UNIVERSITY OF LIVERPOOL

Differences in Phenotypic Features and Hormone Responsiveness between Healthy Eutopic Endometrium and Tubal Epithelium in Women– Functional Relevance in Gynaecological Disease

Thesis submitted in accordance with requirements of University of Liverpool for the degree of MPhil by Eve Bunni

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Abstract

Eve Bunni

Differences in Phenotypic Features and Hormone Responsiveness between Healthy Eutopic Endometrium and Tubal Epithelium in Women– Functional Relevance in Gynaecological Disease.

Introduction: We recently reported a unique phenotypic profile for the epithelial cells of the basalis compartment of the human endometrium, from where the functionalis is regenerated after menstrual shedding. As the human fallopian tube shares the same embryonic origin as the uterus and has the capacity to undergo changes according to the menstrual cycle including regeneration, we wondered whether this unique phenotype was shared with the fallopian tube. Recently it has been thought that the distal fallopian tubal mucosa may also contain a stem cell population - it was found that cells were expressing markers that were known to be stem cell markers in other tissues. This suggested that the distal mucosa may be equivalent to the stem-cell rich *stratum basalis* of the endometrium. Endometrial expression of steroid receptors vary throughout the menstrual cycle in response to the fluctuating steroid hormones in the circulation. Although some evidence exists on steroid receptor presence in human fallopian tube, detailed examination of both the distal and proximal parts of the tube has not been studied. Furthermore even though the androgen receptor has been identified in the human endometrium, the effect of androgens on fallopian tube and endometrial mucosa has not been studied to date.

Aim: To examine the differential expression of phenotypic markers and hormone responsiveness between the endometrium and fallopian tubal epithelium in healthy women across the menstrual cycle and in the postmenopausal period.

Methods: A prospective study examining 22 full-thickness endometrium and matched tubes for the basal endometrial epithelial markers (SOX9, SSEA1, nuclear β-Catenin, BCAM), Sialylated SSEA-1, Steroid receptors and the proliferative marker KI67 with immunohistochemistry and analysed using a modified-Quickscore. Secondary confirmation of data by RT-PCR. Explant cultures of matched

endometrial and fallopian tube tissue (n=3) were treated with DHT for 24 hours and expression of SOX9, AR and Ki67 was assessed with IHC/RT-PCR.

Main results: Trends were observed in the sample populations. The proliferative activity and phenotypic marker expression of distal tubal epithelium correlated with that of the endometrial basalis epithelium. SSEA-1, SOX9 and cytoplasmic beta catenin increased after the menopause in both endometrial and fallopian tubal epithelia. Steroid hormonal expression however, showed a unique pattern in tubal epithelial cells which may play a role in preventing cyclical regeneration in the tube. Proximal and distal portions of the tube had different responses to ER alpha and beta throughout the menstrual cycle indicating a unique function in the isthmic region of the tube in the premenopausal population. Short-term explant culture, DHT treatment up-regulated AR expression with an increase in KI67 in tubal epithelium, but no difference was observed in the matched endometrial epithelium. Increased AR and KI67 expression was observed in the endometrial stroma. Messenger-RNA expression of SOX9 was reduced after DHT treatment. IHC results were confirmed by RT-PCR.

Conclusion: The Tubal Epithelium and Endometrial Mucosa share a phenotypic profile consistent with their shared embryonic origin. Explant analysis suggests a unique role for androgens in the tube. After menopause, it is evident that the tubal epithelia and endometrial glands exist as a continuum. Proximal and distal tubal epithelia have different patterns of steroid receptor expression throughout the menstrual cycle suggesting a unique steroid hormone response in the proximal tube possibly separating these organs and preventing cyclical regeneration in the tube.

