferative phases and postmenopausal uteri.

3.3.3.3 Myometrium

A Menstrual Cycle

The inner zone myometrium was immunopositive for PR-A with 90.0% to 91.8% of the cells in the proliferative phases of the menstrual cycle, showing PR-A immunoreactivity.

The outer myometrium showed immunostaining for PR-A with 84.0% to 89.1% of the cells in the proliferative phases of the menstrual cycle (Table 3.4).

B Postmenopausal group

Eighty five point seven percent of the inner myometrial cells were immunopositive for PR-A. This was similar to the levels seen in the proliferative phases of the menstrual cycle. The outer myometrium had significantly ($p<0.05$) lower (57.7%) PR-A staining compared to the levels observed in the inner zone myometrium (Figure 3.15).

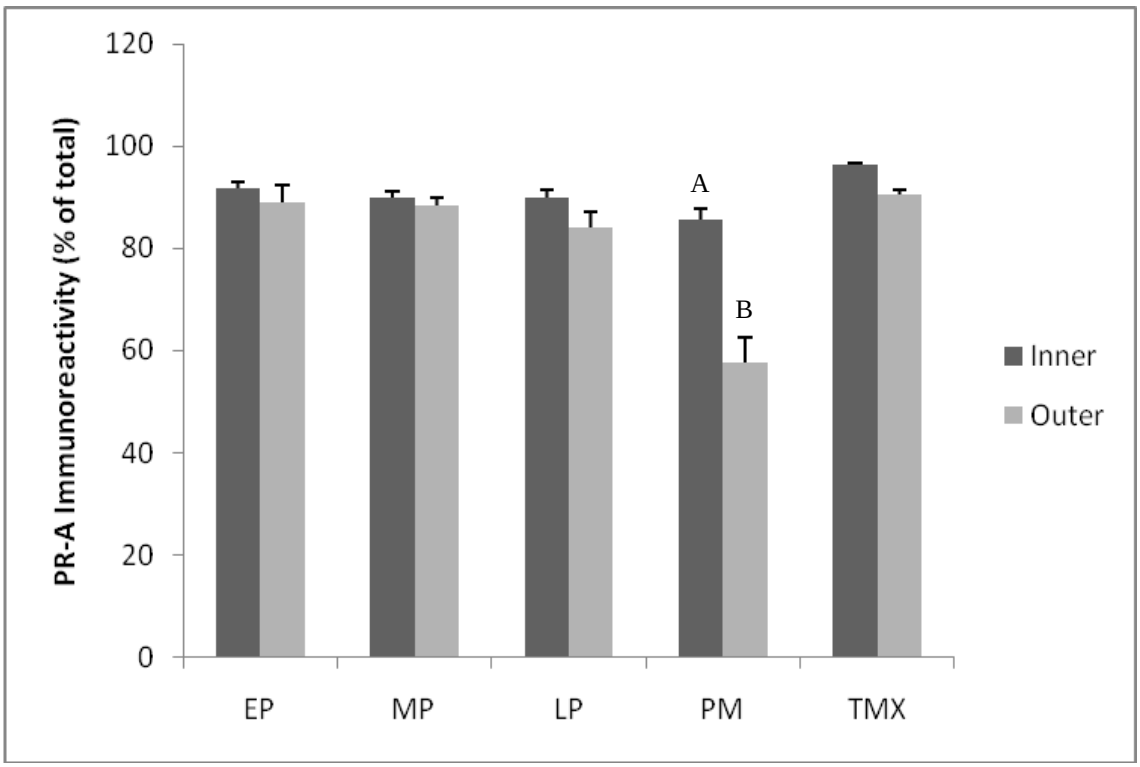


Figure 3.15 Histomorphometric analysis of PR-A expression in the inner and outer myometrium in the proliferative phases of the menstrual cycle, untreated postmenopausal and tamoxifen treated uteri.

PR-A staining was in the tamoxifen myometrium was similar to that of the proliferative phase myometrium. Staining in both the inner and outer parts of the postmenopausal myometrium was significantly reduced when compared equivalent areas in the tamoxifen treated myometrium.

Data are presented as mean percentage and SEM of cells expressing PR-A.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test, A and B $p<0.001$ when using the menstrual cycle samples as the control and the tamoxifen treated sample being the test.

Inner myometrium in the tamoxifen treated samples when compared to PM (A), $p<0.001$.

Outer myometrium in the tamoxifen treated samples compared to PM (B), $p<0.001$.

C Tamoxifen treatment group

The inner zone myometrium demonstrated a higher levels of PR-A immunoreactivity (96.3%), a level that was statistically significant when compared to the postmenopausal myometrium ($p<0.001$, but there was no statistical difference when compared to the proliferative phases of the menstrual cycle myometrium (Table 3.4).

3.3.4 PR-B distribution

PR-B immunoreactivity was primarily confined to the nuclei of cells in all compartments of the endometrium and myometrium (Figure 3.17). This included the stromal and glandular epithelial cell of the endometrium (both within the functionalis and basalis) and the myometrial smooth muscle cells in the inner and outer myometrial layers.

3.3.4.1 Functionalis

A Menstrual cycle

Histomorphometric analysis of the PR-B staining in the glandular epithelium and stromal cells of the functionalis showed cyclical changes (Table 3.5). Both cell types demonstrated greater PR-B immunostaining in the proliferative phases (Table 3.5).

The greatest glandular epithelial staining was observed in the early- (94.9%), mid- (94.3%), late- (94.9%) proliferative phases (Table 3.5). In the stromal cells, the highest PR-B immunoreactivity was observed in the early- and mid-proliferative phases, with 84.8% and 86.9% of the cells being immunopositive, respectively. There was a small decrease in PR-B staining to 81.5% in the late proliferative phase.

B Tamoxifen treatment group

In the functionalis of the tamoxifen treated endometrium, PR-B immunoreactivity was present at high levels in both the stromal and glandular epithelial cells, at 79.5% and 95.1%, respectively (Table 3.5). Both the glandular epithelial and stromal cell staining was similar to that observed in the proliferative phases of the menstrual cycle (Table 3.5).

In the glandular epithelial cells, there was no significant difference between PR-B staining in the tamoxifen treated uteri when compared to the proliferative phases of the menstrual (Table 3.5).

These data indicate that PR-B expression was up-regulated by tamoxifen in the functionalis and similar to that of the proliferative phase of the menstrual cycle (Figure 3.18).

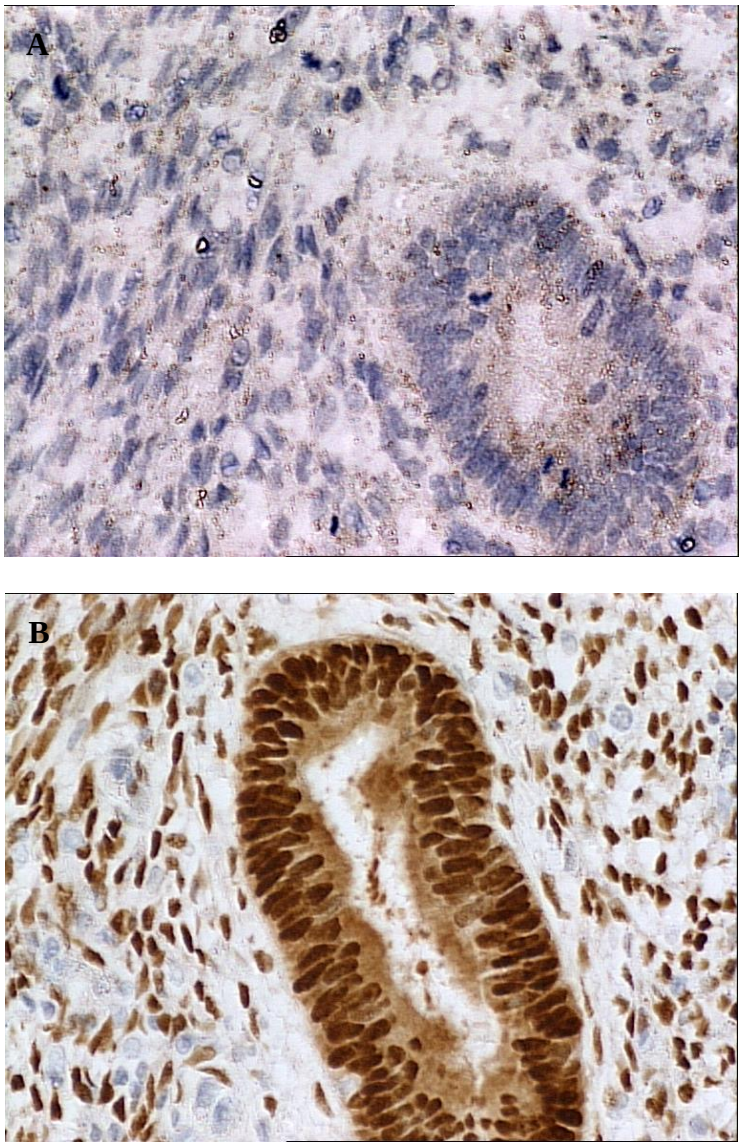


Figure 3.16 Demonstration of PR-B antibody specificity

The tissue sample is an endometrium from the late proliferative phase of the menstrual cycle.

Panel A shows the IgG control (0.167µg/ml) and Panel B shows ERα (0.167µg/ml). Both panels are at x400 magnification.

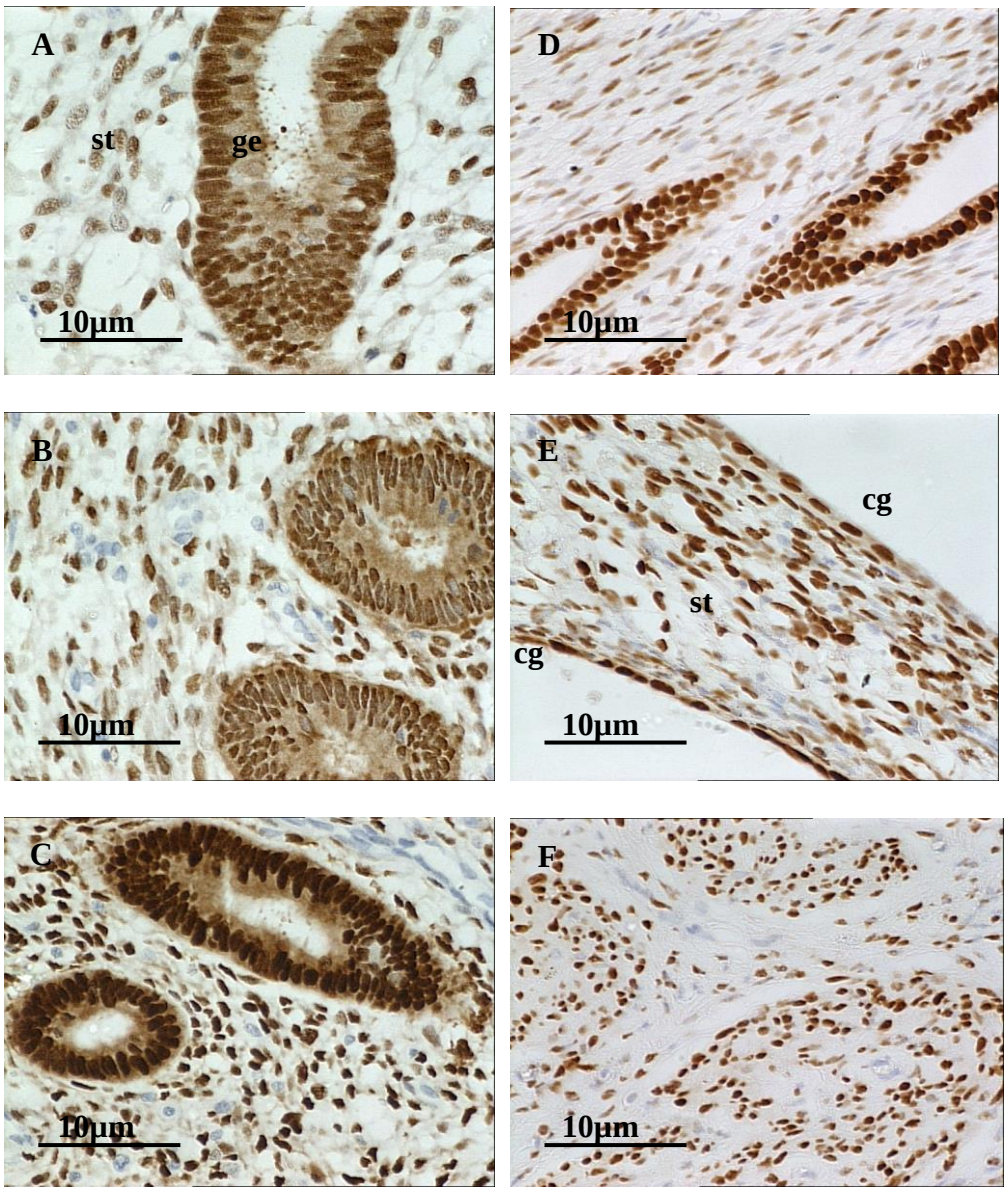


Figure 3.17 PR-B immunoreactivity in the functionalis of the early-, mid- and late-proliferative phase endometrium. PR-B staining was greater in the glandular epithelium (ge) than the stromal (st) cells in the functionalis of the early- (panel A), mid- (panel B) and late-proliferative (panel C) phase endometrium, with staining of both the nucleus and the cytoplasm. The expression of PR-B in the tamoxifen treated endometrium (panel D & E) was similar to that observed in the proliferative endometrium in the stroma and the epithelial cells lining the cystic dilated (cg) glands, although the cytoplasmic staining was less intense. The tamoxifen treated inner myometrium (panel F) demonstrated slightly weaker PR-B staining than that seen in the endometrium. All panels: x 400 magnification.

Category (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Inner	Outer
EP (8)	84.8 (2.4)	94.9 (0.9)	88.1 (2.0)	99.4 (0.1)	88.0 (1.9)	85.8 (3.3)
MP (8)	86.9 (1.1)	94.4 (0.5)	85.8 (1.9)	99.3 (0.1)	81.1 (2.7)	82.9 (2.2)
LP (8)	81.5 (5.1)	94.9 (1.5)	85.2 (3.3)	98.5 (0.7)	77.9 (5.6)	68.6 (8.5)
PM (8)	tissue not present	tissue not present	80.0 (1.8)	99.8 (0.1)	79.1 (2.3)	54.6 (3.2)***
TMX (21)	79.5 (3.6)	95.1 (1.2)	80.0 (3.1)	98.0 (0.4)	90.4 (1.2)	84.9 (2.2)

Table 3.5. PR-B expression in the glandular and stromal cells in the functional and basal endometrium and myometrium.

Data are presented as percentage positive cells (standard error of the mean, SEM).

Numbers of samples assessed in each category are shown in parentheses.

Significance was determined by one way ANOVA analysis with Tukey's post test; ***$p<0.001$ when menstrual cycle and postmenopausal samples were compared to tamoxifen treated samples.

3.3.4.2 Basalis

A Menstrual Cycle

Histomorphometric analysis of the PR-B staining in glandular epithelial cells showed a cyclical trend, (Table 3.5) with staining in the glandular epithelial cells being very high in the early- (99.4%), mid- (99.3%), and late- (98.5%) proliferative phases.

The PR-B staining in the stromal cells demonstrated very little variation in expression and ranged from 85.2% to 88.3% in the proliferative phases of the menstrual cycle (Table 3.5).

B Postmenopausal group

PR-B staining in the postmenopausal glandular epithelium was 99.8% and in the stroma was 80.0%. This level of staining was similar to that observed in the proliferative phases of the menstrual cycle.

C Tamoxifen treatment group

In the tamoxifen basal endometrium, PR-B was present in the glandular epithelial and stromal cells, at 98.0% and 80.0%, respectively (Table 3.5). PR-B immunoreactivity in both the glandular epithelium and stroma were shown to be similar to that observed in the proliferative phases of the menstrual cycle and postmenopausal uteri (Figure 3.19 and Table 3.5).

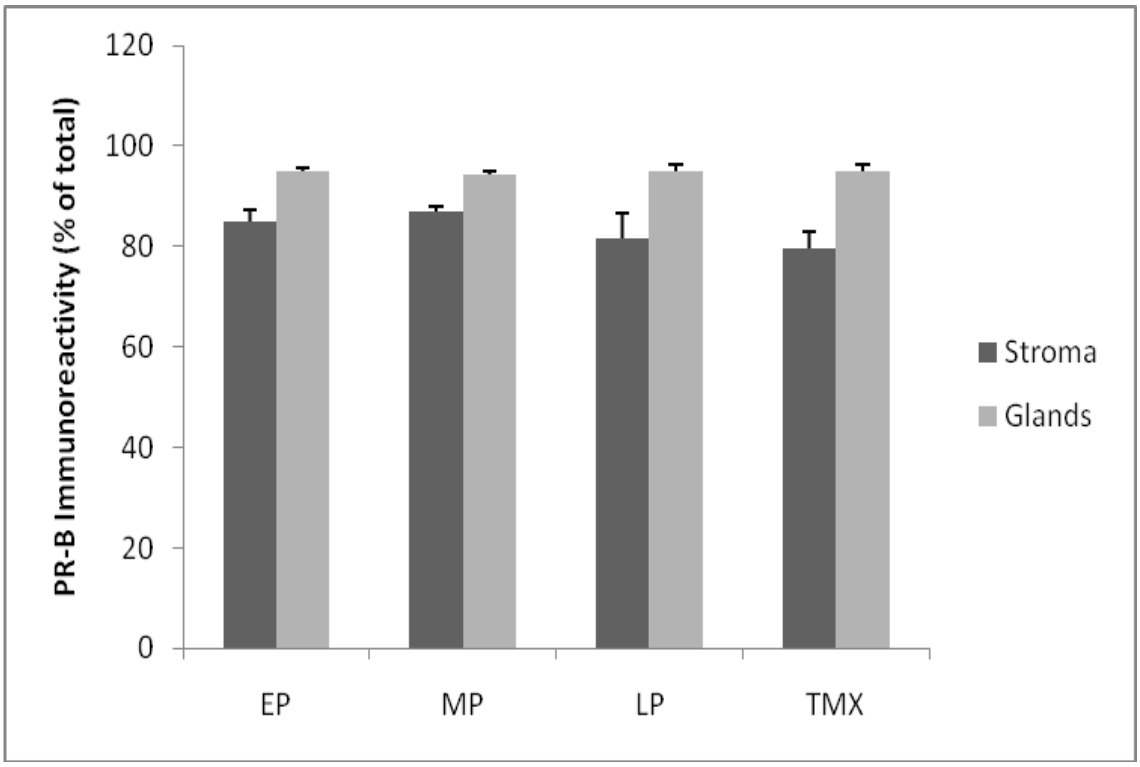


Figure 3.18 Histomorphometric analysis of PR-B expression in the stroma and glandular epithelial in the functionalis in the proliferative phases of the menstrual cycle and in tamoxifen treated uteri.

The number of PR-B positive cells in the tamoxifen treated functionalis was not significantly different from the number of PR-B positive cells observed in the proliferative phase of the menstrual cycle.

Data are presented as mean ± SEM of percent of PR-B positive nuclei.

Statistical significance was determined using one way ANOVA analysis with Tukey's honestly significant difference post test comparing menstrual cycle samples to the tamoxifen treated samples. There were no significant differences.

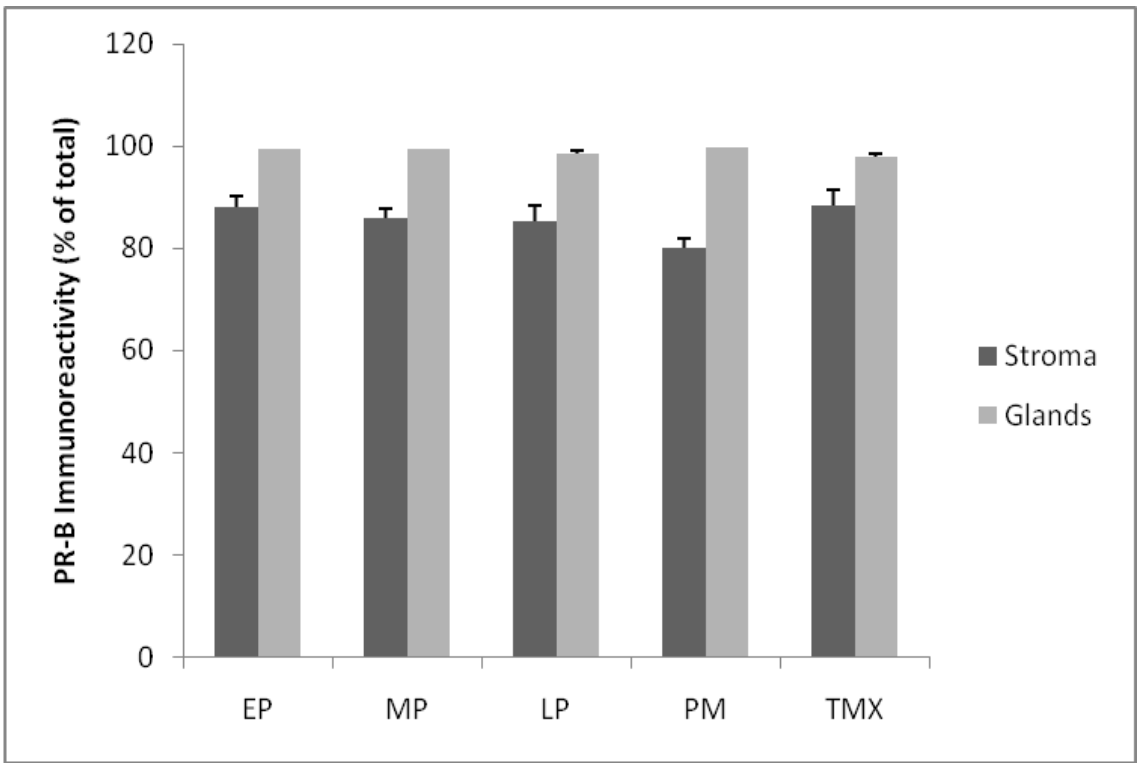


Figure 3.19 Histomorphometric analysis of PR-B expression in the stroma and glandular epithelial in the basalis in the proliferative phases of the menstrual cycle, untreated postmenopausal and tamoxifen treated uteri.

PR-B staining in the tamoxifen treated basalis was not significantly different from the PR-B staining observed in the proliferative phase of the menstrual cycle or in postmenopausal uteri.

Data are presented as mean percentage ± SEM of cells expressing PR-B.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test, with menstrual cycle and postmenopausal stroma and glands compared to the tamoxifen treated stroma and glands. There were no significant differences.

3.3.4.3 Myometrium

A Menstrual Cycle

PR-B immunostaining in the myometrial inner zone was constant throughout the menstrual cycle with 77.9% to 88.0% of the smooth muscle cells being immunopositive for PR-B in the proliferative phase of the menstrual cycle (Table 3.5).

The outer myometrium demonstrated some fluctuation in the staining with 85.8% and 82.9% of the cells being positively stained in the early- and mid-proliferative phases, respectively, with the levels of staining decreasing to 68.6% in the late proliferative phase (Table 3.5).

B Postmenopausal group

The inner zone myometrium was positive for PR-B in 79.1%. This level of staining was similar to that seen in the menstrual cycle (Figure 3.20). The outer myometrial cells had significantly lower PR-B staining compared to the inner myometrium at 54.55% (Table 3.5). This was also significantly lower when compared to any phase of the menstrual cycle (Table 3.5).

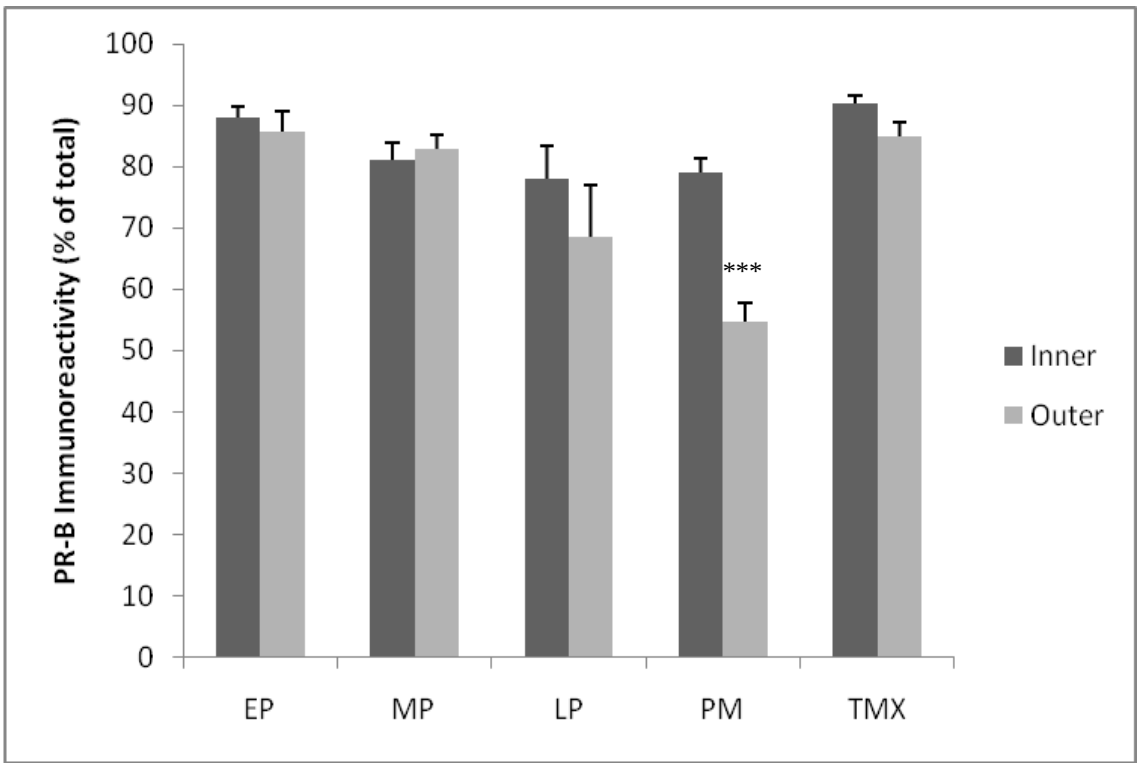


Figure 3.20 Histomorphometric analysis of PR-B expression in the inner and outer myometrium in the proliferative phases of the menstrual cycle, untreated postmenopausal uteri and tamoxifen treated uteri.

The number of PR-B positive cells in the tamoxifen treated myometrium was not different to that observed in the proliferative phase myometrium. The number of PR-B positive cells in the outer myometrium of postmenopausal samples was significantly lower than that observed in the tamoxifen treated myometrium.

Data are presented as mean percentage ± SEM of cells expressing PR-B.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post-test; The inner myometrium demonstrated PR-B immunoreactivity for 90.4% of its cells. This level of staining was not statistically significant when compared to the proliferative phases of the menstrual cycle or the postmenopausal myometrium. The outer myometrial smooth muscle cells showed 84.6% immunostaining for PR-B, which was statistically significant when compared to postmenopausal myometrium (***$p<0.001$), but not different when compared to the menstrual cycle.

3.4 Discussion

The most important finding in this chapter is the demonstration that tamoxifen up-regulates ERβ in the functionalis and basalis endometrium (both in the gland and the stroma), and in the myometrium (both the inner and the outer layers) when compared to the proliferative phase of the menstrual cycle. By contrast, ERβ expression was lower compared to postmenopausal endometrial stroma and outer myometrium, but not the glandular epithelium or the inner myometrium.

In breast cancer, ERβ is an independent marker for a favourable prognosis following tamoxifen treatment in ERα negative breast tumours (Gruvberger-Saal et al., 2007). This could be because tamoxifen has a higher binding affinity to ERβ than ERα, and so tamoxifen may be acting as an anti-proliferative agent via ERβ binding (Bardin et al., 2004).

By contrast, in a report of two patients who developed uterine mesenchymal tumours following tamoxifen therapy, the tumours were ERα negative and ERβ positive (Zafrakas et al., 2004). This suggests that in this tumour type, tamoxifen may exert a stimulatory effect via ERβ not ERα. Another report also found that tamoxifen associated endometrial cancers more frequently expressed ERβ (Wilder et al., 2004). Wilder and co-workers suggest that ERβ negatively regulates ERα in the uterus, if there were absent ERβ then excess endometrial proliferation would occur via ERα if oestradiol was abundant. Wilder and co-workers postulate that tissues expressing more ERβ in an oestrogen deficient environment, as in the postmenopausal state tamoxifen would cause proliferation via the ERβ. My data also shows differential ERβ expression levels and in contrast to the ERα expression were higher than ERβ in all samples examined. It is possible that greater ERβ

levels may modulate the effects of ERα. The difference between my studies and those above is that none of my patients developed endometrial cancer and the tissues may therefore be very different to the mesenchymal tumours examined by Zafrakas and colleagues, or Wilder and colleagues, in that these tissues had higher levels of ERβ than the proliferative phase, but significantly lower levels of ERβ compared to that of the normal postmenopausal uterus.

This study also demonstrated that the expression of ERα, PR-A and PR-B in the tamoxifen endometrium was not significantly different compared to the proliferative phase of the menstrual cycle and postmenopausal controls, in both the basalis and functionalis. In addition, the expression of ERα, PR-A and PR-B in the tamoxifen inner and outer myometrium was also not significantly different to that observed in the myometrium of the proliferative phase of the menstrual cycle. However, there were differences observed between in PR-A expression in the tamoxifen myometrium and postmenopausal myometrium in the inner and outer myometrium, where there was up regulation of PR-A in the inner and outer myometrium with tamoxifen treatment. In addition, there was up regulation of PR-B expression in the outer myometrium of the tamoxifen users when compared to the postmenopausal myometrium. There was no significant difference observed in ERα expression in the tamoxifen treated and postmenopausal myometrium. These data suggest that the tamoxifen endometrium is similar to both a proliferative phase and postmenopausal uterus and likely therefore to respond to progesterone in a similar manner, but that the myometrium has been primed to respond more actively to progesterone, simply because it contains more PR. The endometrium responded to progestagens like the proliferative phase endometrium by exhibiting decidualisation, but

the myometrium did not grow, this may be due to inhibition by high expression of ERβ levels.

It could be argued that some results may be peculiar to the antibody used, but because the results of the distribution of ERα, ERβ, PR-A and PR-B through the menstrual cycle correlate well with previous immunohistochemical studies on the normal menstrual cycle in the endometrial functionalis (Critchley et al., 1993; Taylor et al., 2005) and basalis (Mertens et al., 2001), this seems unlikely. All the receptors were studied throughout the entire normal menstrual cycle to ensure that the assays that were used were valid, and since the findings correlated with previous studies it can be concluded that the distribution of receptors in the tamoxifen treated and postmenopausal uteri are truly representative.

These data demonstrate that in tamoxifen treated uteri, ERα staining in all areas was similar to that observed in the proliferative phase of the menstrual cycle and postmenopausal uteri. Tregon and co-workers observed no statistical difference in ERα and PR-A expression in the glands and stroma of postmenopausal women treated with tamoxifen and those without, except in the glandular epithelium ($p<0.05$) (Tregon et al., 2003). However, Mourits and colleagues found that tamoxifen was associated with up regulation of PR-A in the stromal cells compared to benign endometrium from postmenopausal women, suggesting an agonist effect of tamoxifen on the stroma. The difference between these studies is difficult to explain. My observations support the findings of Tregon and colleagues and suggest that tamoxifen treatment may have an oestrogen agonist effect on endometrial stroma.

In the myometrium there was also differential expression of ERβ in the tamoxifen treated compared to the postmenopausal uteri, ERβ expression was decreased in tamoxifen compared to the postmenopausal uteri in the outer myometrium. PR-B and PR-A were up regulated in the outer myometrium and only PR-A in the inner myometrium. In agreement with my findings, Sakaguchi and colleagues demonstrated greater ERβ expression in the myometrium of postmenopausal women compared to premenopausal women (Sakaguchi et al., 2003). There are no published studies of steroid receptor expression in myometrium of tamoxifen exposed uteri.

Tamoxifen is known to behave as an oestrogen antagonist in the breast and to block the proliferative actions of oestradiol via ERα in that tissue, and to behave like an oestrogen agonist in the endometrium, especially in postmenopausal women who have low circulating oestrogen levels. The data presented in this chapter show that that the expression of ERα, PRA and PRB in the tamoxifen exposed endometrium was up regulated similar to that observed in the proliferative phase of the menstrual cycle, as reported in the literature (Mourits et al., 2002b; Leslie et al., 2007), confirming that tamoxifen is oestrogenic. It is this oestrogenic action on the endometrium which increases a tissue's responsiveness to both oestrogens and progestagens by increasing the expression of oestrogen and progesterone receptors, and promoting cell proliferation. This leads to the increased incidence of endometrial polyps, hyperplasia and cancers in women taking tamoxifen for breast cancer (Ismail, 1994; Cohen, 2004). Other pathologies associated with tamoxifen include an increase in the growth of fibroids and adenomyosis (Cohen et al., 1997a; McCluggage et al., 2000). The mechanism of this tamoxifen action, however, remains unclear. To determine how tamoxifen might induce endometrial pathologies, the

expression of proliferation and apoptosis markers will be described in the following chapter.

Chapter 4

Expression of proliferative (Ki67) and apoptosis markers (BAX & Bcl2) in the tamoxifen treated uterus

4.1 Introduction

Immunohistochemical studies of tamoxifen exposed endometrium versus endometrium not exposed to tamoxifen demonstrates that tamoxifen exposure results in increased epithelial cell proliferation (Mourits et al., 2002b). Thus, it is possible that during tamoxifen exposure proliferation of a subset of cells results in an increased likelihood of the accumulation of mutations, or alternatively, promotes the growth of cells that have already sustained mutations. As a result, tamoxifen exposure could lead to the production of a spectrum of mutations similar to that of sporadic endometrial cancer, thereby explaining the increased incidence of endometrial carcinoma following tamoxifen exposure. It is also possible that tamoxifen exerts a direct neoplastic effect upon the endometrium. In this regard, p53 is a key target molecule, which is directly affected by oestradiol and tamoxifen (Elledge et al., 1997). It should also be noted that p53 is not only a key molecule in proliferation pathways, but also has a controlling role in the regulation of apoptosis (Haupt et al., 2003). Therefore, if tamoxifen is acting like oestradiol then both pathways, proliferation and apoptosis, may be affected in tamoxifen exposed uteri. It is likely that the balance between proliferation and apoptosis increases the predisposition to tamoxifen associated lesions. In the previous chapter, there were changes in the expression of the oestrogen receptor in the tamoxifen exposed uterus, this is likely to have an effect on the proliferation and apoptosis in the endometrium and myometrium of the tamoxifen treated uteri.

The function of the endometrium during the menstrual cycle is dependent on the fine balance between cell death and cell proliferation. This balance is hormone dependent and controlled by endocrine and paracrine factors. Proliferation in the normal endometrium is primarily stimulated by oestrogenic activity, whilst apoptosis allows the removal of unnecessary, damaged or aged cells. Apoptosis is regulated by both anti- and pro-apoptotic factors. Regulators of apoptosis include the B-cell leukaemia/lympohoma 2 protein (Bcl2) family of proteins, these include the anti-apoptotic Bcl2 and pro-apoptotic BAX. Bcl2 is known to protect cells from apoptosis, whereas BAX increases cell susceptibility to apoptosis (Oltvai et al., 1993). This occurs as BAX opposes the anti-apoptotic effects of Bcl2 through the formation of heterodimers of BAX and Bcl2 (Oltvai et al., 1993). Therefore, cell survival or death following stimulus is influenced by the Bcl2/BAX ratio. A high Bcl2/BAX ratio allows cells to resist apoptosis and a low ratio induces cell apoptosis death (Sedlak et al., 1995).

Ki67 is a proliferation marker that is only present in dividing cells (Dahmoun et al., 1999) and is widely used to assess cell proliferation. The endometrial expression of Ki67 is regulated by sex steroid hormones (Shiozawa et al., 1996) and its expression is greater in the proliferative compared to the secretory glandular epithelium (Wahab et al., 1999; Mertens et al., 2002; Maia et al., 2005). The expression of Ki67 in the myometrium during the menstrual cycle and postmenopausal women has been observed to be rare (less than 1%) (Nisolle et al., 1999).

The Bcl2 and BAX gene products have been demonstrated in the endometrium (Tao et al., 1997; Vaskivuo et al., 2002; Harada et al., 2004), and the myometrium (Wu et al., 2002; Kovacs et al., 2003).

The aim of this chapter is to examine the immunohistochemical expression of proliferative indices as determined by using antibodies against the MiB-1 antigen (Ki67) and changes in the expression of the anti- and pro-apoptotic markers, BAX and Bcl2 respectively in women receiving tamoxifen for breast cancer and compare these to the expression observed in the normal menstrual cycle and that of postmenopausal women.

4.2 Methods

The tissue collection, patient groups, sampling technique and histological examination was performed as described in Chapter 3, sections 3.2.1, 3.2.2 and 3.2.3.

4.2.1 Immunohistochemistry

Ki67, BAX and Bcl2 immunoreactivity was visualised using an avidin-biotin complex amplification methodology with 3, 3' diaminobenzidine as the chromogen as described in Chapter 3. Briefly, tissue sections were dewaxed in xylene, rehydrated through graded alcohol to water, antigen retrieval by heating in a microwave for 30 minutes at 800 Watts in 10mM citric acid buffer (pH 6.0). Endogenous peroxidases were suppressed by 6% hydrogen peroxidise. After blocking with Normal Rabbit Serum (10% in TBS-0.1%BSA) at room temperature for 20 minutes, tissue sections were incubated with the primary antibody diluted in TBS-0.1%BSA at the specified dilution (Table 4.1) and incubated overnight in a humidified chamber at 4°C. Antigenic sites were identified using biotinylated rabbit anti-mouse secondary antibody, Vectastain ABC Elite and DAB, counterstained in Mayer's haematoxylin, dehydrated through graded IMS, cleared in xylene and mounted using DPX.

All experiments included a negative control where the primary antibody was replaced with an equivalent concentration of mouse IgG. A positive control tissue for each experiment was also determined and included tonsil for Ki67 and Bcl2 and breast cancer tissue sections for BAX.

Antigen	Clone Name	Manufacturer	Antibody Type	Working Concentration (µg/ml)
Ki67 (Mib1)	NCL-Ki67-MM1	Novocastra	Mouse IgG1	0.8
BAX	B-9	Santa Cruz	Mouse IgG2b	2
Bcl2	C-2	Santa Cruz	Mouse IgG1	0.5

Table 4.1 Details of primary antibodies used to determine proliferation and apoptosis.

4.2.2 Histomorphometric Analysis

The distribution pattern and identities of the positively stained cells were identified by microscopic examination of all slides. Histomorphometric analysis of the staining patterns was determined by counting the number of positively stained stromal and glandular epithelial cells per field from ten randomly selected fields per slide in the basal and functional endometrium, the inner and the outer layer of the myometrium. All images were captured at 400x magnification, with daylight and light grey neutral density filters and with standardised illumination set at just under maximum exposure. The images were captured using an Axioplan microscope (Carl Zeiss, Herts. UK) and a Sony DXC-151P colour camera with the aid of Axiovision Image Capture software version 4.0 (Carl Zeiss, Germany).

For Ki67 assessment, the percentage of immunoreactive cell nuclei was obtained by separately counting the number of positive (brown stained) and negative (blue stained) endometrial and stromal cells and the inner and outer myometrial cells as described in Chapter 3. The mean percentage positive cells was recorded for each specimen and the mean and standard error of the mean was calculated for each phase of the menstrual cycle, for postmenopausal samples and for tamoxifen exposed tissue specimens. Endothelial cells were omitted from the count. The mean percentage positive cells was used because only cells that are actively dividing express Ki67, and since it is a nuclear transcription factor one only has to count the positively stained nuclei to determine the proliferative potential of the tissue in question.

Because BAX and Bcl2 staining were not confined to the nucleus but expressed in the cytoplasm, immunohistomorphometric analysis for BAX and Bcl2 was performed by

calculating a modified H-score. For both antigens, a staining index was generated by multiplying the percentage of positively stained cells observed, where 0-33%=1; 34-66%=2; and 67-100%=3, by the intensity of positive staining in the field ranging from 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (intense staining). This gives a possible staining index from 0 to 9. Staining indices were calculated independently for the stroma and the glandular epithelium of the endometrium and for myometrial cells within the inner and outer layer of the myometrium. The staining index was recorded for each specimen and the mean and a standard error of the mean was then calculated for each phase of the menstrual cycle, for the postmenopausal samples and for the tamoxifen treated tissue specimens.

4.2.3 Statistical Analysis

Histomorphometric data were analysed using the one-way analysis of variance (ANOVA) with Tukey's honestly significant difference post test for Ki67 staining. The non-Gaussian nature of the BAX and Bcl2 data meant they needed to be analysed using non-parametric methods, therefore, Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's post test was used. In both cases $p<0.05$ was accepted as being statistically significant.

4.3 Results

4.3.1 Ki67 distribution

Ki67 was observed in the nuclei of cells within the stroma and glandular epithelium of samples taken from the menstrual cycle and tamoxifen treated uteri (Figure 4.1). Only occasional cells in the postmenopausal samples were positive to Ki67 immunostaining (Figure 4.2) and no immunostaining for Ki67 was observed in the myometrium. Therefore, no further analysis was applied to the myometrial samples.

4.3.1.1 Functionalis

A Menstrual cycle

In the endometrium, Ki67 expression was significantly higher in the functionalis stroma and glands compared to the basalis (Figure 4.2).

Immunostaining for Ki67 in the functionalis stroma was highest in the early- and mid-proliferative phases at 13.7% and 17.3%, respectively (Figure 4.3). The levels of Ki67 then decreased in the late proliferative.

In the glandular epithelium of the functionalis, Ki67 staining was highest in the proliferative.

B Tamoxifen

In the tamoxifen exposed uteri, Ki67 immunoreactivity was low in the stroma (2.4%) but comparatively higher in the glandular epithelium (27.8%, Figures 4.2 and 4.3).

Tamoxifen stromal Ki67 expression was significantly lower compared to the early- and mid-proliferative phases of the menstrual cycle ($p<0.001$ and $p<0.001$ respectively).

In the glandular epithelium of the functionalis the expression of Ki67 was similar to that observed in the normal menstrual proliferative phases (Figure 4.3 and Table 4.2).

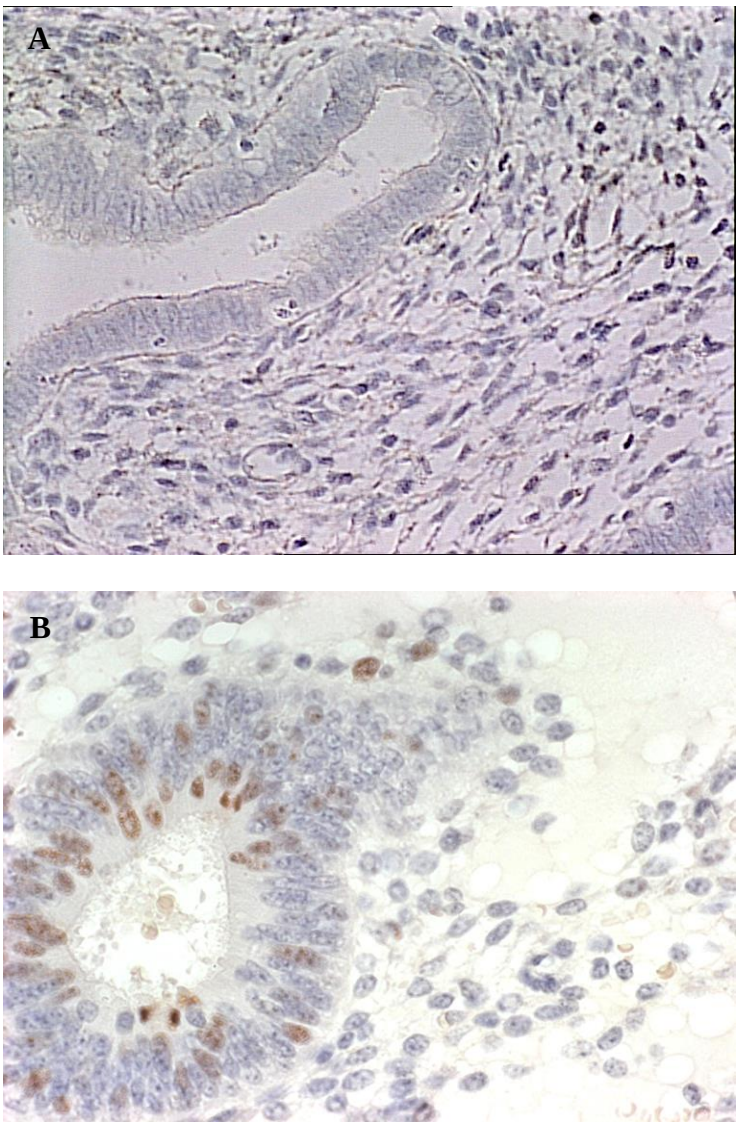


Figure 4.1 Demonstration of Ki67 antibody specificity

The tissue sample is an endometrium from the late proliferative phase of the menstrual cycle.