Contents

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Abbreviations

Abbreviation	In Full
BCAM	Basal Cell Adhesion Molecule
SOX9	Sex Determining Region Y
SSEA-1	Stage-Specific Embryonic Antigen 1
Sialyl SSEA-1	Sialylated Stage-Specific Embryonic Antigen 1
AR	Androgen Receptor
PR	Progesterone Receptor
ER α	Oestrogen Receptor Alpha
ER β	Oestrogen Receptor Beta
PM	Postmenopausal
cDNA	Complimentary Deoxyribonucleic Acid
mRNA	Messenger Ribonucleic Acid
IHC	Immunohistochemistry
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
µl	Microliter
ml	Millilitre
mM	Millimolar
E2	Oestradiol
DHT	Dihydrotestosterone
GCP	Good Clinical Practice
LREC	Local Research Ethics Committee
HmG	High Mobility Group
IgG	Immunoglobulin G
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
MQ	Median Quickscore
MTP	Median Threshold Percentage
MPP	Median Percentage Positive
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
YWHAZ	14-3-3 Protein Zeta/Delta

Chapter 1 Introduction

The fallopian tube and endometrium derives from the same embryological origin.[1] Recent studies investigating endometrial mucosa have revealed that particular markers are strongly expressed in the basalis region of the endometrium. The markers identified were SOX9, SSEA-1 and beta catenin.[2] The presence of these markers has been identified in tissues with regenerative abilities with a subpopulation of progenitor cells residing in these regions. It is thought that progenitor cells give the endometrium the capacity to regenerate and proliferate every month and can thus be found in the basalis region of the endometrium – the regenerative layer.[3] Recently, it has been thought that the distal fallopian tubal mucosa may also contain a stem cell population.[4] The characterisation of the epithelial layer of the fallopian tube is yet to be fully understood.

Endometrium is the main target organ for ovarian steroid hormones. These hormones induce their effects on the endometrium via hormone receptors. The steroid hormone receptors are: Oestrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR). Endometrial expression of the steroid hormone receptors has been investigated in some detail. Expression of the steroid receptors vary throughout the menstrual cycle in response to the fluctuating steroid hormones in the circulation.

Although the general consensus is that the steroid receptor expression in the endometrium is fully characterised, the existing evidence is very limited for the details of steroid receptor expression in functionally very different stratum basalis and functionalis, also no conclusive studies on comparisons of healthy pre and post-menopausal endometrium. For these reasons, from the existing data, it is difficult to conclude on the normal pattern of steroid receptor expression in healthy human endometrium.

Detailed examination of steroid receptor expression in both distal and proximal parts of the tube, across the menstrual cycle of premenopausal women and in

postmenopausal women, is yet to be compared with matched normal endometrial steroid receptor expression - to my knowledge has not been conclusively reported.

Since steroid receptors regulate endometrial and possibly tubal function and their regenerative process hence are implicated in normal function and in many pathological conditions, understanding the above is of significant importance. This thesis therefore provides data, to fill these existing gaps in current knowledge and explore the similarities and differences in the expression of a panel of phenotypical markers and steroid receptors between the endometrium and tubal epithelium examining their differential response to one of the ovarian hormones, androgen.

1.1 Structure and function of human uterus, endometrium and fallopian tube.

1.1.1 Macroscopic structure of the female reproductive system.

The female reproductive system is divided into the internal and external genitalia. The internal genitalia are those that are within the true pelvis. The external genitalia are found outside of the true pelvis.[5]

Table 1-1 – Table of the different organs in the female reproductive system

Internal Genitalia	**External Genitalia**
Vagina	Perineum
Cervix	Mons Pubis
Uterus	Clitoris
Fallopian tubes (Right and left).	Urethral Meatus
Ovaries	Labia Majora
	Labia Minora
	Vestibule
	Vestibular Bulbs
	Bartholin's Glands
	Skene Glands