Panel A shows the IgG control (0.8µg/ml) and Panel B shows Ki67 (0.8µg/ml). Both images were taken at x400 magnification.

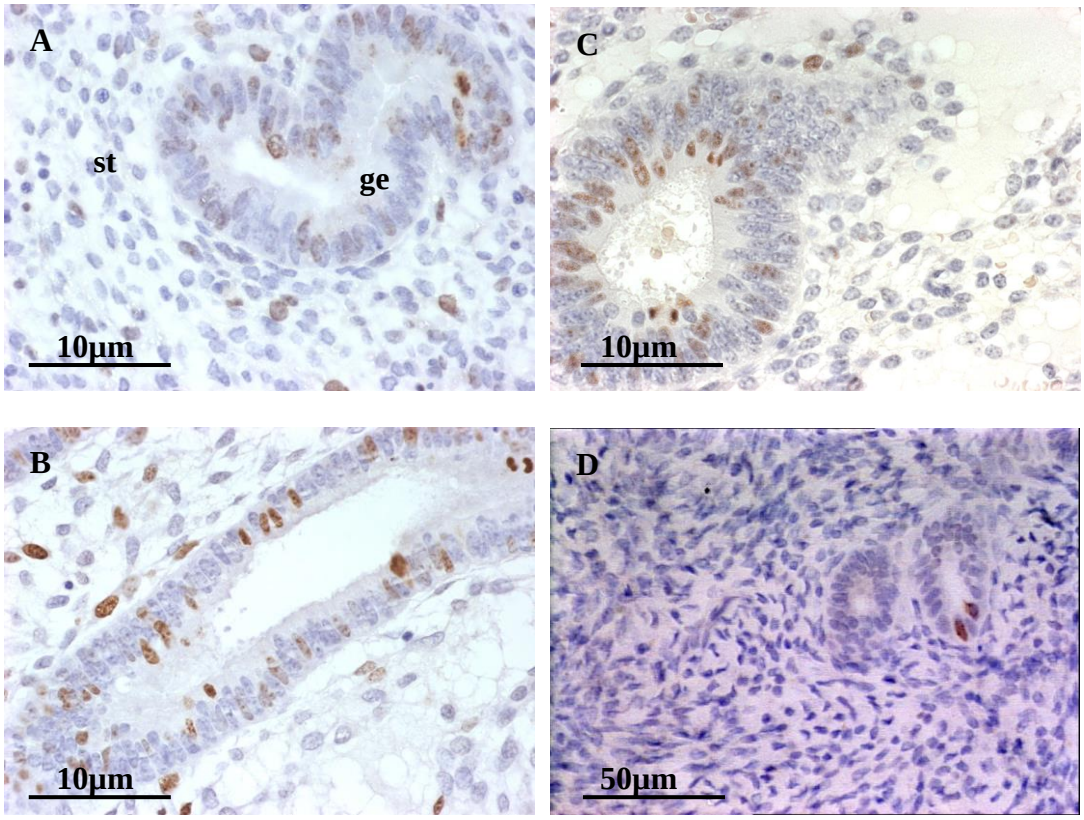


Figure 4.2 Ki67 immunoreactivity in the functionalis of the early-, mid- and late-proliferative phase and postmenopausal endometrium.

The photomicrographs show that Ki67 staining was greater in the early- (panel A) and mid-proliferative (panel B) phase stroma (st) when compared to the late-proliferative phase (panel C) stroma, whilst Ki67 staining levels in the glandular epithelium (ge) were similar throughout the proliferative phase. The Ki67 expression in the postmenopausal endometrium (panel D) was very low in both the stroma and glands, with very few cells being immunopositive. Images in panels A to C were taken at x400 magnification and that in panel D taken at x100 magnification.

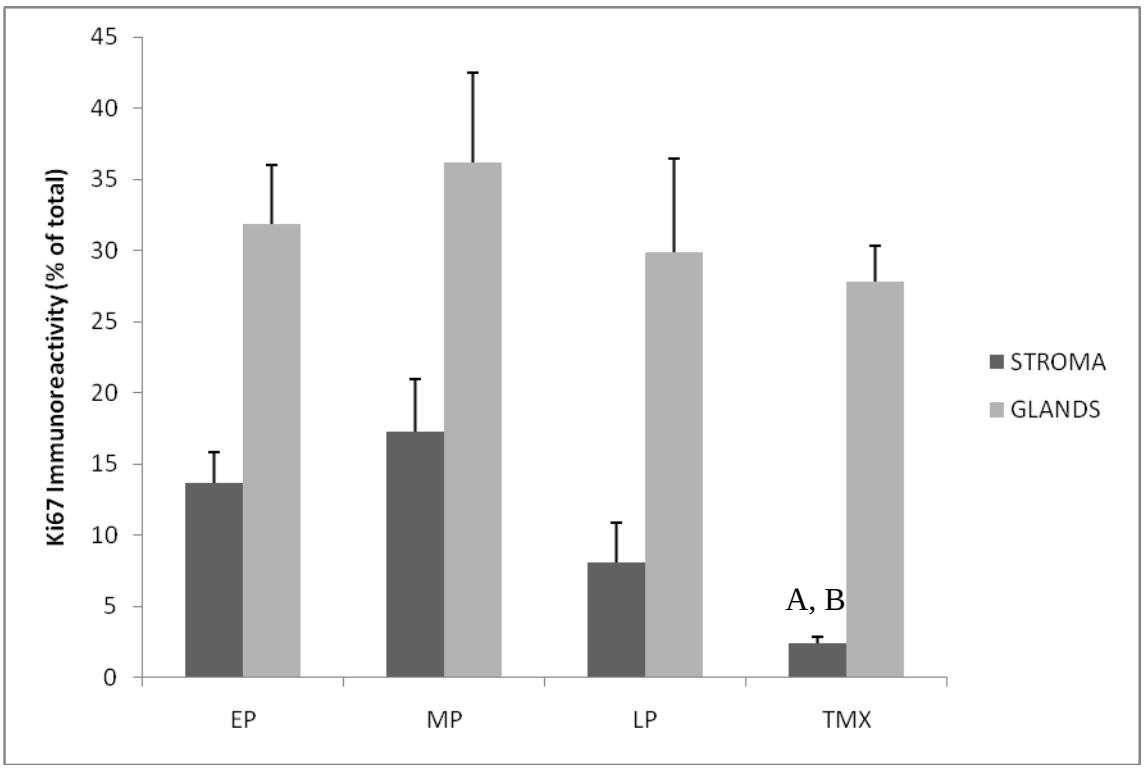


Figure 4.3 Histomorphometric analysis of Ki67 expression in the stroma and glandular epithelium of the functionalis.

The number of Ki67 positive cells in the stroma was lower than in the glands of all samples examined. The number of Ki67 positive cells in the glands of tamoxifen treated uteri was no different to the number of Ki67 positive cells in early- (EP), mid- (MP) and late- (LP) proliferative phases of the menstrual cycle, whilst the numbers of Ki67 positive epithelial cells in the stroma of the early- (EP) and mid- (MP) proliferative phases were significantly higher than that observed in tamoxifen (TMX) treated women, $p<0.001$. Although Ki67 positivity was also elevated in the late- (LP) proliferative gland when compared to the tamoxifen treated gland, the mean value was not statistically significant. Data are presented as mean ± SEM percentage of Ki67 expression. Statistical significance was determined using one way ANOVA analysis with Tukey's post test; A or B; $p<0.001$ when functionalis stroma in the tamoxifen treated samples were compared to EP (A) and MP (B).

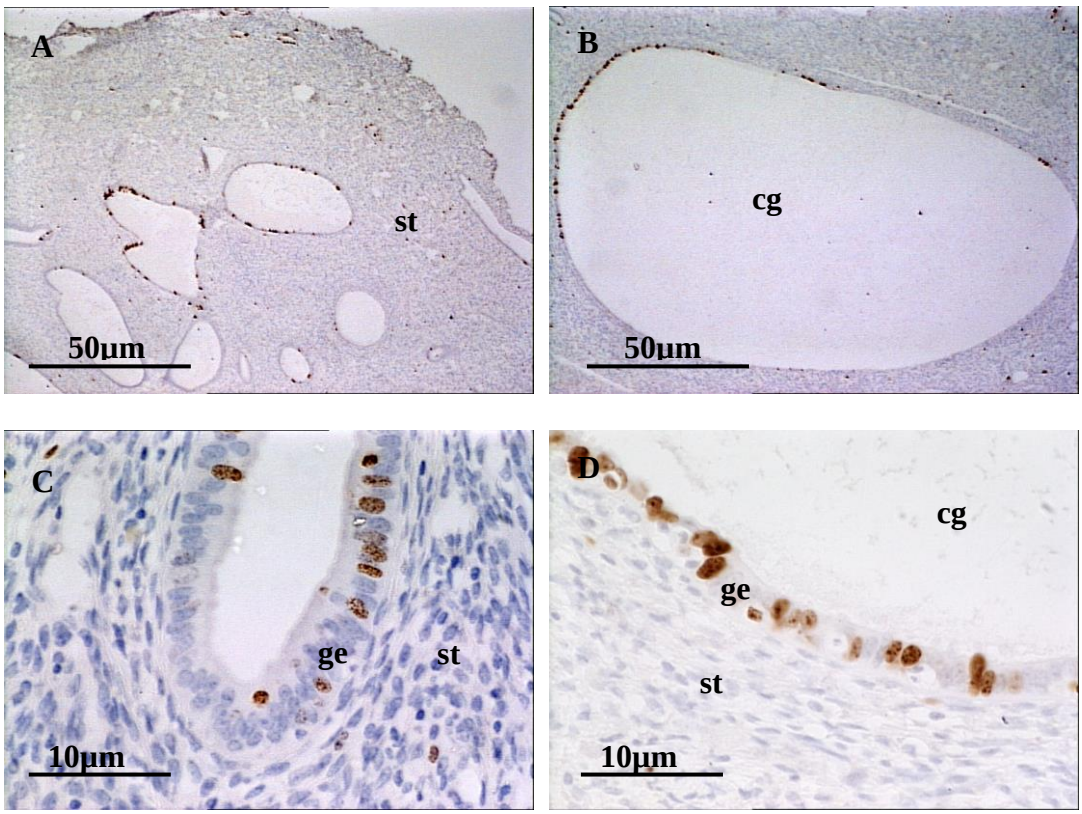


Figure 4.4 Ki67 expression in endometrial functionalis of uteri from women exposed to tamoxifen.

Nuclear Ki67 staining was reduced both in intensity and number of positive cells in the stroma (st) when compared to the staining in glandular epithelium (ge). Panels A & B obtained at low power show diffuse staining patterns in the stroma and cystic dilated (cg) glands. High power images (panels C & D) show the distribution of Ki67 staining in normal sized (ng) and epithelial cells (ge) lining the large cystic dilated (cg) glands. Note the different staining intensities in the various epithelial cells. Images in panels A and B were taken at x50 magnification and those in panels C and D at x400 magnification.

Category (N)	Functionalis		Basalis	
	Stroma	Glands	Stroma	Glands
EP (8)	13.7 (2.1)***	31.9 (4.1)	0.3 (0.1)	12.9 (3.7)
MP (8)	17.3 (3.7)***	36.2 (6.3)	0.4 (0.1)	11.1 (2.2)
LP (8)	8.1 (2.8)	29.9 (6.6)	0.3 (0.08)	3.0 (1.5)**
PM (8)	tissue not present	tissue not present	0.1 (0.02)*	2.0 (0.3)**
TMX (18)	2.4 (0.5)	27.8 (2.5)	1.4 (0.3)	14.0 (1.9)

Table 4.2 Ki67 expression in the glandular and stromal cells within the functionalis and basalis endometrium.

EP = early proliferative; MP = mid proliferative; LP = late proliferative; PM = postmenopausal; TMX = tamoxifen treated uteri.

The data are presented as the mean percentage positive cells (SEM).

Statistical significance was determined using one way ANOVA analysis with Tukey's post test;*$p<0.05$ when comparing postmenopausal levels to tamoxifen-treated levels** $p<0.01$ when TMX was compared to LP and PM levels; and *** $p<0.001$ when TMX was compared to the EP and MP phases of the menstrual cycle.

4.3.1.2 Basalis

A Menstrual Cycle

In the basalis stroma, Ki67 expression was very low ranging from 0.3% in the early proliferative to 0.4% in the mid proliferative phase (Figures 4.1 and 4.4).

Ki67 expression in the basalis glandular epithelium was higher in the early- and mid-proliferative phases, at 12.9% and 11.1%, respectively, compared to the late proliferative (3.0%) phases of the menstrual cycle.

B Postmenopausal Samples

In the basalis region of the postmenopausal endometrium, expression of Ki67 was 0.07% in the stroma and 2.0% in the glandular epithelium (Figure 4.5).

C Tamoxifen treated uteri

In the glandular epithelium of the basalis of uteri exposed to tamoxifen, 14.0% of cells expressed Ki67, and these levels were similar to the proliferative phases of the normal menstrual cycle (Table 4.2 and Figure 4.3). Endometrial stroma cells had comparatively low Ki67 immunoreactivity in the basalis (1.4%) (Table 4.2 and Figure 4.5).

Tamoxifen glandular Ki67 expression was significantly higher when compared to all phases of the menstrual cycle and postmenopausal uterus (Table 4.4).

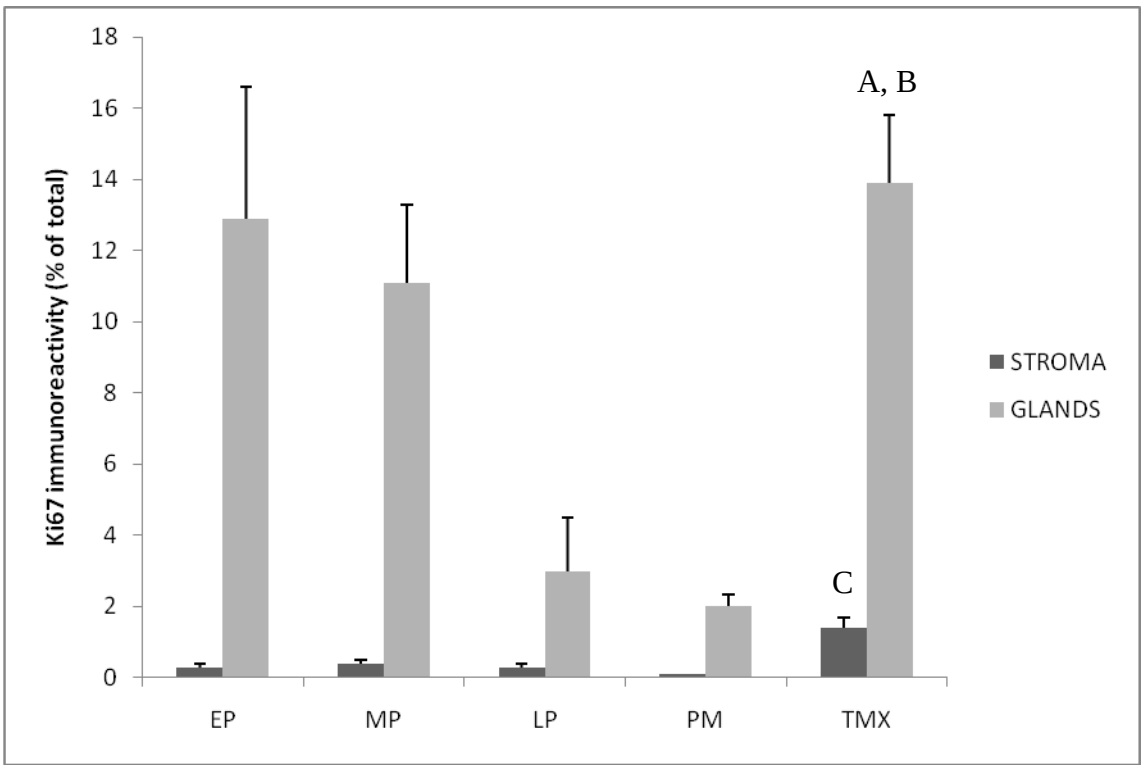


Figure 4.5 Histomorphometric analysis of Ki67 expression in the stroma and glandular epithelium within the basalis endometrium.

Ki-67 staining in the stroma of early- (EP), mid- (MP) and late- (LP) proliferative phase menstrual cycle samples was not different to the levels observed in postmenopausal and tamoxifen treated stroma, whereas the number of Ki-67 positive cells in the postmenopausal uteri (PM) and late-proliferative phase glands were significantly lower than that observed in the glands of uteri from tamoxifen treated women (TMX).

Data are presented as mean percentage of Ki67 expression ± SEM.

Statistical significance was determined using one way ANOVA analysis with Tukey's post test when using the tamoxifen treated sample as the test and the menstrual cycle and postmenopausal samples as the controls; A and B, $p<0.001$ and C, $p<0.005$, when basalis glands in the tamoxifen treated samples were compared to LP (A) and PM (B), $p<0.01$; C, $p<0.05$ when basalis stroma in tamoxifen treated samples were compared to PM samples.

4.3.2 BAX distribution

BAX, was observed in the cytoplasm of stromal, glandular epithelial and myometrial cells in the menstrual cycle and postmenopausal uteri, but absent from the stromal cells in tamoxifen treated uteri (Figures 4.6, 4.7 and 4.8).

4.3.2.1 Functionalis

A Menstrual Cycle

In the functionalis, BAX expression was higher in the glandular epithelial cell compared to the stromal cells (Figure 4.7), and the levels did not vary significantly during the menstrual cycle (Table 4.3).

B Tamoxifen treated uteri

In tamoxifen treated uteri, endometrial stromal cells of the functionalis were negative for BAX (Table 4.3), and the BAX staining index for the glandular epithelial cells (3.5) was comparatively lower in the functionalis when compared to the menstrual cycle (Figures 4.7 and 4.8 and Table 4.3).

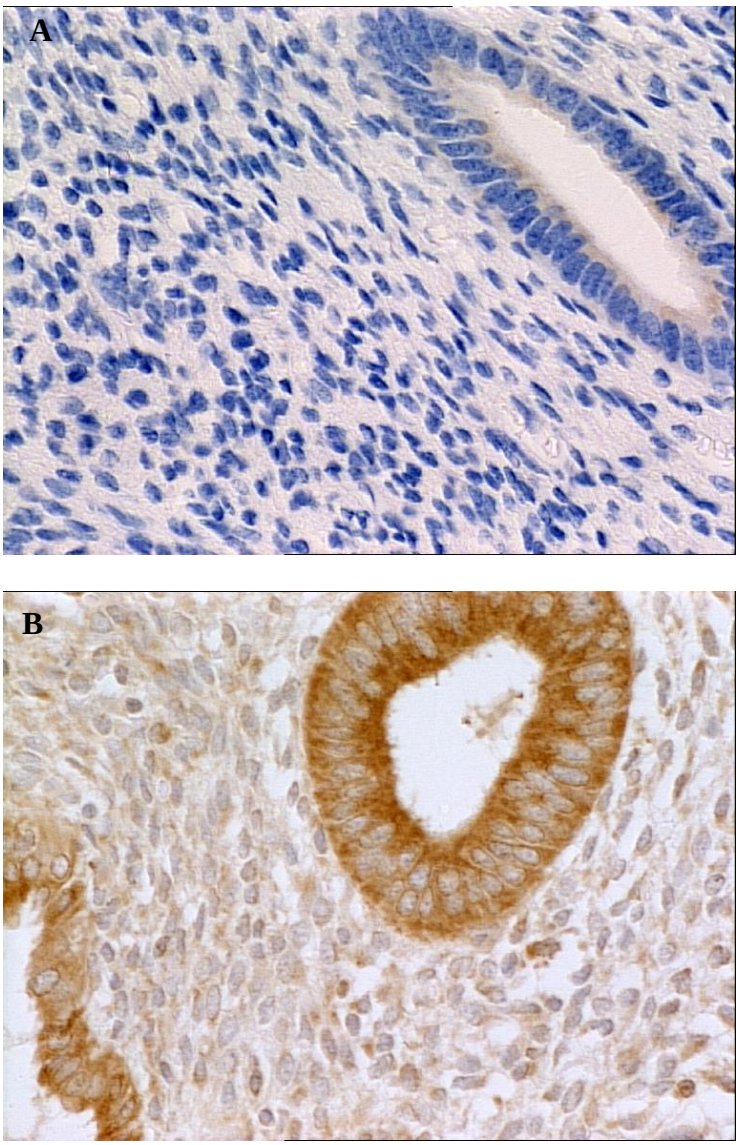


Figure 4.6 Demonstration of BAX antibody specificity

The tissue sample is an endometrium from the early proliferative phase of the menstrual cycle.

Panel A shows the IgG control (2.0 µg/ml) and panel B shows BAX (2.0 µg/ml). Both images were taken at x400 magnification.

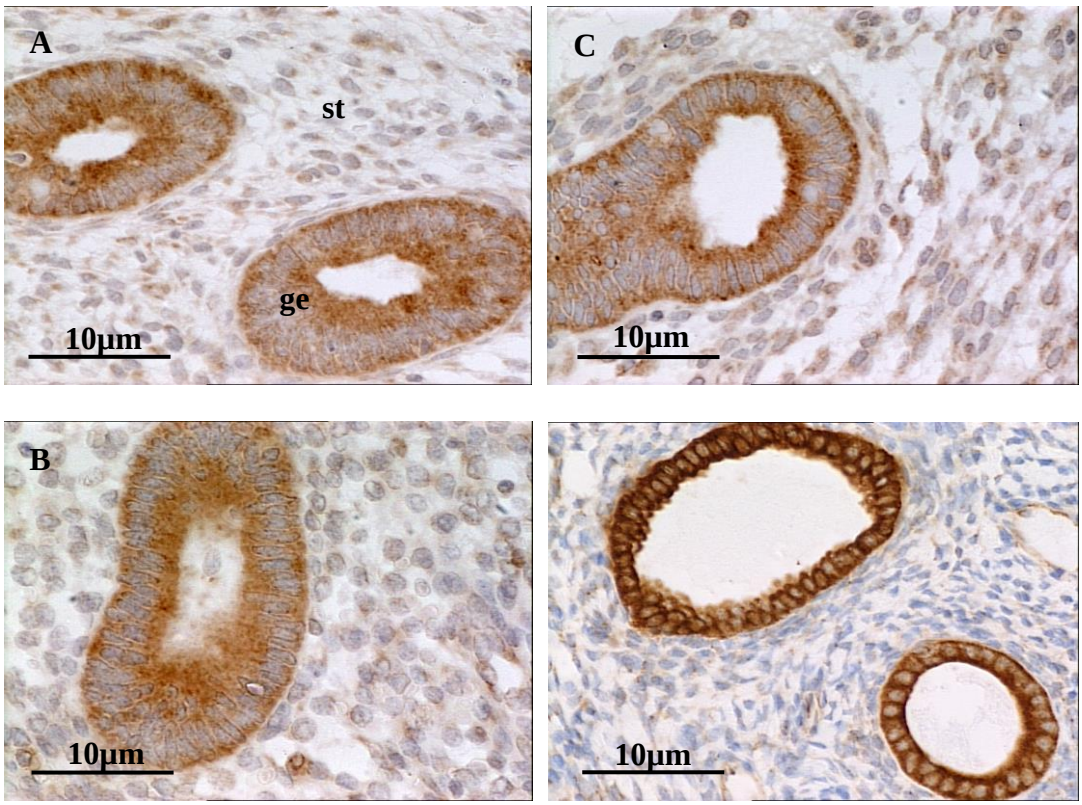


Figure 4.7 BAX immunoreactivity in proliferative phase and postmenopausal endometrium.

In early- (panel A), mid- (panel B) and late- (panel C) proliferative phase endometrium BAX immunoreactivity was present on the basal and apical surfaces of the glandular epithelium (ge) with generalised diffuse cytoplasmic staining in the stromal (st) cells. By contrast, BAX immunoreactivity was of increased intensity and present throughout the glandular epithelial cells whilst being greatly reduced in the stroma of the postmenopausal endometrium (panel D). Note the smaller size of the glandular epithelial cells in the postmenopausal uteri when compared to the proliferative phase of the normal menstrual cycle. All panels show images taken at x 400 magnification.

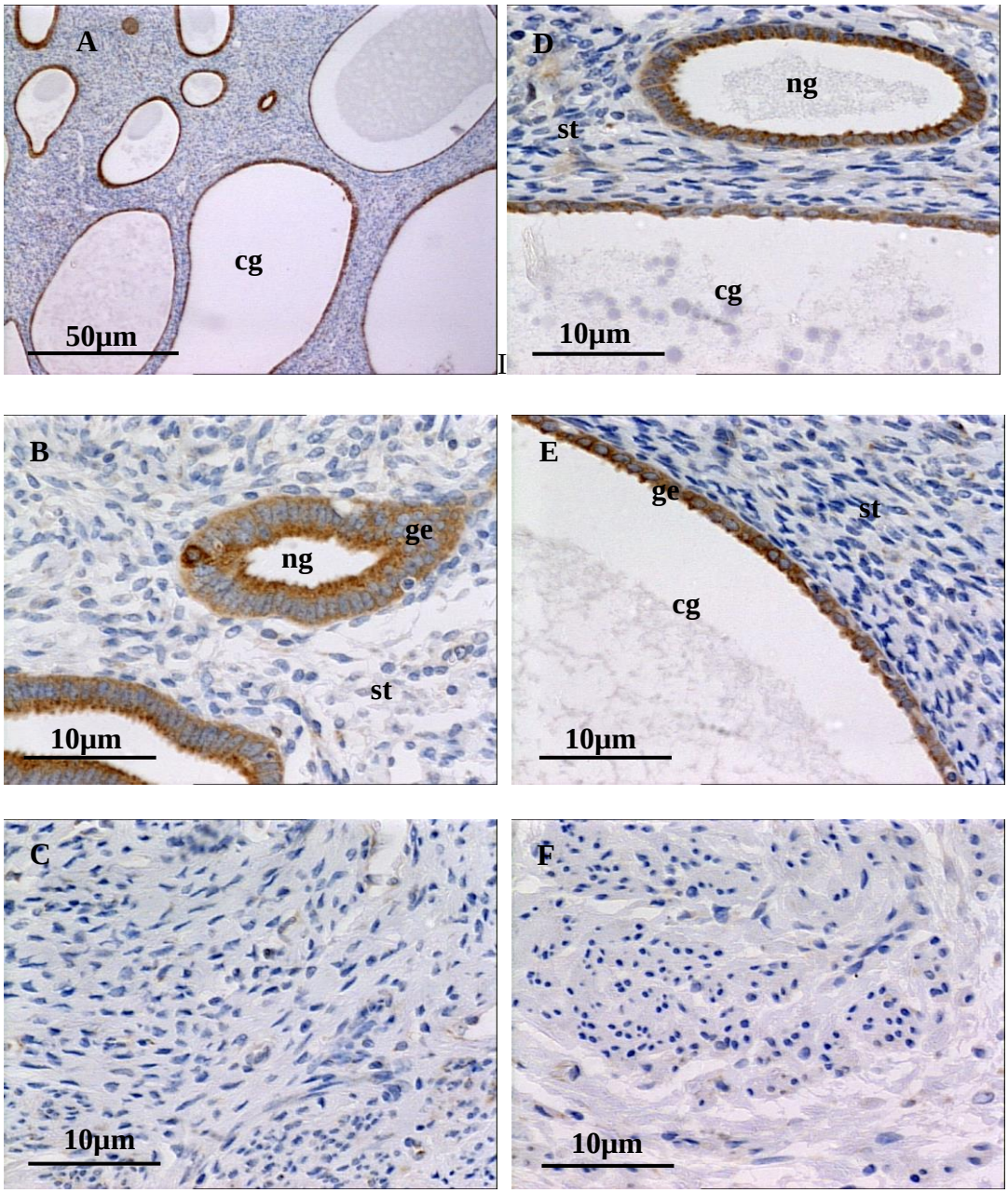


Figure 4.8 BAX immunoreactivity in the functionalis of tamoxifen treated uterus. Low power images of the tamoxifen treated uterus showed that BAX staining was mainly present in the glandular epithelium (panel A) and that the stroma (st) was negative (panels A, B, D & E) Similarly no BAX staining was observed in the inner (panel C) and outer (panel F) myometrium. By contrast, BAX immunoreactivity was present in the glandular epithelium (ge) of normal (ng) and cystic dilated (cg) glands albeit at low levels (panel A, B, D & E) in most of the cystic dilated glands. Panel A was taken at x50 magnification and panels B to F taken at x400 magnification.

Category (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Inner	Outer
EP (8)	2.6 (0.4)***	6 (0.0)**	2.1 (0.4)***	6 (0.0)***	1.8 (0.2)***	2 (0.2)***
MP (8)	2.6 (0.3)***	5.6 (0.4)	1.8 (0.3)***	5.6 (0.4)**	2 (0.0)***	1.9 (0.1)***
LP (8)	2.9 (0.1)***	6 (0.0)**	2.5 (0.3)***	6 (0.0)***	2 (0.0)***	2 (0.0)***
PM (8)	tissue not present	tissue not present	0.4 (0.2)	9 (0.0)***	1 (0.0)	1 (0.0)
TMX (18)	0	3.5 (0.3)	0	2.8 (0.1)	0	0

Table 4.3 BAX expression in the glandular and stromal cells in the functional and basal endometrium and myometrium.

Staining index = % positive cells X Staining intensity of cells.

% positive cells were scored as; 1=0-33%; 2=34-66%; 3=67-100% and Staining intensity; 1=mild; 2=moderate; 3=strong. The data are presented as the mean staining index (SEM).

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test when comparing menstrual cycle and postmenopausal staining to that of the tamoxifen treated uteri ; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

EP = early proliferative; MP =mid proliferative; LP = late proliferative; PM = postmenopausal; TMX = tamoxifen treated uteri.

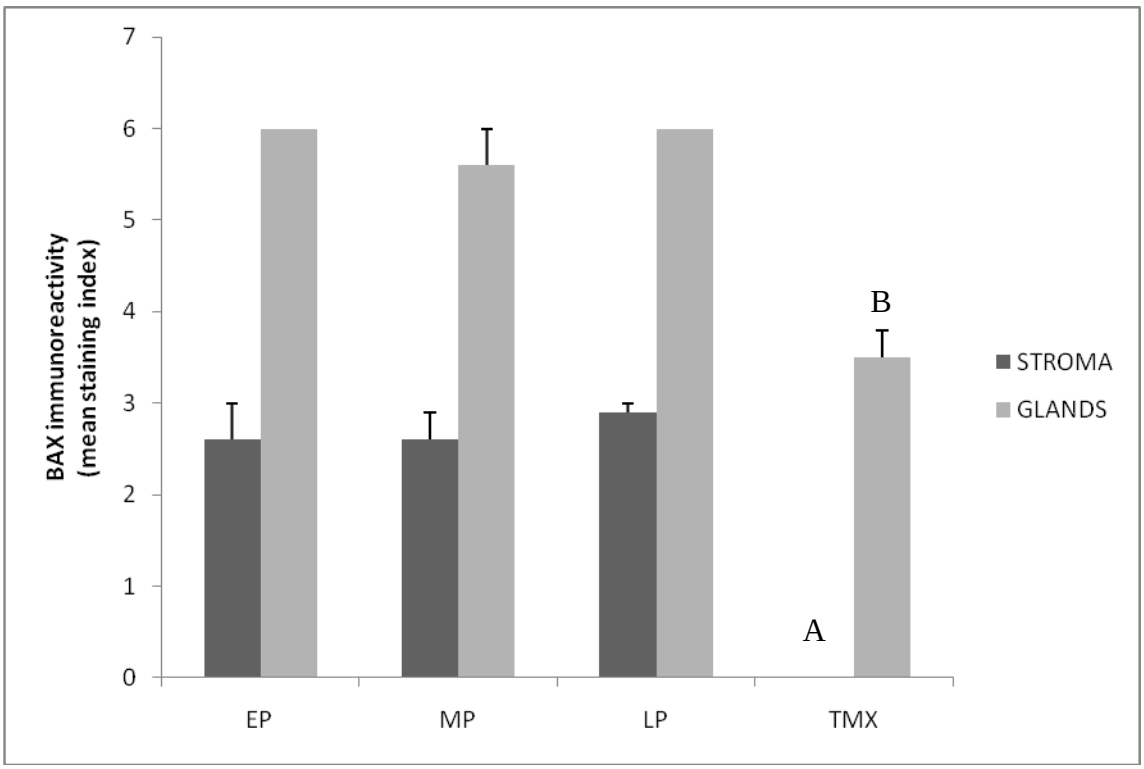


Figure 4.9 Histomorphometric analysis of BAX expression in the stroma and glandular epithelium of the functionalis in the proliferative phases of the menstrual cycle and tamoxifen treated women.

Data are presented as mean BAX staining index ± SEM.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test; A $p<0.001$ comparing the tamoxifen treated samples to the menstrual cycle samples ; B $p<0.01$ when comparing the tamoxifen treated samples to the EP and LP menstrual cycle samples.

Note the complete absence of BAX expression in the tamoxifen treated stroma and the significantly higher levels of BAX expression in the glands of early- (EP) and late- (LP) proliferative phases menstrual cycle. The levels are elevated in mid- (MP) proliferative phase glands but these levels were not statistically significantly different to the levels in the tamoxifen treated glands

4.3.2.2 Basalis

A Menstrual Cycle

In the basalis, BAX expression was higher in the glandular epithelial cell than in stromal cells, but did not vary through the proliferative phases of the menstrual cycle (Tables 4.3 and Figure 4.10).

B Postmenopausal samples

In the basal region of the postmenopausal endometrium, BAX expression was very low in the stroma (0.4), but also showed the maximum staining index in the glandular epithelium at 9. This was greater than that observed in the menstrual cycle and tamoxifen treated uteri (Figure 4.7 & 4.10).

C Tamoxifen treated uteri

The stromal cells of the basalis of uteri exposed to tamoxifen treated were negative for BAX (Table 4.3). In the glandular epithelium of tamoxifen treated uteri BAX expression was lower (2.8) than in the functionalis. Endometrial glandular epithelium had significantly lower BAX ($p<0.05$) compared to the postmenopausal and proliferative phases of the menstrual cycle (Figure 4.10).

4.3.2.3 Myometrium

A Menstrual Cycle

The inner myometrial cells BAX expression ranged between 1.8 and 2.0 in the menstrual cycle uteri (Table 4.3). BAX expression in the outer myometrium was very similar to the inner myometrium and menstrual cycle ranging between 1.9 and 2.0 (Table 4.3).

B Postmenopausal samples

The BAX expression was 1.0 in both the inner and outer zones of the myometrium (Table 4.3). This was not significantly different from the BAX expression in the tamoxifen treated uteri.

C Tamoxifen treated uteri

The inner and outer myometrial cells of uteri exposed to tamoxifen were BAX negative (Table 4.3 and Figure 4.8). This value was significantly different ($p<0.01$) when compared to all phases of the menstrual cycle (Table 4.3).

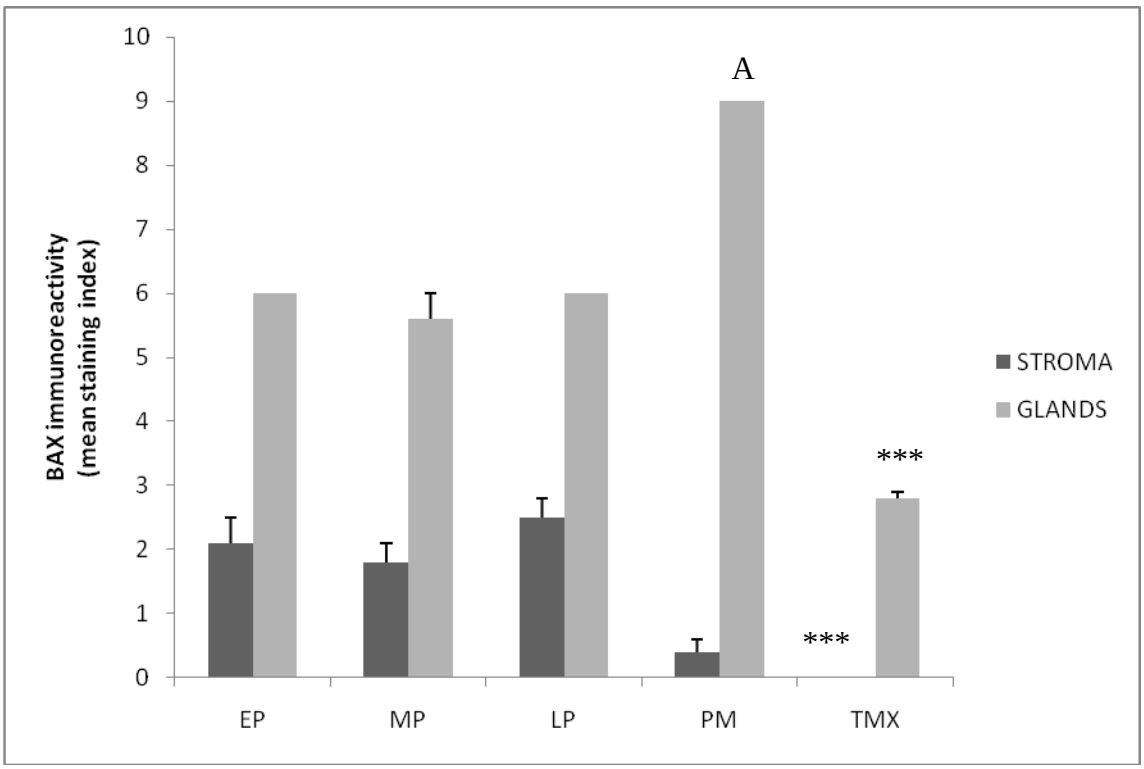


Figure 4.10 Histomorphometric analysis of BAX expression in the stroma and glandular epithelium of the basalis in the proliferative phases of the menstrual cycle, postmenopausal and tamoxifen treated women.

Data are presented as mean staining index ± SEM.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test; ** $p<0.01$ and ***$p=0.001$ when comparing the staining of tamoxifen treated uteri to that of the menstrual cycle samples; ** $p<0.01$ versus MP; ***$p=0.001$ versus EP and LP.

A, $p<0.001$ when comparing the BAX staining in functionalis glands of tamoxifen treated samples with that in the glands of postmenopausal samples.

4.3.3 Bcl2 distribution

Bcl2 was observed in the cytoplasm of stromal, glandular epithelial (Figures 4.11 and 4.12) and myometrial cells of samples taken from the menstrual cycle, and was variable in its expression in the postmenopausal (Figure 4.12) and tamoxifen treated uteri (Figure 4.13).

4.3.3.1 Functionalis

A Menstrual Cycle

Throughout the menstrual cycle the Bcl2 staining index was higher in the glands than in the stroma in the functionalis (Table 4.4).

Stromal cells Bcl2 expression ranged between 0.1 and 0.6, and in the glandular epithelial cells between 2.6 and 4.0 through the proliferative phases of the menstrual cycle (Table 4.4).

B Tamoxifen treated uteri

The Bcl2 expression in the glandular epithelial cells of the functionalis of tamoxifen treated endometrium was 4.7 (Table 4.4), this was similar to that observed in the proliferative phases (Figure 4.11).

The tamoxifen treated endometrium demonstrated no Bcl2 expression in the stromal cells of the functionalis (Table 4.4 & Figure 4.13).

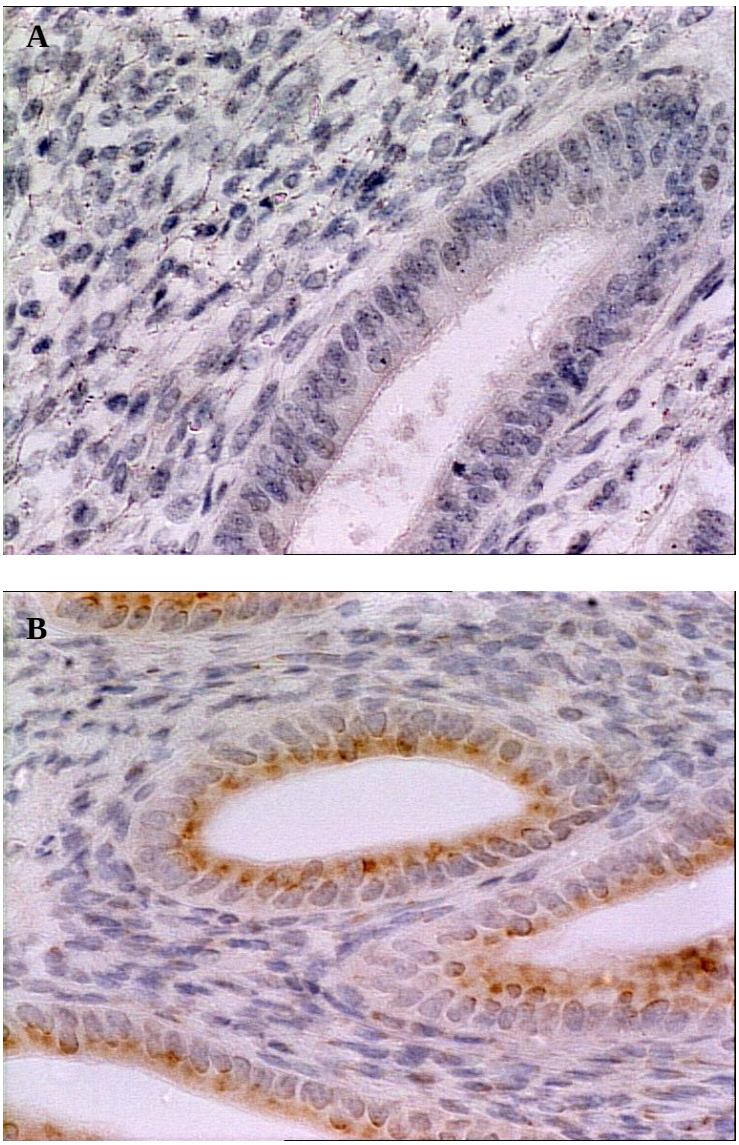


Figure 4.11 Demonstration of Bcl-2 antibody specificity

The tissue sample is an endometrium sample from the early proliferative phase of the menstrual cycle.

Panel A shows the IgG control (0.5 µg/ml) and panel B shows Bcl2 (0.5 µg/ml). Both images were taken at x400 magnification.

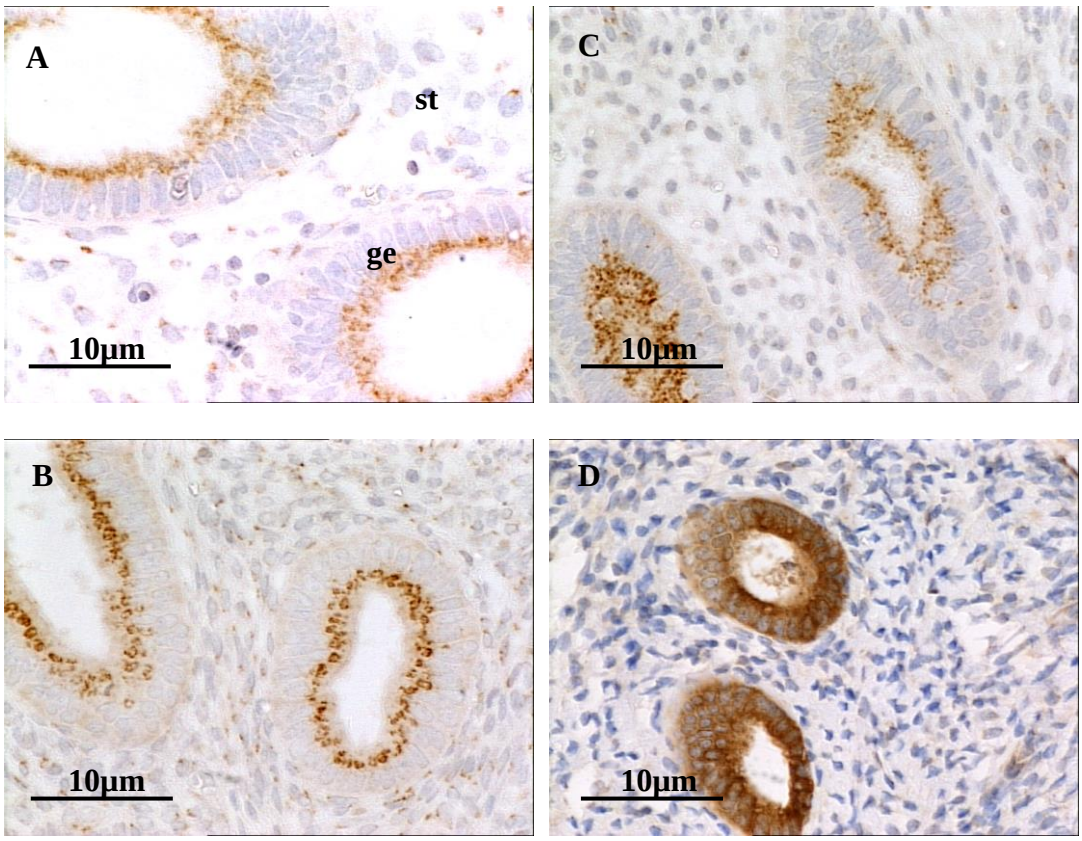


Figure 4.12 Bcl2 immunoreactivity in the functionalis of proliferative phase and postmenopausal basal endometrium.