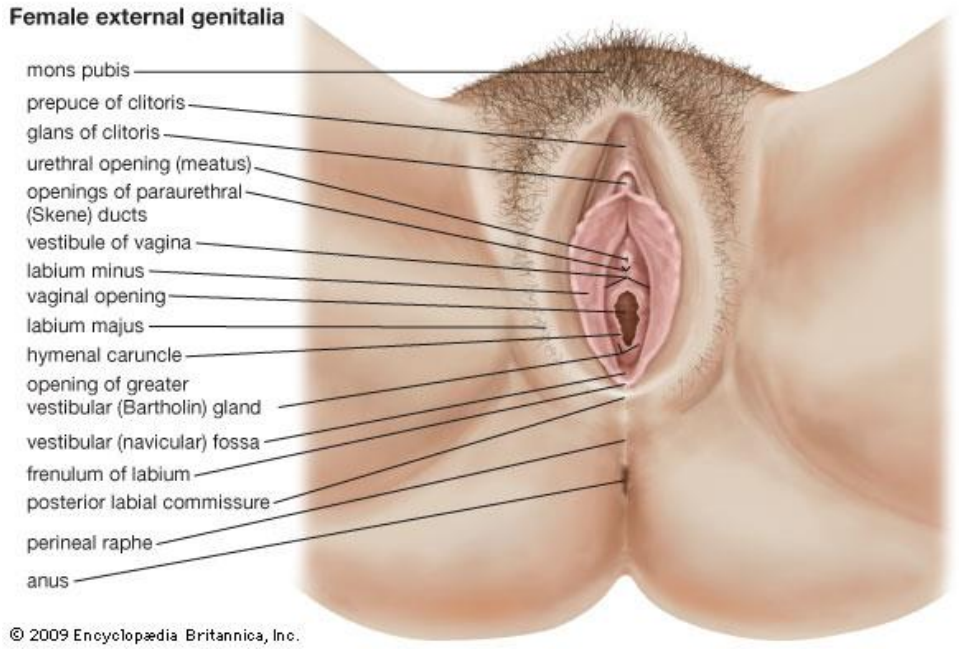


Figure 1-1 - The external genitalia[6]

1.1.1.1 The Vagina

The vagina connects the cervix to the outside environment. It is a muscular tube of approximately 6.5-7cm in length anteriorly and 9cm posteriorly. It is situated anterior to the rectum and posterior to the urinary bladder. The vagina is lined with mucosal folds called rugae. In-between a layer of erectile tissue can be found with a large venous plexus within it. The vaginal portion of the cervix is found towards the apex of the vagina. It protrudes into the vagina leaving a recess surrounding it called the anterior, posterior and lateral fornices.[7] The vaginal arteries supply the vagina; these branch off the internal iliac artery.

Its function is a reservoir for sperm; to facilitate their ascent towards the uterus and up towards the fallopian tubes to fertilise an oocyte. It is also the canal in which the neonate descends during labour and delivery.

1.1.1.2 The Cervix

This is the most inferior portion of the uterus separating the vagina from the uterus. There is an opening to allow sperm to enter the cervical canal called the external os. The sperm exit the canal, into the uterine cavity via the internal os. Branches of the

descending uterine arteries supply the cervix. The nerve supply to the cervix is via the parasympathetic nervous system (S2-S4). Lymphatic drainage is complex.[1]

1.1.1.3 The Uterus

The uterus is a pear-shaped, hollow, muscular organ lying between the urinary bladder and the rectum. It begins with the cervix most inferiorly then becomes the body; in which the blastocyst implants. Superiorly the fallopian tubes project laterally either side of the organ – the lumen is continuous with the endometrial lining. The fallopian tubes, most distally, then open adjacently to the ovaries in the peritoneal cavity. The uterus expands enormously during the pregnant-state of the female.[8] The uterine arteries supply the main body of the uterus. As they enter the myometrium they branch to become the arcuate arteries. They then branch further as they enter the endometrial layer, becoming the spiral arteries. The venous system usually runs below the arteries. The nervous system supplying the body of the uterus is via sympathetic nerve fibres originating from thoracic vertebrae 11 and 12 (T11, 12). Lymphatic drainage eventually drains into the internal iliac, external iliac and sacral groups.[9]

1.1.1.4 The Fallopian Tubes

The fallopian tubes (also referred to as; uterine tubes and oviducts) exit the uterus bilaterally via the cornua and are considered a link between the endometrium and the peritoneal cavity. There are three parts to the tube each believed to have different, important roles. The most proximal part is named the Isthmus. This is thought to be a reservoir for sperm as they enter the oviduct.[10] The middle part is called the Ampulla. This is the main site of fertilisation. The most distal part of the fallopian tube is called the Infundibulum where finger-like projections called fimbriae can be found. These fimbriae receive the oocyte that is released during ovulation from the ovary. The arterial supply to the fallopian tube originates from the ovarian and uterine arteries. The nerve supply comes from thoracic and lumbar vertebrae (T11-L1) via sympathetic and parasympathetic fibres.[11] Lymphatic drainage is into to the external iliac and lateral aortic nodes.[12] The main function of the fallopian tube is to facilitate fertilisation by promoting contact between the

sperm and the oocyte. After successful fertilisation, the fallopian tube enables the transport of the fertilised zygote toward the endometrium for subsequent implantation.

1.1.1.5 The Ovaries

The ovaries are approximately 3-5cm in length. They are located either side of the uterus and lie within the ovarian fossa. The ovarian fossa is a shallow depression encased by the external iliac arteries superiorly, the obliterated umbilical artery anteriorly, and posterior is the ureter.[13] The ovaries are held within the broad ligament which also covers the anterior aspect of the uterus. The ovaries have an uneven surface and contain 1-3 million immature oocytes, of which only 0.01% will mature and be released at ovulation. The main purpose of the ovary is to house the ova so that under the influence of pituitary hormones FSH and LH, a follicle can develop and an oocyte can mature and be released for the purpose of fertilising with a spermatozoa. During the menstrual cycle the ovary produces steroid hormones; oestrogen and progesterone (see 1.1.5 for full explanation). The blood supply to the ovary is via the ovarian artery originating directly from the descending aorta. The venous drainage is slightly different in that the left ovarian vein empties into the left renal vein and the right ovarian vein drains directly into the vena cava. Lymphatic drainage is into the aortic nodes as well as some of the iliac nodes. The nerve supply is via the ovarian, hypogastric and aortic plexuses and runs with the vessels into the hilum of the ovary in the suspensory ligament.

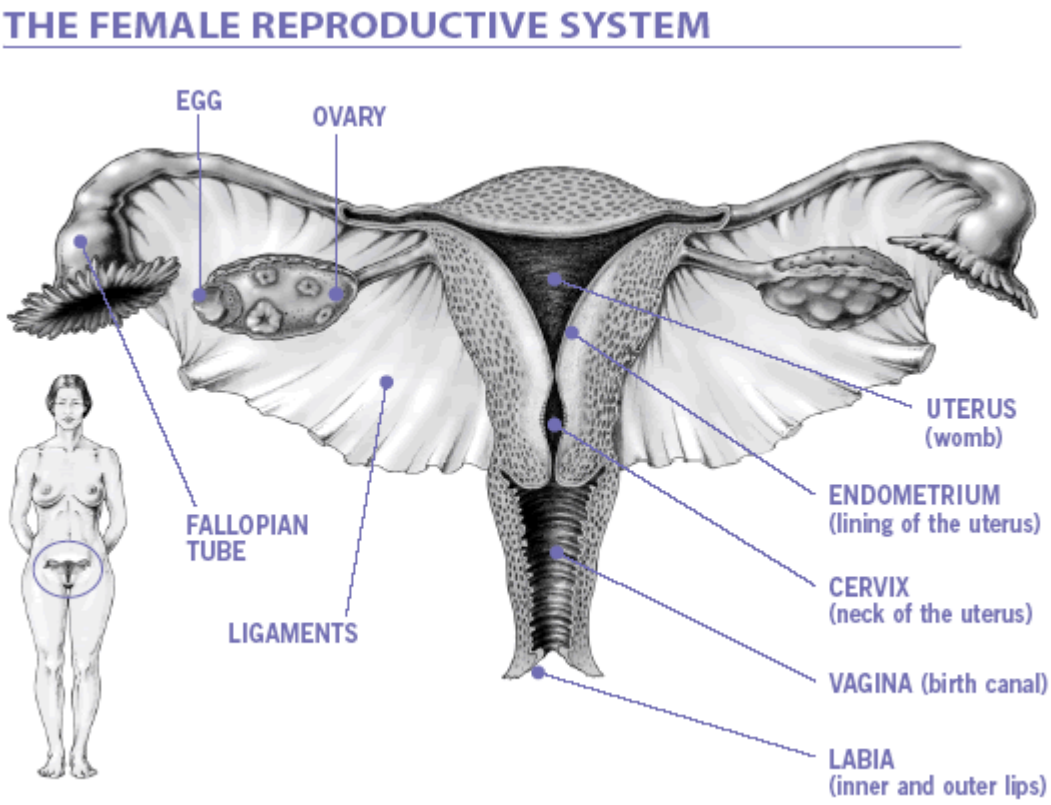


Figure 1-2 - The female reproductive system[14]