Bcl2 staining was observed to be present on the apical surfaces of the glandular epithelium (ge) with low level staining in the stromal (st) cells of the early- (panel A), mid- (panel B) and late- (panel C) proliferative phase basal endometrium. By contrast, Bcl2 immunoreactivity was of increased intensity and present throughout the glandular epithelial cell and absent in the stroma of postmenopausal basal endometrium (panel D). In all panels the magnification is x400.

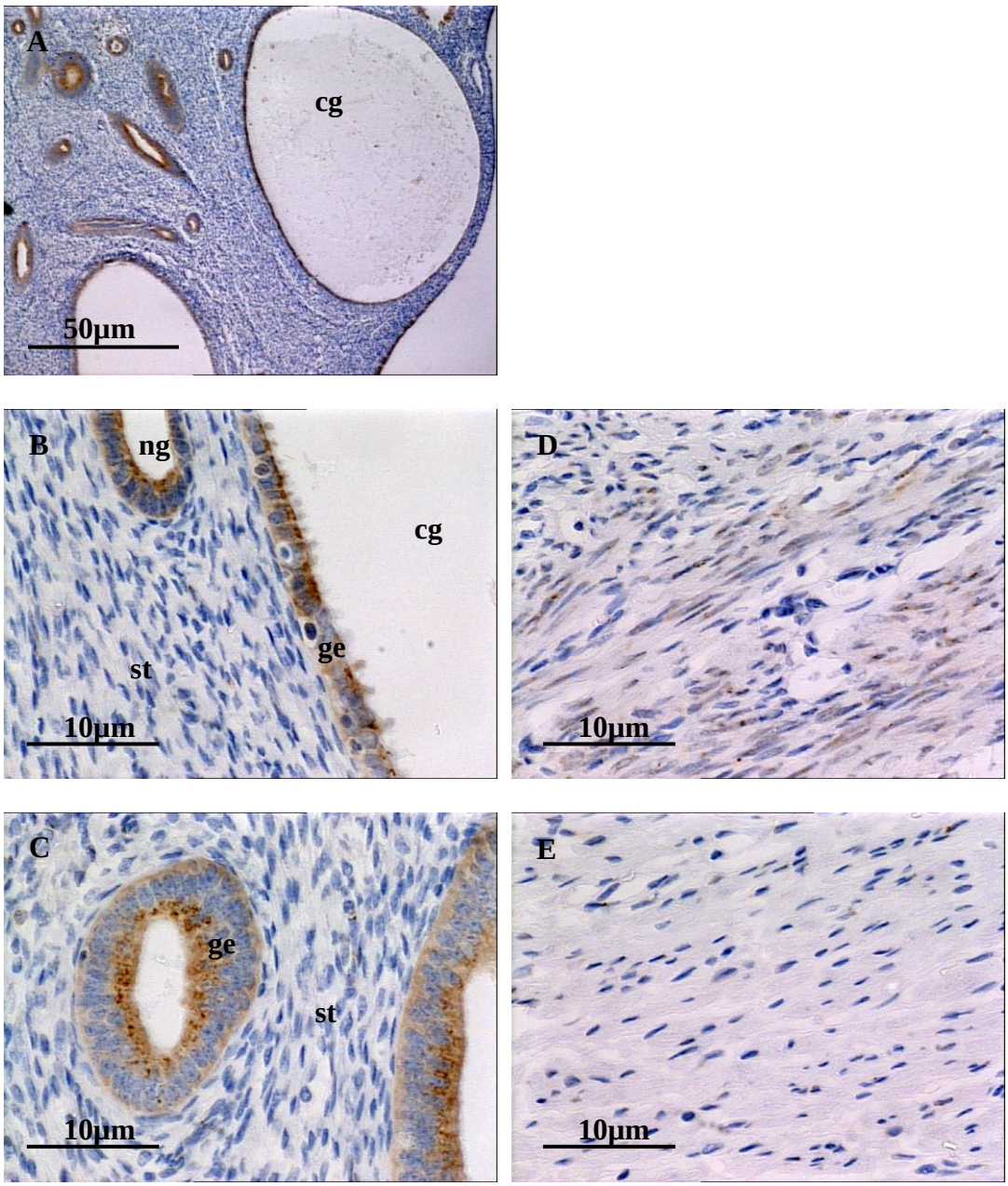


Figure 4.13 Bcl2 immunoreactivity in the functionalis and myometrium of the tamoxifen treated uterus. Bcl2 staining was absent from the stroma (st) (panels A, B & C) and outer (panel E) myometrium, whilst showing a low level of Bcl2 staining in the inner (panel D) myometrium. Bcl2 immunoreactivity was present in the glandular epithelium (ge) of normal (ng) and cystic dilated (cg) glands (panel A, B, & C). The image in panel A was taken at x50 magnification and panels B to F taken at x400 magnification.

Category (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Junctional	Outer
EP (8)	0.1 (0.1)	2.6 (0.2)	0.5 (0.2)	5.3 (0.5)	0	0.1 (0.1)
MP (8)	0.6 0.2)***	4.0 (0.6)	1.3 (0.3)***	6 (0.0)	0.8 (0.2)	0.1 (0.1)
LP (8)	0.3 (0.2)	3.9 (0.6)	0.6 (0.3)	4.9 (0.6)	0.8 (0.3)	0.1 (0.1)
PM (8)	tissue not present	tissue not present	0	3.3 (0.6)	0	0
TMX (18)	0	4.7 (0.3)	0	4.7 (0.3)	0.2 (0.1)	0

Table 4.4 Bcl2 expression in the glandular and stromal cells in the functional and basal endometrium and myometrium.

Significance was determined by one way ANOVA with Tukey's honestly significant difference post test when comparing tamoxifen treated to menstrual cycle and postmenopausal women; *** $p<0.001$; ** $p<0.01$; * $p<0.05$. The data are presented as the mean Bcl2 staining index (SEM).

EP = early proliferative; MP = mid proliferative; LP = late proliferative; PM = postmenopausal; TMX = tamoxifen treated uteri. Numbers of samples (N) are shown in parantheses within each category.

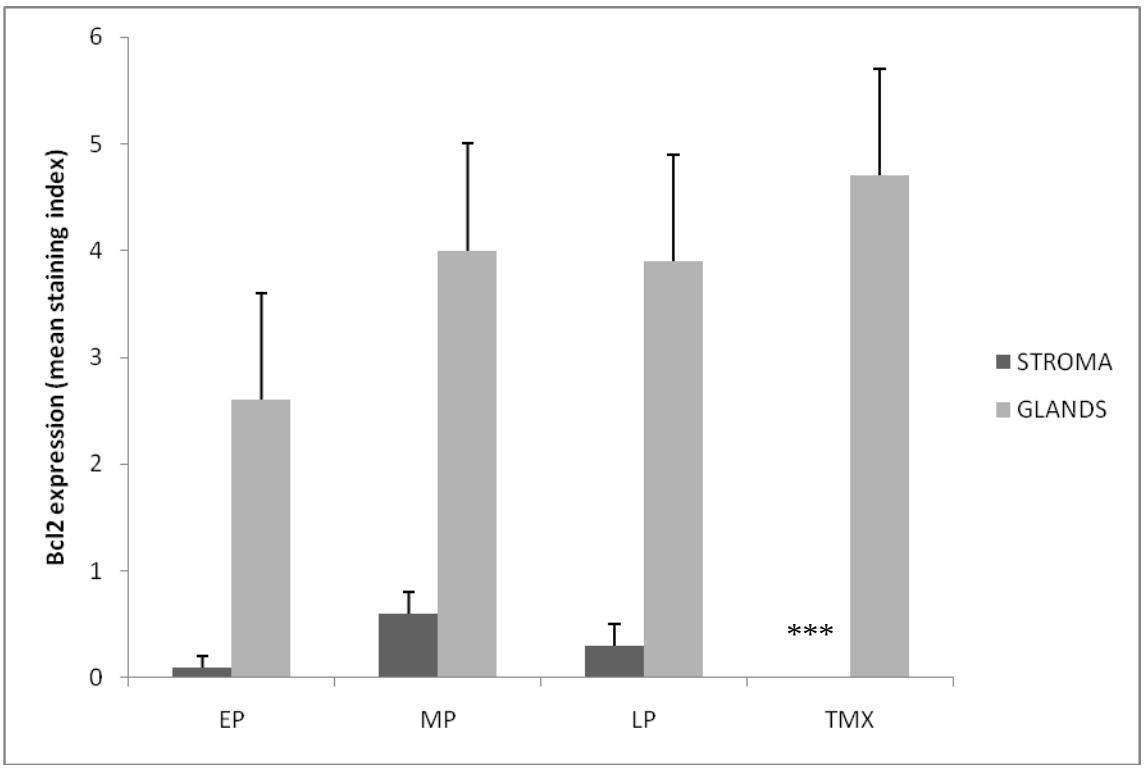


Figure 4.14 Histomorphometric analysis of Bcl2 expression in the stroma and glandular epithelium within the functionalis in the proliferative phases of the menstrual cycle and tamoxifen treated women.

Data are presented as mean Bcl2 staining index ± SEM.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test; ***$p<0.001$ when the tamoxifen treated samples were compared to MP sample. All other tests were not statistically significantly different

4.3.3.2 Basalis

A Menstrual Cycle

Throughout the menstrual cycle the Bcl2 staining index was greater in the glandular epithelium than in the stroma in the basalis (Figure 4.15).

In the stromal cells Bcl2 expression ranged between 0.5 and 1.3, and in the glandular epithelium ranged between 4.9 and 6.0 through the proliferative phases of the menstrual cycle (Table 4.4). The Bcl2 expression in the glandular epithelium was greater in the basal than in the functional endometrium (Table 4.4).

B Postmenopausal samples

In the basal region of the postmenopausal endometrium, Bcl2 staining was not observed in the stromal cells, the glandular epithelium expressed Bcl2 with a staining index of 3.3, which was lower than that observed in the proliferative phases of the menstrual cycle or in tamoxifen treated uteri (Table 4.12).

C Tamoxifen treated uteri

In the glandular epithelium of the basalis of tamoxifen exposed uteri Bcl2 staining index was 4.7 (Table 4.4). There was no difference when tamoxifen exposed endometrium was compared to the postmenopausal or menstrual uteri (Table 4.4). The tamoxifen treated endometrium demonstrated no staining for Bcl2 in the stromal cells of the basalis (Table 4.4).

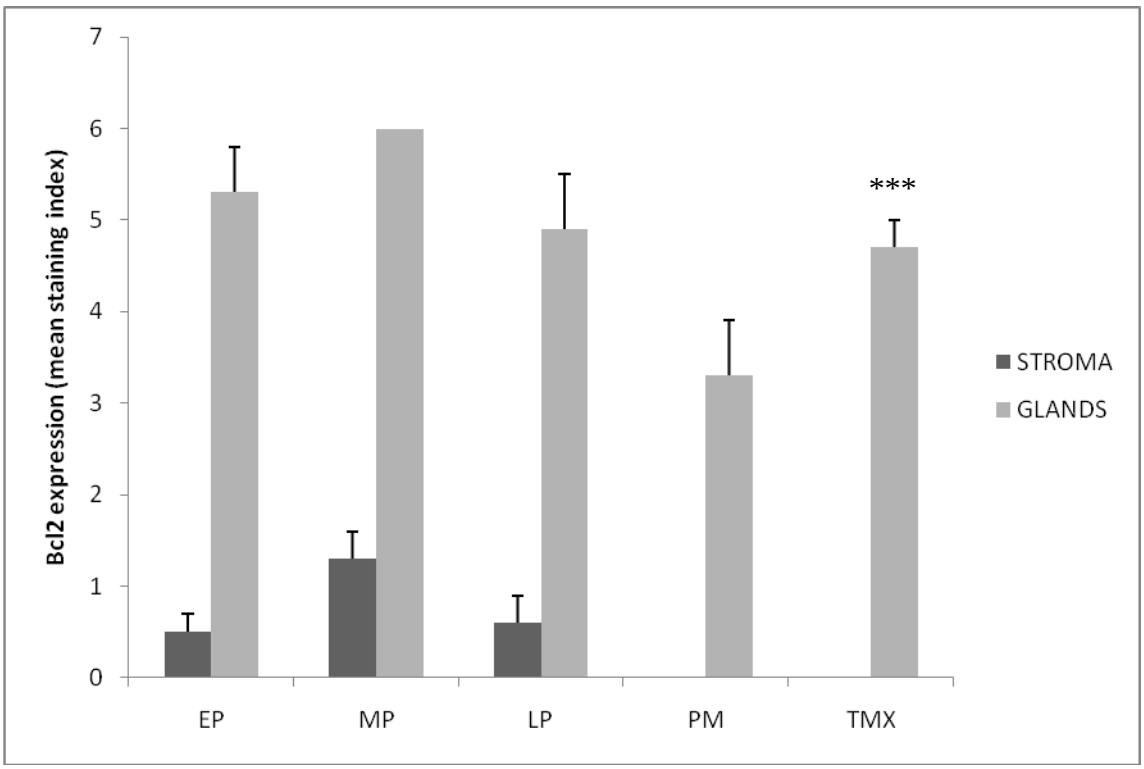


Figure 4.15 Histomorphometric analysis of Bcl2 expression in the stroma and glandular epithelium in the basalis of the proliferative phases of the menstrual cycle, postmenopausal and tamoxifen treated uteri.

Data are presented as mean Bcl2 expression ± SEM.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test; *** $p<0.001$ when comparing the tamoxifen treated uteri to that of the MP menstrual cycle glandular staining ,. Note the absence of Bcl2 staining in the stroma of the tamoxifen treated and postmenopausal uteri and lack of difference in Bcl2 scores in the glands throughout the control samples.

4.3.3.3 Myometrium

A Menstrual Cycle

Bcl2 expression in the inner myometrial cells ranged between 0 and 0.8 through the menstrual cycle. In the outer myometrium, Bcl2 expression was uniform at 0.1 throughout the proliferative phase (Table 4.4).

B Postmenopausal samples

The inner and outer zones of the myometrium of postmenopausal uteri were negative for Bcl2 (Table 4.4).

C Tamoxifen treated uteri

The inner myometrium in the tamoxifen uteri expressed low levels of Bcl2 (0.2), whereas it was negative for Bcl2 in the outer myometrial cells (Table 4.4 & Figure 4.13).

4.3.4 BAX/Bcl2 Ratios

This section describes the results of the pro-apoptotic/anti-apoptotic (BAX/Bcl2) ratio, which is thought to be an important index in describing the state of apoptosis within the endometrium as well as in other tissues (Tao et al., 1997; Vaskivuo et al., 2002).

4.3.4.1 Functionalis

A Menstrual Cycle

The BAX/Bcl2 ratio was higher in the stroma compared to the glandular epithelium in the functionalis throughout the cycle. In the early proliferative phase the ratio was 21, and decreased by about half to 10.5 and 11.5 in the mid- and late-proliferative phases, respectively.

B Tamoxifen treated uteri

In the tamoxifen exposed functionalis stromal cells, the BAX/Bcl2 ratio was zero. However, the BAX/Bcl2 ratio in the glandular epithelium was 0.7, and this ratio was considerably lower than that in any phase of the menstrual cycle (Table 4.5).

4.3.4.2 Basalis

A Menstrual Cycle

In the basalis the BAX/Bcl2 ratio was higher in the stroma than in the glandular epithelium, and lower than that observed in the functionalis. In the stromal cells there was no distinct pattern to the BAX/Bcl2 ratio, with the ratio ranging from the 1.4 to 4.3 in the proliferative phase (Table 4.5).

In the glandular epithelial cells, the BAX/Bcl2 ratio was similar to that observed in the functionalis and ranged between 0.9 and 1.1 in the proliferative phase (Table 4.5).

B Postmenopausal samples

The BAX/Bcl2 ratio in the postmenopausal stromal cells was 0.4/0, which I have interpreted as being infinitely high BAX expression. In the glandular epithelium the BAX/Bcl2 ratio was greater (2.8) than the proliferative phases of the cycle and tamoxifen exposed uteri (Table 4.5).

C Tamoxifen treated uteri

In the tamoxifen exposed basalis stromal cells, the BAX/Bcl2 ratio was zero. The BAX/Bcl2 ratio in the glandular epithelium was 0.7 and this was considerably lower than that observed in the menstrual cycle or postmenopausal uteri (Table 4.5).

4.3.4.3 Myometrium

A Menstrual Cycle

In the inner myometrium, there was no distinct pattern to the BAX/Bcl2 ratio (Table 4.5). In the proliferative phase, the range was 2.7 to 1.8/0 (+ infinity). The outer myometrium started at a higher BAX/Bcl2 ratio than that observed in the inner myometrium ranging from 15.0 to 16.0 (Table 4.5).

B Postmenopausal samples

The inner and outer myometrial cells had a BAX and Bcl2 ratio of 1/0 (+ infinity).

C Tamoxifen treated uteri

The inner and outer myometrial cells were negative for both BAX and Bcl2 and therefore the ratio was – infinity. Because – infinity could be considered a very small number equivalent to zero, for comparison purposes I set 0/0 to be equal to zero.

Category (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Inner	Outer
EP (8)	21	2.3	4.3	1.1	1.8/0	16
MP (8)	10.5	1.4	1.4	0.9	2.7	15.0
LP (8)	11.5	1.6	4.0	1.2	2.7	16.0
PM (8)	tissue not present	tissue not present	0.4/0	2.8	1/0	1/0
TMX (18)	0/0	0.7	0/0	0.7	0.2/0	0/0

Table 4.5 BAX/Bcl2 ratios for glandular and stromal cells within the functional and basal endometrium and for the inner and outer myometrium.

4.4 Discussion

In this chapter, I have shown that there is a difference in the expression of the proliferation marker Ki-67, the pro-apoptotic marker BAX and the anti-apoptotic marker Bcl2, such that cell density increases in the tamoxifen treated uterus. These data support the observations in chapter 2, where the uterus increased in size and density when treated with tamoxifen. The increase in Ki-67 expression also supports the role of tamoxifen as in inducer of cell proliferation in the endometrial glands. The data for the menstrual cycle correlated well with previously published data indicating that the methodology was appropriate (Mourits et al., 2002b).

Tamoxifen has oestrogenic actions on the endometrium and is associated with proliferative effects on the endometrium causing polyps, hyperplasia and carcinoma (Fisher et al., 1998). The risk of endometrial carcinoma increases two to seven fold; this risk is cumulative with long term use (Fornander et al., 1989; Fisher et al., 1994; Fisher et al., 1998; Mignotte et al., 1998; Bernstein et al., 1999; Bergman et al., 2000).

This study demonstrated that tamoxifen increased proliferation in the glandular epithelium within both the functionalis and basalis. This is in keeping with its proposed pro-proliferative effect and its pro-oncogenic potential (Mourits et al., 2002b). By contrast, the pro-apoptotic factor BAX was expressed at very low levels in the tamoxifen uteri when compared to the atrophic, postmenopausal control or the proliferative phase of the menstrual cycle. At the same time, Bcl2, an anti-apoptotic factor, was at its highest levels in both the functionalis and basalis, even higher than that of the proliferative phase of the menstrual cycle. These data suggest that the alteration in the apoptosis and anti-apoptosis balance within these cells affect the size of the uterus when exposed to tamoxifen.

Oestrogen has been demonstrated to increase both Ki-67 and Bcl2 in endometrial glands (Gompel et al., 1994; Critchley et al., 1999; Mertens et al., 2002), whilst inhibiting BAX expression (Vaskivuo et al., 2002), suggesting similarities between the effects of oestrogen and tamoxifen in the glandular epithelium.

The pattern of Ki67 expression in the functionalis and basalis is in agreement with published literature (Garry et al., 2010). The highest Ki67 expression was noted in the glandular epithelium in the functionalis in the proliferative phases of the menstrual cycle (Hachisuga et al., 1999; Elkas et al., 2000; Duffy and Taylor, 2004; Cirpan et al., 2008).

The endometrial stroma showed lower proliferative activity compared to the glandular epithelium. The basalis stroma showed low proliferative activity as did the postmenopausal glandular epithelial and stromal cells. These findings are consistent with low cell turnover and low levels of oestrogen and are in agreement with published literature (Duffy and Taylor, 2004). Tamoxifen treated endometrium showed Ki67 immunostaining similar to that in the proliferative phase both in the functionalis and basalis. The stroma showed little proliferative activity in the functionalis and basalis. These results confirm that tamoxifen promotes proliferation of the glandular epithelial cells. There are reports of increased cell proliferation in benign tamoxifen associated endometrium (Mourits et al., 2002b; Tregon et al., 2003) and it is this increase in epithelial proiliferative activity which may increase the risk of developing endometrial cancer during tamoxifen administration. These data also showed that in the tamoxifen endometrium the ratio of Ki67 in the glandular epithelial to stromal cells was higher than the corresponding ratio in the normal proliferative endometrium, and this may explain the development of the

cystic dilated glands and the appearance of stromal fibrosis in the tamoxifen treated endometrium.

BAX is a member of the Bcl2 family of proteins that promotes cell apoptosis, whilst another member of this family Bcl2, is a cell death repressor. The two factors thus have opposite effects. It is the balance between the expression of cell apoptosis and cell survival factors that regulates apoptosis, and in turn, tissue homeostasis and remodelling.

In the present study, I have shown that BAX expression is similar in the basal and functional endometrium in the stromal and epithelial cells. The BAX expression in the glandular epithelial cells of the menstrual cycle showed no significant variation through the proliferative phase of the cycle, and a similar staining index was noted in the basal glands. The BAX expression in this study differs from the literature which states BAX expression is greater in the secretory phase endometrium compared to the proliferative phases in the glandular epithelium (Tao et al., 1997; Harada et al., 2004; Wei et al., 2005; Narkar et al., 2006). It is worth noting that studies by Narkar and colleagues and Wei and co-workers' were conducted in the primate endometrium. On the other hand, Vaskivuo and co-workers observed that BAX staining in the glandular epithelium was highest in the late proliferative and early secretory phases of the menstrual cycle and lower in the secretory phase (Vaskivuo et al., 2002). They observed greater immunostaining of BAX in the stroma of the proliferative compared to the secretory phase of the menstrual cycle. The stroma showed lower BAX expression in all phases compared to the epithelial cells in the basal and functional endometrium. The low BAX activity in the stroma was also shown by Wei and co-workers and Narkar and colleagues (Wei et al., 2005; Narkar et al., 2006) suggesting that the work in primate studies is applicable to the human studies.

Postmenopausal glandular epithelial cells had the highest BAX staining index and this coupled with the low proliferation indicates no cell growth but possible cell loss. In the tamoxifen endometrium there was no BAX detected in the stroma and low staining index for BAX in the glandular epithelium indicating low apoptotic activity. No study to date has described BAX expression in the tamoxifen treated endometrium, although others have tried to evaluate apoptosis using other methods. For example, Mourits and co-workers evaluated apoptosis in postmenopausal endometrium exposed to tamoxifen immunohistochemically using monoclonal antibodies to M30 and caspase-3. They found that both caspase-3 and M30 were negative in benign tamoxifen endometrium (Mourits et al., 2002a) and observed no difference in the apoptotic index in benign and malignant endometrium in tamoxifen users compared to non users. They also noted that the ratio of apoptotic index to proliferative index was lower in benign endometrium from tamoxifen user compared to non users, indicating that tamoxifen induced proliferation is not matched with an increase in cell death.

Recently, Cirpan and colleagues showed that the apoptotic markers, PTEN and CD95, were not significantly affected in the uteri of rats treated with tamoxifen for 20 days compared to non treated rats (Cirpan et al., 2008). However, they did show an increase in Ki67 expression following tamoxifen administration compared to the control group, confirming Mourits' conclusion of proliferation induction by tamoxifen. Moreover, these data also indicate that there was no increase in apoptosis in the tamoxifen treated rat endometrium, supporting the data presented in this chapter.

I also examined the expression of Bcl2 in the menstrual cycle, postmenopausal and tamoxifen treated uteri and showed that Bcl2 staining was higher in the glandular

epithelium than the stroma. In agreement with published literature, the proliferative phase showed high levels of Bcl2 expression (Tao et al., 1997; Duffy and Taylor, 2004; Wei et al., 2005). Bcl2 expression was slightly higher in the glandular endometrium of the basal compared to the functional endometrium; this was also noted by Rogers and colleagues (Rogers et al., 2000). My interpretation of these data is that because the basal endometrium remains constant throughout the menstrual cycle then there is a lower requirement for apoptosis to occur in this layer. The postmenopausal endometrium was negative for Bcl2 in the stroma and showed a low staining index in the glandular epithelium. Duffy and colleagues also noted the stroma in inactive atrophic endometrium to be mostly negative or have low Bcl2 in the stroma (Duffy and Taylor, 2004), supporting my findings. The endometrial stroma from tamoxifen treated uteri was negative for Bcl2, although Bcl2 staining index in the glandular epithelium in the basalis and the functionalis was similar to the proliferative phase of the menstrual cycle, again supporting the findings in this study. There is no known link between vaginal bleeding and the expression of Bcl2 (Habiba et al., 1996).

The BAX/Bcl2 rheostat is thought to be an important factor in regulating apoptosis in the endometrium (Gompel et al., 1994; Tao et al., 1997; Critchley et al., 1999). Therefore, it was important to study the BAX/Bcl2 ratio, which determines cell survival or death following an apoptotic stimulus. BAX's pro-apoptotic signal is dependent on the formation of BAX homodimers. When BAX forms heterodimers with Bcl2 this prevents formation of the BAX homodimer and thus blocks apoptosis (Oltvai et al., 1993; Sedlak et al., 1995). In the present study, I have shown that the BAX/Bcl2 ratio in the glandular epithelium was at low levels in the proliferative phase of the menstrual cycle indicating an decrease in BAX activity. Tao and colleagues also reported an decrease in BAX/Bcl2 ratio

in the proliferative phase of the menstrual cycle, (Tao et al., 1997). The findings in my study also correlate with that of Vaskivuo and co-workers, who observed an decrease in the BAX/Bcl2 ratio supporting overall anti-apoptotic activity in the proliferative phase of the menstrual cycle (Vaskivuo et al., 2000).

The BAX/Bcl2 ratio in the postmenopausal glandular epithelium and stroma was high, supporting a predisposition to cellular loss. The tamoxifen endometrial stromal cells were negative for both BAX and Bcl2, suggesting that cells numbers in this part of the endometrium is not under apoptotic control by BAX and Bcl2 when compared to other parts of the tamoxifen treated uterus, suggesting tamoxifen has differential effect on the endometrium. For example, in the glandular epithelium the BAX/Bcl2 ratio was 0.7 in the functionalis and basalis, lower than that observed in the menstrual cycle and postmenopausal uteri, indicating an increase in Bcl2 expression confirming the cells resistance to apoptosis.

This is the first study describing the BAX/Bcl2 ratio in the tamoxifen treated endometrium. It is important to study the combined apoptotic and anti-apoptotic signal simultaneously to understand what is happening in a tissue at any given time, it also gives an overall understanding of whether a tissue is exhibiting increased or decreased tissue loss. Dysregulation of apoptosis has been observed in the endometrial hyperplasia-atypia-carcinoma pathway and associated with the genetic changes that in turn drive this proposed neoplastic path (Arends, 1999). This change in apoptosis regulation may account for the various endometrial pathologies associated with tamoxifen therapy.

In this study, the myometrium did not express the proliferative marker Ki67 in either the menstrual cycle, the postmenopausal or tamoxifen treated myometrium. Nisolle and colleagues observed low Ki67 immunostaining in the myometrium, with 0.98% of the cells being immunopositive in the proliferative phases of the menstrual cycle (Nisolle et al., 1999). Wu and colleagues showed greater Ki67 activity in the secretory phase compared to the proliferative phase, they also observed very low Ki67 activity in postmenopausal myometrium (Wu et al., 2000).

In this study, BAX expression in both the inner and outer myometrium was constant throughout the menstrual cycle at 1.8 to 2.0 (H-score), in the postmenopausal myometrium BAX immunoreactivity was lower, and negative in the tamoxifen myometrium. The findings in the literature in one report demonstrated lower BAX expression in the proliferative phase endometrium compared to the findings of this report and negative BAX in the secretory phase (Wu et al., 2002). Kovacs and colleagues observed similar BAX expression in the proliferative and secretory phases and slightly greater BAX activity in the postmenopausal myometrium, they used immunoblotting not immunohistochemistry (Kovacs et al., 2003). The Bcl2 immunostaining was less than 1% in all phases of the menstrual cycle myometrium, the outer myometrium showed lower Bcl2 staining than the junctional zone. The postmenopausal myometrium was negative for Bcl2, as was the tamoxifen outer myometrium, whereas the junctional zone had minimal Bcl2 activity at 0.2. The BAX/Bcl2 ratios in the myometrium in all phases of the menstrual cycle were higher in the outer myometrium than the junctional zone, suggesting greater BAX expression in the outer myometrium. Overall, there was greater apoptosis in the myometrium of the menstrual cycle and postmenopausal myometrium in both the inner and outer myometrium. In the tamoxifen treated uteri, the inner myometrium showed only

Bcl2 and no BAX expression, therefore the ratio would suggest anti-apoptosis or resistance to apoptosis occurs in the inner myometrium, as was observed in the endometrium.

This study has shown an increase in Ki67 expression, low BAX and high Bcl2 expression in tamoxifen treated uteri. This suggests that there is increased proliferation and simultaneous apoptosis failure which in turn prolongs the survival of cells after cell DNA damage and may therefore predispose this group of women to proliferative lesions associated with tamoxifen, such as endometrial polyps and cancers. Such changes must require a measure of tissue remodelling to accommodate the increased cell mass and this idea will be examined in the following chapter.

Chapter 5

Mesenchymal cell markers in the endometrium of tamoxifen treated postmenopausal women

5.1 Introduction

Because tamoxifen use is known to be associated with several endometrial pathologies, such as endometrial hyperplasia, adenomyosis (Cohen et al., 1997a), endometrial polyps and several types of cancers (Ismail, 1994), then a hypothesis that springs forth is that such endometrial changes, including the formation of large cystic glands, occurs through mechanisms that involve tissue remodelling. Previously it has been demonstrated that tamoxifen-induced adenomyosis and its associated stromal fibrosis is probably a consequence of disordered mesenchymal differentiation (Parrott et al., 2001). This has led to a new idea that during mesenchymal differentiation, the myometrial cells are derived from the transition of the stromal fibroblast through an intermediary myofibroblast to the fully differentiated myocyte (Parrott et al., 2001). The key molecules for defining this fibroblast-myofibroblast-smooth muscle cell transition are, vimentin, an intermediate filament protein only found in mesenchymal cells, α-smooth muscle actin (α-SMA), which is a marker for the smooth muscle cell and can distinguish myocytes from the stromal fibroblast, and tenascin-C, which is an extracellular glycoprotein that is up-regulated in some tissues that are undergoing tissue remodelling, such as those cells that are de-differentiated and proliferating. Examples of this are myofibroblasts in certain types of breast cancer (Pas et al., 2006; Hancox et al., 2009) and cells of the fetal membranes (Castellucci et al., 1991; Bell et al., 1999). Thus, tenascin-C may be considered as a marker of the myofibroblast.

To test the hypothesis that tamoxifen is affecting the fibroblast-myofibroblast-smooth muscle cell transition pathway in the uterus, the expression of a range of mesenchymal markers that have been used to differentiate cell types in that pathway were investigated in the normal menstrual cycle to establish the normal distribution and examined further in uteri obtained from untreated postmenopausal uteri and uteri from women treated with tamoxifen. The data generated may point towards a mechanism for the observed pathologies associated with tamoxifen use.

5.2 Materials & Methods

5.2.1 Antibodies

The primary antibodies used within this Chapter are summarized in Table 5.1.

5.2.2 Tissue collection, Patient groups and Sampling technique

The uterine endometrial and myometrial samples used have been detailed in Chapters 3, section 3.2.1, 3.2.2 and 3.2.3.

5.2.3 Immunohistochemistry

Immunohistochemistry for vimentin, α-smooth muscle actin and tenascin-C was performed as described in Chapter 3, section 3.2.6 with the exception that no antigen retrieval step was used for vimentin or α-smooth muscle actin methods, and that pepsin digestion was used for the staining of tenascin-C. For pepsin digestion, tissue sections were incubated with 0.4% pepsin in 10mM HCl at 37°C for 20 minutes.

5.2.4 Controls samples

All experiments included a negative control where the primary antibody was replaced with an equivalent concentration of mouse IgG. A positive control tissue for each experiment was also used for each experiment and included breast cancer for tenascin C, uterine myometrium for α-SMA and tonsil for vimentin.

5.2.5 Histomorphometric Analyses

Images of stained sections were captured using an Axioplan microscope (Carl Zeiss, Herts. UK) and a Sony DXC-151P colour camera system with illumination set at just under maximum exposure in the presence of daylight and light grey neutral density filters.

The immunostaining of the tissue sections was assessed for the staining distribution and intensity using a visual scoring system as follows:

Absent (-), very weak (-/+), weak (+), moderate (++), intense staining (+++) of the positively stained cells was recorded by image capture of 10 randomly selected fields per slide in the basal and functional endometrium, the inner and the outer myometrium.

Because staining of tenascin-C and α-smooth muscle actin was observed immediately surrounding the glands, the percentage of glands with this type of staining was calculated by counting the number of positive and negative glands.

Endothelial cells in the stromal compartment were omitted from the count.

Antigen	Clone Name	Antibody Type	Working Concentration (µg/ml)	Manufacturer
α-smooth muscle actin	1A4	Mouse monoclonal IgG_2a, kappa	1.4	Dako
Vimentin	V9	Mouse monoclonal IgG_1	3.87	Sigma
Tenascin	BC-24	Mouse monoclonal IgG_1	1.6	Sigma

Table 5.1 Sources of antibodies used for mesenchymal markers.

The clone name, antibody type and working concentrations are shown along with the supplier of the antibody.

5.3 Results

Vimentin, α-SMA and tenascin-C were all expressed in the uterus of all patient groups. The differences in the staining patterns for these markers are shown in Table 5.3 and will be described in detail here.

5.3.1 Vimentin distribution

5.3.1.1 Functionalis

A Menstrual Cycle

Specific vimentin immunoreactivity (Figure 5.1) was observed in the cytoplasm of the luminal epithelial cell in the proliferative of the menstrual cycle (Figure 5.2).

In the endometrial functionalis, vimentin immunoreactivity was observed in the cytoplasm of the glandular epithelium in all phases of the menstrual cycle. In the proliferative phases the staining was intense.

In the early proliferative phase stromal cell, immunoreactivity was "patchy" and noted to be concentrated subluminally in approximately half of the samples. The stromal staining intensified in the mid- and late-proliferative phases (Figure 5.2).

Category (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Inner	Outer
EP (8)	++	+	+/-	++	+	+
MP (8)	+++	++	+	++	+	+
LP (8)	+++	++	+	++	+	+
PM (8)	tissue not present	tissue not present	+	++	+	+
TMX (18)	+/-	+	+	++	+	+

Table 5.2 Vimentin expression in the glandular and stromal cells in the functionalis, basalis and myometrium.

Data are presented as (-) absent, (+/-) very weak, (+) weak, (++) moderate, or (+++) intense staining.

Numbers of samples (N) assessed in each category are shown in parentheses.

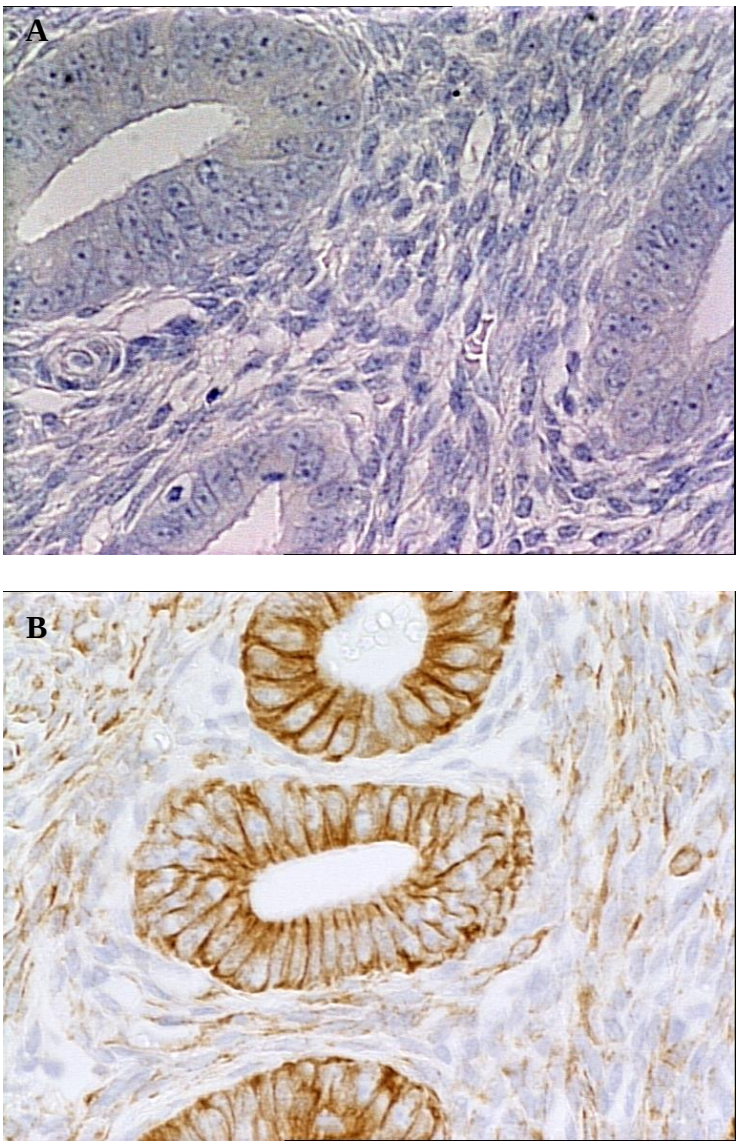


Figure 5.1 Demonstration of Vimentin antibody specificity

The tissue sample is an endometrium from the early proliferative phase of the menstrual cycle.

Panel A shows the IgG control (3.87 µg/ml) and Panel B shows vimentin (3.87 µg/ml). Both images were taken at x400 magnification.

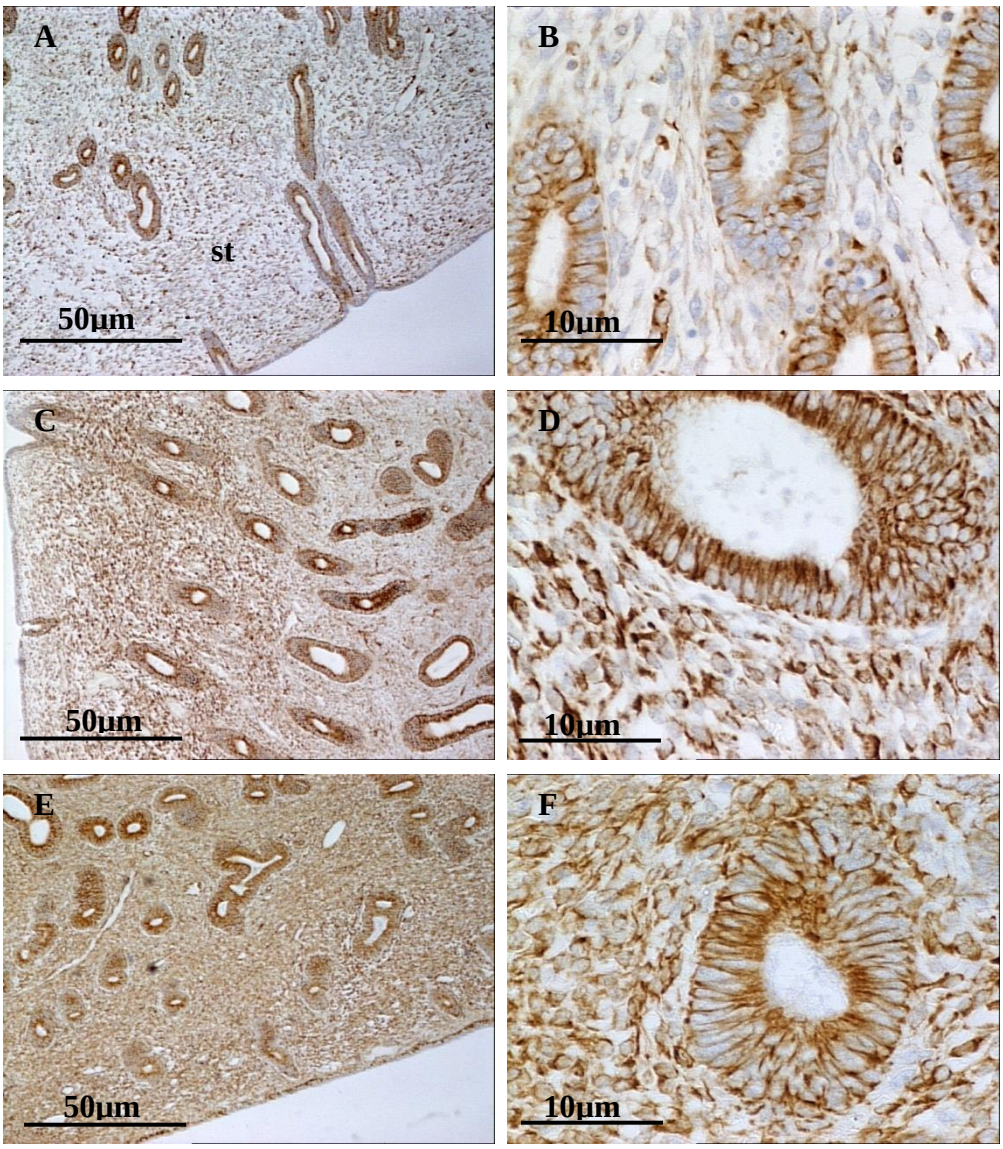


Figure 5.2 Vimentin expression in the proliferative phase functional endometrium. Immunoreactive vimentin was present in the early- (panels A & B), mid- (panels C & D) and late- (panels E & F) proliferative phase functional endometrium, with intense generalised diffuse cytoplasmic staining in the cytoplasm of the glandular epithelium (ge). There was no nuclear staining. In the stroma (st) the vimentin staining was apparently more "patchy" in the early-proliferative than in the mid- and late-proliferative phases. The images shown in panels A, C and E were taken at x50 magnification and those in panels B, D and F at x400 magnification.

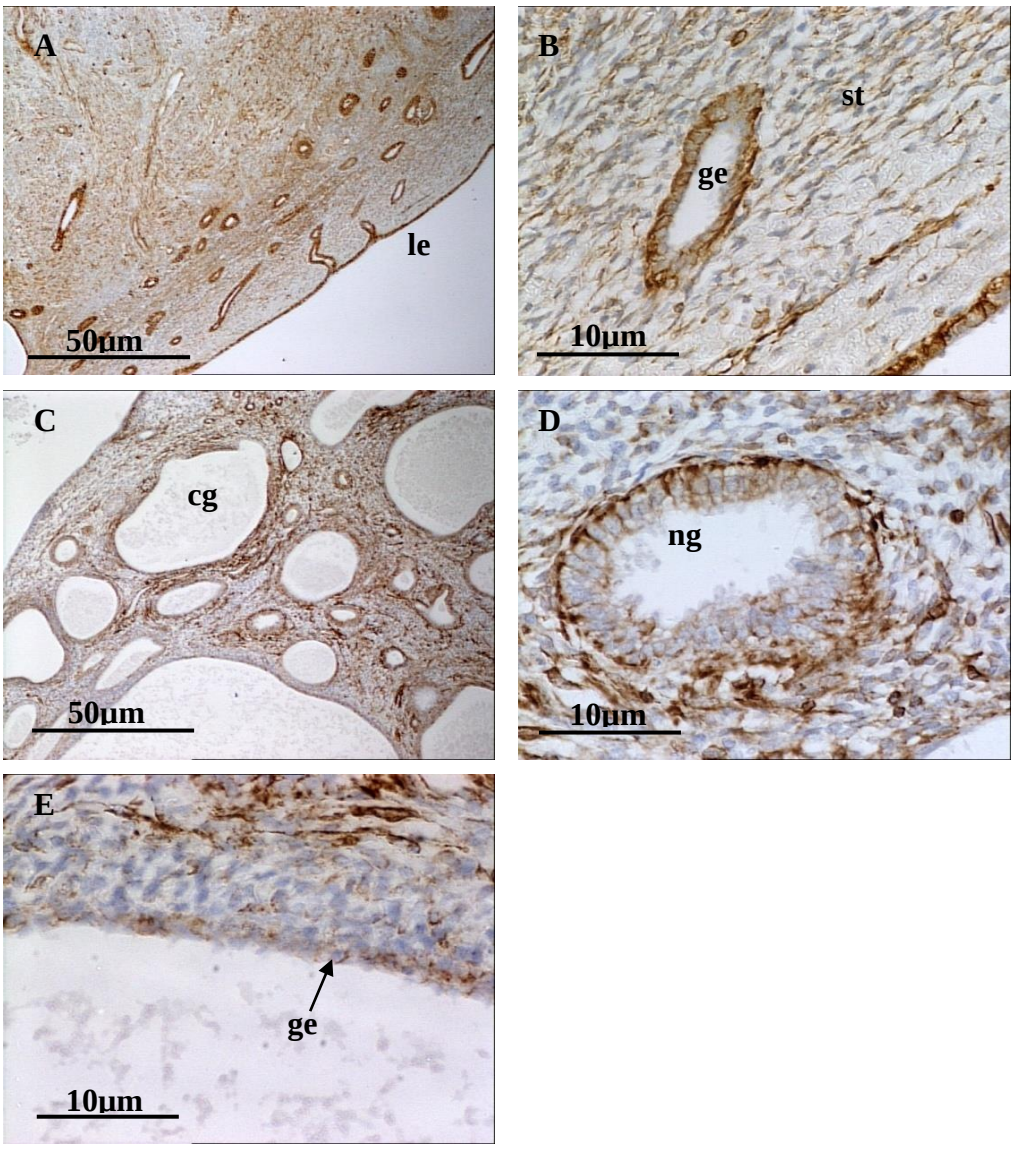


Figure 5.3 Vimentin expression in the functional endometrium of postmenopausal and tamoxifen treated uteri.

Vimentin immunoreactivity in the functional endometrium of postmenopausal women (panels A & B) showed moderate staining of the glandular epithelium (ge) and the luminal epithelium (le) with weaker staining in the stroma (st). In women who took tamoxifen (panels C, D & E) the vimentin immunoreactivity was diffuse and cytoplasmic in the epithelial cells lining cystically dilated glands (cg) and mainly basal in the normal sized glands (ng). The stromal staining in the tamoxifen uteri was weak, but noted to be present near to the pericytes surrounding the normal sized but not the cystic glands. The images

shown in panels A and C were taken at x50 magnification and those in panels B, D and E at x400 magnification.

B Tamoxifen treated uteri

In the tamoxifen treated uteri, vimentin immunoreactivity was observed in the luminal epithelium, the stromal cells and in the glandular epithelial cells (Figure 5.3).

Staining in the large cystic glands was diffuse and cytoplasmic, whilst in the normal sized glands vimentin immunoreactivity was primarily concentrated on the basal surface near to the pericytes. The immunoreactivity in the stromal cytoplasm of the tamoxifen treated uteri was weak. Staining was observed around the normal glands but absent from the stromal cells immediately surrounding the large cystic glands, especially those in close contact with the pericytes and glandular epithelial cells.

5.3.1.2 Basalis

Vimentin immunoreactivity was seen in the cytoplasm of the glandular epithelium in all the phases of the menstrual cycle and the postmenopausal uteri; staining was similar to that observed in the functionalis of the same samples. By contrast, the cytoplasm of the stromal cells also showed vimentin immunoreactivity throughout the menstrual cycle and, in the postmenopausal and tamoxifen treated uteri, but the staining was less when compared to the levels observed in the functionalis.

A Menstrual Cycle

In the endometrial basalis, vimentin immunoreactivity was also observed in the cytoplasm of the glandular epithelium in all phases of the menstrual cycle with a staining pattern no

different to that of the functionalis. In the proliferative phases, the staining was intense (Figure 5.2).

In the early proliferative phase stromal cell, immunoreactivity was very weak. The stromal staining in the mid- and late- proliferative was weak (Figure 5.2).

B Postmenopausal

Vimentin immunoreactivity in the glandular cell cytoplasm was of moderate intensity in the postmenopausal uteri. In the postmenopausal stromal cell, immunoreactivity was weak, this staining was uniform (Table 5.2).

C Tamoxifen

In the tamoxifen treated uteri, the normal sized glands showed staining similar to that seen in the proliferative phase menstrual cycle. However, the cystic glands showed "patchy" weak staining in the glandular cells although the staining appeared to be less patchy and more consistent between glands when compared to the staining observed in comparable cystic glands in the functionalis.

In the tamoxifen treated uteri, vimentin immunoreactivity was observed in the cytoplasm of the stromal cells surrounding normal sized glands but not for the stromal cells immediately, around the cystic glands. The pattern of distribution was similar to that seen in the functionalis.

5.2.1.3 Myometrium

A Menstrual Cycle

The myometrium showed immunoreactivity of the same weak intensity throughout the menstrual cycle. There were no observed differences in relation to the menstrual cycle (Table 5.2).

B Postmenopausal

There was no difference observed in the staining of vimentin in the postmenopausal myometrium when compared to the staining levels in the myometrium observed through the menstrual cycle (Table 5.2).

C Tamoxifen

There was no difference observed in the myometrium of the tamoxifen treated uteri when compared to either the menstrual cycle or the postmenopausal uteri (Table 5.2).

5.3.2 Tenascin-C

5.3.2.1 Luminal and Functional Glandular Epithelium

No immunoreactivity for tenascin-C was observed in the luminal epithelium of samples taken from the menstrual cycle, or for postmenopausal or tamoxifen treated uteri (Figure 5.4).

The luminal and glandular epithelium did not express tenascin-C in any of the sections examined, as expression was confined to the stroma (Table 5.3, Figures 5.5, and 5.6).

5.3.2.2 Functional Stroma

A Menstrual Cycle

In the proliferative phase the stroma showed positive staining for tenascin-C. The subluminal stroma was more immunopositive than the rest of the functionalis stroma and tenascin-C staining was also observed as a distinct band surrounding the functionalis endometrial glands. Tenascin-C immunoreactivity in the stroma was highest in the mid proliferative phase of the cycle. The average percentage of positive tenascin-C stroma surrounding the epithelial glands in the proliferative phases was 60% in the early-proliferative phase (range 60-100%; 2 samples were negative), 70% in the mid-proliferative phase (range 40-100%; all samples positive) and 40% in the late-proliferative phase (range 10-100%; 3 samples were negative).

B Tamoxifen

In the tamoxifen treated uteri, subluminal stroma tenascin-C staining was observed in 26/28 (93%) cases. There was positive staining noted around the cystic dilated tamoxifen

associated endometrial glands and their surrounding stroma. The normal endometrial glands and surrounding stroma showed no immunoreactivity for tenascin-C.

5.3.2.3 Basalis Glandular Epithelium

In keeping with the staining patterns in the functionalis, no immunostaining for tenascin-C was observed in the glandular epithelial cell of the basalis during the menstrual cycle, or in postmenopausal or tamoxifen treated uteri.

5.3.2.4 Basalis Stroma

A Menstrual Cycle

Contrary to the staining pattern in the functionalis, tenascin-C immunoreactivity was not observed in the stroma of the basal endometrium in the early proliferative phase. In the mid proliferative phase, there was patchy staining in 5/8 cases 35% of the glands having surrounding staining positive for tenascin-C (range 40-70%; 3 samples were negative). In the late-proliferative phase, the stroma was negative for tenascin-C, and 2/8 cases showed 100% staining around the endometrial glands.

B Postmenopausal

In the postmenopausal uteri, tenascin-C immunoreactivity was confined to the subluminal stroma in 50% of cases and absent completely from the other 50%. In all cases, tenascin-C staining was absent from the rest of the stroma or the areas surrounding the endometrial glands that were immunopositive in the proliferative phase of the menstrual cycle.

C Tamoxifen

In the tamoxifen treated uteri, there was positive staining noted around the cystic glands and their surrounding stroma. The normal endometrial glands and surrounding stroma showed no immunoreactivity for tenascin-C (Figure 5.7).

5.2.2.4 Myometrium

All the myometrium and blood vessels showed immunoreactivity throughout the menstrual cycle and in the postmenopausal and tamoxifen treated uteri. There were no differences observed in the staining patterns in the menstrual cycle, or in postmenopausal or tamoxifen treated uteri.

Category	Functionalis			Basalis			Myometrium
	Stroma	SAG (%)	Glands	Stroma	SAG (%)	Glands	Inner & Outer
EP (8)	+	70	-	-	0	-	+
MP (8)	++	40	-	+/-	35	-	+
LP (8)	+	0	-	-	100 (2/8 cases)	-	+
PM (8)	tissue not present-	tissue not present-	-	+/-	0	-	+
TMX (18)	+	Cystic 100	-	-	Cystic 100	-	+

Table 5.3 Tenascin-C expression in the glandular and stromal cells within the functionalis, basalis and myometrium.

Data are presented as (-) absent, (+/-) very weak, (+) weak, (++) moderate, (+++) intense staining and the percentage of stroma surrounding endometrial glands (% SAG) that was immunopositive, as determined by counting the number of glands that had tenascin-C staining surrounding them and those that did not. The % SAG data are the mean values from the numbers of samples assessed in each category (shown in parentheses).

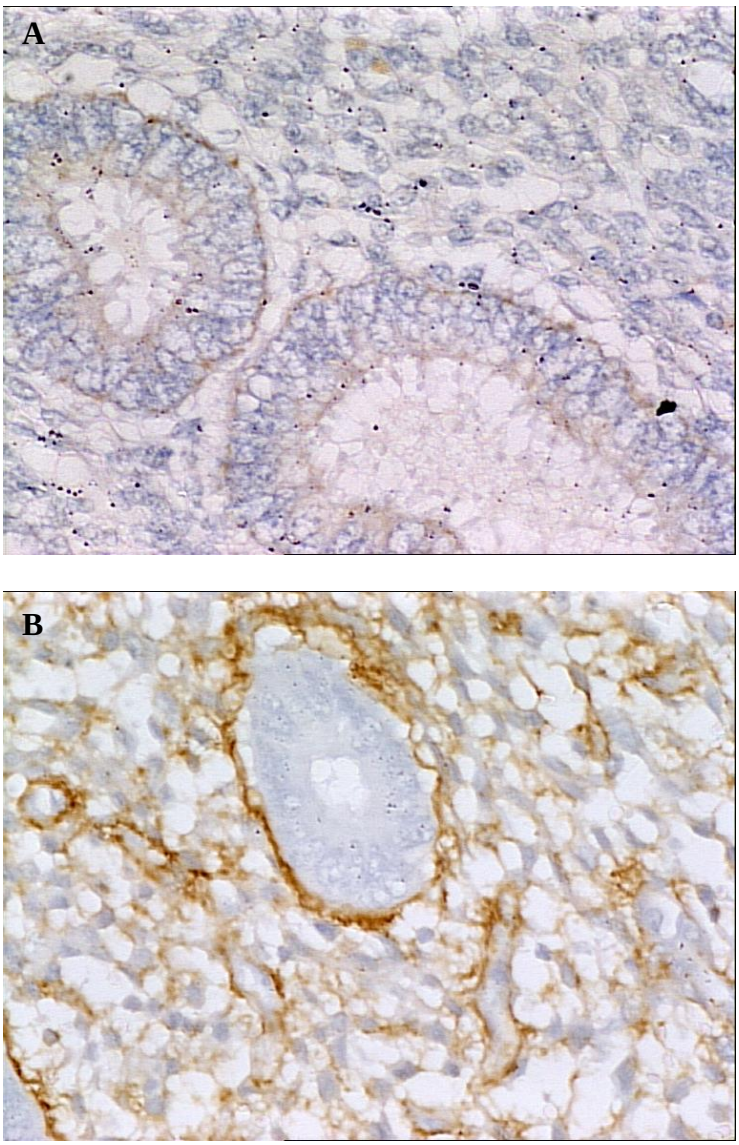


Figure 5.4 Demonstration of Tenascin-C antibody specificity

The tissue sample is an endometrium from the late proliferative phase of the menstrual cycle.

Panel A shows the IgG control (1.6 µg/ml) and Panel B shows tenascin-C (1.6 µg/ml). Both images were taken at x400 magnification.

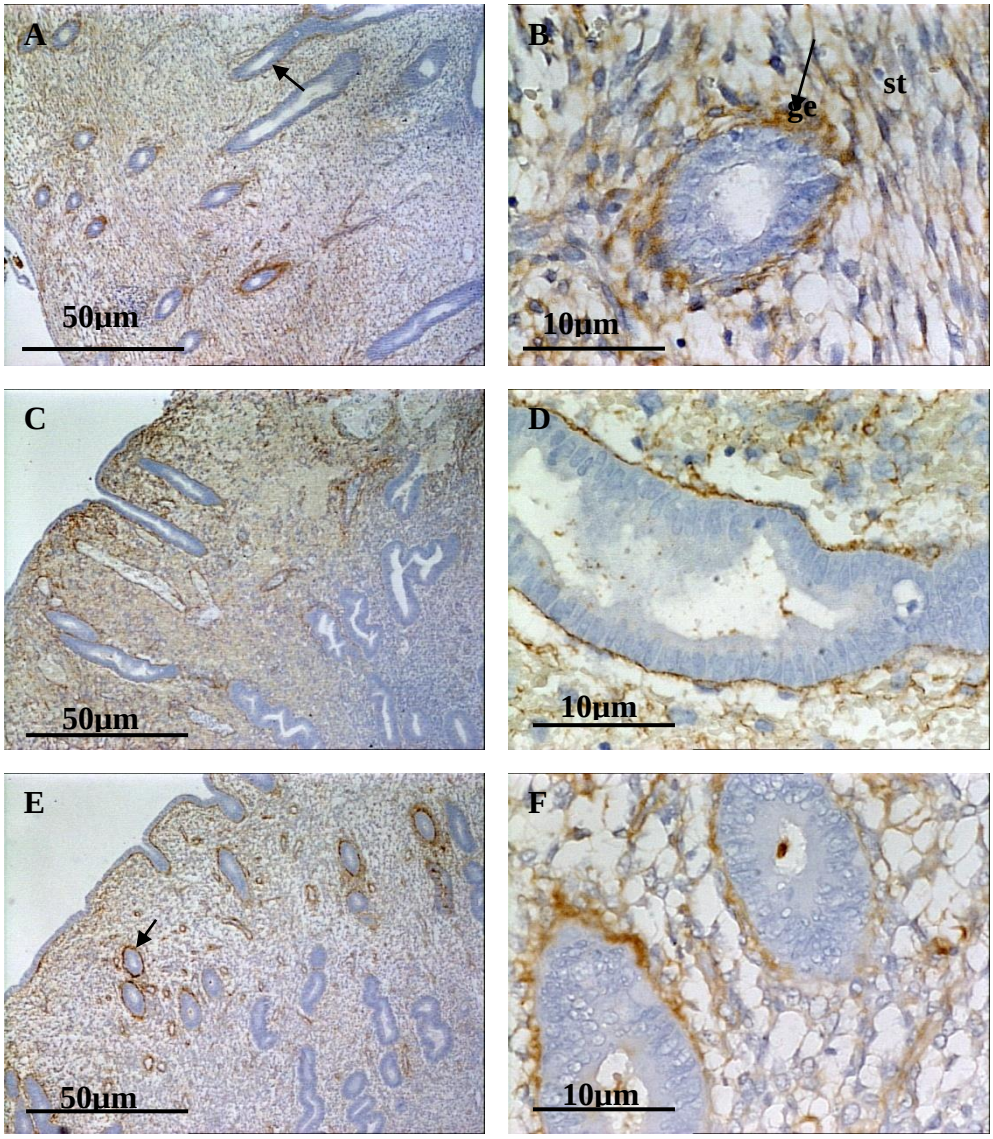


Figure 5.5 Tenascin-C expression in the proliferative phase endometrial functionalis. Tenascin-C staining in the early- (panels A & B), mid- (panels C & D) and late- (panels E & F) proliferative phase functional layer was absent in the glandular epithelium (ge). By contrast, the stroma (st) was immunopositive for tenascin-C, with distinct bands of staining noted around the endometrial glands (arrows), the greatest stromal staining was seen in the mid-proliferative phase. The images shown in panels A, C and E were taken at x50 magnification and those in panels B, D and F at x400 magnification.

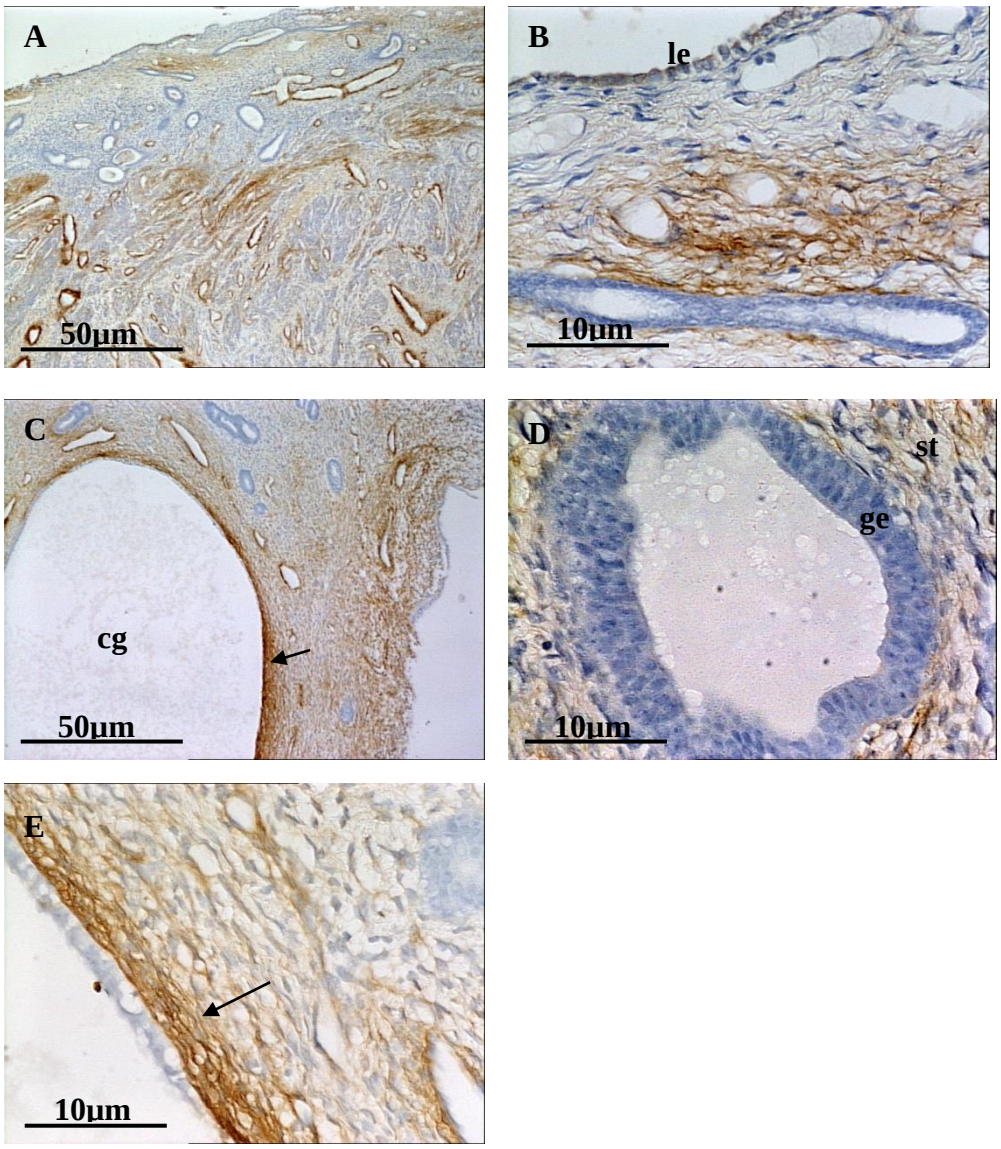


Figure 5.6 Tenascin-C expressiom in the endometrium of postmenopausal women and women treated with tamoxifen.

Tenascin-C immunoreactivity in functional layer of uterus obtained from postmenopausal women (panels A & B) showing no staining in the glandular epithelium (ge), whilst staining in the stroma (st) was present in 50% of the subluminal area and absent from the stroma surrounding the glands. In women who took tamoxifen (panels C, D & E), tenascin-C staining was also absent in the glandular epithelium. The stromal staining in the tamoxifen uteri was greater in the subluminal space than in the remaining stroma. The

stromal staining was noted to be present surrounding the cystic dilated (cg) (arrows) but not the normal sized (panel D) glands.

5.3.3 Alpha-Smooth Muscle Actin

5.3.3.1 Luminal Epithelium

Alpha smooth muscle actin (α-SMA) immunoreactivity (Figure 5.7) was not observed in the luminal epithelium of any samples taken from the menstrual cycle, postmenopausal or tamoxifen treated uteri (Figure 5.8 and 5.9). Staining was also absent from the glandular epithelium and was confined to endometrial stroma and the myometrial layers (Table 5.4).

5.3.3.2 Functionalis

A Menstrual Cycle

In the proliferative phase, the stroma showed positive staining for α-SMA. In the early proliferative phase, the stromal staining was "patchy"; this disappeared in the mid proliferative phase and staining was only seen in the subluminal stroma in 3/8 cases in the late proliferative phase. α-SMA immunoreactivity was also observed surrounding the endometrial glands within the endometrial functionalis in the proliferative phases. The mean percentage of positive periglandular immunoreactivity was 29.1%, 16.5% and 12.3% in the early-, mid- and late-proliferative phases, respectively (Figure 5.10).

B Tamoxifen

There was no immunostaining for α-SMA in the glandular epithelial cell of the functionalis of tamoxifen treated uteri, nor staining for α-SMA in the stromal cells within the functionalis. There was periglandular immunostaining observed in 4 out of 28 cases ranging from 0 to 8.6% (mean 0.9%) and this staining was only observed in the normal glands. The stroma surrounding cystically dilated glands showed no periglandular α-SMA cuffing.

5.3.3.3 Basalis

A Menstrual Cycle

There was no immunostaining for α-SMA in the glandular epithelium of the basalis in menstrual cycle uteri. Immunoreactivity for α-SMA was observed in the stroma of the basalis in all phases of the menstrual cycle. Periglandular α-SMA immunoreactivity was observed in all phases of the menstrual cycle.

B Postmenopausal

There was no immunoreactivity for α-SMA observed in the glandular epithelium of the postmenopausal uteri, but there was immunostaining in the subluminal stroma in 25% of cases; the rest of the stroma showed no immunoreactivity.

Periglandular immunoreactivity was seen in 10.3% of the total number of glands present in both the functionalis and basalis. The whole endometrium was assessed due to the negligible amount of functionalis available for study in these cases (Figure 5.10). This periglandular cuffing was prominent in the cystic dilated glands (Figure 5.9).

C Tamoxifen

There was no immunostaining for α-SMA in the glandular epithelium of the basalis in the tamoxifen treated uteri. There was, however, some minimal α-SMA periglandular staining observed in 2.6% of the normal but not the dilated cystic glands.

Category (N)	Functionalis		Basalis		Myometrium	
	Stroma	Glands	Stroma	Glands	Inner	Outer
EP (8)	+/-	-	+	-	+	+
MP (8)	-	-	+	-	+	+
LP (8)	+/- (3)	-	+	-	+	+
PM (8)	tissue not present-	tissue not present-	+	-	+	+
TMX (18)	-	-	+	-	+	+

Table 5.4 Alpha smooth muscle actin expression in the glandular and stromal cells in the functionalis, basalis and in the inner and outer myometrium.

Data are presented as (-) absent, (+/-) very weak, (+) weak staining.

Numbers (N) of samples assessed in each category are shown in parentheses. Only 3 out of 8 samples in the LP phase showed any level of staining.

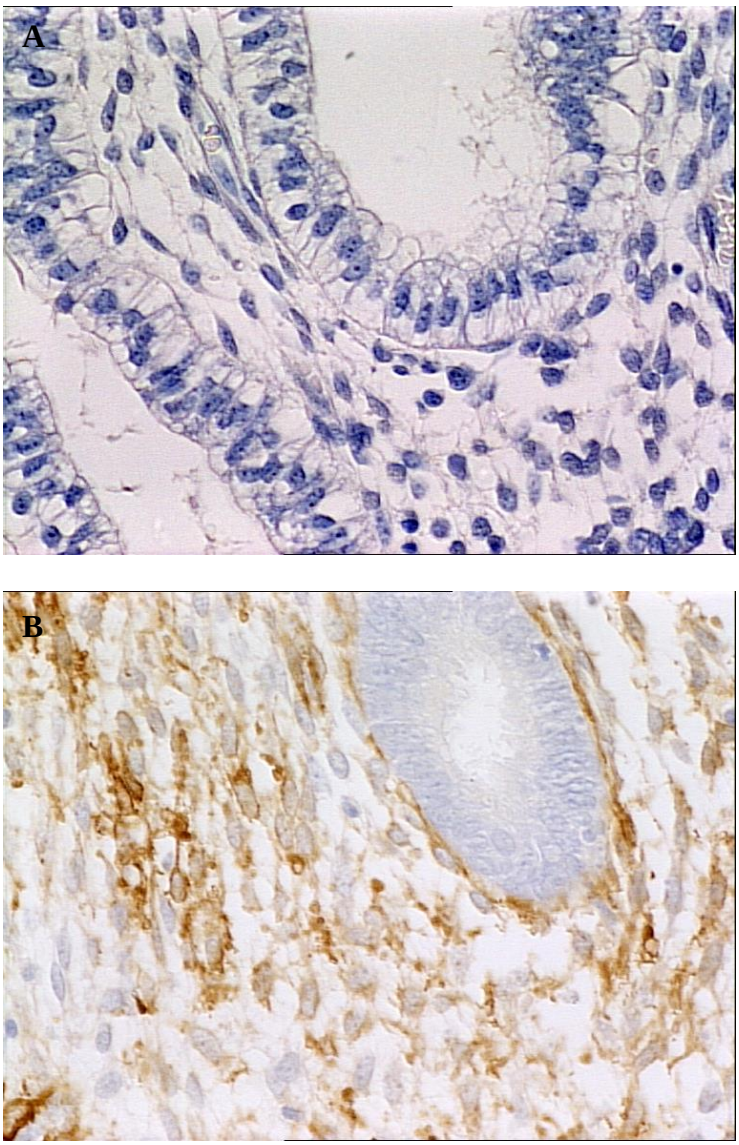


Figure 5.7 Demonstration of α-SMA antibody specificity

The tissue sample is an endometrium from the early proliferative phase of the menstrual cycle.

Panel A shows the IgG control (1.4 µg/ml) and Panel B shows α-SMA (1.4µg/ml). Both images were taken at x400 magnification.

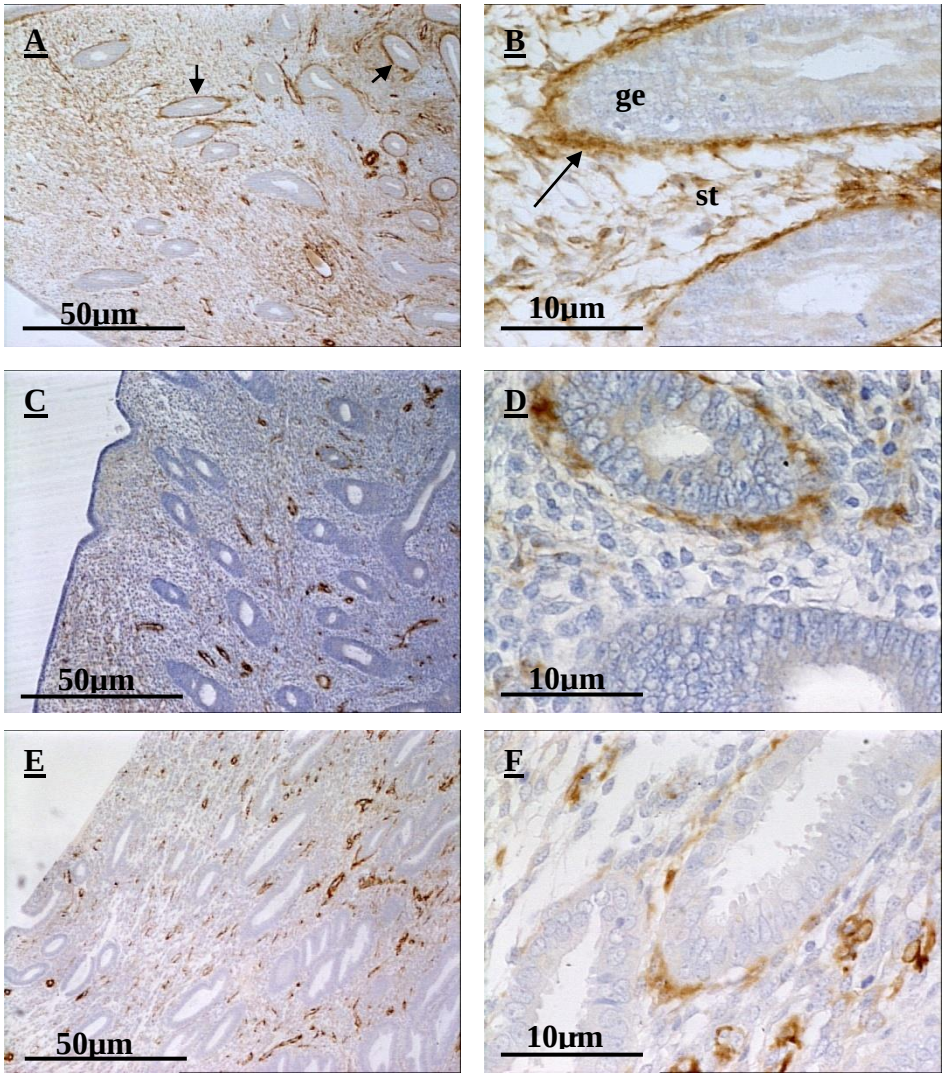


Figure 5.8 α-SMA expression in proliferative phase endometrial functionalis.

α-SMA immunoreactivity in the early- (panels A & B), mid- (panels C & D) and late- (panels E & F) proliferative phases of the menstrual cycle was absent from the glandular epithelium (ge). In the stroma (st), α-SMA immunoreactivity was weakly expressed in the early- and late-proliferative phases but absent in the mid-proliferative phase. The images in panels A, C and E were taken at x50 magnification and those in panels B, D and F at x400 magnification.

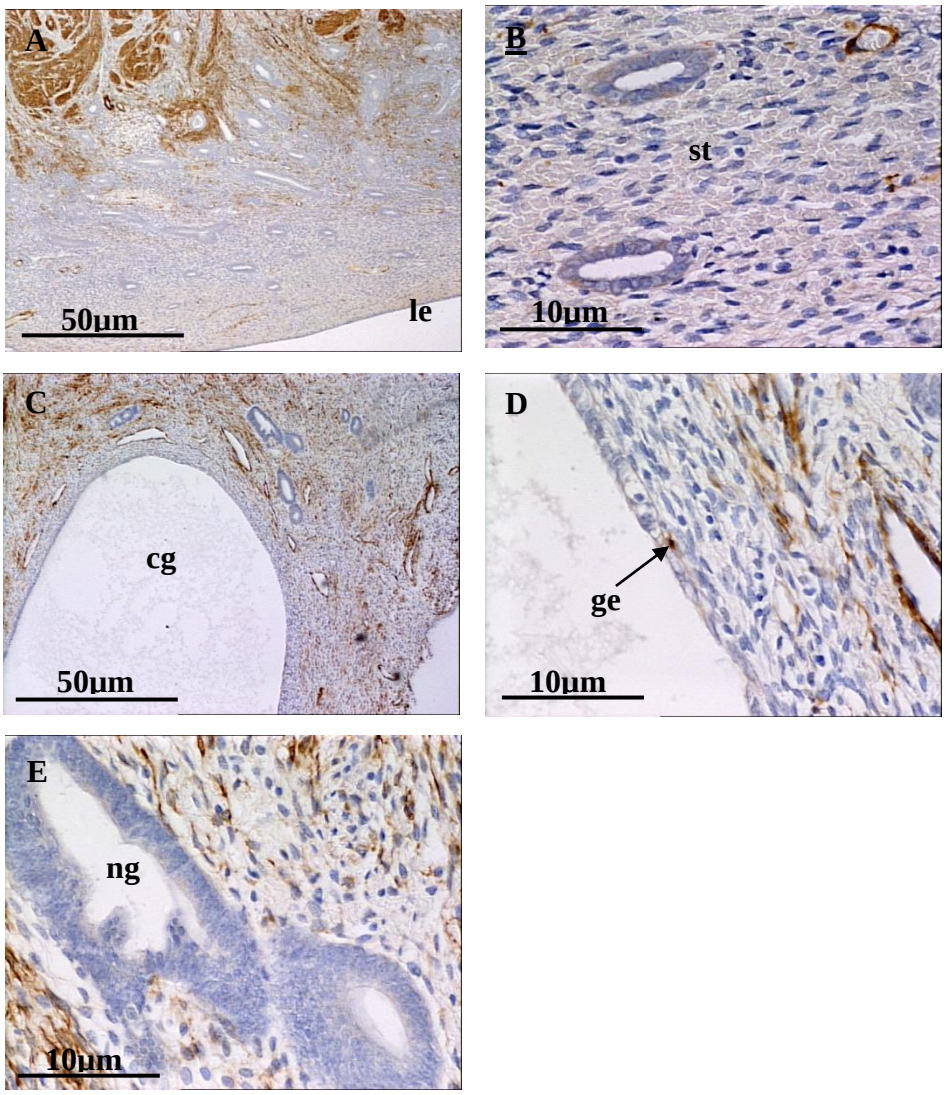


Figure 5.9 α-SMA in expression in the endometrium of postmenopausal women and women treated with tamoxifen.

α-SMA immunoreactivity was absent from the glandular epithelium or periglandular area (ge), with some weak stroma staining subluminally but not the rest of the stroma (st) in postmenopausal uteri (panels A & B). In women who took tamoxifen (panels C, D & E), α-SMA staining was also absent from the glandular epithelium and stromal cells. There was low level of α-SMA immunoreactivity in the periglandular region and this was only noted to occur around the normal sized glands (ng) but not the cystic dilated glands (cg).

The images in panels A, C and E were taken at x50 magnification and those in panels B, D and F at x400 magnification.

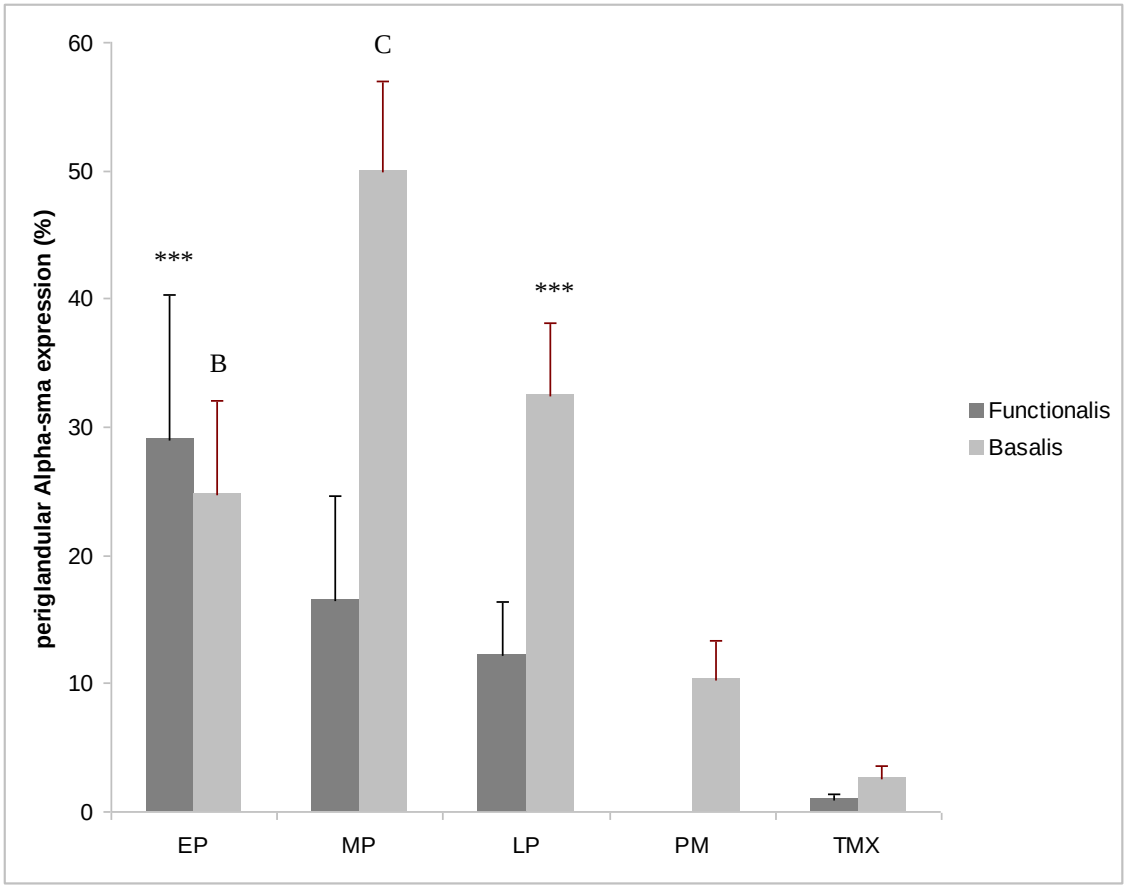


Figure 5.10 Histomorphometric analyses of the α-SMA expression in the functional and basal endometrium throughout the menstrual cycle in postmenopausal and tamoxifen treated women.

Data are presented as mean % periglandular α-SMA expressing glands ± SEM.

Statistical significance was determined using one way ANOVA with Tukey's honestly significant difference post test; *** $p<0.001$ when comparing the tamoxifen treated uteri staining to that of a specific phase of the menstrual cycle. Note the absence of α-SMA staining and low levels of periglandular staining in the functionalis and basalis glands of the postmenopausal uteri. The number of positive glands in the tamoxifen treated uteri was similar to that of the postmenopausal uteri.

5.4 Discussion

Tamoxifen is associated with several endometrial pathologies, including an increased risk of endometrial cancer (Fisher et al., 1994; Fisher et al., 1998; Bernstein et al., 1999; Bergman et al., 2000). Such changes are considered to come about through mechanisms that involve tissue remodelling in which there is a transition of the mesenchyme through an intermediary myofibroblast stage that ends in the production of the myocyte or *vice versa* (Desmoulière et al., 2004). This study assessed the expression of the mesenchymal marker vimentin (Leader et al., 1987; Song et al., 1998), the myofibroblast marker, tenascin-C (Pas et al., 2006) and the smooth muscle cell and myofibroblast marker, α-smooth muscle actin (Christensen et al., 1995; Chaponnier and Gabbiani, 2004) through the normal menstrual cycle and in postmenopausal uteri taken from women either treated with or without tamoxifen.

The data showed that in tamoxifen treated uteri, endometrial glands stained for vimentin, a mesenchymal marker, suggesting that the cells are undergoing an epithelial to mesenchymal transition (EMT), whereby they contain a mixed phenotype that includes epithelial and mesenchymal proteins. During EMT the epithelial cells loose there cell adhesive properties and the cytoskeleton remodels in order to allow the cells to migrate and become invasive, this has been also shown in endometrial carcinoma (Colas et al., 2012). This has been reported for other cell types, such as the myoepithelial cells in the breast (Korsching et al., 2005) and more recently in the uterus (Colas et al., 2012). This observation suggests that endometrial cells can not only undergo a transition from a mesenchymal cell through a myofibroblast to a myocyte (as suggested for the stromal cell), but that epithelial cells may also go through a mesenchymal-epithelial cell transition that involves an intermediary cell that has both mesenchymal and epithelial cell phenotypes.

The stroma surrounding the large cystic glands in the tamoxifen uteri were positive for all three markers, vimentin, tenascin-C and α-SMA, whereas the normal sized endometrial glands in the same samples were negative for tenascin-C, suggesting either the cystic glands are exerting gene modulation in the stroma to bring about the changes associated with tamoxifen treatment, or tamoxifen may be causing modifications in tenascin-C and α-SMA resulting in the formation of the cystic glands, or that the normal sized glands are somehow protected from this effect. It is well known that tenascin-C is regulated by the actions of both tension and stretch associated with tissue remodelling (Chiquet-Ehrismann and Chiquet, 2003) and it seems likely that the expression of tenascin-C around the large cystic glands and not around the normal sized glands is caused by the enlargement of the glands themselves.

Tenascin-C is a cell and tissue adhesion modulating extracellular protein that is often described as being a tissue lubricant allowing cells and tissues to slide over each other (Chiquet-Ehrismann and Chiquet, 2003). The data presented here indicates that the stromal staining in the proliferative phase of the menstrual cycle, that was prominent as a distinct band surrounding the functionalis endometrial glands, probably has some role in the regulation of cell movement and growth during proliferation. The staining pattern observed correlates with that presented in the literature (Vollmer et al., 1990; Sasano et al., 1993; Yamanaka et al., 1996), and as it was not observed in the postmenopausal uteri but present in the tamoxifen treated uteri, then this suggests tenascin-C in the uterus is induced by cell proliferating states.

This idea is supported by data showing that tenascin-C is up regulated in the extracellular space in endometrial adenocarcinoma (Vollmer et al., 1990; Sasano et al., 1993). Vollmer

and co-workers showed tenascin-C was induced by proliferation and low degrees of cell differentiation. Furthermore, they also showed that tenascin-C expression was "shut down" when epithelial differentiation was achieved in mammary tumour cells (Vollmer et al., 1992).

In the tamoxifen treated uteri, tenascin-C expression was observed in the subluminal stroma in 26 out of 28 cases. There was immunostaining around the cystically dilated glands and their surrounding stroma, whilst normal sized endometrial glands and stroma were negative for tenascin-C in the functional and basal endometrium. Tenascin-C has been described as an acute phase protein produced in response to mechanical stress (Chiquet-Ehrismann and Chiquet, 2003), it is possible that cystically dilated glands associated with tamoxifen treatment exert a mechanical force inducing tenascin-C expression and allowing their expansion. The data suggest that tamoxifen acts either by altering the stromal expression of α-SMA and tenascin-C and this, in turn, makes the stroma less resistant to expansion and growth of the endometrial glands, or that the primary defect may be glandular. These endometrial glands contain thick mucoid material that may therefore accumulate and through tension pressure result in the loss of α-SMA expression and an increase in tenascin-C expression. The cystic glands in the tamoxifen endometrium are lined by cuboidal cells rather than tall columnar cells suggesting pressure within the cystic glands causes this phenotypic change.

Because vimentin is known to have a structural role, providing mechanical support to the cell (Leader et al., 1987), it was as expected present in the cytoplasm of cells. Nevertheless, the decrease in vimentin staining in the secretory phase has been suggested

to reflect the changing glandular function in preparation for implantation (Song et al., 1998).

There was also stromal immunoreactivity of vimentin in all phases of the menstrual cycle, with greatest expression observed in the mid- and late-proliferative phases. These data are in agreement with Tabibzabeh and co-workers (Tabibzadeh, 1991) who observed strong vimentin immunostaining throughout the menstrual cycle, but not in agreement with the findings of Song and co-workers (Song et al., 1998) who observed greatest stromal staining in the secretory phase. This difference may be due to patient selection because the patients Song and co-workers studied had either adenomyosis or ovarian endometrioma, whereas Tabibzabeh's patients had no such pathology.

In tamoxifen treated uteri, the glandular epithelium showed positive staining for vimentin similar to that in the proliferative phase of the menstrual cycle, but the large cystic glands had "patchy", less intense staining. At the same time, the stromal staining was positive around the normal glands but negative around the cystic glands. Because the epithelial cells in the large cystic glands were cuboidal as opposed to tall columnar cells, the decrease in vimentin staining around these glands may be a result of decreased functional activity, secondary to a pressure effect, as observed in epithelial structures in endometriomas (Song et al., 1998). Bindels and colleagues showed vimentin expression correlates with a migratory and invasive phenotype in epithelial breast tumour cells (Bindels et al., 2006), suggesting that reduced cell migration might be occurring around the cystic glands in the tamoxifen treated uteri.

Stromal staining for α-SMA was observed along with periglandular staining throughout the menstrual cycle with greater α-SMA staining in the secretory phase when compared to the proliferative phase of the menstrual cycle. This is in contrast to the observation by Czernobilsky and co-workers (Czernobilsky et al., 1993) who found periglandular α-SMA cuffing mainly in the proliferative phases. The reason for this difference remains unclear.

Postmenopausal uteri whether treated with tamoxifen or not, showed very minimal periglandular α-SMA staining. The observation of low or absent α-SMA in all uterine cell types has been observed in ovariectomized baboons (Christensen et al., 1995) and correlates with the staining patterns observed in the postmenopausal uteri. I am unaware of any literature describing α-SMA staining in tamoxifen treated uteri, and so the periglandular staining in tamoxifen uteri appears to be the first report describing α-SMA expression in tamoxifen treated uteri. The work by Christensen in the baboon indicated that initial changes in decidualization might be the cause of the increased α-SMA expression. It is well-known that during decidualization, the stromal fibroblasts undergo a series of transformations including a change in cell shape and change in protein synthetic capacity, at least in the baboon (Fazleabas et al., 1989). The observation of increased periglandular α-SMA cuffing during the secretory phases probably indicates a similar cell and protein synthesis transformation in the human uterus. However, it is also possible that the dilated cystic glands associated with tamoxifen-treatment only express α-SMA transiently.