1.1.2 Histology of The Uterus

The uterus has three layers:

- The endometrium
- The myometrium
- The perimetrium

The endometrium or mucosa of the uterus is the innermost part of the uterus. It is divided into two zones: the functional layer (*stratum funtionale*) and the basal layer (*stratum basale*). The functional layer proliferates and then sloughs off during menstruation. The basal layer is thought to be the source of regeneration of the functional layer; it remains constant throughout the female menstrual cycle. The endometrium builds up during the menstrual cycle to prepare for implantation of the blastocyst. The endometrium is lined by simple columnar epithelium made up of a mixture of secretory and ciliated cells. The endometrial tissue is made up of

stroma and glands. The glands are lined also by columnar epithelium. The stroma contains many cells and bears a resemblance to mesenchyme.

The myometrium is a thick muscular layer in the uterus. There is a thick middle layer consisting of smooth muscle bundles arranged in a circular and spiral pattern with large numbers of blood and lymphatic vessels running through it. This layer is called the vascular layer (*stratum vasculare*). The inner and outer layers surrounding this are also made up of smooth muscle bundles that run in parallel to the uterine axis. These smooth muscle cells undergo massive hypertrophy in the pregnant-state. They begin at 50 micrometres and enlarge to 500 micrometres. The myometrial fibres are arranged randomly for the very important reason that during uterine contraction, the layers of musculature can work together to expel material through the very narrow cervical lumen.

The microvasculature of the endometrium is abundant. The uterine artery divides into the arcuate arteries in the myometrium. These further divide and become the radial arteries which then enter the basal layer of the endometrium - the radial arteries branch to become straight arteries supplying the *stratum basale* of the endometrium. The radial arteries coil when they enter the functional layer and become the spiral arteries. The spiral arteries anastomose and make a richly vascularised functional endometrium.

The perimetrium is the peritoneal outer-lining of the uterus. This becomes continuous with the abdominal peritoneum. It is comprised of mesothelium, connective tissue and some elastic tissue. The perimetrium covers the uterus posteriorly and partially anteriorly.[15]

1.1.3 Histology of the Uterine Tube

The fallopian tubes have three layers, histologically. The innermost layer is the luminal epithelium, the middle layer is the muscularis layer and the outermost layer is the serosal layer. Starting most distally, the infundibulum has a row of ciliated columnar epithelium coating the fimbriae. This helps to capture the oocyte as it is released from the ovary and sweep it along the tube. The ampulla has numerous

longitudinal folds with a layer of epithelial cells comprising of ciliated, secretory and peg cells that are claimed to be more abundant around day 14 of the menstrual cycle, the time of ovulation.[16] The isthmus – the most proximal part, is small in diameter and an increased proportion of secretory cells are seen in the epithelium. The muscularis layer surrounding the epithelium gradually increases more proximally. The smooth muscle layer is made up of circular, oblique and longitudinal fibres and is thought to be involved in the movement of the oocyte towards the uterus through regular peristaltic movements. It is also thought that the infundibular muscular layer relaxes and distends towards the ovary to facilitate oocyte capture. The outer layer, the serosa is made up of mesothelium and is continuous with the broad ligament.[17]

1.1.4 Embryology of the Fallopian Tube

In the second month of embryologic development the genital tract begins to develop. It begins with a pair of genital ducts: the mesonephric (Wolffian) ducts and paramesonephric (Mullerian) ducts. The Wolffian duct regresses and the mullerian ducts are what form the female reproductive tract.[18] After meeting in the midline, the most superior part of the paramesonephric ducts are what form the fallopian tubes.[19] These open into the coelomic cavity. The caudal part of these ducts fuse together forming the uterus and upper vagina. Tissue lateral to the middle portion forms the broad ligament.[20]