From these data, it is difficult to conclude that tamoxifen is indeed promoting or inhibiting the mesenchyme-myofibroblast-myocyte transition, although the differential expression of all three markers surrounding the large cystically dilated glands, but not the normal sized

glands suggest that the phenotypes of these two types of glands differs, with the large cystically dilated glands being closer to the proposed transitional state and that the tamoxifen treated uterus is primarily in a 'myofibroblast' phase. There is increased presence of tenascin-C in cases of breast cancer and in endometrial adenocarcinoma (Vollmer et al., 1990; Sasano et al., 1993), there is also α-SMA expression in the stromal cells of mammary tumour myofibroblasts (Sappino et al., 1988). The co-expression of α-SMA and tenascin C suggest that there is tissue reorganization occurring, and that the large cystically dilated glands could be the primary precursor lesion in tamoxifen induced endometrial cancer, as such lesions are also observed in tamoxifen induced polyps that eventually develop into malignancy. The observation that there were lower levels of vimentin (i.e. less mechanical support), more tenascin C, due to mechanical stretch in cystic glands and so increased migratory potential, tend to support this idea.

In conclusion, the cystically dilated epithelial glands in tamoxifen treated uteri may exert a gene modulatory function on the surrounding stromal cells or the other way around that is unique to this drug, alternatively, the drug has a unique gene modulatory function that results in the formation of this unique pathology. This will be examined in the following chapter.

Chapter 6

Tamoxifen activates oestrogenic-specific, anti-oestrogenic and tamoxifen-unique pathways in the human uterus

6.1 Introduction

At the end of Chapter 5, I suggested that the specific mesenchymal changes observed in the tamoxifen treated uteri, such as the presence of cystically dilated epithelial glands, might either exert a gene modulatory function on the surrounding stromal cells that is unique to this drug, or alternatively, that tamoxifen might have a unique gene modulatory action that results in the formation of this pathology. Although many studies that have tried to discover the molecular mechanism of tamoxifen action in the endometrium very few studies have focussed on what tamoxifen does in the myometrium.

Breast cancer patients treated with adjuvant tamoxifen therapy have higher expression of both ER and PR in benign endometrium compared to women who are not taking tamoxifen (Elkas et al., 2000; Mourits et al., 2002b). In chapter 3 I showed that the tamoxifen treated endometrium has higher ERβ expression, and this increased expression of steroid receptor may indicate that the uterotrophic effects of tamoxifen are possibly due to the potentiation of ER signalling. Since PR is a well established oestrogen-responsive gene, up-regulation of the PR in the uterus of tamoxifen users strengthens this hypothesis. In benign endometrium (including those that are atrophic, proliferative, contain polyps or are hyperplastic) the expression of TGF-β1, p27, cathepsin D and Ca-125 are different in tamoxifen users when compared to non-users (Carmichael et al., 2000; Mylonas et al., 2003). In addition, the proliferation and apoptosis index, as measured by examining the expression of Ki67, Fas, FasL and Bcl2 is higher in benign endometrium from tamoxifen-

users when compared to non-users (Mourits et al., 2002a; Mourits et al., 2002b). In endometrial cancer specimens, there were no differences in gene expression profiles between matched and staged tamoxifen-associated tumours compared to sporadic tumours (Ferguson et al., 2004; Prasad et al., 2005). All of these data suggest that tamoxifen may act as a weak oestrogen. Indeed, Gielen and colleagues compared the modulation of gene expression in an endometrial cancer cell line (ECC1) treated with tamoxifen and oestrogen, and not only revealed a common gene expression profile between tamoxifen and oestrogen, but also a specific set of genes regulated by tamoxifen alone (Gielen et al., 2005a). This was confirmed *in vivo*, where 256 genes products were shown to be either up- or down-regulated in the endometrium from tamoxifen treated patients compared to non-treated postmenopausal patients without breast cancer (Gielen et al., 2005b). The function of the tamoxifen regulated genes were involvement in apoptosis, proliferation, differentiation and cell-cell adhesion. A comparison of tamoxifen and oestrogen signalling concluded that tamoxifen-induced proliferation occurs via the same set of genes as regulated by oestradiol, but it is the tamoxifen specific gene regulation which gives rise to an increased incidence of endometrial carcinoma in these patients (Gielen et al., 2008). These data led to the conclusion that in the benign endometrium, tamoxifen induces the expression of specific genes and pathways, resulting in an increased incidence of endometrial pathologies that may eventually lead to the development of endometrial neoplasia. There is currently no literature examining the gene expression profile in tamoxifen treated patients in both the myometrium and endometrium compared to oestrogen deprived tissue.

The aim of this Chapter is therefore to further investigate endometrial and myometrial gene expression after tamoxifen treatment, and compare this to the changes induced by

oestradiol. Therefore, gene expression profiles from endometrial and myometrial samples from postmenopausal women using tamoxifen are compared to the profiles of postmenopausal atrophic uteri and those from the proliferative phase of the menstrual cycle, which is the oestrogen dominant phase of the cycle.

6.2 Materials & Methods

6.2.1 Acknowledgements

At this point, I would like to take the opportunity to acknowledge the contributions of individuals who performed some of the work presented in this chapter. This includes the staff at Geneservice who performed the microarray experiment, Dr Taylor who did the preliminary analysis of the data and produced the graphs and tables, and Mrs Muna Abbas who completed the confirmatory RT-PCR experiments. Due to several personal problems, I could not do these tasks myself, but I was responsible for the identification and collection of all of the tissue samples, I extracted and processed all the RNA and produced the complimentary DNA required. I liaised with all those involved in helping me to complete this Chapter and I re-confirmed the analyses (with the aid of Dr Taylor). There are two sections that are mine, and mine alone, and they are the presentation of the data and their subsequent discussion.

6.2.2 Patient groups

Endometrial and myometrial tissue was obtained from women undergoing hysterectomy. The postmenopausal patients not exposed to tamoxifen treatment and those exposed to tamoxifen for primary breast cancer used for this part of the study are the same as those used in Chapters 3, sections 3.2.2.2. and 3.2.2.3. The third group were uteri from pre-menopausal women in the proliferative phase of the menstrual cycle. Each group was represented by 6 patient samples in each group.

6.2.3 Sample collection

In all cases, the uterine sample was obtained immediately after the uterus had been removed from the patient. The uteri were transferred to a sterile area where the uterus was opened sagitally and a full thickness piece of uterus including the luminal epithelial to the serosal surface and placed in 10% formalin for histological examination as previously described in Chapter 3, section 3.2.1 and 3.2.3.

To prepare the endometrium sample, an adjacent piece of uterus was selected and the endometrium scraped away from the myometrium with a sharp curette, placed in a sterile container and snap-frozen in liquid nitrogen. The innermost piece of myometrium was dissected free and discarded and a piece of myometrium representing the middle layer of the myometrium was placed in a separate pot and snap frozen in liquid nitrogen. Samples were stored at -80°C for later RNA preparation.

6.2.4 Histological examination

The histological assessment of the uterine tissue for this part of the study was the same as that described in Chapters 3, section 3.2.5.

6.2.5 Total RNA extraction, cDNA synthesis and microarray hybridization

Total RNA was extracted using a combination of TRIZOL reagent (Invitrogen, Paisley, UK), RNAeasy mini kit (Qiagen Ltd., Crawley, UK) and ethanol precipitation. Tissues were weighed in a liquid nitrogen cooled mortar and pulverized under liquid nitrogen. The powdered tissue was further homogenised in 1ml of TRIZOL reagent per 100mg of tissue using a high-speed blade homogeniser and 20 strokes of hand-held Dounce homogenisers. The homogenate was transferred to RNase-free 1.5ml microfuge tubes (1ml per tube) and

incubated at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Chloroform (200µl per ml of TRIZOL) was added, the sample shaken vigorously by hand for 15 seconds and allowed to rest for 10 minutes at room temperature. The aqueous and organic phases were separated by centrifugation at 7,000 rpm at 4°C for 15 minutes. The upper aqueous phase was transferred to a new RNase-free 1.5mL microfuge tube and 0.5ml isopropanol added per ml of TRIZOL used. The samples were vortexed and incubated at room temperature for 10 minutes, and then the RNA was pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C. The supernatant from each tube was discarded by inversion of the microfuge tube onto a clean paper tissue, taking care not to disturb the RNA pellet. The pellets were washed by adding 0.5ml of 75% ethanol/DEPC-treated water and then vortexed and collected at 13,000 rpm for 5 minutes. The pellets were then allowed to air-dry inverted for 5 minutes, any excess liquid above the pellet was carefully removed with paper tissue, and then the RNA pellet was re-dissolved in 100µl DEPC-treated water by heating at 55°C for 5 minutes.

To prepare the RNA for microarray analysis, aqueous total RNA from the TRIZOL reagent procedure was mixed with ethanol, applied to the RNAeasy columns, and subsequently purified and concentrated according to the manufacturer's instructions (Qiagen, Crawley, West Sussex, UK). RNA integrity was assessed using agarose gel electrophoresis and Agilent 2100 Bioanalyser (Agilent Technologies, UK, South Queensferry, UK). Due to the small amounts of RNA obtained from the atrophic uteri, the RNA from all 6 samples were pooled and then submitted to Geneservice Ltd. (Babraham, Cambridge, UK) for further purification and production of biotinylated cRNA. Similar RNA pools for the tamoxifen-treated and proliferative uteri were created and also forwarded to Geneservice Ltd. Biotinylated cRNA was prepared from 10µg of total RNA according to Affymetrix protocols. The integrity of the labelled cRNA and fragmentation products were assessed

on an Agilent 2100 bioanalyser. Next, 15µg of biotinylated cRNA fragments were hybridized to Affymetrix human HU133_plus 2 microarray chips overnight and then stringently washed, stained and scanned using a Gene-Array scanner (Agilent Technologies, Palo Alto, CA, USA), according to Affymetrix protocols.

6.2.6 Gene expression analysis

Fluorescence data were corrected for background fluorescence, reduced in intensity values before normalization against internal standards. All arrays were scaled to the same target intensity and were normalized to housekeeping genes on the U133_plus 2 arrays and analysed using dChip Analyzer software version 1.4 (Harvard School of Public Health and Dana-Farber Cancer Research Institute, Boston, MA, USA, 2004; http://biosun1.harvard.edu/complab/dchip/) using a 1.6-fold change in expression with an α of 0.90 and a β of 0.05 being the statistical cut-off points for real expression changes. Hierarchical clustering was used to obtain gene expression patterns and ontology information was obtained by using Ingenuity Pathway Analysis (http://www.ingenuity.com) and PathViso software (http://www.pathvisio.org). Lists of modulated probesets are included on the CD-ROM at the back of this thesis.

6.2.7 Confirmatory RT–PCR

Oligonucleotide primers were synthesized (Sigma-Genosys Ltd., Haverhill, Suffolk, UK) and desalted before reconstitution in sterile de-ionised water (dH_2O) at 200pmol/µl and stored at –20°C. The mRNA specific primer sets and sequences are listed in Table 6.1.

End-point RT–PCR was used to determine amplicon product sizes and integrity of the samples used in these experiments. Briefly, one microgram of total RNA was reversed

transcribed with avian myeloblastosis virus-reverse transcriptase (AMV-RT 5 Units; Promega, Southampton, UK) at 42°C for 60 minutes in the presence of 24.8 Units of RNase inhibitor (RNasin; Promega), 1µl of anchored oligo $(dT)_{23}$ (40µM), 2.5µl of 10mM dNTP's and 5µl of (5x) AMV buffer in 25µl reaction. A minus RT reaction was obtained by omitting the AMV-RT enzyme and replacing with DEPC- treated dH_2O. At the end of the reaction, the enzymes were denatured by heating at 95°C for 5 minutes and the cDNA stored at 4°C. One microlitre of cDNA was then subjected to PCR using 10 pmol/µl of specific primers for target genes and the 'housekeeping gene' glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the annealing temperatures and thermal cycler conditions shown in Table 6.1.

The RNA for quantitative RT-PCR was treated with RNAse-free DNAse 1 (Promega) before reverse transcription and PCR amplification. For gene targets where the primers were designed 'in house' (Table 6.1), PCR was performed using a Genius thermal cycler (Techne Corp., Duxford, UK) and 1 µl of cDNA. Reactions included 5µl of 10x AJ Buffer (450mM Tris-HCl (pH 8.8), 110mM $(NH_4)_2SO_4$, 45mM $MgCl_2$, 2mM of each dNTP, 1.1 mg/ml acetylated BSA (Roche Diagnostics Ltd., Lewes, UK; 110mM β-mercaptoethanol; 4.4 mM EDTA), 10 pmol of gene-specific primers (Table 6.1) combined with 1.0 Unit of GO Taq polymerase (Promega) diluted in 1x AJ Buffer.

The thermal cycler conditions for all reactions were an initial denaturation step at 95°C for 2 minutes, followed by 35 cycles of 95°C for 45 seconds, the annealing temperature (see Table 6.1) for 1 minute, 72°C for 1 minute with a final extension time of 10 minutes at 72°C. The absence of genomic DNA was confirmed using samples where the AMV-RT

enzyme had been omitted. The amplification profiles for each gene were all within the linear range of detection (data not shown).

After PCR, the reaction mixtures were separated through 3% TAE-agarose gels (1.5g agarose (Bioline, London, UK) dissolved in 50ml of tris-acetate-EDTA buffer (40mM tris-acetate, 1mM EDTA (pH 8.0) plus 0.05% (v/v) ethidium bromide (ICN Biomedicals, Brussels, Belgium) at ~5 V/cm for 1 hour. Visualization and image capture was performed on a Gene Genius gel documentation and analysis system (Syngene, Cambridge, UK).

The expected sizes for the amplicons are also shown in Table 6.1. No PCR products were detected when the reverse transcriptase was omitted (data not shown). The relative levels of transcripts in the three tissue types were quantified using quantitative real-time RT-PCR.

Table 6.1 Primer sequences and PCR conditions. The primers labelled [a] were designed 'in-house' using Primer Premier Version 5.0 software; Premier Biosoft International, Palo Alto, CA, USA. www.PremierBiosoft.com.

mRNA target	GenBank accession No.	Primers	Conditions	Product size (bp)	Ref.
CA12	NM_001218	Sense 5'-gagacttactgggaggtgc-3' Antisense 5'-agtgtggttccttggagc-3'	95ºC, 30 sec 54ºC, 15 sec 72ºC, 30 sec	134	[a]
CD24	NM_013230	Sense 5'-gctcctacccacgcagat-3' Antisense 5'-ggacttccagacgccatt-3'	95ºC, 30 sec 55ºC, 15 sec 72ºC, 30 sec	263	[a]
ERα	NM_000125	Sense 5'- cagacatgagagctgccaac-3' Antisense 5'- ccaagagcaagttaggagca-3'	95ºC, 12 sec 60ºC, 12 sec 72ºC, 24 sec	381	
ERβ	NM_001437	Sense 5'- tccctggtgtgaagcaagatc-3' Antisense 5'- cgccggtttttatcgattgt-3'	95ºC, 12 sec 60ºC, 12 sec 72ºC, 24 sec	279	(Mesiano et al. 2002)
FosB	NM_005252	Sense 5'- ctgattgttcccgatggt-3' Antisense 5'- cacccagaattgtcaaagata-3'	95ºC, 30 sec 53ºC, 15 sec 72ºC, 30 sec	212	[a]
GAPDH	X01677	Sense 5'- agaacatcatccctgcctc-3' Antisense 5'- gccaaattcgttgtcatacc-3'	95ºC, 30 sec 46ºC, 15 sec 72ºC, 30 sec	347	[Hall et al., 1998]
GRIA2	NM_000826	Sense 5'-gaggcatccagtatctt-3' Antisense 5'-atgtccaccacgcttt-3'	95ºC, 25 sec 46ºC, 15 sec 72ºC, 25 sec	139	[a]
Hox A11	M75952	Sense 5'- tcggccaacgtctaccaccacc-3' Antisense 5'- ctgaagaagaactcccgttccagct-3'	95ºC, 15 sec 59ºC, 15 sec 72ºC, 30 sec	458	[a]
IGFBP-3	NM_000598	Sense 5'- ggcactctgggaacctat-3' Antisense 5'- tctgaatgtggaggctga-3'	95ºC, 30 sec 55ºC, 15 sec 72ºC, 30 sec	157	[a]
PENK	NM_001135690	Sense 5'- acggaggatttatgagatt-3' Antisense 5'- atacaaggcaagcaacac-3'	95ºC, 30 sec 53ºC, 15 sec 72ºC, 30 sec	126	[a]
PR (all isoforms)	NM_000926	Sense 5'- ctgcaggtctacccgccctatc-3' Antisense 5'- gaattcatttggaacgcccactgg-3'	95ºC, 12 sec 60ºC, 12 sec 72ºC, 24 sec	396	[Taylor et al., 2008] [Hirata et al.,2000]
PR-B	NM_000926	Sense 5'- cctgaagtttcggccatacct-3' Antisense 5'- agcagtccgctgtccttttct-3'	95ºC, 12 sec 60ºC, 12 sec 72ºC, 24 sec	197	(Mesiano et al. 2002)
α-SMA	NM_001141945	Sense 5'- gctggcatccatgaacc-3' Antisense 5'- ggacattcacagttgtgtgc-3'	95ºC, 12 sec 60ºC, 9 sec 72ºC, 12 sec	363	[McParland, 2002]
SMTN	NM_134270	Sense 5'- tccgacaaaggaagagacc-3' Antisense 5'- ggtcgaagatgctgcccatctt-3'	95ºC, 10 sec 56ºC, 5 sec 72ºC, 13 sec	327	(Nui et al. 2001)
VIM	NM_00380	Sense 5'- agaactttgccgttgaagctg-3' Antisense 5'- ccagagggagtgaatccagatta-3'	95ºC, 12 sec 58ºC, 9 sec 72ºC, 12 sec	255	[a]
WFDC1	NM_021197	Sense 5'- accctcacagttcacattcage-3' Antisense 5'- acatacctcccagagccaga-3'	95ºC, 12 sec 58ºC, 9 sec 72ºC, 12 sec	299	[a]
WFDC2	NM_006103	Sense 5'- tctgccccaatgataaggag-3' Antisense 5'- caccccaagagggaatacag-3'	95ºC, 12 sec 58ºC, 9 sec 72ºC, 12 sec	280	[a]
Wif-1	NM_007191	Sense 5'- aaaatgcgtaagtcacaag-3' Antisense 5'- ttaaatgccactaccaca-3'	95ºC, 30 sec 46ºC, 15 sec 72ºC, 30 sec	211	[a]
WISP2	NM_003881	Sense 5'- ctgggctgatggaagatg-3' Antisense 5'- taggcagtgagttagaggaaag-3'	95ºC, 30 sec 57ºC, 15 sec 72ºC, 30 sec	167	[a]

6.2.8 Quantitative RT–PCR (qPCR)

For quantitative PCR, gene specific primers (Table 6.1) were used at 10pmol/µl in a SYBR green system (Roche Diagnostics, Lewes, UK) with 1 µl of cDNA as template in a Roche Lightcycler 1.2. The PCR conditions in all cases started with a denaturation step at 95ºC for 10 minutes and followed by up to 50 cycles of denaturation, annealing and primer extension (Table 6.1). Standard curves of diluted cDNA pools were constructed for each gene target and the expression levels corrected for the levels of human GAPDH using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). These data were compared to repeats using pools of cDNA prepared from the atrophic, proliferative and tamoxifen treated uteri samples.

6.2.9 Data analysis

The PCR data were normalized to the levels of GAPDH transcript found in each sample and expressed relative to the mean of the atrophic normalised value (Control). All data were analysed for differences using one-way ANOVA with Tukey's honest significant difference (HSD) test with the InStat version 3.0 software package (GraphPad Software, San Diego, CA, USA, 1998; http://www.graphpad.com). Statistical significance was accepted as $p<0.05$. Comparisons were then made to the fold-change in transcript levels found on the microarrays.

6.2.10 Pathway Analysis

The data from the microarray were examined further by subjecting the entire dataset to pathway analysis using PathVisio software (van Iersel et al., 2008) including Wikipathways (Pico et al., 2008). Using the Z-score algorithm, the numbers of genes significantly regulated in each of the 144 human pathways were calculated. To determine

significantly over/under-representation of genes in the various pathways, χ^2-tests were applied to the lists, on the basis that each regulated gene could be present at a constant rate (Doniger et al., 2003). Pathways were then compared across tissues and treatments and significant patterns identified.

6.3 Results

6.3.1 Microarray analysis: identification of oestradiol and tamoxifen regulated genes

The use of microarray analysis aids the global assessment of the transcriptional profiles of cells and tissue samples. Utilizing the Affymetrix HU133A_Plus 2 arrays, which represents 54,613 characterized transcripts and expressed sequence tags (ESTs), this study found a number of genes that were induced and repressed in the proliferative phase endometrium at the 1.6-fold level to be 796 and 865, respectively. The number of probe sets induced and repressed within the tamoxifen endometrium was 753 and 1170, respectively. The endometrium had a measurement rate of 3.04% and 3.52%, respectively. In the proliferative phase myometrium, the number of probe sets induced and repressed was 406 and 151, respectively. In the tamoxifen treated myometrium, the number of probe sets induced was 407 and repressed were 98. The measurement rate in the myometrium was lower at 1.03% and 0.92%, respectively.

Concentrating on genes showing an increase in expression using the 1.6-fold change cut-off in the endometrial and myometrial arrays, 616 genes were identified in the proliferative endometrium, 583 in the tamoxifen treated endometrium, 307 genes in the proliferative myometrium and finally 293 genes in the tamoxifen treated myometrium. The top 30 genes with an increased level of expression are shown in Table 6.2.

Table 6.2. List of the top 30 genes up-regulated in the proliferative and tamoxifen-treated endometrium and myometrium.

The gene symbol, accession number and gene name are listed. Fold change = fluorescence levels relative to the levels found in the relevant atrophic tissue

Gene Symbol	Accession Number	Gene Name	Fold Change
Proliferative endometrium			
OLFM4	AL390736	olfactomedin 4	58.78
PENK	NM_006211	Proenkephalin	41.08
DIO2	U53506	deiodinase, iodothyronine, type II	27.94
KMO	AI074145	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	23.00
IGF1	AU144912	insulin-like growth factor 1 (somatomedin C)	22.20
EDNRA	AU118882	endothelin receptor type A	20.68
THY1	AA218868	Thy-1 cell surface antigen	19.98
MGC16121	AV728999	hypothetical protein MGC16121	19.18
ADAM12	W46291	ADAM metallopeptidase domain 12	18.11
WFDC1	NM_021197	WAP four-disulfide core domain 1	17.30
LOC100505967	BC042069	LOC100505967 gene	16.05
SFRP4	NM_003014	secreted frizzled-related protein 4	15.54
PCSK5	NM_006200	proprotein convertase subtilisin/kexin type 5	15.52
GNLY	NM_006433	Granulysin	15.23

CPM	NM_001874	carboxypeptidase M	13.56
COL1A1	AI743621	collagen, type I, alpha 1	11.54
MMP10	NM_002425	matrix metallopeptidase 10 (stromelysin 2)	10.84
C5orf13	U36189	chromosome 5 open reading frame 13	10.49
7b67f11.x1	BE551038	CDNA FLJ25178 fis, clone CBR09176	10.25
SLC46A2	AF242557	thymic stromal co-transporter	10.21
PPP1R14A	AA156998	protein phosphatase 1, regulatory (inhibitor) subunit 14A	10.12
LOC100507043	AU151635	Homo sapiens, clone IMAGE:4214962, mRNA	10.10
ADAMTS2	BF058422	ADAM metallopeptidase with thrombospondin type 1 motif, 2	9.96
PKD1L2	AW082870	polycystic kidney disease 1-like 2	9.88
WNT4	NM_030761	wingless-type MMTV integration site family, member 4	9.72
COL5A1	AI983428	collagen, type V, alpha 1	9.63
COL5A2	NM_000393	collagen, type V, alpha 2	9.42
PCOLCE	NM_002593	procollagen C-endopeptidase enhancer	9.29
RRM2	BC001886	ribonucleotide reductase M2 polypeptide	9.28
ADAMTS9	AI431730	ADAM metallopeptidase with thrombospondin type 1 motif, 9	9.21
Proliferative myometrium			

SCGB1D2	NM_006551	secretoglobin, family 1D, member 2	14.68
AGTR2	AI911273	angiotensin II receptor, type 2	10.41
PENK	NM_006211	Proenkephalin	9.24
ADAMTS2	BF058422	ADAM metallopeptidase with thrombospondin type 1 motif, 2	9.15
RASSF2	NM_014737	Ras association (RalGDS/AF-6) domain family 2	7.76
MMP23A	NM_004659	matrix metallopeptidase 23A & B	7.33
KCNE4	AI002715	potassium voltage-gated channel, Isk-related family, member 4	7.01
PLP1	BC002665	proteolipid protein 1 (Pelizaeus-Merzbacher disease, spastic paraplegia 2, uncomplicated)	6.80
OLFM4	AL390736	olfactomedin 4	6.72
SEL1L2	AL137678	sel-1 suppressor of lin-12-like 2 (C. elegans)	6.25
MACROD2	BF032500	MACRO domain containing 2	6.21
PGBD5	NM_024554	piggyBac transposable element derived 5	6.19
MXRA5	AF245505	matrix-remodelling associated 5	6.04
WISP2	NM_003881	WNT1 inducible signalling pathway protein 2	5.94
GREM1	AF154054	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	5.93
CPM	NM_001874	carboxypeptidase M	5.81

DIO2	NM_013989	deiodinase, iodothyronine, type II	5.41
KCNIP4	AI732844	Kv channel interacting protein 4	5.37
THY1	AL558479	Thy-1 cell surface antigen	5.22
CA3	NM_005181	carbonic anhydrase III, muscle specific	4.93
WFDC1	NM_021197	WAP four-disulfide core domain 1	4.89
AGTR2	NM_000686	angiotensin II receptor, type 2	4.78
CA12	AL050025	carbonic anhydrase XII	4.71
DAPP1	AI632216	dual adaptor of phosphotyrosine and 3-phosphoinositides	4.61
NRXN3	AI129949	neurexin 3	4.59
SYNC	NM_030786	syncoilin, intermediate filament 1	4.45
CCL21	NM_002989	chemokine (C-C motif) ligand 21	4.39
PTPLA	NM_014241	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	4.37
SYT1	AV723167	synaptotagmin I	4.36
C13orf33	AW084730	chromosome 13 open reading frame 33	4.32
Tamoxifen endometrium			
OLFM4	AL390736	olfactomedin 4	111.18
PI15	AI088609	peptidase inhibitor 15	51.45
GJB6	AI694073	gap junction protein, beta 6 (connexin 30)	50.21
WFDC1	NM_021197	WAP four-disulfide core domain 1	26.21
PENK	NM_006211	Proenkephalin	25.97

SLC46A2	AF242557	thymic stromal co-transporter	24.76
PITX1	NM_002653	paired-like homeodomain transcription factor 1	20.48
PTGDS	M61900	prostaglandin D2 synthase 21kDa (brain)	19.24
TMEM158	BF062629	Ras-induced senescence 1	18.00
GJC1	AA430014	gap junction protein, alpha 7, 45kDa (connexin 45)	17.15
WNT4	NM_030761	wingless-type MMTV integration site family, member 4	17.06
PDZK1	NM_002614	PDZ domain containing 1	16.57
EDNRA	AU118882	endothelin receptor type A	16.55
IGFBP5	AW157548	insulin-like growth factor binding protein 5	16.22
IGF1	AU144912	insulin-like growth factor 1 (somatomedin C)	14.65
ADAM12	W46291	ADAM metallopeptidase domain 12	14.34
ASCL1	BC001638	achaete-scute complex-like 1 (Drosophila)	14.03
ACTG2	NM_001615	actin, gamma 2, smooth muscle, enteric	12.95
SFRP4	NM_003014	secreted frizzled-related protein 4	12.53
zl50d02.s1	AA147933	CDNA FLJ31066 fis, clone HSYRA2001153	12.38
PPP2R2C	AI669212	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), gamma	12.30

		isoform	
ACCN1	NM_001094	amiloride-sensitive cation channel 1, neuronal (degenerin)	11.95
MRVI1	N66571	Murine retrovirus integration site 1 homolog	10.74
SDK2	N23651	sidekick homolog 2 (chicken)	10.49
IGFBP4	NM_001552	insulin-like growth factor binding protein 4	10.37
COL15A1	NM_001855	collagen, type XV, alpha 1	9.24
GJB2	M86849	gap junction protein, beta 2, 26kDa (connexin 26)	9.14
PTGER3	AW242315	prostaglandin E receptor 3 (subtype EP3)	9.04
COL21A1	NM_030820	collagen, type XXI, alpha 1	8.68
PAEP	NM_002571	progestagen-associated endometrial protein (placental protein 14, pregnancy-associated endometrial alpha-2-globulin, alpha uterine protein)	8.64
Tamoxifen myometrium			
KRT13	NM_002274	keratin 13	16.52
AGTR2	AI911273	angiotensin II receptor, type 2	16.18
FNDC1	AI345957	fibronectin type III domain containing 1	11.91
PENK	NM_006211	proenkephalin	11.03
PCDH20	AA040057	protocadherin 20	9.85

GREM1	AF154054	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	9.43
PI15	AI088609	peptidase inhibitor 15	9.03
DOCK10	NM_014689	dedicator of cytokinesis 10	8.35
DCLK1	BE672499	doublecortin-like kinase 1	7.76
ACCN1	NM_001094	amiloride-sensitive cation channel 1, neuronal (degenerin)	7.42
ADAMTS2	BF058422	ADAM metallopeptidase with thrombospondin type 1 motif, 2	7.03
KCNE4	AI002715	potassium voltage-gated channel, Isk-related family, member 4	6.20
NELL2	NM_006159	NEL-like 2 (chicken)	6.13
KCNIP4	AI732844	Kv channel interacting protein 4	6.08
SCG5	NM_003020	secretogranin V (7B2 protein)	6.03
RASSF2	NM_014737	Ras association (RalGDS/AF-6) domain family 2	5.62
CBLN4	AA868507	cerebellin 4 precursor	5.41
KCNE4	NM_080671	potassium voltage-gated channel, Isk-related family, member 4	5.15
KIAA1211	BE855799	KIAA1211 protein	4.91
PTPLA	NM_014241	protein tyrosine phosphatase-like (proline instead of catalytic arginine), member A	4.89
PGR	NM_000926	progesterone receptor	4.77
CITED1	NM_004143	Cbp/p300-interacting transactivator,	4.72

		with Glu/Asp-rich carboxy-terminal domain, 1	
ZCCHC12	AV725825	zinc finger, CCHC domain containing 12	4.7
UNC5D	NM_080872	Unc-5 homolog D (C. elegans)	4.64
AMIGO2	AC004010	adhesion molecule with Ig-like domain 2	4.51
JPH1	AI202201	junctophilin 1	4.51
IMAGE:5273964	AW298375	CDNA clone IMAGE:5273964	4.44
MCHR1	AI934819	melanin-concentrating hormone receptor 1	4.38
MXRA5	AF245505	matrix-remodelling associated 5	4.36
MFAP5	NM_003480	microfibrillar associated protein 5	4.34

Of the genes demonstrating a decreased level of expression on the endometrial and myometrial arrays with a 1.6-fold change cut-off (38.5% repression), 652 genes were identified in the proliferative endometrium, 893 genes in the tamoxifen treated endometrium, 132 genes in the proliferative myometrium and 88 genes in the tamoxifen treated myometrium. See Table 6.3 for the top 30 genes with decreased expression.

In both the proliferative and tamoxifen treated endometrium, 277 genes were induced and 436 were repressed, whilst in the myometrium 167 genes were induced and 49 genes were repressed (Figure 6.1). The noise, percentage of present or absent calls and scaling factors showed minor variations between the arrays; this is essential when comparing microarrays. These gene chip experiments revealed an overall correlation ($r2 = 0.89$) with an average of only 0.004% of probe sets showing more than a two-fold change between experiments. These are within the boundaries of a well conducted microarray experiment.

6.3.2 Confirmation of microarray data

To validate the microarray analysis data, I chose eight candidate genes for the endometrium and 16 candidate genes for the myometrium (Table 6.2 and 6.3). In the endometrium, genes were chosen that were already known to be affected by oestrogen, e.g. the ERα, PR, PR-B, the transcription factor homeobox A11 (HoxA11) and the protease whey acidic protein (WAP) four disulphide core domain 2 (WFDC2) and genes unknown to be affected but identified on microarray to be regulated, e.g. ERβ, the ionotrophic glutamate receptor 2 (GRIA2), the protease WAP four disulphide core domain 1 (WFDC1) and the precursor opiod peptide producer, proenkephalin (PENK). PENK has been shown to be expressed in the Ishikawa cell line (Hatzoglou et al., 1995), rat and monkey uterus but not in the normal human endometrium.

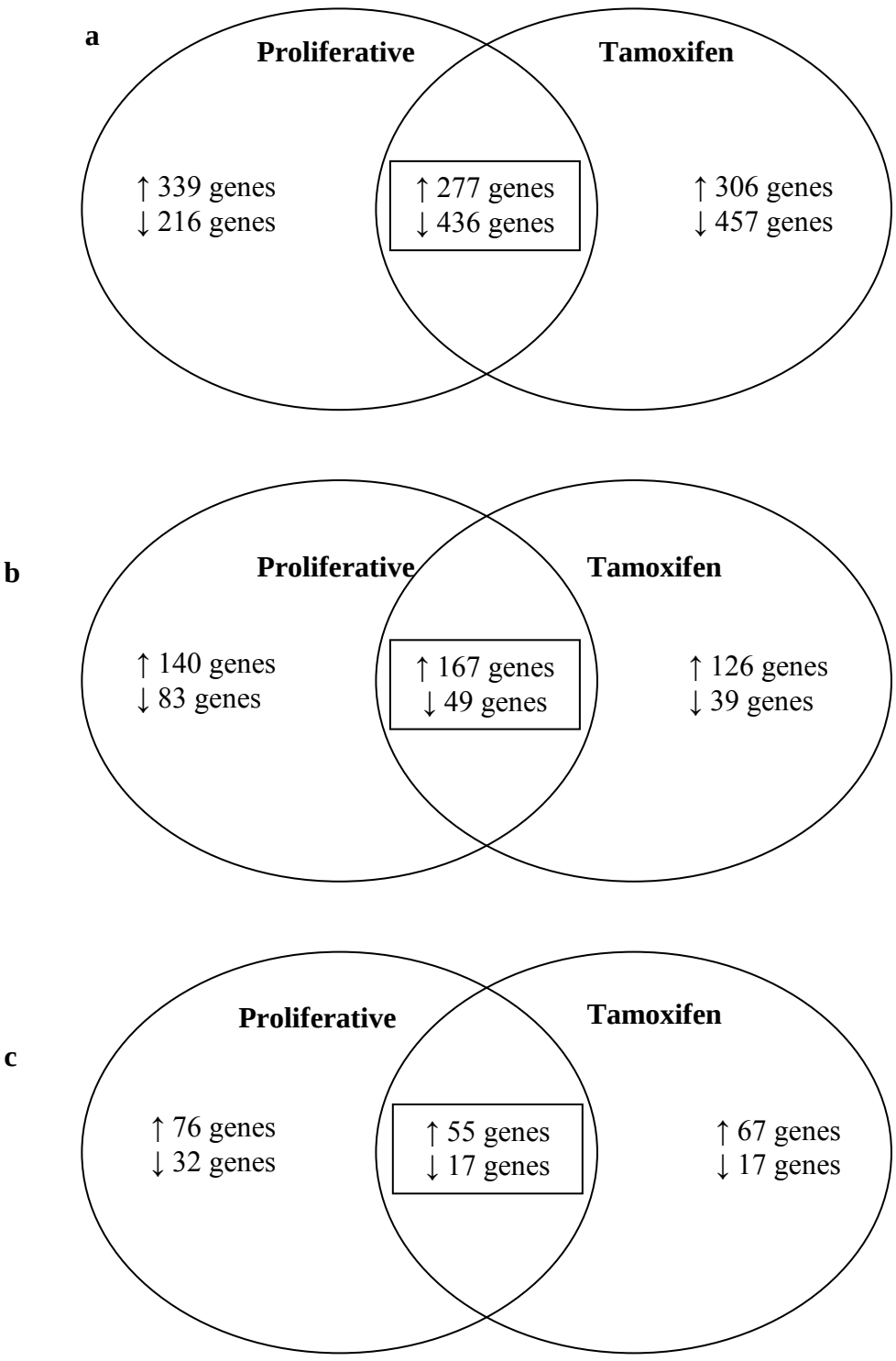


Figure 6.1. Venn-diagrams of gene expression profiles.

Data are, (a) the number of genes modulated in the proliferative or tamoxifen endometrium compared to atrophic endometrium, (b) the number of genes modulated in the proliferative or tamoxifen myometrium compared to atrophic myometrium, and (c) the number of genes

modulated in the tamoxifen or proliferative uterus (endometrium and myometrium combined). Genes within the intersections are genes regulated by both in the proliferative and tamoxifen treated uteri, genes outside this area are unique to that tissue.

Table 6.3. List of the top 30 genes down-regulated in the proliferative and tamoxifen-treated endometrium and myometrium.

The gene symbol, accession number and gene name are listed. Fold change = fluorescence levels relative to the levels found in the relevant atrophic tissue.

Gene Symbol	Accession Number	Gene Name	Fold Change
Proliferative endometrium			
IGHA1	S55735	immunoglobulin heavy constant alpha 1 & 2	-286.47
CSH2	NM_022645	chorionic somatomammotropin hormone 2	-142.89
CSH1	NM_022642	chorionic somatomammotropin hormone 1 (placental lactogen)	-110.73
CGA	NM_000735	glycoprotein hormones, alpha polypeptide	-95.02
IGH@	M87789	immunoglobulin heavy locus	-80.19
CHL1	NM_006614	cell adhesion molecule with homology to L1CAM (close homolog of L1)	-72.47
LIX1	N29837	Lix1 homolog (mouse)	-63.58
COL9A1	NM_001851	collagen, type IX, alpha 1	-59.37
LTF	NM_002343	Lactotransferrin	-55.06
CSHL1	NM_022579	chorionic somatomammotropin hormone-like 1	-49.67
IGKC	BG485135	Immunoglobulin kappa constant	-47.37
IGJ	AV733266	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu	-45.44

		polypeptides	
IGL@	M87790	Immunoglobulin lambda locus	-44.47
CLU	M25915	Clusterin	-38.26
KISS1	NM_002256	KiSS-1 metastasis-suppressor	-35.45
IGKV@	AW404894	similar to Ig kappa chain V-I region Walker precursor	-31.38
IGKC	BG536224	immunoglobulin kappa constant	-30.05
MSLN	NM_005823	Mesothelin	-28.26
GH2	NM_022557	growth hormone 2	-27.37
GPX3	NM_002084	glutathione peroxidase 3 (plasma)	-25.87
FAM107A	AL050264	family with sequence similarity 107, member A	-25.58
GH1	AF185611	growth hormone 1	-25.41
PSG7	NM_002783	pregnancy specific beta-1-glycoprotein 7	-24.84
RARRES1	NM_002888	retinoic acid receptor responder (tazarotene induced) 1	-24.15
C2CD4A	BE218239	Nuclear localized factor 1	-22.72
SOX9	NM_000346	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	-22.03
UBD	NM_006398	gamma-aminobutyric acid (GABA) B receptor	-20.49
CXCL2	M57731	chemokine (C-X-C motif) ligand 2	-20.06
IGKV1-5	BG540628	(clone TR1.6VL) anti-thyroid peroxidase monoclonal autoantibody IgK chain, V	-19.91

		region	
CGB5/7	NM_000737	chorionic gonadotropin, beta polypeptide 5 & 7	-19.77
Proliferative myometrium			
FosB	NM_006732	FBJ murine osteosarcoma viral oncogene homolog B	-42.01
GRIA2	NM_000826	glutamate receptor, ionotropic, AMPA 2	-15.21
ah68c03.s1	AA758105	Transcribed locus (EST)	-10.48
ATF3	NM_001674	activating transcription factor 3	-8.84
LGR5	AL524520	leucine-rich repeat-containing G protein-coupled receptor 5	-7.90
SOCS3	AI244908	suppressor of cytokine signalling 3	-7.70
th79b10.x1	AI435590	gb:AI435590 /EST	-7.37
NR4A1	NM_002135	nuclear receptor subfamily 4, group A, member 1	-6.01
v-fos	BC004490	v-fos FBJ murine osteosarcoma viral oncogene homolog	-5.87
Wif-1	NM_007191	WNT inhibitory factor 1	-5.81
PLAG1	NM_002655	pleiomorphic adenoma gene 1	-5.35
7o64e09.x1	BF449053	hypothetical protein LOC285016	-5.20
BEX2	AF251053	brain expressed X-linked 2	-5.11
LOC572558	BF677651	hypothetical locus LOC572558	-5.02
EGR1	AV733950	early growth response 1	-5.00
NR4A2	S77154	nuclear receptor subfamily 4,	-4.99

		group A, member 2	
ZEB1	NM_030751	zinc finger E-box binding homeobox 1	-4.81
JUNB	NM_002229	jun B proto-oncogene	-4.67
IGFBP3	BF340228	insulin-like growth factor binding protein 3	-4.54
F3	NM_001993	coagulation factor III (thromboplastin, tissue factor)	-4.53
HRC13275	BC003629	CDNA: FLJ23438 fis, clone HRC13275	-4.41
CSRNP1	NM_033027	cysteine-serine-rich nuclear protein 1	-4.23
NUAK2	AL831884	SNF1-like kinase 2	-3.98
BEX5	AV726956	NGFRAP1-like 1	-3.93
Hh02e12.x1	AW612461	ERBB receptor feedback inhibitor 1	-3.91
AGS1	AF069506	RAS, dexamethasone-induced 1	-3.91
DUSP1	AA530892	dual specificity phosphatase 1	-3.89
ZFP36	NM_003407	zinc finger protein 36, C3H type, homolog (mouse)	-3.83
EGR3	NM_004430	early growth response 3	-3.69
v-MAF	AL021977	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	-3.67
Tamoxifen treated endometrium			
CGA	NM_000735	glycoprotein hormones, alpha polypeptide	-205.31
CSH1	NM_022641	chorionic somatomammotropin hormone 1 (placental lactogen)	-201.51
CSH2	NM_022645	chorionic somatomammotropin hormone 2	-147.03
IGHA1	S55735	immunoglobulin heavy constant alpha 1	-122.44

CSHL1	NM_022579	chorionic somatomammotropin hormone-like 1	-47.34
FLJ12005	AK022067	gb:AK022067.1 / Homo sapiens cDNA FLJ12005 fis, clone HEMBB1001565.	-44.69
COL9A1	NM_001851	collagen, type IX, alpha 1	-39.65
IGL@	X57812	immunoglobulin lambda locus	-32.87
LTF	NM_002343	Lactotransferrin	-28.99
GH2	NM_022557	growth hormone 2	-28.67
IGH@	M87789	immunoglobulin heavy locus	-28.26
yb29h11.s1	T52027	KIAA1217	-26.17
IGK@	AW575927	immunoglobulin kappa constant	-24.76
IGKC	BG485135	Immunoglobulin kappa constant	-24.75
CGB5/7	NM_000737	chorionic gonadotropin, beta polypeptide	-24.72
IGL@	AA680302	immunoglobulin lambda locus	-23.15
PSG7	NM_002783	pregnancy specific beta-1-glycoprotein 7	-22.41
CHL1	NM_006614	cell adhesion molecule with homology to L1CAM (close homolog of L1)	-22.39
CDR1/2/3	M87790	Immunoglobulin lambda locus	-22.26
KIAA1217	AU146385	KIAA1217	-21.23
GH1	AF185611	growth hormone 1	-20.69
PSG2	NM_031246	pregnancy specific beta-1-glycoprotein 2	-20.13
KISS1	NM_002256	KiSS-1 metastasis-suppressor	-19.58
GPCR5A	BE815259	G protein-coupled receptor, family C, group 5, member A	-18.15
RARRES1	NM_002888	retinoic acid receptor responder (tazarotene	-17.81

		induced) 1	
LIX1	N29837	Lix1 homolog (mouse)	-17.78
CD9	AK025016	CD9 molecule	-17.68
THBS1	AK024132	Thrombospondin, type I, domain containing 4	-16.65
DHRS3	T68858	Dehydrogenase/reductase (SDR family) member 3	-16.52
PSG3	NM_021016	pregnancy specific beta-1-glycoprotein 3	-16.05
Tamoxifen treated myometrium			
Wif-1	NM_007191	WNT inhibitory factor 1	-5.89
IGH1/2	S55735	immunoglobulin heavy constant alpha 1 & 2	-5.73
FosB	NM_006732	FBJ murine osteosarcoma viral oncogene homolog B	-5.69
SCGB2A1	NM_002407	secretoglobin, family 2A, member 1	-5.62
LGR5	AL524520	leucine-rich repeat-containing G protein-coupled receptor 5	-5.49
IGJ	AV733266	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	-5.04
PLAG1	NM_002655	pleiomorphic adenoma gene 1	-4.86
CLDN1	AW264204	claudin 11 (oligodendrocyte transmembrane protein)	-4.78
SOCS3	AI244908	suppressor of cytokine signalling 3	-4.53

KIAA1324	AB037745	KIAA1324	-4.49
PROM1	NM_006017	prominin 1	-4.38
F3	NM_001993	coagulation factor III (thromboplastin, tissue factor)	-4.16
th79b10.x1	AI435590	gb:AI435590 /EST	-4.09
CDH1	NM_004360	cadherin 1, type 1, E-cadherin (epithelial)	-3.93
oe93h05.s1	AA838075	polymeric immunoglobulin receptor	-3.92
FAM150B	BF449053	hypothetical protein LOC285016	-3.83
GRIA2	NM_000826	glutamate receptor, ionotropic, AMPA 2	-3.68
TSPAN8	NM_004616	tetraspanin 8	-3.56
IGFBP3	BF340228	insulin-like growth factor binding protein 3	-3.55
IGKV@	AW404894	similar to Ig kappa chain V-I region Walker precursor	-3.52
NR4A2	NM_006186	nuclear receptor subfamily 4, group A, member 2	-3.46
TMEM184B	AL021977	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	-3.45
IGKC	AW575927	immunoglobulin kappa constant	-3.42
CCL2	S69738	chemokine (C-C motif) ligand 2	-3.27
IGHG3	M87789	immunoglobulin heavy constant gamma 3 (G3m marker)	-3.21
GPX3	NM_002084	glutathione peroxidase 3 (plasma)	-3.09
BHLHE41	BE857425	basic helix-loop-helix domain containing, class B, 3	-3.08
FLJ23438	BC003629	CDNA: FLJ23438 fis, clone HRC13275	-3.02

SLC6A6	BG150485	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	-2.99
EPCAM	NM_002354	epithelial cell adhesion molecule	-2.93

To confirm the myometrial expression data, all the above mentioned genes were used in addition to myometrial smooth muscle cell genes, e.g. α-smooth muscle actin, smoothelin and vimentin. I also selected genes that were differentially regulated on the microarray, e.g. carbonic anhydrase 12 (CA12), CD24, FosB, IGFBP3, Wif-1 and WISP2, these genes were represented by several probe sets on the HU133_plus 2 microarray chip and were modulated more than 2-fold. CD24 has been associated with tamoxifen associated breast cancer treatment (Surowiak et al., 2006) and is thought to be down regulated *via* ERα. FosB is a component of the activator protein 1 complex and is known to be involved in myometrial cell remodelling, especially during the latter stages of pregnancy (Mitchell and Lye, 2005), whereas IGFBP3 expression in the breast has been shown to inhibit cellular proliferation (Schernhammer et al., 2003) and induce apoptosis that is independent of IGF-I and IGF-1 receptor, especially in the lungs (London et al., 2002). FosB is also known to be regulated by sex steroids (Moggs et al., 2004). The WNT signalling pathway proteins, WNT inhibitory factor 1 (Wif-1) and the WNT1 inducible signalling pathway protein 2 (WISP2) products are intimately involved in tissue remodelling and breast cancer generation, and are thought to be oestrogen regulated (Banerjee et al., 2005). Wif-1 has been shown to be down regulated in numerous breast cancer cell lines (Ai et al., 2006) and hormonally regulated tumours (Wissman et al., 2003).

The cycle numbers for each primer set were obtained empirically with an untreated endometrial or myometrial extract and each amplicon was of the expected size (Figure 6.2). In quantitative RT-PCR, all genes apart from Wif1 provided a single melting curve peak at the expected melting temperature for the amplified product (Table 6.1). The presence of Wif1 of higher molecular weight did not hinder the qPCR process as it represented only 3% of the amplified product.

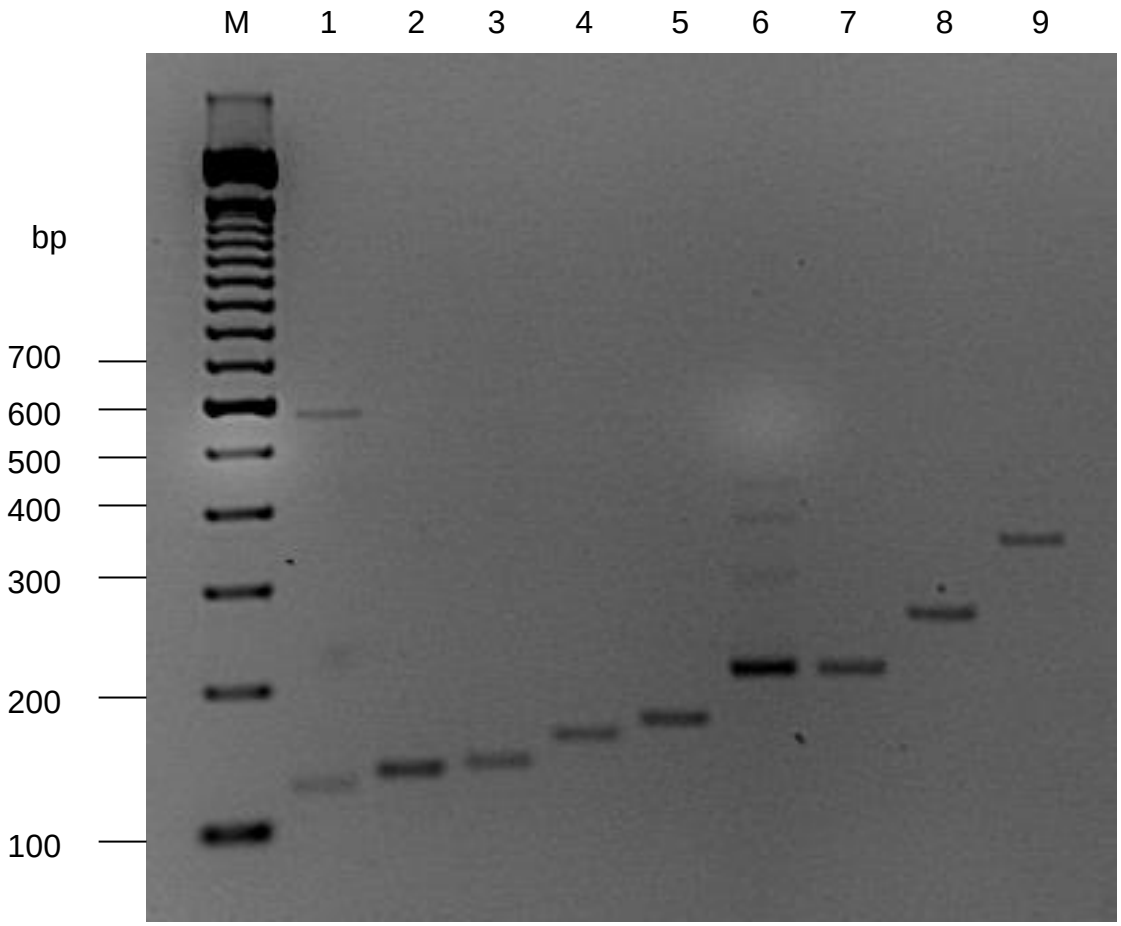
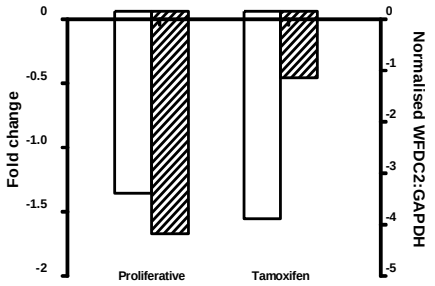


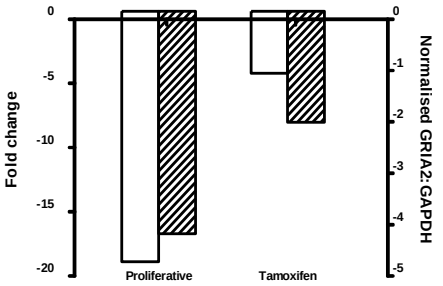
Figure 6.2. EtBr-stained agarose gel indicating the amplicon sizes of products generated with the unpublished gene-specific primers listed in Table 6.1.

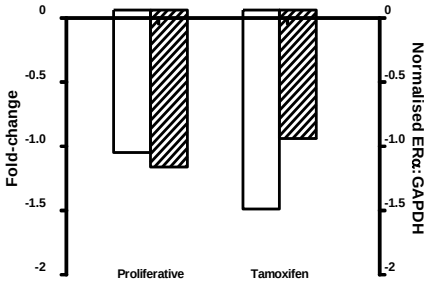
Lane M=100bp ladder; lane 1=PENK; lane 2=CA12; lane 3=GRIA2; lane 4=IGFBP3; lane 5=WISP2; lane 6=WIF1; lane 7=FOSB; lane 8=CD24; lane 9=GAPDH. bp = base-pairs.

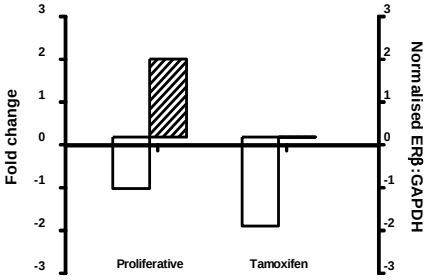
In most cases, an increase or decrease in transcript expression for the various genes on the microarray was confirmed by qPCR analysis (Figures 6.3 & 6.4). There was only one gene that did not follow the same pattern and that was the PR-B isoform. This could be due to the fact that the sequences on the HU133_Plus2 array cannot distinguish between the two main isoforms of the progesterone receptors A and B, whereas the primers used can (Table 6.1). These data provide additional information which suggests that in the proliferative endometrium progesterone receptor levels are increasing when compared to the postmenopausal endometrium, thus it is the PR-A isoform that is increasing. In the tamoxifen uterus, both PR-A and PR-B isoforms are being induced.

a

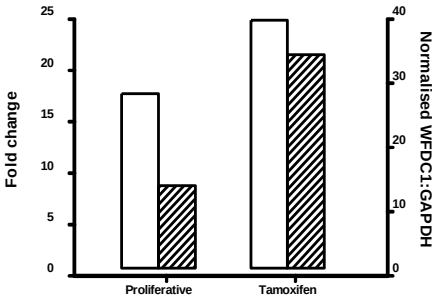

Fold change
Normalised WFDC2:GAPDH
Proliferative
Tamoxifen

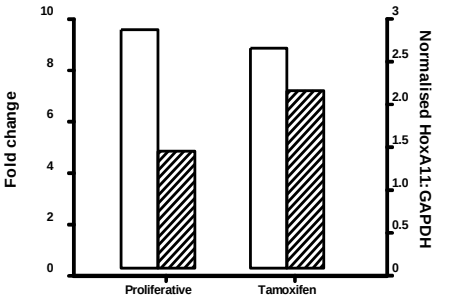

Fold change
Normalised GRIA2:GAPDH
Proliferative
Tamoxifen

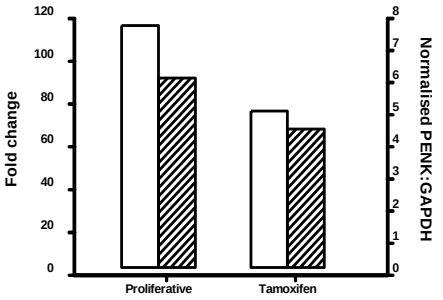

Fold-change
Normalised ERα:GAPDH
Proliferative
Tamoxifen

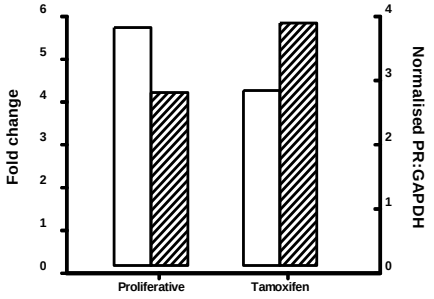

Fold change
Normalised ERβ:GAPDH
Proliferative
Tamoxifen

b


Fold change
Normalised WFDC1:GAPDH
Proliferative
Tamoxifen


Fold change
Normalised HoxA11:GAPDH
Proliferative
Tamoxifen


Fold change
Normalised PENK:GAPDH
Proliferative
Tamoxifen


Fold change
Normalised PR:GAPDH
Proliferative
Tamoxifen

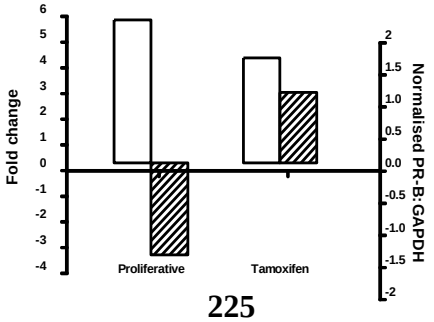

Fold change
Normalised PR-B:GAPDH
Proliferative
Tamoxifen

Figure 6.3 Validation of the endometrial microarray data by qPCR. The graphs show a selection of genes that were (a) down regulated in both the proliferative and tamoxifen endometrium (open bars) and the mean levels of transcript measured by qPCR (hatched bars). Panel (b) shows a selection of genes up regulated in both the proliferative and tamoxifen endometrium. Data are from 11 fluorescence probes (microarray data) and 6 individual biopsies (qPCR data).

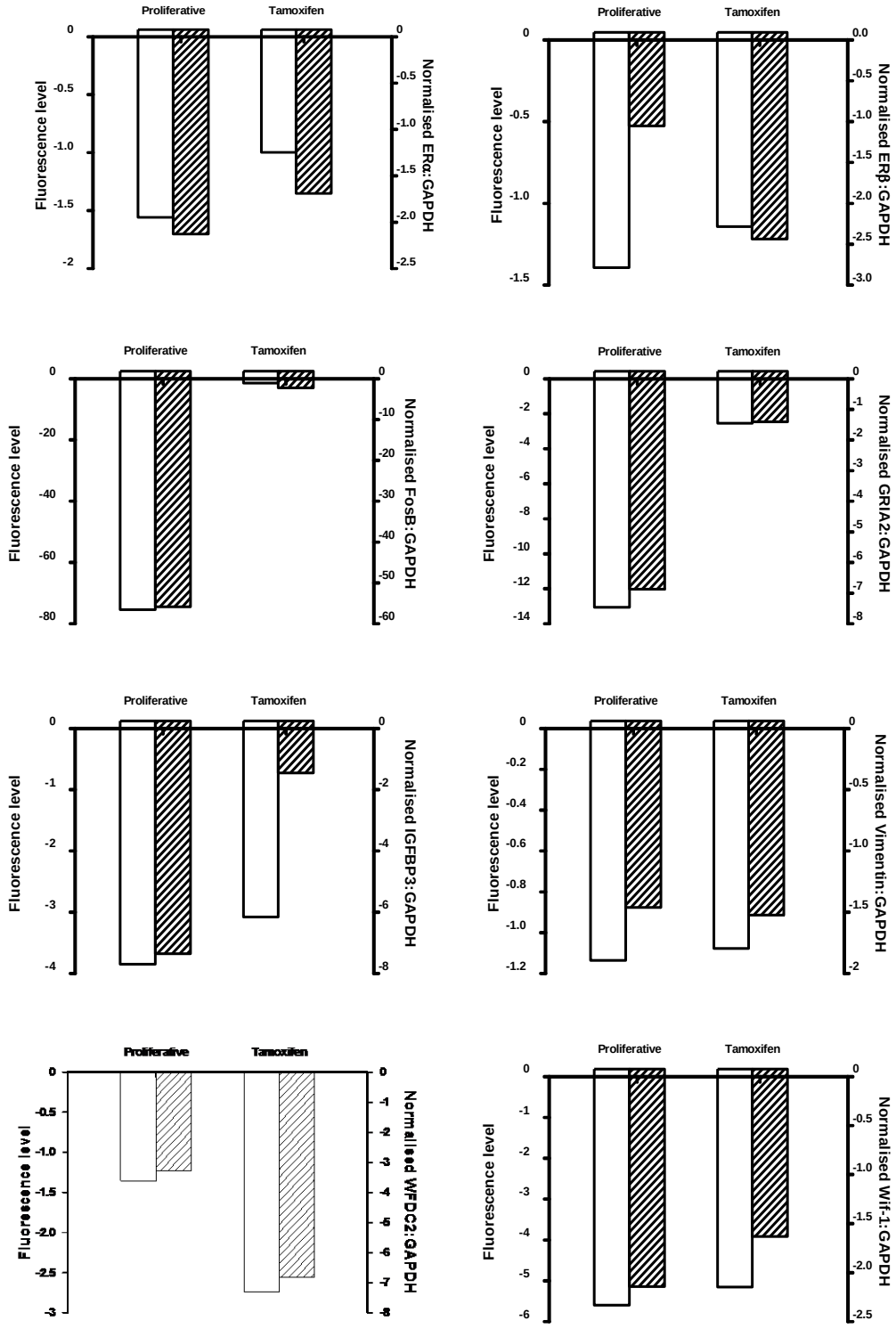


Figure 6.4a Validation of the myometrial microarray data by qPCR.

The graphs show a selection of genes down regulated in both the proliferative and tamoxifen myometrium (open bars) and the mean levels of transcript measured by qPCR (hatched bars).

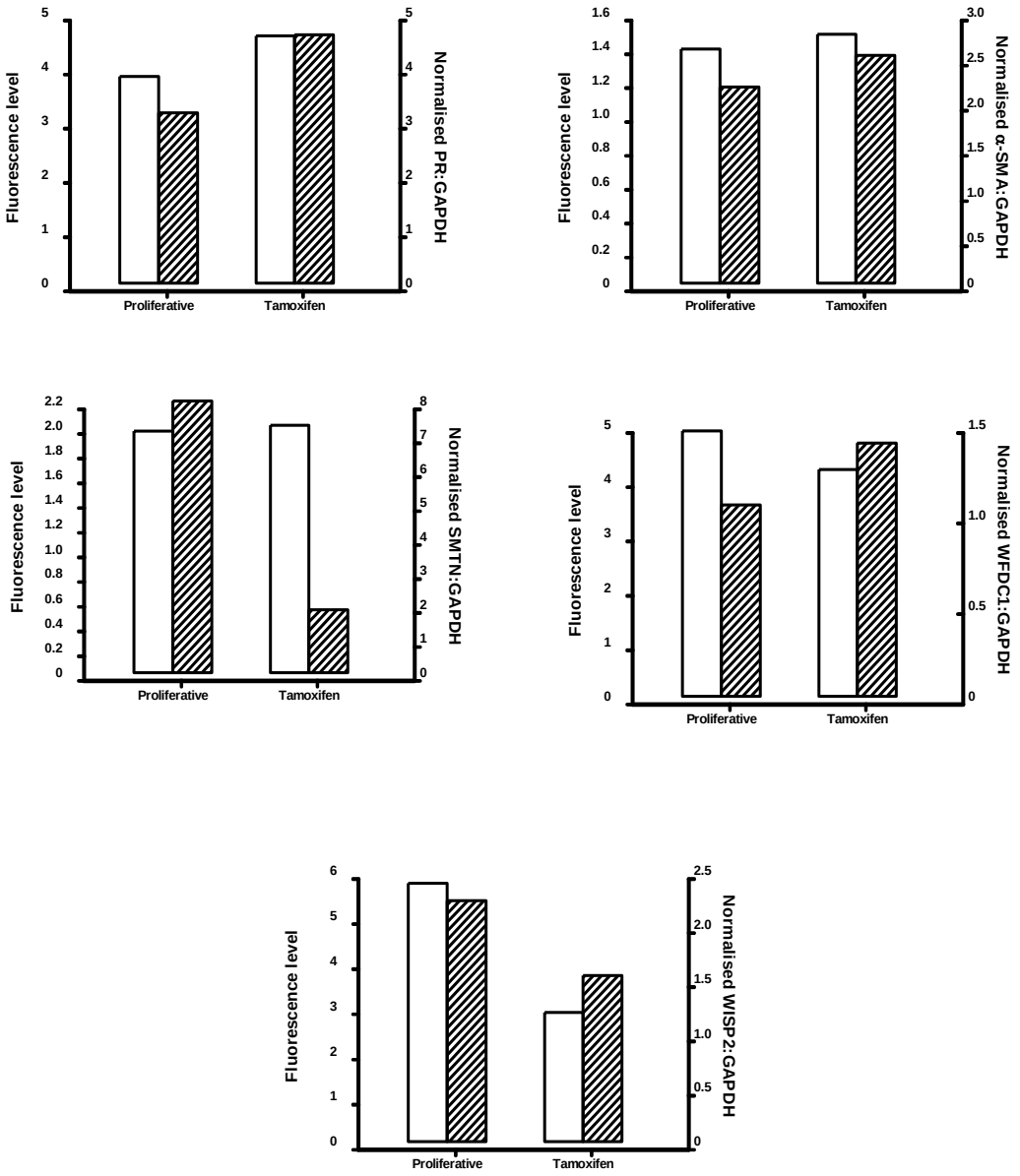


Figure 6.4b Validation of the myometrial microarray data by qPCR.

The graphs show a selection of genes up regulated in both the proliferative and tamoxifen myometrium. Data are from 11 fluorescence probes (microarray data) and 6 individual biopsies (qPCR data).

6.3.4 Pathway analyses

In the proliferative endometrium, a total of 2390 probe sets out of 54613 satisfied the 1.6 fold filtering criteria, with a possible 5892 possible genes listed. This gave an expected pathway frequency of 0.403. In the proliferative myometrium, a total of 1715 probe sets out of 54613 satisfied the 1.6 fold filtering criteria, with 5892 possible listed genes. This gave an expected pathway frequency of 0.277. In the tamoxifen treated endometrium, a total of 1670 probe sets out of 54613 meet the 1.6 fold filtering criteria, with 5892 possible genes listed. This gave an expected pathway frequency of 0.263.

A total of 108 different pathways were affected in the proliferative and tamoxifen treated endometrium, and 75 different pathways were affected in the proliferative and tamoxifen treated myometrium when compared to atrophic controls. Of these, 81 were affected in the proliferative endometrium, 85 in the tamoxifen treated endometrium, 78 in the proliferative myometrium and 71 in the tamoxifen treated myometrium (Table 6.4). Detailed analysis of these pathways showed that only a small proportion of the pathways were affected in the proliferative uteri alone (Table 6.5). The common pathways affected were limited to 47 pathways in the endometrium and 48 in the myometrium. Of these pathways, only the calcium signalling, drug metabolism cytochrome P450, ECM-receptor interaction, haemopoietic cell lineage, metabolism of xenobiotics by cytochrome P450, monoamine GPCRs, neuroactive ligand-receptor interaction, oxidative phosphorylation and tamoxifen metabolism pathways were over represented in all four tissue groups (Table 6.5), whereas the Alzheimer's disease, citrate (TCA) cycle, cytoplasmic ribosomal protein, electron transport chain, eukaryotic transcription initiation, Huntington's disease, mRNA processing, nucleotide excision repair, propanoate metabolism, proteasome degradation, pyruvate metabolism, ribosome RNA polymerase, TNF-alpha/NK-κB signalling,

translation factors and the valine leucine and isoleucine degradation pathways were all underrepresented in all four tissue groups (Table 6.5). These represent common uterine oestrogen/tamoxifen affected pathways.

There were only seven pathways affected in both the proliferative and tamoxifen treated endometrium of which autoimmune thyroid disease, cytokines and inflammatory response (BioCarta), the Irinotecan pathway and prostaglandin synthesis and regulation pathways were over represented and three pathways, alanine and aspartate metabolism , basal transcription factors and cholesterol biosynthesis had fewer genes than predicted (Table 6.5).

In the tamoxifen treated endometrium, only arylamine metabolism, the Blakely Network, GnRH signalling pathway, insulin signalling, leukocyte transendothelial migration, the MAPK signalling and TGF-Beta signalling pathways had more genes modulated than anticipated and the apoptosis modulation by HSP70, arginine and proline metabolism, base excision repair, fructose and mannose metabolism, glutathione metabolism and glycosaminoglycan degradation pathways had less modulated genes than anticipated. These represent 7 over represented pathways and 6 underrepresented pathways unique within the tamoxifen treated endometrium (Table 6.5).

In the tamoxifen treated myometrium, only adherens junction, G1 to S cell cycle control, Jak-STAT signalling pathway, linoleic acid metabolism, long-term depression, melanogenesis, melanoma, non-small cell lung cancer, peptide GPCRs, the statin pathway (PharmGKB) and sulphur metabolism pathways had more genes modulated than was anticipated and the delta-Notch signalling, DNA replication, folate biosynthesis, glycolysis

and gluconeogenesis, non-homologous end-joining and protein export pathways contained less modulated genes than anticipated. These represent 11 over represented pathways and 6 underrepresented pathways unique within the tamoxifen treated endometrium (Table 6.5).

Finally, the cytokine-cytokine receptor interaction, fatty acid omega oxidation, inflammatory response and nuclear receptors in lipid metabolism and toxicity pathways contained more genes than was anticipated in both the endometrium and myometrium and thus represent tamoxifen unique pathways affecting the entire uterus (Table 6.5). There were no pathways that contained fewer modulated genes than was predicted.

Feature	Proliferative Endometrium	Tamoxifen treated endometrium	Proliferative myometrium	Tamoxifen treated myometrium
Number of pathways affected	81	85	71	78
↑ genes than expected	42	39	40	46
↓ genes than expected	39	36	31	34
Oestrogen-specific pathways	33	0	23	0
Tamoxifen-specific pathways	0	26	0	30
Common (uterotrophic pathways)	47	47	48	48

Table 6.4. The number of pathways affected in proliferative and tamoxifen-treated uteri.

The total number of pathways affected was 106 in the endometrium and 101 in the myometrium.

↑ genes than expected = number of pathways where the genes were over-represented;

↓ genes than expected = number of pathways where the genes were under-represented;

oestrogen-specific pathways = pathways only affected in the proliferative uterus;

tamoxifen-specific pathways = pathways only affected in the tamoxifen treated uterus;

common (uterotrophic pathways) = pathways affected in both the proliferative and tamoxifen treated uterus.

Table 6.5. Lists of pathways affected in the proliferative and tamoxifen treated uteri.

Pathways with more (M) genes modulated than expected and pathways with fewer genes (F) modulated than expected. Pathways where genes were either not modulated or numbers were not significantly different from the expected values are not shown. A p-value of <0.05 was considered significant using χ^2-test. The expected gene frequencies in the different tissues were calculated using PathVisio and were 0.385 for proliferative endometrium, 0.403 for tamoxifen endometrium, 0.277 for proliferative myometrium and 0.263 for tamoxifen myometrium. The pathways were then clustered to identify common and dissimilar pathways in the endometrium and myometrium that are commonly affected by oestrogen and tamoxifen and those that were uniquely oestrogenic or tamoxifenogenic.

	Proliferative endometrium	Tamoxifen treated endometrium	Proliferative myometrium	Tamoxifen treated myometrium
Calcium signalling pathway	M	M	M	M
Drug metabolism - cytochrome P450	M	M	M	M
ECM-receptor interaction	M	M	M	M
Hematopoietic cell lineage	M	M	M	M
Metabolism of xenobiotics by	M	M	M	M

cytochrome P450				
Monoamine GPCRs	M	M	M	M
Neuroactive ligand-receptor interaction	M	M	M	M
Tamoxifen metabolism	M	M	M	M
Nuclear Receptors	M	M	M	
Small Ligand GPCRs	M	M	M	
Systemic lupus erythematosus	M	M	M	
Cell adhesion molecules (CAMs)	M	M	M	
Endochondral Ossification	M	M	M	
Asthma	M	M		
Autoimmune thyroid disease	M	M		
Axon guidance	M	M		
Cytokines and Inflammatory	M	M		

Response (BioCarta)				
Irinotecan Pathway	M	M		
Prostaglandin Synthesis and Regulation	M	M		
Complement and coagulation cascades	M	M		M
Pathways in cancer	M	M		M
Pentose and glucuronate interconversions	M	M		M
Drug metabolism - other enzymes	M		M	M
Estrogen metabolism	M		M	M
Glucuronidation	M		M	M
Metapathway biotransformation	M		M	M
Matrix Metalloproteinases	M		M	

p53 signalling pathway	M		M	
Starch and sucrose metabolism	M		M	
Benzo[a]pyrene metabolism	M		F	M
Adipogenesis	M			M
Allograft rejection	M			
Antigen processing and presentation	M			
Ascorbate and aldarate metabolism	M			
Cell cycle	M			
Complement Activation, Classical Pathway	M			
GPCRs, Class B Secretin-like	M			
Graft-versus-host disease	M			
Hedgehog	M			

Signalling Pathway				
T cell receptor signalling pathway	M			
Type I diabetes mellitus	M			
Alzheimer's disease	F	F	F	F
Citrate cycle (TCA cycle)	F	F	F	F
Cytoplasmic Ribosomal Proteins	F	F	F	F
Electron Transport Chain	F	F	F	F
Eukaryotic Transcription Initiation	F	F	F	F
Huntington's disease	F	F	F	F
mRNA processing	F	F	F	F
Nucleotide excision repair	F	F	F	F

Oxidative phosphorylation	F	F	F	F
Parkinson's disease	F	F	F	F
Propanoate metabolism	F	F	F	F
Proteasome	F	F	F	F
Proteasome Degradation	F	F	F	F
Pyruvate metabolism	F	F	F	F
Ribosome	F	F	F	F
RNA polymerase	F	F	F	F
TNF-alpha/NF-kB Signalling Pathway	F	F	F	F
Translation Factors	F	F	F	F
Valine, leucine and isoleucine degradation	F	F	F	F
Alanine and aspartate metabolism	F	F		
TCA Cycle	F	F		

Cholesterol Biosynthesis	F	F		
Aminoacyl-tRNA biosynthesis	F	F		F
Estrogen signalling	F	F		F
Basal transcription factors	F	F		
Pyrimidine metabolism	F		F	F
Aminophosphonate metabolism	F			
Arachidonate Epoxygenase & Epoxide Hydrolase	F			
Benzoate degradation via CoA ligation	F			
Butanoate metabolism	F			
Fatty Acid Beta Oxidation	F			
Glycerolipid	F			

metabolism				
Glycogen Metabolism	F			
Glyoxylate and dicarboxylate metabolism	F			
Hs Selenium metabolism/Selenoprotein	F			
Inositol phosphate metabolism	F			
N-Glycan biosynthesis	F			
Phosphatidyl-inositol signalling system	F			
Focal adhesion		M	M	M
GPCRs, Class A Rhodopsin-like		M	M	M
Arylamine metabolism		M		
Blakely Network		M		
Cytokine-cytokine receptor interaction		M		M

Fatty Acid Omega Oxidation		M		M
Inflammatory Response Pathway		M		M
Nuclear receptors in lipid metabolism and toxicity		M		M
PPAR signalling pathway		M		M
GnRH signalling pathway		M		
GPCRs, Other		M		
Insulin Signalling		M		
Integrin-mediated cell adhesion		M		
Leukocyte transendothelial migration		M		
MAPK signalling pathway		M		
TGF Beta Signalling Pathway		M		F

Base excision repair		F	M	F
Mismatch repair		F	F	
Aminosugars metabolism		F		
Apoptosis Modulation by HSP70		F		
Arginine and proline metabolism		F		
Atrazine degradation		F		
Fructose and mannose metabolism		F		
Glutathione metabolism		F		
Glycosaminoglycan degradation		F		
Selenoamino acid metabolism		F		
Androgen and estrogen metabolism			M	M

Ascorbate and aldarate metabolism			M	M
Calcium Regulation in the Cardiac Cell			M	M
Glycosphingolipid biosynthesis - lacto and neolact			M	M
Hypothetical Network for Drug Addiction			M	M
Retinol metabolism			M	M
Small cell lung cancer			M	M
Striated Muscle Contraction			M	M
Blood Clotting Cascade			M	
Caffeine metabolism			M	
D-Glutamine and D-glutamate metabolism			M	

IL-5 Signalling Pathway			M	
Myometrial Relaxation and Contraction Pathways			M	
Ovarian Infertility Genes			M	
Regulation of actin cytoskeleton			M	
Sulphation			M	
Taurine and hypotaurine metabolism			M	
Acute myeloid leukaemia			F	
Circadian			F	
Glycine, serine and threonine metabolism			F	
Notch signalling pathway			F	F
Nucleotide Metabolism			F	F
Purine			F	F

metabolism				
Regulation of autophagy			F	
Ubiquitin mediated proteolysis			F	
Delta-Notch Signalling Pathway				F
DNA Replication				F
Folate biosynthesis				F
Glycolysis and Gluconeogenesis				F
Non-homologous end-joining				F
Protein export				F
Adherens junction				M
Cytochrome P450				M
G1 to S cell cycle control				M
Jak-STAT signalling pathway				M
Linoleic acid				M

metabolism				
Long-term depression				M
Melanogenesis				M
Melanoma				M
Non-small cell lung cancer				M
Peptide GPCRs				M
Statin Pathway (PharmGKB)				M
Sulphur metabolism				M

6.4 Discussion

Microarray analysis has demonstrated that there are distinct changes in the transcription pattern in the uterus in response to proliferative phase signals and in response to tamoxifen. I have taken the proliferative phase uterus to represent oestrogen stimulated tissue, whilst the tamoxifen treated uterus represents a tamoxifen stimulated tissue. It is not surprising that the transcript patterns in the myometrium are different from that of the endometrium. There are, though, some genes that are similarly regulated in both tissues and several pathways that were altered in both the endometrium and myometrium by both oestradiol and tamoxifen. The gene expression profile of both the endometrium and myometrium was different between oestrogen and tamoxifen. Whilst there were pathways that were similarly affected (induced or inhibited) by oestrogen and tamoxifen, each of these agents had its own unique pattern (Tables 6.4 and 6.5).

These findings suggest that what defines oestrogen and tamoxifen are some similarities and other features that are unique to each. Tamoxifen is best known for its oestrogen antagonist effects and it is this oestrogen antagonist action of tamoxifen is what has made the compound a useful treatment for women diagnosed with hormone positive breast carcinoma. However, in addition to the oestrogen antagonist effects, tamoxifen also displays oestrogen agonist properties. Tamoxifen is known for its beneficial effect on bone density (Chang et al., 1996), colonic and bladder cancer, has been shown to lower cholesterol concentrations in the blood stream (Schapira et al., 1990). The major disadvantage of tamoxifen use for breast cancer therapy occurs in the endometrium, where it causes a 2 to 7-fold increased risk of endometrial cancer (Fisher et al., 1998; Mignotte et al., 1998; Bergman et al., 2000).

Previously, microarray analyses using a human cell line (ECC1), showed that tamoxifen gene expression modulation when compared to oestradiol gene modulation not only overlapped but also that tamoxifen regulated its own specific set of genes (Gielen et al., 2005b). Gielen and colleagues confirmed this concept *in vivo*, when they identified 256 gene products that were regulated in the endometrium from tamoxifen treated patients (Gielen et al., 2005b). Importantly, a number of these gene products were observed to be part of pathways which could play a role in endometrial carcinogenesis. It is thought that tamoxifen induction of imbalances in several of these pathways may explain why tamoxifen induced cancers behave more aggressively than sporadic endometrial tumours (Prasad et al., 2005) and as such these genes and pathways are unique to tamoxifen.

The study presented in this Chapter supports the findings of the previous *in vivo* data by Gielen and colleagues (Gielen et al., 2005b) in that many of the genes modulated by tamoxifen were similar in both studies, with several carcinogenic pathways altered by tamoxifen (Table 6.5). Although a much higher number were modulated in the present study, this could be related to different methodologies used. The previous study had a conservative 5-fold cut-off point for gene expression, whilst the current study used a 1.6-fold change in gene expression. The reason this threshold was used was due to recent discussions in the literature that a 50% change in transcript level could be significant to the physiology of any target cell or tissue (Kahlem et al., 2004; Curtis et al., 2005). Comparison of the tamoxifen gene lists generated in this study with that produced by Gielen and co-workers who studied the expression profiles in human endometrial cancer cell line ECC-1 treated with oestradiol and tamoxifen, showed that all the previously described genes are encompassed in the present gene list (Table 6.2) (Gielen et al., 2005b). The data in this study also indicate that several pathways involved in cell proliferation and

apoptosis regulation were significantly affected by both tamoxifen and also in the oestradiol dominant proliferative phase endometrium.

For example, the expression of CD24, an important diagnostic and prognostic marker in breast, ovarian, colon and prostate cancers (Kim et al., 2009) has been shown to be inversely correlated to oestrogen and progesterone levels and, is down regulated in the proliferative and up regulated in the secretory phase of the menstrual cycle. Its expression is decreased in endometrial hyperplasia, whereas it is increased in endometrial carcinoma (Kim et al., 2009) suggesting not only a role in tumour progression but also as a diagnostic marker. Furthermore, the reciprocal expression between CD24 and ER expression in endometrial carcinoma suggest a strong link between CD24 and ER-dependent disease progression. In my microarray, CD24 was down regulated in the tamoxifen myometrium (Figure 6.4) which may correlate with the up regulation of ERβ protein staining observed in Chapter 3 and the ERβ transcript levels obtained by qPCR (Figure 6.4).

Furthermore, pathway analysis of the microarray data showed that Wnt inhibitory factor 1 (Wif-1) was suppressed and WISP2 was induced significantly in the myometrium. Both of these genes are located in the delta notch and Wnt signalling pathways and WISP2 has been shown to be induced in the rat uterus by oestrogen (Mason et al., 2004). Dysregulation of WISP2 expression, or a change in oestrogen responsiveness, may lead to increased proliferation and predispose an individual to endometrial cancer (Mason et al., 2004), with the suggestion that WISP2 may be an important proliferation and motility regulating gene. In the myometrium, WISP2 is thought to act to limit myometrial cell proliferation, and in the endometrium, it is involved in the regulation of proliferation, cell migration and remodelling of the vasculature, glands and endometrial cells (Mason et al.,

2004). These authors suggest that all of these processes are regulated by oestrogen. The involvement of notch (Cobellis et al., 2008) and Wnt signalling pathways (Rider et al., 2006) within uterine remodelling could thus provide the missing link between tamoxifen action and the uterine growth observed in Chapter 2 and perhaps also the changes in the expression of the mesenchymal markers (used to demonstrate tissue remodelling), as shown in Chapter 5. It may be that tamoxifen has an effect on the aforementioned processes but less efficiently than that of oestrogen. Indeed, the qPCR confirmation studies showing that a number of genes (such as FosB, GRIA-2, and IGFBP3) were up-regulated or down-regulated in the proliferative uterus more than in the tamoxifen treated uterus (Figures 6.3 and 6.4) support that conclusion. Conversely, the presence of genes where regulation was greater in the tamoxifen treated uterus than in the proliferative uterus, such as CA12, WFDC1 (in the endometrium) and WFDC2 (in the myometrium), may indicate that the differences in the effects of these compounds can be quantitative as well as qualitative. These data need further confirmation and so will be studied in Chapter 7.

Chapter 7

The effect of oestradiol on the expression of WFDC1 and WFDC2 in primary uterine cell cultures

7.1 Introduction

As identified in Chapter 6, many genes and pathways are uniquely altered by tamoxifen and others are altered in both the proliferative phase uterus and in the tamoxifen treated uterus. The latter group include the whey acid protein four core domain proteins, WFDC1 and WFDC2. The mechanism(s) by which tamoxifen induces uterine abnormalities such as its uterotrophic effect, generalised growth and the development of endometrial polyps is not fully understood. It is possible that paracrine regulatory factors that are growth inhibitory may be involved in the normal coordinated growth of the uterus. Such factors may allow normal uterine development by being inhibited by oestradiol, and because tamoxifen has weak oestrogenic properties, it may not fully suppress these regulators resulting in disordered uterine growth. WFDC1 has previously been shown not only to be anti-proliferative, but also to have antiprotease activity in the extracellular matrix. This may mediate the regulation of growth and differentiation.

WFDC1, also known as ps20, was identified as a prostatic stromal mesenchymal-derived paracrine-acting factor (Rowley et al., 1995) and it has been shown to inhibit the proliferation of prostate carcinoma cells (PC3) (Larsen et al., 1998). WFDC1 is thought to be a possible marker for prostate cancer progression, as loss of WFDC1 in the stroma and increased expression in the epithelial cell indicate an aggressive epithelial tumour as well as the presence of an epithelial-mesenchymal cell transition (McAlhany et al., 2004).

The expression of WFDC1, in the rat uterus is inversely correlated to the levels of oestradiol in vivo and in vitro. Hung observed high WFDC1 expression in the rat uterine smooth muscle cells but faint expression in the luminal epithelial and glandular cells (Hung, 2005). They also showed that tamoxifen decreased WFDC1 expression, but less effectively (approximately 800 to 1500-fold less potent) when compared to oestradiol in the control of rat uterine cell proliferation, suggesting that WFDC1 may function as a mediator of growth in other tissues (Hung, 2005). Furthermore, previous evidence from my research group demonstrated that WFDC1 and its sister protein, WFDC2 (HE4) are expressed by primary cultures of human uterine smooth muscle cells, and that their expression was suppressed by oestradiol (Taylor & Bell, unpublished observations).

WFDC2 is a protease inhibitor and has been shown to be expressed by the epithelial cells in the human endometrium (Yanaihara et al., 2004), Yanaihara and co-workers observed WFDC2 as the most upregulated gene (44 fold) in the secretory epithelium when compared to the proliferative epithelium. However, it is unknown whether WFDC2 is expressed by other cellular components in the human uterus. WFDC2 is expressed in healthy oral and nasal mucosa, epididymis, lung, kidney and prostate (Bouchard and Tremblay, 2006). The function of WFDC2 is yet to be defined, but is has been shown to be up regulated in ovarian and pancreatic cancer and down regulated in prostate cancer (Bouchard and Tremblay, 2006).

The aim of this chapter is to confirm whether WFDC1 and WFDC2 are expressed by human uterine myometrial and stromal cells, and to examine their regulation by oestradiol.

7.2 Materials and Methods

The tissue collection, patient groups, sampling technique and histological examination was performed as described in Chapter 3.

7.2.1 Preparation of human myometrial smooth muscle and endometrial stromal cells

Before the tissue for culture was collected from the operating theatre, collagenase (50mg in 25mls of DMEM F12 medium) and growth media (500mls DMEM F12 medium, 50mls fetal calf serum and 5ml antibiotic antimycotic solution containing penicillin and streptomycin) was prepared and heated to 37°C in a water bath.

7.2.1.1. Myometrial cells

The fresh myometrial tissue was collected from women undergoing hysterectomy for benign pathology. A 2cm x 1.5cm full thickness piece of uterus was collected. The endometrium was set aside for stromal cell preparation and serosal surfaces were discarded. The remaining myometrium was cut into 1 x 1 x 1mm cubes with sterile scalpels and transferred to the prewarmed collagenase solution. The tubes were transferred to a 37°C water bath rotating at maximum rotations for 3 hours. The tube containing the cells was then allowed to stand for 5 minutes before the top cloudy layer was transferred to a fresh tube, leaving all the undigested material at the bottom of the digestion tube. Fifty millilitres of fresh media was added to the cloudy cell mixture, mixed and centrifuged at 300g at 4°C for 5 minutes to collect the cells. The clear supernatant was discarded without disturbing the cell pellet, which was then resuspended in fresh media before repeating the centrifugation step. The supernatant was removed and the cell pellet resuspended in 10ml of cell culture media, put in a T75 flask with a further 15ml of growth medium and incubated at 37ºC in 5% CO_2 in air. The cells were grown to ~90% confluence when they

were used in experiments. The myometrial cells were sub cultured to 6 well plates at 3.5 x 10^5 cells per well.

7.2.1.2 Stromal cells

For stromal cells, the endometrium set aside in step 2 of the myometrial cell preparation above, was minced with scalpels and transferred to a separate tube of pre-warmed collagenase solution. The digestion and isolation of the stromal cells was as per the smooth myometrial cells, except that digestion was for only 2 hours and stromal cells were plated to 6 well plates at 1 x 10^5 cells per well.

7.2.2 Human UtSMC (Clonetics) cells

To confirm the experiment, commercially available human myometrial cells were also used (UtSMC, Clonetics, Cambrex BioScience, Walkersville, MD, USA). These cells were provided with special growth conditions and media. The media serum and additives (part of the SmBm Bullet kit) were warmed in a water bath at 37° for 20 to 30 minutes and then aseptically transferred into the basal media, the contents mixed by swirling to create the growth media. Twenty five millilitres of this was transferred into two T75 flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 30 minutes to allow the media to equilibrate. A vial of human UtSMC containing 5 x 10^5 cells was removed from the liquid nitrogen tank and rapidly thawed in a 37ºC water bath with shaking. The vial contents were then transferred to the two flasks of basal media giving ~3,500 cell/cm^2 and incubated at 37ºC for 18 to 24 hours. The medium was changed every 48 hours until the cultures were ~90% confluent, when the cells were sub cultured onto 6 well plates coated with either plastic or collagen I, at 3.5 x 10^5 cells per well. The plate(s) were incubated at 37ºC in 5% CO_2 in air for 48 hours and then treated with drugs.

7.2.3 Cell treatments

Initial experiments examined the effect of oestradiol (E2) on WFDC1 and WFDC2 transcript levels by both primary myometrial smooth muscle cells and endometrial stromal cells. Cells that had reached ~80% confluence were deprived of oestrogen signal, by having the medium changed from DMEM or Bullet kit containing 10% fetal bovine serum (FBS) to one containing 10% charcoal stripped FBS (oestrogen free) supplemented with 0.1% ethanol for 4 days. At this point cell proliferation had stopped at ~90-95% confluency. The myometrial cells were then treated with either control (0.1% ethanol) or 10^{-8}M oestradiol in 0.1% ethanol for 4 days. Endometrial stromal cells were treated in exactly the same way, except the concentration of oestradiol used was 10^{-6}M.

7.2.4 RNA extraction from the cells

The culture medium within each well was aspirated and 1ml of Tri-reagent (Sigma Aldrich, Poole, UK) added for 5 to 10 minutes during which time each well was scraped using a long-handled plastic scraper. The well contents were then transferred to an RNase-free 1.5 ml microfuge tube and left for a further 3 min for cell lysis to complete. Next, 200µl of chloroform was added to the samples, shaken vigorously by hand for 15 seconds and left to settle for 10 minutes to aid phase separation. After centrifugation at 13000 rpm for 15 minutes at 4°C, the supernatant was transferred a fresh RNase-free 1.5 ml microfuge tube. Isopropanol (0.5 ml) was then added, the samples mixed on a vortexer and incubated at room temperature for 10 min. The RNA was then collected by centrifugation at 13000 rpm for 10 minutes at 4°C. The supernatant was removed carefully without losing the small white pellet at the bottom of the tube. One ml of 75% ethanol/DEPC-treated water was added to the samples and vortexed, and then centrifuged at 13000 rpm for 5 minutes. Care was taken to remove the supernatant from the RNA pellets and the tube was inverted

on a sheet of tissue for 7 to 10 minutes in order to air dry the pellet, and any fluid remaining on the microfuge tube was removed by wiping the upper inside surface with a clean, rolled paper tissue. Pellets were then diluted in 100µl of DEPC-treated water, heated at 55°C for 5 minutes to dissolve the RNA and centrifuged briefly before being stored at-20°C freezer until required.

7.2.5 DNase I treatment

Instead of aliquoting individual treatment constituents into each microfuge tube, a master mix containing correct proportional volumes was prepared as follows (Table 7.1), and multiplied by the number of samples:

Ingredient	**Volume**
Reaction buffer x10	15µl
DNase I	10µl
RNasin	3.15µl
DEPC-treated water	21.85µl
Total volume	50µl

Table 7.1 Proportions of ingredients used per sample to make a DNase I treatment mastermix.

RNA samples (100µl) were thawed slowly on ice, 50µl of the above master mix was added to each sample, the samples gently mixed and then incubated at 37°C for 60 minutes.

7.2.5.1 Phenol Chloroform Extraction

At the end of the DNase I treatment, 150µl of phenol:chloroform:isoamylalcohol (25:24:1) was added to each sample, vortexed on high power for 1 minute and then centrifuged at 13000 rpm for 2 minutes at 4°C. The supernatant was transferred to a new RNase-free microfuge tube and an equivalent amount of chloroform: isoamylalcohol (24:1) added. Samples were vortexed again for 1 minute and the centrifugation step repeated. The supernatant was transferred to new microfuge tube and a volume of isopropanol equivalent to 60% of the supernatant was added. Samples were then incubated at –20°C for at least 1 hour to allow the RNA to precipitate. RNA was then collected by centrifugation at 13000 rpm for 5 minutes at 4°C and the pellets washed with 75% ethanol/DEPC-treated water as described in the original extraction procedure (section 7.2.4). The RNA was allowed to air-dry for 10 minutes at room temperature inverted over a paper towel. After removal of any residual ethanol/DEPC-treated water, the inside of the microfuge tube was wiped with a clean rolled-up piece of paper tissue, the RNA pellets were diluted in 50µl of DEPC-treated water and heated at 56°C for 5 minutes, before being stored at -20°C for further processing.

7.2.6. RNA Quantification

An Ultrospec 3000 UV Visible Spectrophotometer was used for RNA quantification. The RNA samples were diluted 1 in 10 with DEPC-treated water. The machine was 'zeroed' at 260nm and 280nm using 50µl of DEPC-treated water and then 50µl of each sample was quantified in turn, rinsing the cuvette with DEPC-treated water twice between sample measurements.

7.2.7. RNA quality analysis by agarose gel electrophoresis

All equipment was soaked for at least 24 hours in a tank containing 3% hydrogen peroxide and then rinsed with DEPC-treated water immediately prior to use. Gel cassettes were sealed using tape and well forming combs inserted. A 1% agarose gel containing 0.05% ethidium bromide was then prepared (a large gel (123mm x 114mm) required 2ml of 50x TAE buffer, 98ml of DEPC water and 1g of low melting point agarose; a mini gel (85mm x 73mm), used half of those values). The mixture was boiled in a microwave and the agarose was swirled to carefully aid clarification of the solution. The mixture was allowed to cool slightly and ethidium bromide (1mg/ml; 5µl for large gels and 2.5µl for small gels) added and the mixture poured into the cassette. The gel was allowed to set at 4°C.

Meanwhile, a 1x TAE buffer solution was made by mixing 980ml of DEPC water and 20ml of 50x TAE buffer (large gel) or 294ml DEPC water and 6ml 50x TAE buffer (for a small gel). Ethidium bromide (1mg/ml: 0.05% v/v) was added to the TAE buffer and the mixture poured over the set gel now placed in the electrophoresis tank.

Five µl of each RNA sample was added to 13µl of DEPC-treated water and 2µl of 10x loading buffer in an RNase-free 0.5ml microfuge tube, mixed and heated at 65°C for 5 minutes and then allowed to cool. The samples (19.5µl) were then loaded onto the gel and electrophoresed at 100-180V until the bromophenol blue dye travelled 2/3rds the distance of the gel. The 28S and 18S rRNA bands were visualised on a GeneGenie gel documentation system under UV light (Syngene Gene Genius Bio Imaging gel system). Only samples with a good 28S/18S ratio were used in subsequent RT-PCR.

7.2.8 Reverse transcriptase reaction

One μg of RNA was converted to cDNA using avian myelomablastosis virus reverse transcriptase (AMV-RT; Promega Corporation, Southampton). The reaction was performed on a 15.38μl mixture of RNA diluted with DEPC-treated water. The volume of each individual RNA sample required was calculated using the equation:

Volume of RNA sample required (μl) =

(1/concentration of RNA sample, μg/μl)*(2/(260/280 OD ratio))

To produce cDNA, separate master mixes for both the positive and negative reverse transcriptase (RT) reactions were prepared, as shown in Table 7.2:

Ingredient	**+RT (Volume)**	**-RT (Volume)**
5 x AMV buffer	5µl	5µl
RNasin	0.62µl	0.62µl
Anchored oligo$(dT)_{23}$	1µl	1µl
10mM dNTPs	2.5µl	2.5µl
AMV RT	0.5µl	-
DEPC-treated water	-	0.5µl
Total Volume	9.62µl	9.62µl

Table 7.2 Proportions of ingredients used per sample to make reverse transcriptase treatment mastermixes.

As negative RT reactions contain no AMV-RT enzyme, they were employed as a control for any DNA contamination that occurred during the extraction procedure. Once the RNA and reaction mixes were combined to make 25µl solutions in 500µl microfuge tubes, they were then incubated at 42°C for 1 hour in a thermal cycler. The samples were then briefly centrifuged to collect at the bottom of the tube and then stored at 4°C for PCR.

7.2.9 Semi-quantitative polymerase chain reaction (PCR)

A PCR master mix was made, consisting of the following proportional volumes:

Ingredient	**Volume**
Sterile Pure Water	41.8µl
10X AJ Buffer	5µl
Forward primer	1µl
Reverse primer	1µl
Taq. DNA Polymerase	0.2µl
Total Volume	49µl

Table 7.3 Proportions of ingredients used per sample to make endpoint PCR mastermixes.

Prior to the very first PCR on a set of samples, the cDNA was heated at 95°C for 2 minutes in order to destroy the RT enzyme. In most reactions, 1μl of cDNA was added to a 49μl aliquot of the PCR mastermix to produce a total volume of 50μl of PCR reaction mixture. The forward and reverse primers are shown in Table 7.4. The prepared samples were then put in the thermal cycler set to an appropriate optimised program that differed for each target. The thermal cycler conditions for WFDC1 and WFDC2 were: 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 58°C for 45 seconds, 72°C for 1 minute, followed by 72°C for 10 minutes and 4°C for 5 minutes. For GAPDH and PR the conditions were 95°C for 1 minute, 60°C for 45 seconds (1 minute for PR), 72°C for 1 minute, followed by 72°C for 10 minutes and 4°C for 5 minutes. All samples were stored for 4°C for agarose gel electrophoresis.

Primer	Product Size (bp)	Sequence of primers (5'-3')
GAPDH	347	Forward: AGAACATCATCCCTGCCTC Reverse: GCCAAATTCGTTGTCATACC
WFDC1	299	Forward: ACCCTCACAGTTCACATTCAGC Reverse: ACATACCTCCCAGAGCCAGA
WFDC2	280	Forward: TCTGCCCCAATGATAAGGAG Reverse: CACCCCAAGAGGGAATACAG
PR	298	Forward: AGGAGTTTGTCAAGCTTCAA Reverse: CTGCAGGGACTGGATAAATGTATTC

Table 7.4 The product size and primer sequence for each gene used in the PCR. GAPDH is glyceraldehyde-3-phosphate dehydrogenase; WFDC1 is whey acidic protein four core domain 1 (ps20); WFDC2 is whey acidic protein four core domain 2 (HE4) and PR is the progesterone receptor.

7.2.10. Agarose Gel Electrophoresis

PCR products were separated through 3% agarose gels buffered with 1x TAE and containing 0.05% ethidium bromide, bathed in a 1x TAE buffer solution containing 0.05% ethidium bromide at between 100-180V for ~ 45 to 60 minutes. Samples were prepared by adding 18μl of each PCR product to 2μl 10x DNA loading buffer (0.25g bromophenol blue dye dissolved in 123.75mM Tris-HCl (pH7.6) buffer) and heating at 65°C for 5 minutes. Samples were then loaded onto the gel together with a 3 μl sample of 100bp DNA size ladder (Invitrogen, Paisley, UK) in the end well of each row.

7.2.11 Syngene gel documentation

Gel images were captured using Gene Snap version 6.0 software on a Syngene Gene Genius Bio Imaging gel documentation system.

7.2.12 Densitometric quantification

Measurements of the band densities were made using the NIH Image (Scion Corporation) computer program. Values for each sample were standardised against the housekeeping gene, GAPDH, to eliminate any variability not due to the treatment effect. Data are presented as the ratio of the gene of interest to GAPDH.

7.3 Results

7.3.1 Primary myometrial cells

Agarose gel electrophoresis indicated that the amplicons produced using the WFDC1 and WFDC2 primers (Table 7.4) were of the expected sizes of 299bp for WFDC1 and 280bp for WFDC2 (Figure 7.1). Primary myometrial cells treated with 10^{-8}M oestradiol showed no overall response with regard to WFDC1 or WFDC2 transcript levels (Figure 7.2) and when each experiment was examined individually (Figure 7.3), the results were quite variable with the first experiment showing increased WFDC1 and WFDC2 expression, the second showing increased WFDC1 but decreased WFDC2 expression and the third experiment showing decreased WFDC1 and WFDC2 expression. This was perplexing and view of previous more consistent results obtained in our laboratory (Dr A Taylor, personal communication) it was decided to rerun the experiment using commercial cells from Clonetics cultured on collagen I coated plates. These two possible explanations for a lack of effect were therefore examined further.

In human primary myometrial cells grown on collagen I plates, there also was no down regulation of WFDC1 with oestradiol but a slight increase (Figure 7.4). Similarly, there was no real modulation of WFDC2 transcript levels in response to oestradiol treatment (Figure 7.4), only a small up regulation. Examination of the individual experiments (Figure 7.5) revealed no change in either WFDC1 or WFDC2 expression for myometrial cells grown on collagen I plates and that transcript levels differed greatly from one culture to another with WFDC2 transcripts barely detectable in experiment 3.

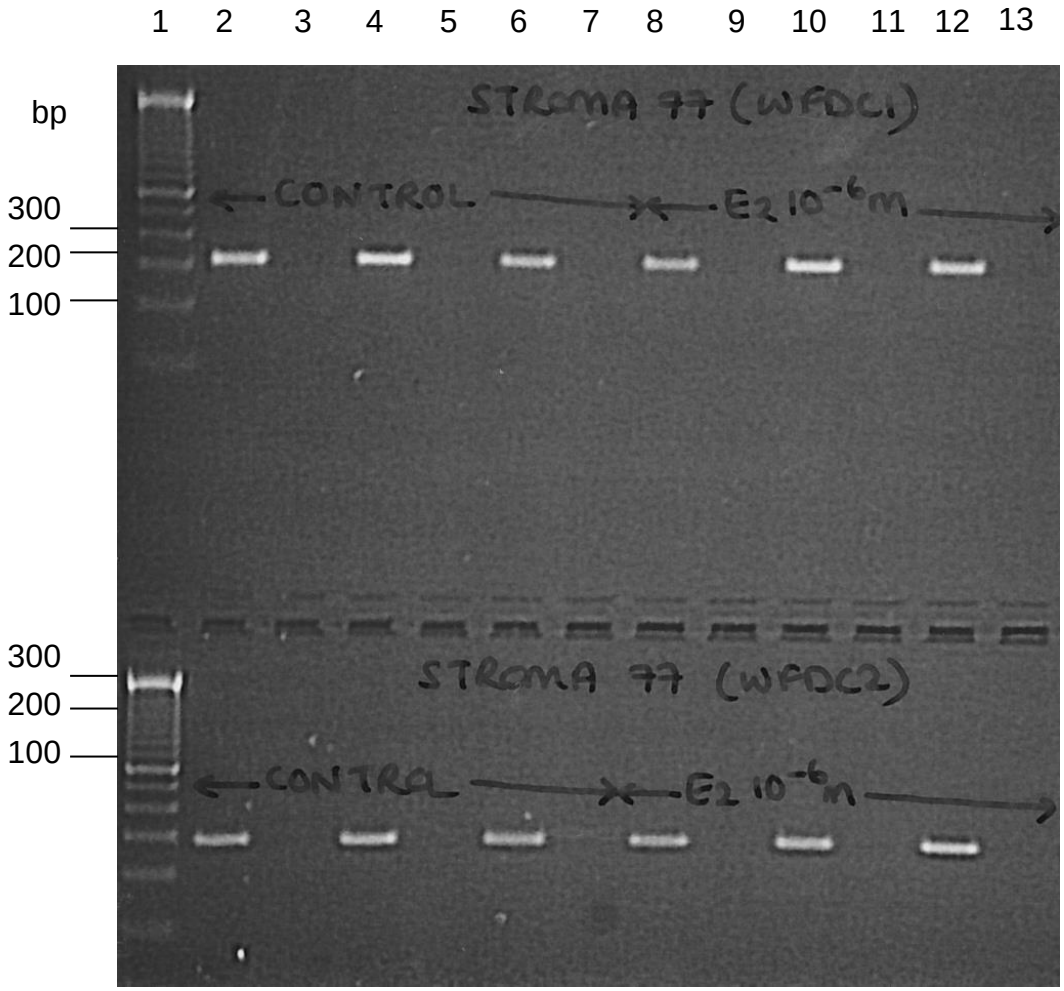


Figure 7.1 Ethidium bromide stained gel showing amplicons for WFDC1 (upper panel) and WFDC2 (lower panel). Lane 1 contains the 100bp ladder; lanes 2-7 are ethanol controls from stromal cell cultures; lanes 8-13 are from cells treated with 10^{-6}M oestradiol. Lanes 2, 4, 6, 8, 10 and 12 are +RT samples and lanes 3,5, 7, 9, 11 and 13 are RT samples; bp = basepair.

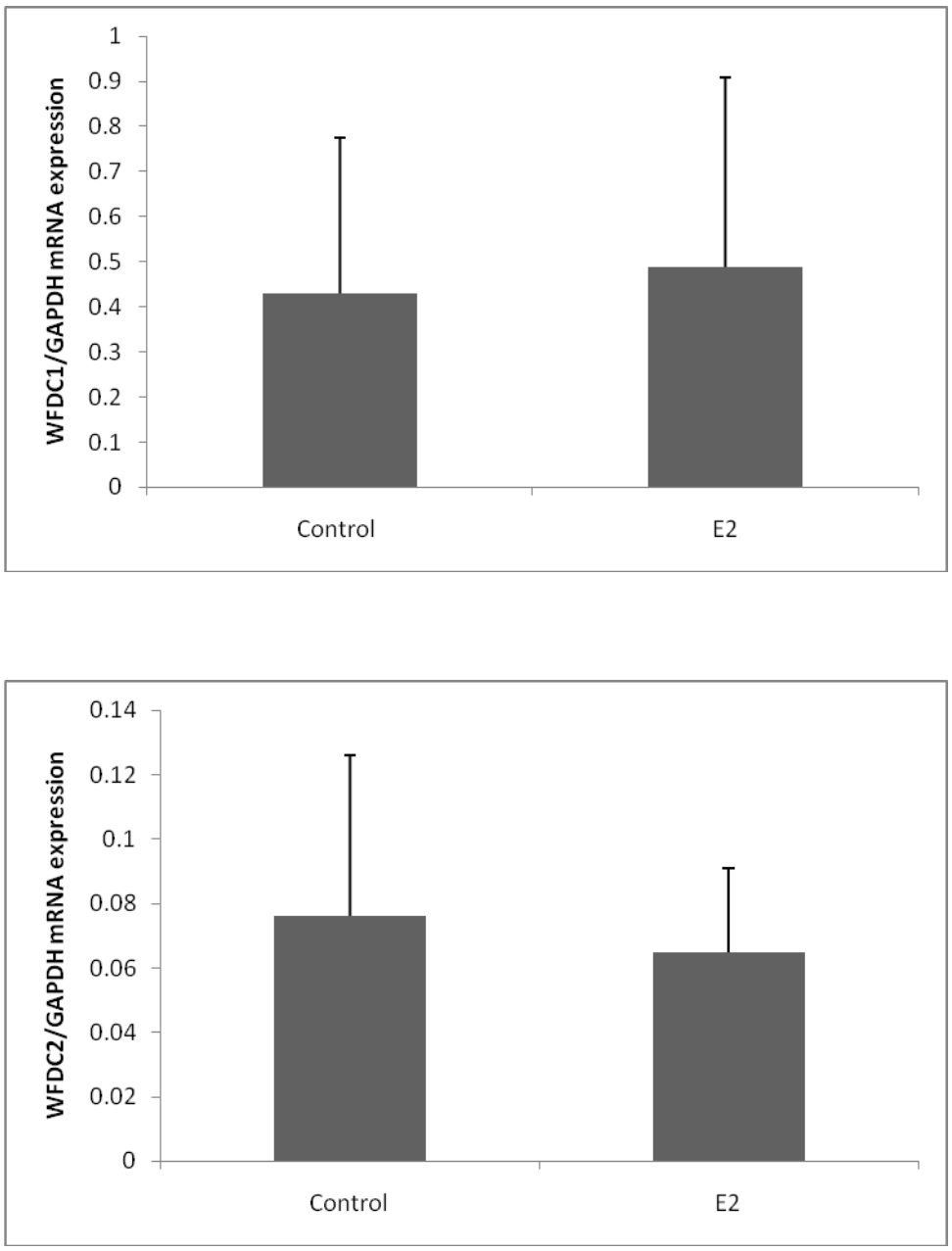


Figure 7.2 Expression of WFDC1 and WFDC2 transcripts from primary myometrial smooth muscle cells.

Cells were grown in 6-well plates with 10% CSS and treated with 10^{-8}M oestradiol (E2) for 4 days. The data are presented as the mean ± SEM for 3 experiments performed in triplicate.

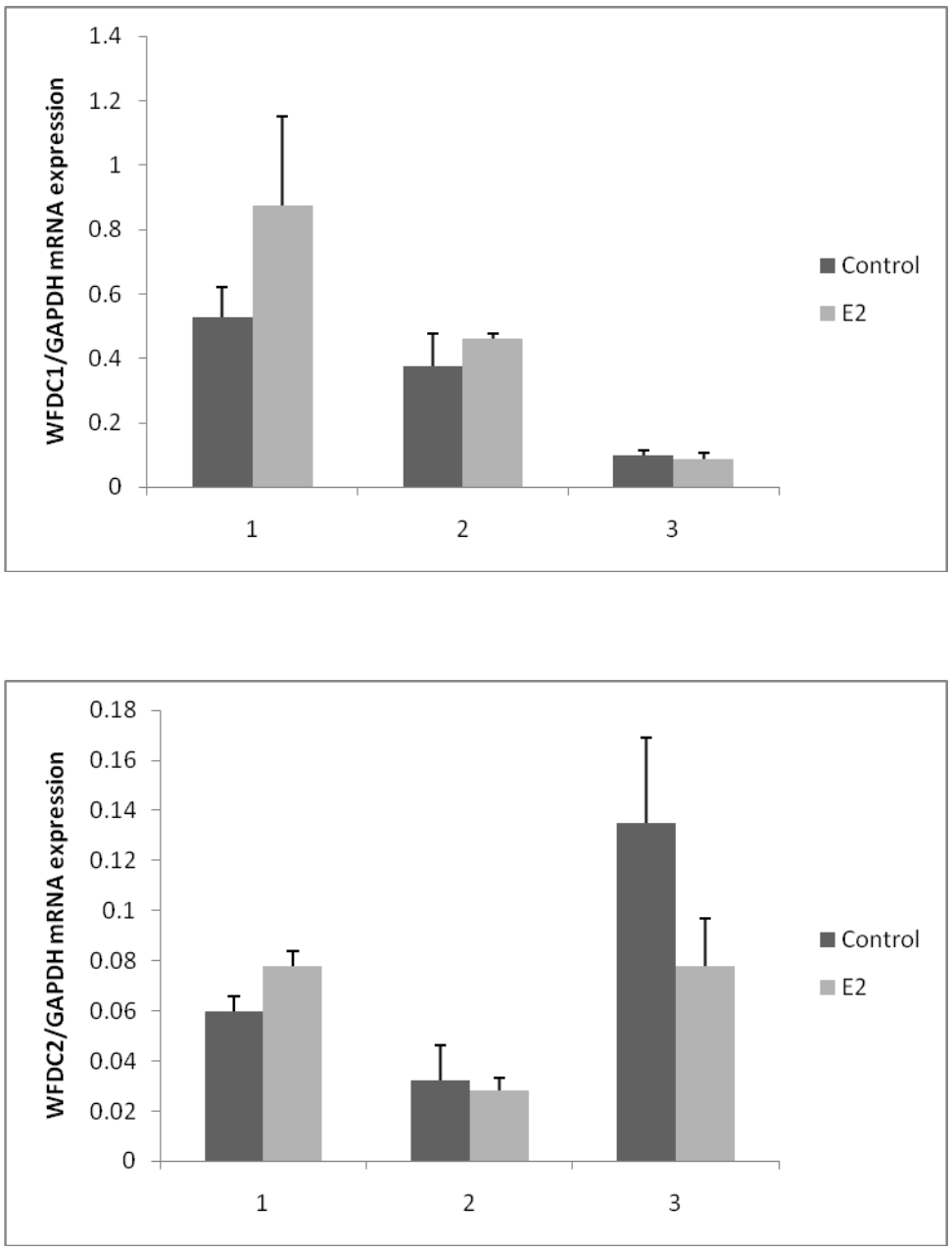


Figure 7.3 Examination of the individual experiments for the expression of WFDC1 and WFDC2 transcripts from primary myometrial smooth muscle cells.

The data are presented as the mean ± SEM for triplicate replicate wells.

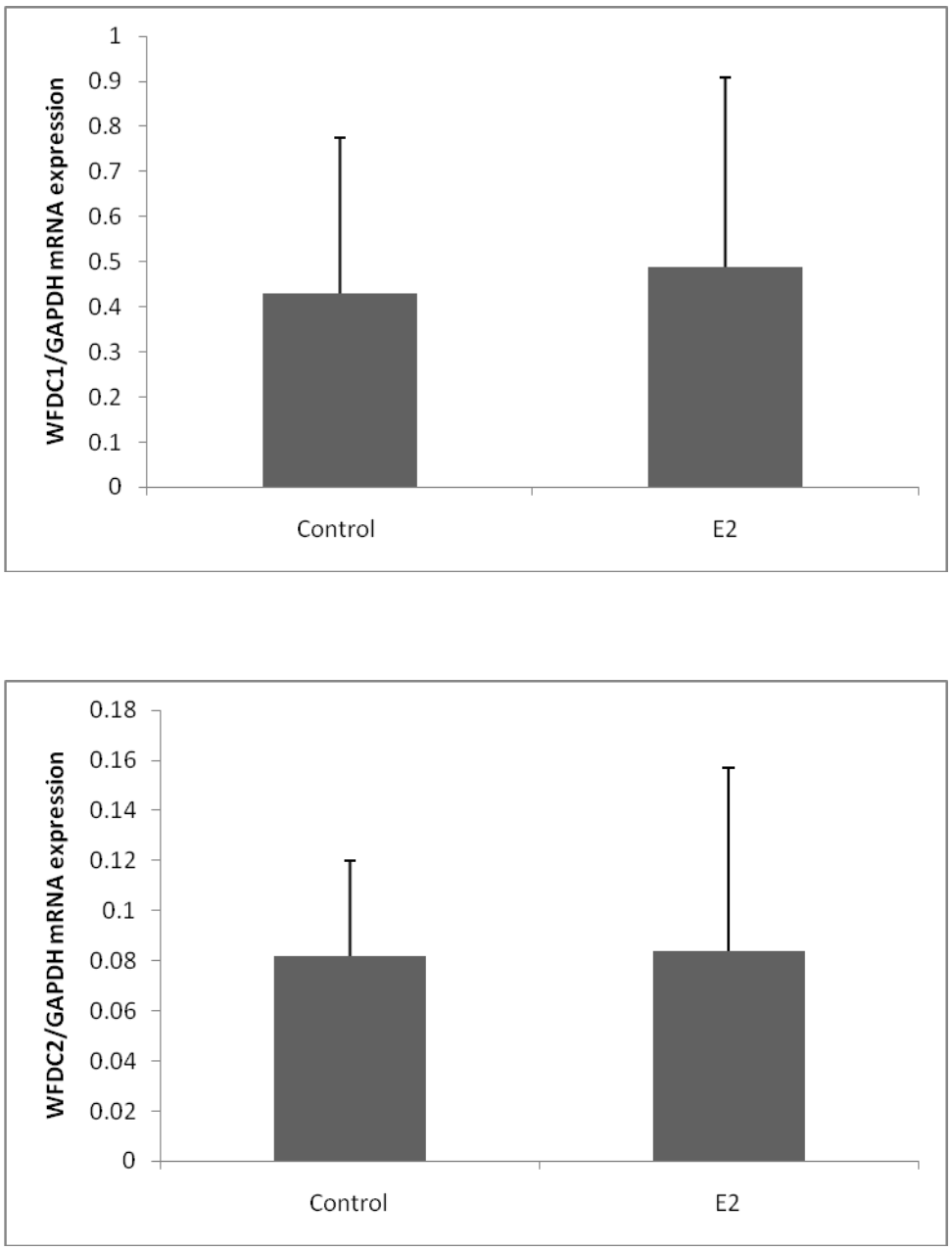
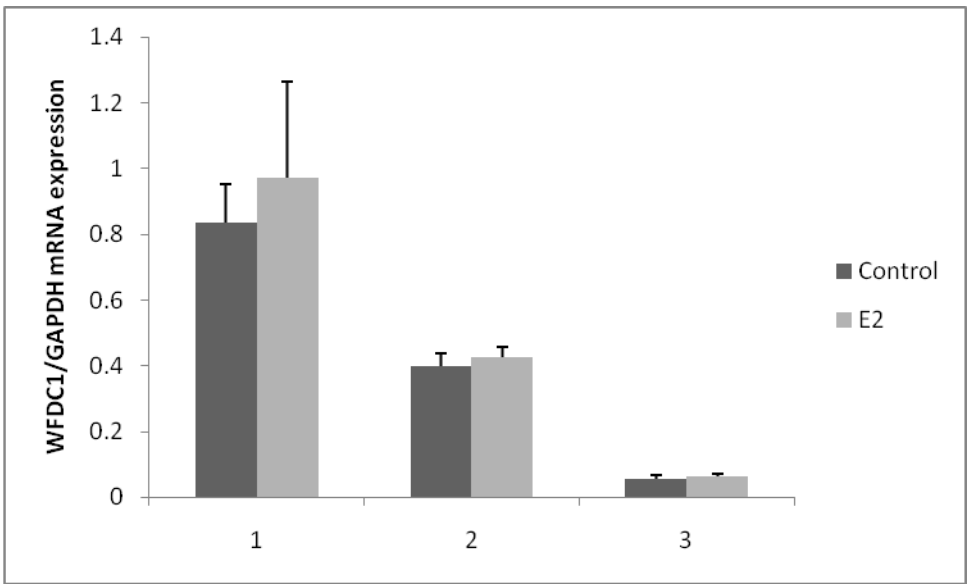


Figure 7.4 Expression of WFDC1 and WFDC2 transcripts from primary myometrial smooth muscle cells grown on collagen I plates.

Cells were grown in 6-well plates with 10% CSS before treatment with 0.1% ethanol (control) and 10^{-8}M oestradiol (E2) for 4 days. The data are presented as the mean ± SEM for 3 experiments performed in triplicate.



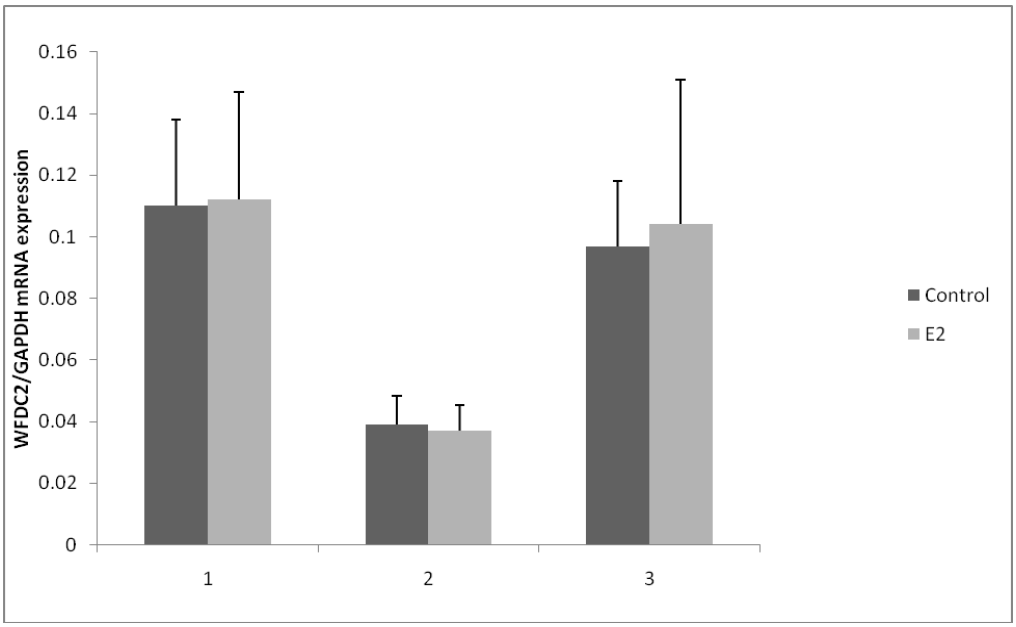


Figure 7.5 Examination of the individual experiments for the expression of WFDC1 and WFDC2 transcripts from primary myometrial smooth muscle cells grown on collagen I plates. The data are presented as the mean ± SEM for triplicate replicate wells.

In a single trial experiment, where primary myometrial smooth muscle cells were grown on Collagen-I coated plates and treated with 10^{-8}M oestradiol or 10^{-6}M tamoxifen or a combination of the two compounds, oestradiol or tamoxifen alone had no effect on WFDC1 transcript levels, whereas a combination of both compounds reduced WFDC1 transcript levels by 21.7% (Figure 7.6).

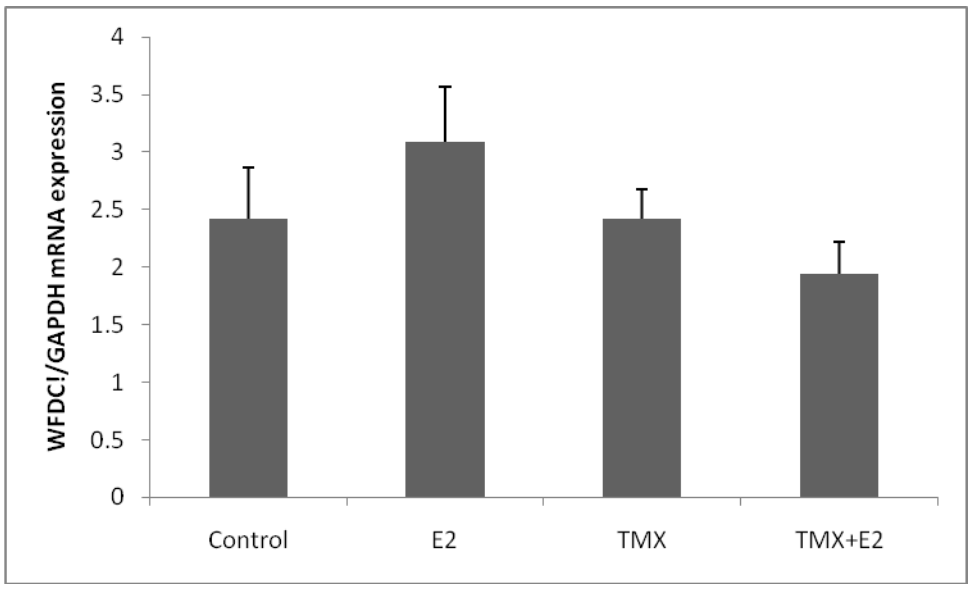
Similarly, oestradiol had a minimal effect on WFDC1 and WFDC2 transcript levels in the commercially available uterine smooth muscle cells (UtSMC), regardless of whether the cells were grown on collagen I coated plates or not (Figure 7.7). There was, however, a small decrease in WFDC1 and WFDC2 transcript levels in the UtSMC cultures in response to 10^{-8}M oestradiol (Figure 7.7). Examination of the individual experiments revealed that WFDC1 transcript levels were decreased in the first experiment, and then not altered in the remaining 3 experiments (plastic plates) and that WFDC2 transcript levels were decreased by oestradiol in 3 of the 4 experiments, but those changes did not reach statistical significance (Figure 7.8). When these cells were cultured on collagen I coated plates, the transcript levels for WFDC1 and WFDC2 were suppressed in the first 2 experiments, but then not altered in the remaining 3 experiments (Figure 7.9). What was notable in all of these experiments the WFDC1:GAPDH and WFDC2:GAPDH ratios were very low.

7.3.2 Primary endometrial stromal cells

Similar experiments performed on endometrial stromal cell expression of WFDC1 and WFDC2 showed that expression of these two proteins was not modulated in these cells by the addition of oestradiol (Figure 7.10). Examination of the individual experiments showed that WFDC1 expression was suppressed by ~50% in two of the three experiments, whilst the first experiment showed increased transcript levels (Figure 7.11). At the same

time, WFDC2 expression was inconsistent with suppression only occurring in the final experiment (Figure 7.11).

To test whether that the stromal cells were indeed responsive to the applied oestradiol, levels of the progesterone receptor transcript were also measured (Figure 7.12). The data indicated that only the first culture increased progesterone receptor transcript levels in response to oestradiol, whereas in the second and third cultures there was a decrease in progesterone receptor expression. When the data were summarized (Figure 7.12), oestradiol was shown to have no effect on progesterone receptor expression.



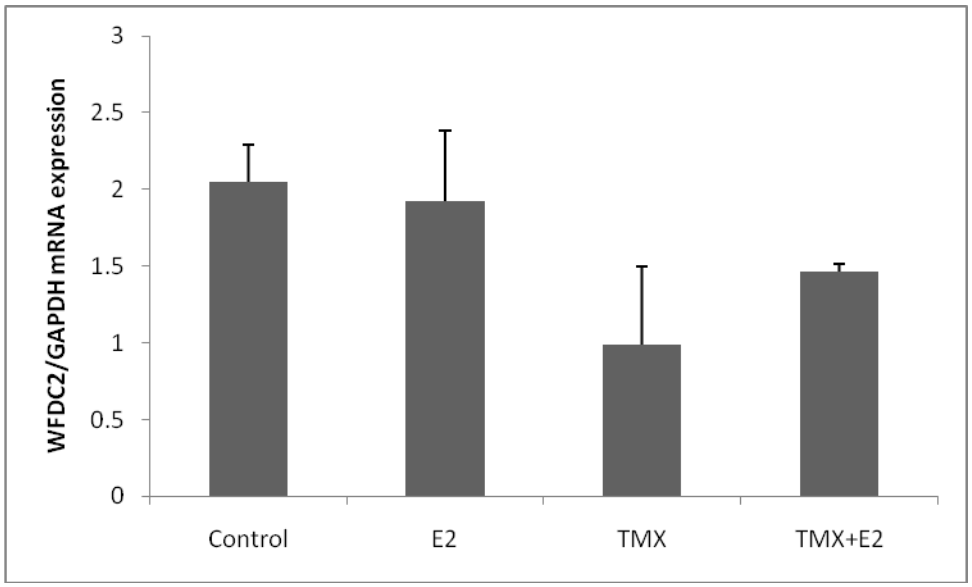
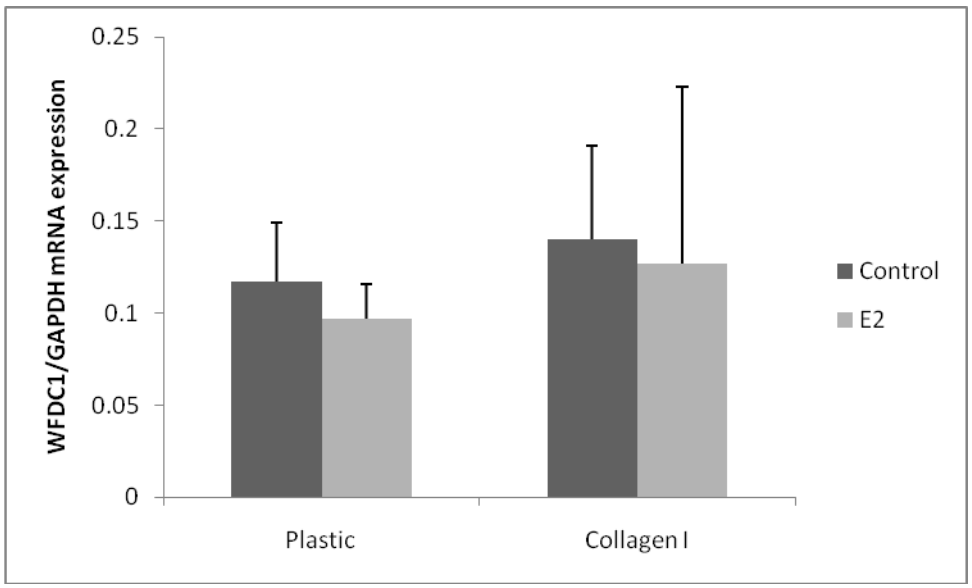


Figure 7.6 Expression of WFDC1 and WFDC2 transcripts from primary myometrial smooth muscle cells grown on collagen I plates in response to oestradiol, tamoxifen or a combination of both compounds. Cells were grown in 6-well plates with 10% CSS containing 10^{-8}M oestradiol (E2), 10^{-6}M tamoxifen (TMX) or a combination of both compounds for 4 days. The control consisted of 0.2% ethanol in medium containing 10% CSS. The data are presented as the mean ± SEM for one experiment performed in triplicate.



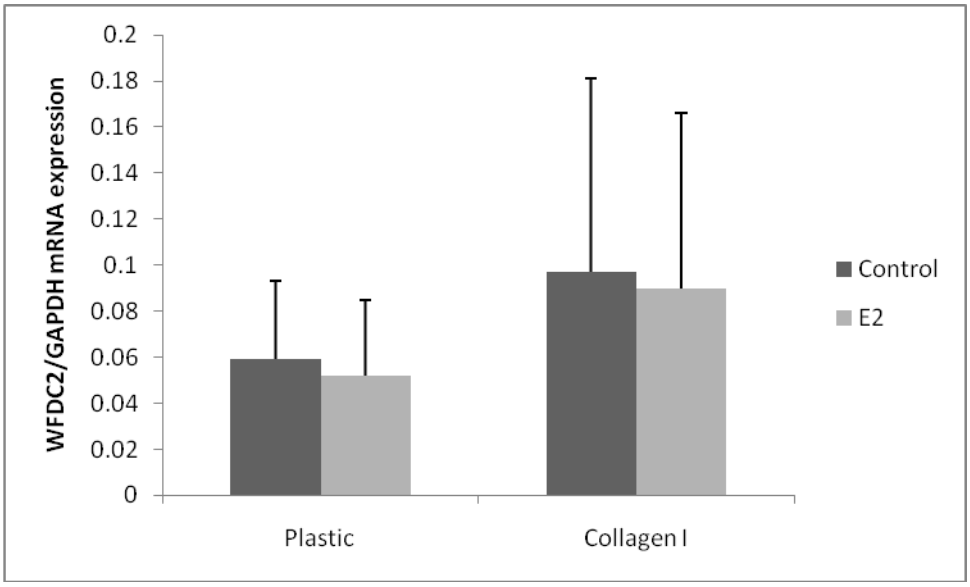


Figure 7.7 Expression of WFDC1 and WFDC2 transcripts from Clonetics uterine smooth muscle cells grown on plastic plates. Cells were grown in 6-well plates with 10% CSS containing 10^{-8}M oestradiol (E2) for 4 days. The data are presented as the mean ± SEM for 4 experiments performed in triplicate (plastic plates) and 5 experiments performed in triplicate (collagen-I plates).

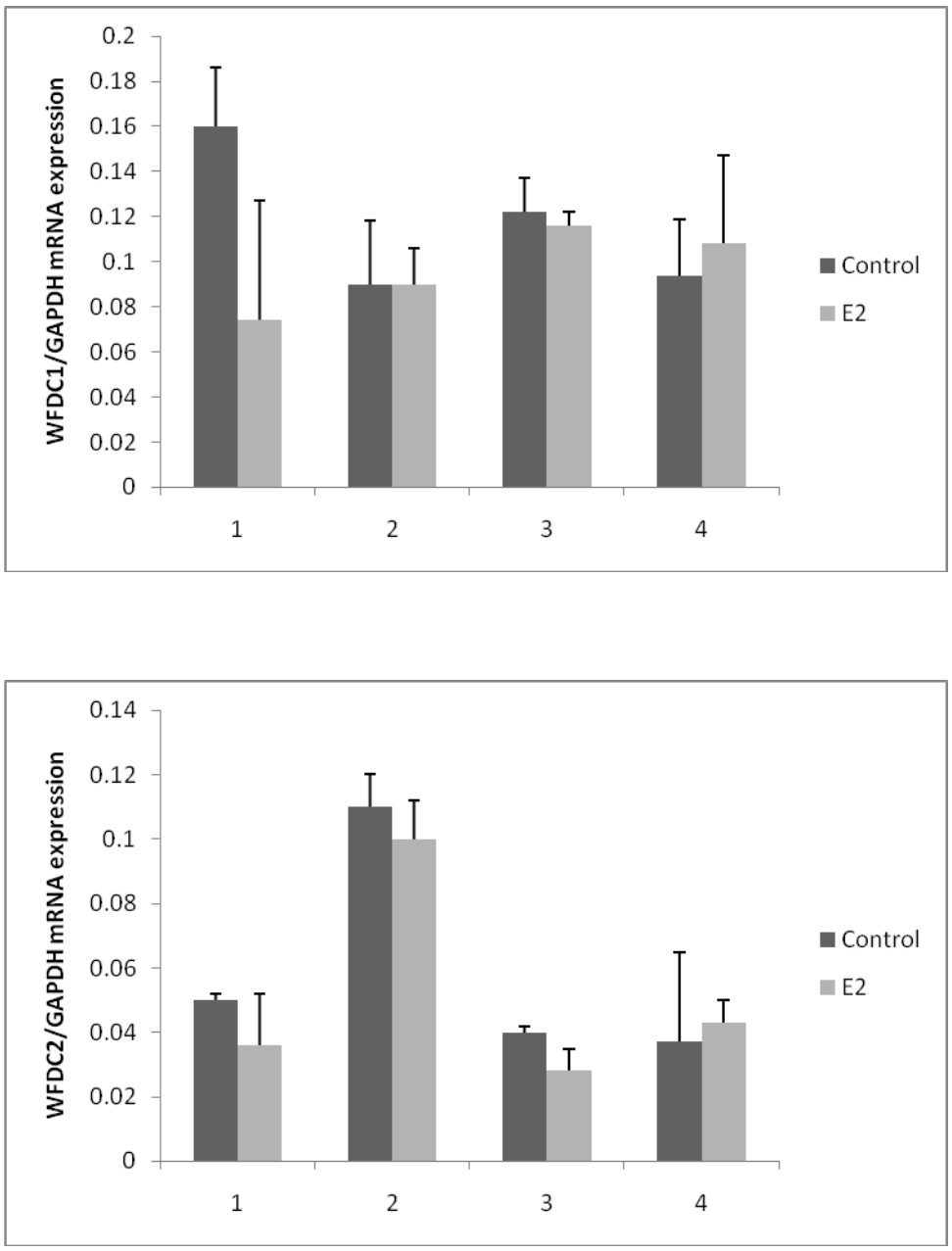


Figure 7.8 Examination of the individual experiments for the expression of WFDC1 and WFDC2 transcripts from Clonetics uterine smooth muscle cells grown on plastic plates. The data are presented as the mean ± SEM for triplicate replicate wells.

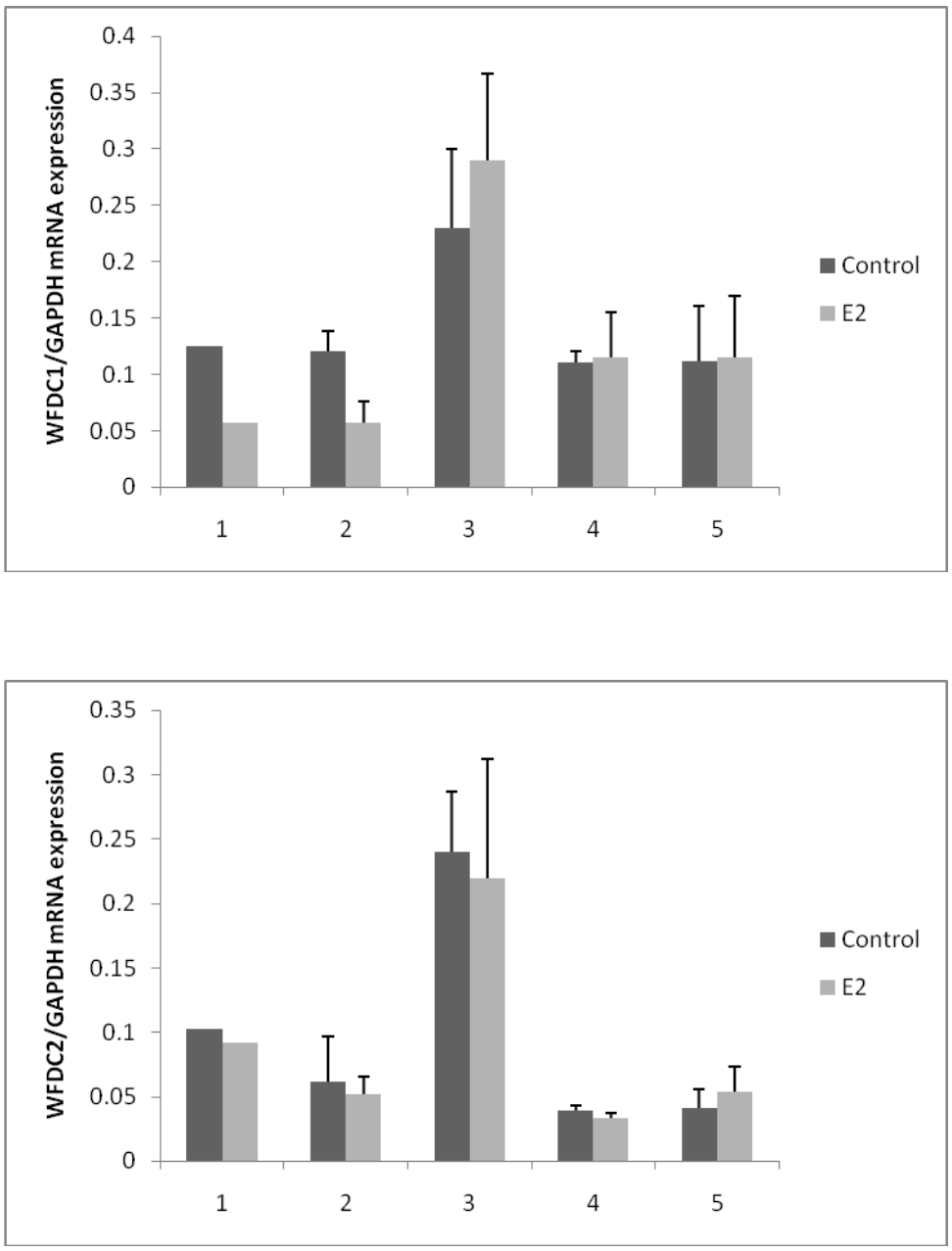


Figure 7.9 Examination of the individual experiments for the expression of WFDC1 and WFDC2 transcripts from Clonetics uterine smooth muscle cells grown on collagen-I plates. The data are presented as the mean ± SEM for triplicate replicate wells.

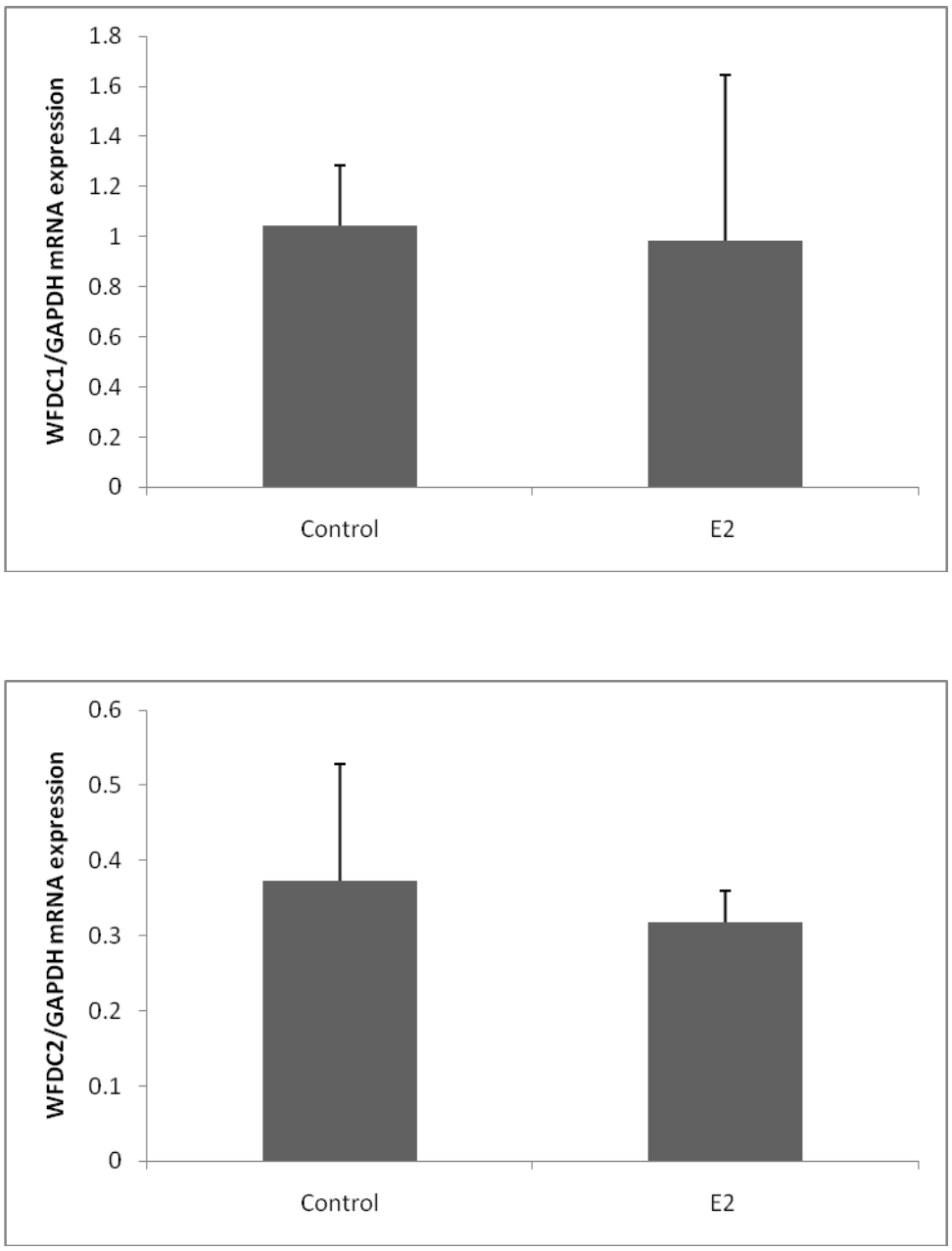


Figure 7.10 Expression of WFDC1 and WFDC2 transcripts from primary endometrial stromal cells grown on plastic plates. Cells were grown in 6-well plates with 10% CSS and treated with a higher dose of E2 (10^{-6}M E2) than the myometrial cells. The data are presented as the mean ± SEM for 3 experiments performed in triplicate.

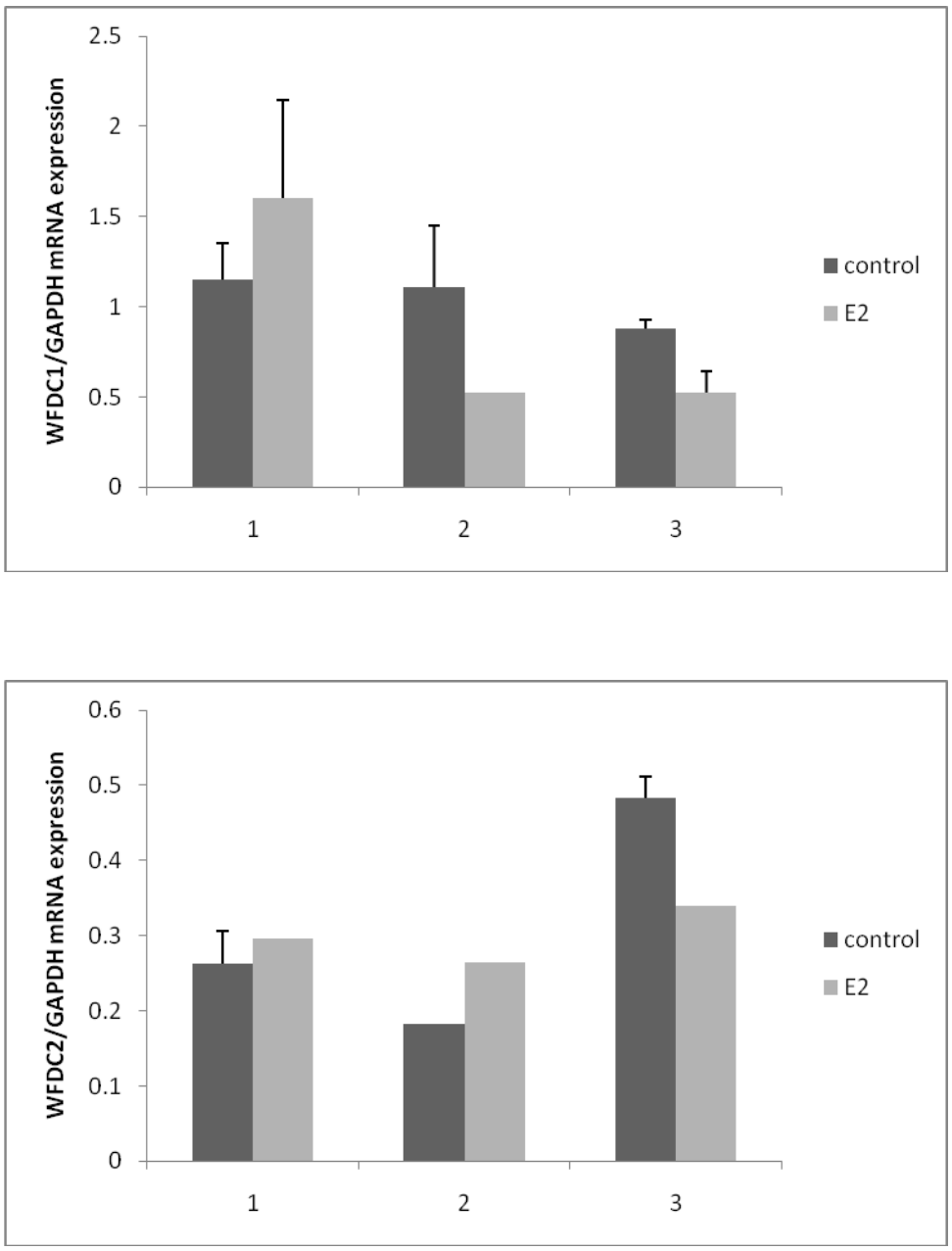


Figure 7.11 Examination of the individual experiments from primary endometrial stromal cells grown on plastic plates. The data are presented as the mean ± SEM for triplicate replicate wells.

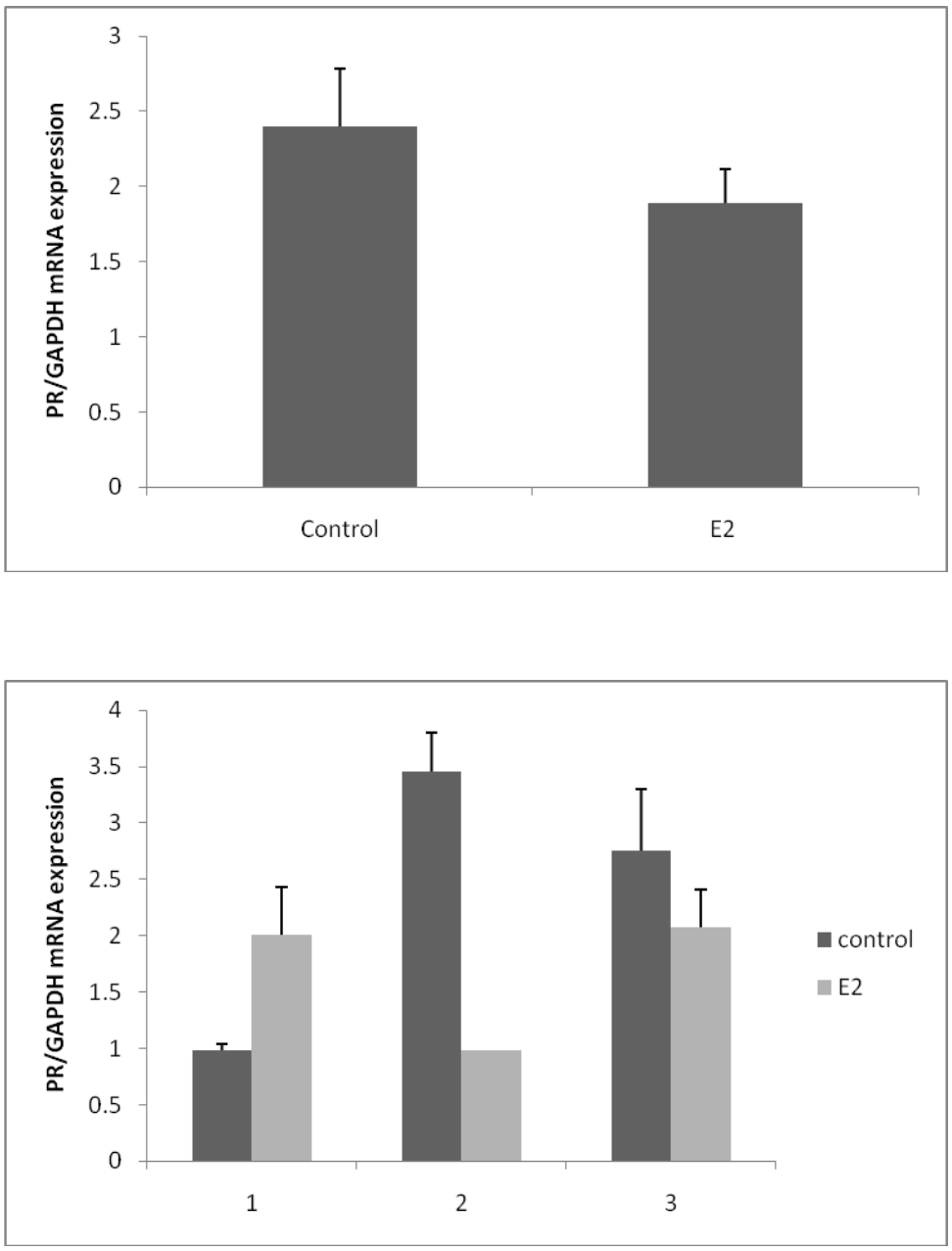


Figure 7.12 Expression of PR transcripts from primary endometrial stromal cells grown on plastic plates. Cells were grown in 6-well plates with 10% CSS and treated with a higher dose of E2 (10^{-6}M) than the myometrial cells. The data are presented as the mean ± SEM for 3 experiments performed in triplicate (upper panel) and the individual experiments (lower panel). Two RNA samples in experiment 2 were degraded and therefore the E2 bar represents a single sample.

7.4 Discussion

The data is this chapter showed that there was no change observed in either WFDC1 or WFDC2 mRNA expression in response to oestradiol on either plastic or collagen coated plates in either the myometrial cells or stromal cells. This does not fit with the microarray data, with the assumption that the major cell type in the endometrium is the stromal cell and the major cell type in the myometrium is the smooth muscle cell.

Previous data from other members of the research group indicated that E2 had a dramatic effect on WFDC1 and WFDC2 expression in both UtSMC cells grown on collagen I plates and stroma cells grown on plastic. The UtSMCs showed a 49% reduction in WFDC1 transcript level and a 43% reduction in WFDC2 transcript levels on microarray in response to 10^{-8}M oestradiol (Taylor and Bell, unpublished observations; Figure 7.13). In addition, these workers demonstrated similar decreases in WFDC1 and WFDC2 expression in primary myometrial smooth muscles cultured on collagen I coated plates and plastic plates and that there was a ~80% reduction in WFDC1 transcript levels in primary stromal cell cultures treated with 10^{-6}M oestradiol.

These data differ from those generated by my experiments presented in this chapter, where no consistent effect of oestradiol was observed in the expression of WFDC1 or WFDC2 in any of the cell types tested or in the presence or absence of collagen-I. The reason for this discrepancy is not clear. In some of my experiments, I did indeed observe decreased expression for both WFDC1 and WFDC2 transcripts, but in others I observed increased expression of transcript for these genes. One possibility is that the cells I used were obtained from different phases of the menstrual cycle and that the cells retain some type of resistance due to the particular phase of the cycle that they were obtained from, or the cells

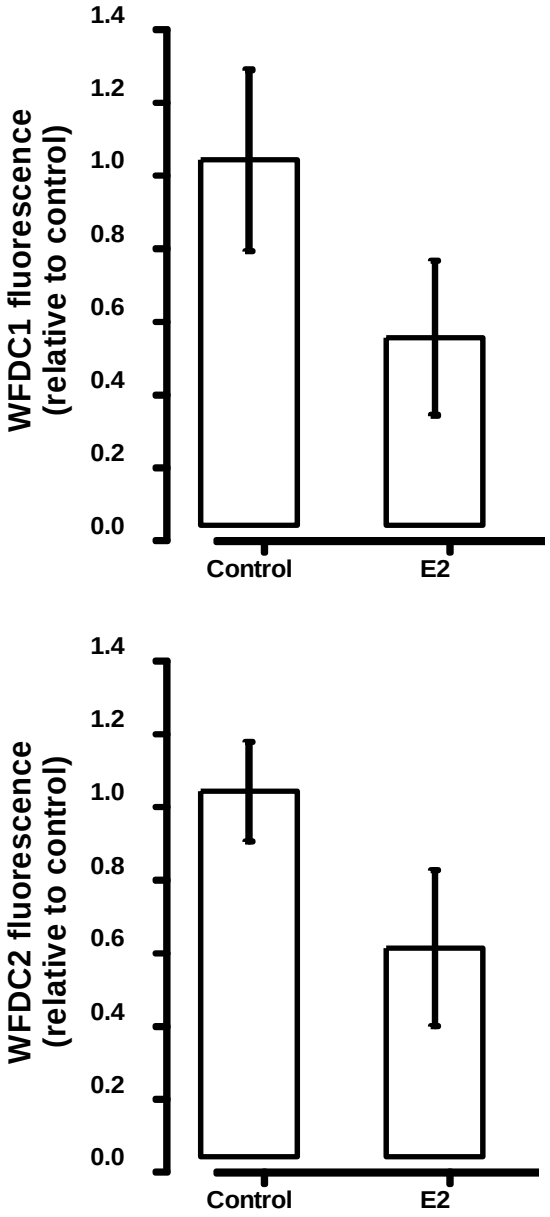


Figure 7.13 The effect of oestradiol on WFDC1 (upper panel) and WFDC2 (lower panel) transcript levels from UtSMCs grown on collagen I coated plates. The data are the mean ± SEM from 9 samples pooled from 3 separate experiments subjected to cDNA microarray analysis with the controls labelled with Cy-3' (green) and the oestradiol treated RNA pools labelled with Cy-5' (red) fluorescent labels. (Data are unpublished observations by Taylor and Bell, personal communication).

were not responding to oestradiol. For example, in Chapter 3, I demonstrated that the levels of ER isoforms changes from the proliferative to the secretory phase of the cycle. This change could alter the responsiveness of the resultant cultures to oestradiol and to tamoxifen. If the changes in receptor expression were the cause of the discrepancy between experiments, then I could have studied the expression of the oestrogen receptors in these cultures. This is an unlikely explanation because the literature does not support this.

Also, it is well known that myometrial cells rapidly lose their ability to produce the oestrogen receptor once placed into culture (Zaitseva et al., 2006) and this may also contribute to the inconsistent findings. Recent studies by the Gynaecology Endocrinology Research Group (Mehasseb and Habiba, unpublished observations) have indicated that the expression of WFDC1 and WFDC2 is also related to menstrual cycle phase and presence of adenomyosis. This could be the reason for the discrepancy between experiments, if uterine cells 'remember' which phase of the cycle they arise from (Ohata et al., 2001; Loughney and Redfern, 1009).

Recent work by Gielen and co-workers, examined the genome wide pattern of gene expression in the normal and tamoxifen treated endometrium (Gielen et al., 2005b; Gielen et al., 2008). Analysis of their microarray data with respect to WFDC1 and WFDC2 expression indicated that WFDC1 was unaffected by tamoxifen treatment, but that WFDC2 was up-regulated 1.93-fold by tamoxifen treatment. Unfortunately, they did not examine the proliferative endometrium; they had 4 patient groups, atrophic/inactive endometrial tissues from tamoxifen users and non tamoxifen users and polyps from tamoxifen users and non tamoxifen users. That there were differences in the three cultures examined is also

difficult to explain, but could be related to the same reasons why the myometrial smooth muscle cells did not respond as outlined above. It is unlikely that it is due to the oestrogen used, because Taylor and Bell used the same oestradiol and obtained 3 consecutive repeats in their stromal cell cultures, with strong PR responses and strong inhibition of WFDC1 and WFDC2 levels. Conversations with those workers revealed that they used cultures that were obtained from uteri of women in the proliferative phase of the cycle. It is well known that such cultures respond well to E2 and that low levels of E2 are required to maintain not only the ER, but also to prime the cells for the expression of PR.

In conclusion, these data did not indicate that WFDC1 and WFDC2 were down regulated by oestradiol as has been shown previously; more work in this area is required. In view of the inconsistent results I did not proceed to examine the effects of the addition of progestogens, as planned.

Chapter 8

Discussion and Future Directions

8.1 Discussion

The key hypothesis being tested in this thesis was that the abnormal features of uterine growth and development associated with the use of tamoxifen, is due to its failure to replicate all the effects of oestradiol in either inducing or repressing the expression of key paracrine acting regulators of uterine cellular populations or their gene expression profiles.

At the outset it was important to demonstrate that tamoxifen does indeed induce uterine growth. To do that the size and structure of the tamoxifen treated uterus of women being treated for breast cancer was compared and contrasted with that of postmenopausal uteri and uteri from the proliferative phase of the menstrual cycle. The data indeed confirmed that tamoxifen acted as a weak oestrogen in generating uterotrophic effects. Furthermore, (as was shown in Chapter 2), the application of the LNG-IUS device at the onset of tamoxifen treatment prevented those uterotrophic effects of tamoxifen when examined after 12 months continuous treatment. I also observed that the formation of endometrial polyps was prevented, possibly through endometrial decidualisation, an observation also reported by Gardner and colleagues (Gardner et al., 2000), but this was not uniformly observed by Chan and colleagues (Chan et al., 2007). Although the mechanism of how levonorgestrel prevents tamoxifen induced uterine growth was not a primary hypothesis of this thesis, it pointed to an answer to the second hypothesis, "Could the abnormal features of uterine growth and development associated with the use of tamoxifen be prevented by the administration of a progestagen-containing levonorgestrel intrauterine device from the onset of tamoxifen treatment?. The answer was "partially" yes because, although I showed

that the LNG-IUS prevented the uterotrophic effects of tamoxifen and prevented the development of endometrial polyps it did not return the uterus to an 'atrophic' state but caused endometrial decidualisation, which is reminiscent of the secretory phase of the menstrual cycle and early stages of pregnancy. This is probably due to the 'oestrogenic' effect of tamoxifen on PR expression, which is a well known oestrogen responsive gene (Chapter 3 and 6), allowing the levonorgestrel to activate decidualisation pathways, as has been suggested previously (Gardner et al., 2000). The very fact that the LNG-IUS did not return the tamoxifen treated uterus to an atrophic state points to tamoxifen having other effects on the endometrium and myometrium. One of those effects was increased uterine volume and production of endometrial pathologies.

How LNG-IUS can apparently reverse the uterotrophic effect of tamoxifen on the uterus (by preventing uterotrophy) but not in the endometrium (where tamoxifen use is associated with decidualisation) was a main focus of the thesis and it could have been a result of differential steroid receptor expression. When studying the expression of steroid receptors, I showed the expression of ERα, PRA and PRB in the tamoxifen endometrium was similar to that observed in the proliferative phase of the menstrual cycle, as reported (Mourits et al., 2002b; Leslie et al., 2007). It is thought that it this particular oestrogenic pathway in the endometrium that increases its responsiveness to both oestrogens and progestagens by increasing oestrogen and progesterone receptor expression, thereby promoting oestrogenic cell proliferation. This then leads to the increased incidence of endometrial polyps, hyperplasia and cancers in women taking tamoxifen for breast cancer (Ismail, 1994; Cohen, 2004). Other pathologies associated with tamoxifen include an increase in the growth of fibroids and adenomyosis (Cohen et al., 1997b; McCluggage et al., 2000), suggesting a direct effect of tamoxifen on the myometrium as well as the endometrium.

In the tamoxifen treated uteri, ERβ was up regulated in the functionalis and basalis endometrium and the myometrium (both within the inner and the outer myometrium) when compared to the proliferative phase of the menstrual cycle. In breast cancer, ERβ is an independent marker for a favourable prognosis following tamoxifen treatment in ERα negative breast tumours (Gruvberger-Saal et al., 2007). It may be that tamoxifen binds to ERβ with a slightly greater affinity than ERα, and so tamoxifen may function as an anti-proliferative signal *via* ERβ binding (Bardin et al., 2004). There have been reports of two cases where two patients with uterine mesenchymal tumours following tamoxifen therapy, observed the tumours to be ERα negative and positive for ERβ (Zafrakas et al., 2004). They suggest that tamoxifen exerts its stimulatory effect on the uterus *via* ERβ not ERα. Other reports have also found that tamoxifen associated endometrial cancers more frequently expressed ERβ (Wilder et al., 2004). It has been suggested that ERβ negatively regulates ERα in the uterus, therefore in the absence of ERβ there would be excess proliferation *via* ERα if oestradiol was abundant. The suggestion is that tissues expressing more ERβ in an oestrogen deficient environment, such as in the postmenopausal state, tamoxifen would cause proliferation *via* the ERβ. My finding of increased ERβ expression supports the idea that in an oestrogen-deficient state, proliferation occurs *via* activation of ERβ, as was shown in HEC-1b cells, when tamoxifen was applied to cells that no longer expressed ERα (Zhang et al., 2006). The difference between my studies and those mentioned above, is that none of the patients enrolled in this study developed endometrial cancer, and the tissues may therefore be very different to the mesenchymal tumours examined. These data suggest that in the uterus, ERβ is not the mediator of anti-proliferative signals as it is in the breast (Hodges-Gallagher et al., 2008; Treeck et al., 2010), but the main mediator of tamoxifen induced proliferation.

How the increase in uterine size in response to tamoxifen seen in Chapter 2 occurred was partially resolved in the study by examining the expression of proliferation and apoptosis markers (Chapter 4), where there was an increase in Ki67 expression, high Bcl2 and low BAX expression in the glandular epithelium of the tamoxifen endometrium. In this scenario, a tissue would have increased cellular proliferation coupled to apoptosis failure, thereby potentially prolonging cell survival after cell DNA damage; this may therefore predispose this group of patients to the proliferative lesions associated with tamoxifen, including large cystically dilated glands, endometrial polyps, hyperplasia and cancers (Ismail, 1994; Kedar et al., 1994; Cohen, 2004). The presence of the large cystically dilated glands was of particular interest, and to determine how the responses and phenotype of these cystically dilated glands differed from the normally sized glands the expression of mesenchymal markers were examined. The expression of vimentin, tenascin-C and α-SMA was studied to determine how tamoxifen may induce the appearance of the dilated, cystic glands observed in the endometrium of these patients.

I hypothesised that endometrial changes, including the formation of large cystic glands, occur *via* mechanisms that involve tissue remodelling. During mesenchymal differentiation, myometrial cells are thought to be derived from the transition of the stromal fibroblast through an intermediary myofibroblast to the fully differentiated myocyte (Parrott et al., 2001). The key molecules for defining this fibroblast-myofibroblast-smooth muscle cell pathway are, vimentin, an intermediate filament protein only found in mesenchymal cells, α-smooth muscle actin (α-SMA), which is a marker for the smooth muscle cell and can distinguish myocytes from the stromal fibroblast, and tenascin-C, which is an extracellular glycoprotein that is up-regulated in some tissues that are undergoing tissue remodelling such as those cells that are de-differentiated and

proliferating. The data in Chapter 5 showed that the endometrial glands in the tamoxifen uteri stained for vimentin. Vimentin does not normally stain epithelial cells, so this was a surprising finding and suggests that the cells are not entirely epithelioid, but contain a mixed phenotype that includes epithelial and mesenchymal proteins. This has been reported for other cell types, such as the myoepithelial cells in the breast (Korsching et al., 2005) and in breast mammary epithelial cells treated with tamoxifen, where there was not only an increase in vimentin staining, but an increase in CD24 expression (as seen in the tamoxifen treated myometrium (Chapter 6) and markers of stem cells, during their immortalization (Mani et al., 2008). The findings described in Chapter 5 suggest that endometrial stromal cells can thus undergo a transition from a mesenchymal cell through a myofibroblast to a myocyte **and** through a mesenchymal-epithelial cell transition that involves an intermediary cell that has both mesenchymal and epithelial cell phenotypes. I cannot conclude from my data whether tamoxifen is indeed promoting or inhibiting the mesenchyme-myofibroblast-myocyte transition, although the presence of all three markers surrounding the large cystically dilated glands, but not the normal sized glands suggest that the phenotypes of these two types of glands differ, with the large cystically dilated glands being closer to the suggested transitional state and that the tamoxifen treated uterus is primarily in a 'myofibroblast' phase. The expression of the mesenchymal markers in the tamoxifen treated uteri, lead to the possibility that the cystically dilated epithelial glands in tamoxifen treated uteri may exert a gene modulatory function on the surrounding stromal cells that is unique to tamoxifen, or that tamoxifen has a unique gene modulatory function that results in the formation of this unique pathology. To find out if the latter were true, I performed a microarray comparing the gene expression profiles from postmenopausal women taking tamoxifen to that of postmenopausal atrophic uteri and the proliferative phase of the menstrual cycle.

The microarray analysis revealed that there were changes in the transcription pattern of the uterus in response to the oestrogenic proliferative signals and in the tamoxifen treated uteri. There were differences and similarities in the transcript patterns in the myometrium and endometrium, supporting previous observations that tamoxifen behaves both as an agonist and an antagonist. Tamoxifen has oestrogen antagonistic activity in the breast (Shang, 2006), and oestrogen agonistic effects that are beneficial in the bone and cardiovascular system (Chang et al., 1996; Jordan et al., 2001) and detrimental in the uterus by increasing the risk of cancer development (Fornander et al., 1989; Fisher et al., 1994; Mignotte et al., 1998; Bernstein et al., 1999; Bergman et al., 2000; Jordan, 2004). There were pathways that were affected by tamoxifen alone, and pathways that were altered in a similar manner and the opposite direction to the proliferative phase (Table 6.4).

It has been shown that tamoxifen gene modulation when compared to oestradiol gene modulation in a human cell line (ECC1) overlapped and that tamoxifen regulated its own individual set of genes (Gielen et al., 2005b). This observation suggests that some, if not all, of the uterine pathologies associated with tamoxifen therapy in post-menopausal women (Chapter 2) is due to a unique action of the drug itself, because if it were due to either an anti-oestrogenic or pro-oestrogenic action, then it follows that others would have observed similar gene changes in hyper-oestrogenic states, or in patients treated with other ant-oestrogens. For example, Gielen and colleagues identified 256 gene products that were regulated in the endometrium from tamoxifen treated patients *in vivo*. They observed that a number of these gene products were part of pathways which could play a role in endometrial carcinogenesis based on their normal physiological function. Therefore, it may be that tamoxifen induction of imbalances in several of these pathways could

contribute to why tamoxifen induced cancers behave more aggressively than sporadic endometrial tumours (Prasad et al., 2005).

The microarray findings in Chapter 6 support the findings of the previous *in vivo* data (Gielen et al., 2005b). When the gene list generated in this study was compared to that produced by Gielen and co-workers, it showed that all the previously described genes are included in the present gene list (Table 6.2) (Gielen et al., 2005b). The data in this thesis also indicated that several pathways involved in cell proliferation and apoptosis regulation were significantly affected by both tamoxifen and also by the oestradiol dominant proliferative phase endometrium. For example, the WFDC1 and WFDC2 genes were affected in this way with differential effects for WFDC1 in the endometrium and myometrium. Such observations led to a new hypothesis that some of the uterotrophic effects of tamoxifen may be due to a loss of the normal regulatory factors involved in uterine growth. It is possible that paracrine regulatory factors that are growth inhibitory may be involved in the normal coordinated growth of the uterus and such factors may allow normal uterine development by being inhibited by oestradiol. In the tamoxifen treated uterus, tamoxifen may not fully suppress these regulators as it may only have weak oestrogenic properties and so results in disordered uterine growth. In this regard, WFDC1, which has previously been shown to be anti-proliferative and possess antiprotease activity in the extracellular matrix seemed a good candidate gene to further study. The loss of WFDC1 in the extracellular matrix might therefore allow cell proliferation and protease activity to occur, adding to local regulation of growth and differentiation, thus addressing the concept that tamoxifen suppresses the expression of these proteins leading to increased proliferation, and increased invasiveness of the cells.

The staining pattern of the mesenchymal markers in the tamoxifen treated uterus suggests that it is in a myofibroblast phase and based on the limited literature concerning WFDC proteins, these molecules could be involved in this expression of this phenotype. For example, in the rat uterus, WFDC1 expression is inversely correlated to the levels of oestradiol (Hung, 2005). Tamoxifen also decreases WFDC1 expression, but is 800- to 1500-fold less effective when compared to oestradiol in the control of rat uterine cell proliferation. WFDC1 may be a mediator of growth in the uterus (Hung, 2005). Unpublished data from my research group showed that WFDC1 and its sister protein, WFDC2 (HE4) are expressed by primary cultures of human uterine smooth muscle cells, and that their expression was suppressed by oestradiol (Taylor & Bell, unpublished observations). The function of WFDC2 has not be ascertained, but it is down regulated in prostate cancer and up regulated in ovarian and pancreatic cancer (Bouchard and Tremblay, 2006). I showed that both WFDC1 and WFDC2 were indeed expressed in both human uterine myometrial and stromal cells; however, I did not observe the down regulation of these proteins by oestradiol or tamoxifen. The reason for this is not clear to me and is something that needs to be examined further.

8.2 Future Directions

The results of the studies within this thesis have highlighted some of the mechanisms whereby tamoxifen induces uterine growth and, in particular, that some of these effects are oestrogen-like and some are due directly to the actions of tamoxifen itself. Although some of the effects of tamoxifen on the human uterus are becoming clearer, others need additional work. Here are some suggestions for future research projects:-

1. The effect of progestagens on tamoxifen-induced genes. In this series of studies the gene expression profiles from endometrial and myometrial cells treated with tamoxifen plus levonorgestrel would be examined and compared to those from tamoxifen treatment alone.

2. An examination of steroid receptors expression, along with the expression of proliferation, apoptosis and mesenchymal markers in tamoxifen-induced endometrial polyps with a comparison of that expression to those in endometrial polyps in non-tamoxifen users and tamoxifen-treated and non-treated uteri. The effect of levonorgestrel on the expected differential expression would be useful here.

3. A series of studies into the role of vimentin in the epithelial to mesenchymal transition as a possible mediator of tamoxifen-induced uterine pathology. In these experiments, it would be anticipated that epithelial cells would be induced to undergo EMT by tamoxifen, and that effect prevented by the application of levonorgestrel. Additional experiments would involve the over expression of vimentin in uterine epithelial cells by transfection and examining their ability to remain in close contact with each other. Proteins involved in cell-to-cell adhesion could also be examined.

Appendices

1. Patient information leaflet
2. Consent form
3. Ethical approval letter

Appendix 1

Investigators: Dr Rina Panchal, Mr Marwan Habiba, Professor Justin Konje
Division of Obstetrics and Gynaecology
Department of Cancer Studies and Molecular Medicine
Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Leicester, LE2 7LX

PATIENT INFORMATION LEAFLET

Title of study

The application of the Levonorgestrel containing intrauterine device in the prevention of endometrial changes induced by Tamoxifen in women undergoing adjuvant therapy for breast cancer.

This study is sponsored by Schering Healthcare.

We would like to invite you to participate in the above study being undertaken at the University Hospitals of Leicester NHS Trust.

1. What is the purpose of the study?
This study is aimed at examining the effects of a locally administered hormone (levonorgestrel) on the changes induced in the lining of the womb (endometrium) by Tamoxifen - a drug used in women with breast cancer to prevent the progression of the disease or the development of new disease. This drug may cause changes in the lining of the womb, which in some patients may require further investigation to exclude cancer.
In a previous trial in Leicester in women who have been on Tamoxifen for at least 12 months, we showed that this drug reversed the changes in the endometrium induced by tamoxifen. Such effects will be very beneficial in women on this drug worrying about the risk of cancer of the endometrium. An important question, which we wish to address, is whether the use of this locally administered hormone from the initiation of treatment with tamoxifen will prevent the development of these changes.

2. What is the name of the drug*?*
The drug to be used in this study is called a Levonorgestrel intrauterine contraceptive device (Mirena). This device is commonly used as a form of contraception and also to treat very heavy periods. The most common side effect is irregular vaginal bleeding. However, after about 4-6

months, this complication all but disappears. Other side effects include slight weight gain, breast discomfort and occasional mood swings. In our previous study, this device was well tolerated by all the women recruited into the study.

3.What will be involved if I take part in the study?
If you agree to take part in this study you will be randomised (similar to the tossing of a coin) into one of two groups. One group will receive the intrauterine device while the other will not. However, each patient will have an ultrasound scan of the uterus and ovaries and a camera examination of the inside of the uterus (hysteroscopy). At the time of these investigations a small sample of the lining of the womb (biopsy) and a small blood sample (10ml – approximately 2 teaspoonfuls) will be collected from you. At the time of the camera examination (to be done with local anaesthesia and as an outpatient procedure), the intrauterine device will be inserted into your womb if you have been randomised to receive it. You will be asked to keep a diary of abnormal vaginal bleeding and any other side effects of the device, which you may experience.

Follow up visits will be at 1, 6, 12, 18 and 24 months after the device. Those in whom the device was not inserted will keep similar diaries and will also be seen at the same intervals. At the follow-up visits at 6, 12, 18 and 24 months, a repeat ultrasound scan of the uterus and ovaries will be undertaken and another small blood samples (10ml) will be collected. At 12 months, another camera examination undertaken. Again, at this time, a small biopsy of the endometrium will be taken. The follow-up visits and sampling will be same in both groups of patients. With your permission, the telescopic examination of your womb may be video recorded. You will not be identified on the video and it will be destroyed after the research project is complete.

You will be followed up in the Breast Clinic as decided by the clinic doctors.

4. Will information obtained in the study be confidential?
All information obtained in relation to the study will be confidential. You will have a unique trial identification number (ID), which will not contain any of your details. You will not be identified in any documents relating to the trial. Only the unique trial IDs will be used. The Data Protection Act will form the bases of the confidentiality of the data from the trial. Participation in the study will be recorded in your medical notes although the results will not be recorded in your notes. However, you will have access to the data from the study if you so wish.

Your GP will be informed of your participation in the study.

5. What if I am harmed by the study?
Medical research is covered for mishaps in the same way, as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

6. Will I receive out of pocket expenses for taking part in the study?
Yes, a bus, train or taxi fare will be paid for all the study related visits to the hospital. These will be able to be claimed at each visit.

Version No.2 dated February 2004

7. What happens if I do not wish to participate in this study or wish to withdraw from the study?

If you do not wish to participate in this study or if you wish to withdraw from the study you may do so without justifying your decision and your future treatment will not be affected.

If you have any questions please contact:

Dr. Rina Panchal

Tel: 0116 2523149, 0116 2523170
Mobile phone 07813 643449

Mr M Habiba

Tel: 0116 2523170

Professor J Konje

Tel: 0116 2525826

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Department of Cancer Studies & Molecular Medicine
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Version No.2 dated February 2004

Appendix 2

Investigators: Dr Rina Panchal, Mr Marwan Habiba, Professor Justin Konje
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Leicester Royal Infirmary
Leicester, LE2 7LX

PATIENT CONSENT FORM

Title of study

The application of the Levonorgestrel containing intrauterine device in the prevention of endometrial changes induced by Tamoxifen in women undergoing adjuvant therapy for breast cancer.

This leaflet should be read in conjunction with patient information sheet version no 2 dated February 2004.

I agree to take part in the above study as described in the Patient Information Sheet.

I understand that I may withdraw from the study at any time without justifying my decision and without affecting my normal care and medical management.

I understand that members of the research team may wish to view relevant sections of my medical records, but that all the information will be treated as confidential.

At the termination of this trial I understand that there is no guarantee that the drug treatment received during this trial will continue.

I understand medical research is covered for mishaps in the same way, as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.

I agree* / do not agree* to have the telescopic examination of my womb video recorded. I understand that if I agree, I will not be identified in any way in this recording and that it will be destroyed after the research project is completed.

I have read the patient information leaflet on the above study and have had the opportunity to discuss the details with Dr Rina Panchal and ask any questions. The nature and the purpose of the tests to be undertaken have been explained to me and I understand what will be required if I take part in the study.

* Please delete as appropriate

Signature of patient .....Date.....
(Name in BLOCK LETTERS below)

.....

I confirm I have explained the nature of the Trial, as detailed in the Patient Information Sheet, in terms, which in my judgement are suited to the understanding of the patient.
Signature of Investigator .....Date.....
(Name in BLOCK LETTERS below

Version No2 dated February 2004

Appendix 3

University Hospitals of Leicester NHS
NHS Trust

DIRECTORATE OF RESEARCH AND DEVELOPMENT
Director: Professor D Rowbotham
Co-ordinator: C Cannaby
Direct Dial: 0116 258 4614
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11 August 2004

Mr Habiba
Senior Lecturer in Obstetrics and Gynaecology
Directorate of Women's, Perinatal and Sexual Health Services
LRI

Dear ~~Dr~~ Mr. Habiba

RE: **UHL Ref. 7517** *[Please quote this number in all correspondence]*
The application of the Levonorgestrel containing intrauterine contraceptive device in the prevention of endometrial changes induced by tamoxifen in women undergoing adjuvant therapy for breast cancer

We have now been notified by the Ethical Committee that the protocol concerning the above titled project has been given favourable ethical opinion subject to receiving a complete response to the requested information listed in the Ethics letter dated 06-08-04.

In order to secure full Trust approval of the amendment, please send one hard copy of the amended paperwork together with an electronic copy to the Research Office as soon as possible. We will pass this on to the Ethics Committee on your behalf.

Since all other aspects of your R+D Notification have been completed, I anticipate that there will be no further barrier to the approval of your amendment by the Trust as soon as full Ethical authorisation has been granted.

However, please note that the Trust has NOT yet given approval to this amendment, nor indemnified it, until you receive the full, formal approval letter from this office.

I look forward to hearing from you.

Yours sincerely

Chris Cannaby
R & D Programme Board Co-ordinator

13 AUG 2004

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