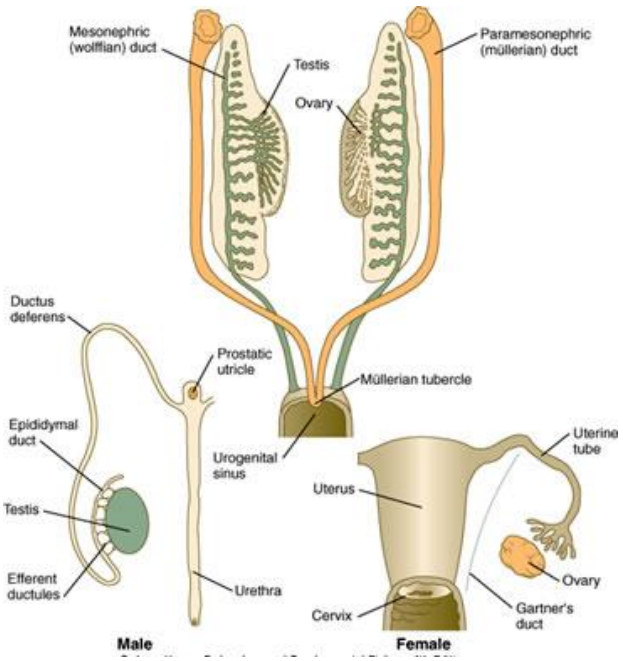


Figure 1-3 - Reproductive System Development Overview[21]

1.1.5 The Human Menstrual Cycle

The female menstrual cycle typically lasts 28 days. It begins on the first day of a menstrual bleed, this process is termed menstruation. Bleeding will slow and eventually stop after 3-7 days and proliferative/follicular phase will begin. During this phase, the endometrial lining builds up or proliferates to create a hospitable environment for an embryo to implant. Follicular growth occurs in the ovary in response to a rise in circulating gonadotrophin, FSH. At around day 14 of the

menstrual cycle another phase begins, the ovulatory phase. The follicle releases the matured oocyte into the fallopian tube ready to be fertilised by spermatozoa. After follicular rupture, a corpus luteum remains hence marking the beginning of the luteal phase of the menstrual cycle. The corpus luteal hormones help to maintain the endometrium for the arrival of the embryo. If fertilisation does not take place, the corpus luteum breaks down and the lining of the uterus, no longer being maintained, is shed. This marks the beginning of the next cycle.

1.1.5.1 Hormonal regulation during the menstrual cycle

Beginning in the follicular phase, follicle-stimulating hormone (FSH) is released by the anterior pituitary gland. The rise in FSH levels causes the development of antral ovarian follicles. It also causes proliferation of oestrogen-secreting granulosa cells and stimulates expression of luteinising hormone (LH) receptors. As oestrogen levels inevitably rise, this stimulates release of gonadotropin-releasing hormone (GnRH). GnRH increases the production of LH. At this stage, LH stimulates proliferation and generation of follicular thecal cells and induces androgen synthesis by these cells.

Serum oestrogen levels rise during this phase promoting growth and proliferation of the endometrium and myometrium in the uterus. They also promote progesterone receptor production in preparation for the luteal phase. 2-3 days before the LH surge; one or occasionally two follicles emerge as dominant. Oestrogen levels then continue to rise from the follicle. As well as causing endometrial changes, oestrogen stimulates mucus production in the crypts of the cervix. This provides a less acidic and hence more hospitable environment for sperm in the vagina.

Mid-cycle there is a surge in LH levels. This causes completion of the first meiotic division of the oocyte. About 30 hours after the beginning of the LH surge, ovulation occurs. There is also a spike in FSH causing cumulus expansion just before ovulation. After ovulation the corpus luteum is formed – this is stimulated by FSH and LH. During the luteal phase of the cycle the main hormone that is produced by the corpus luteum is progesterone. Progesterone plays a key role in making the

endometrium receptive to the implantation of the blastocyst. It also causes a rise in basal body temperature.

Oestrogen is also produced by the corpus luteum. There is usually a "secondary oestrogen surge" a few days after ovulation, this can lower basal body temperature and create fertile cervical mucus.

The corpus luteal hormones suppress production of LH and FSH. The fall of these hormones causes the death of the corpus luteum. With the death of the corpus luteum, levels of progesterone and oestrogen fall. The subsequent lack of suppression of the pituitary hormones (LH and FSH) causes a rise in FSH levels stimulating follicular growth – the next cycle begins.

In the event of fertilisation and implantation – the embryo produces human chorionic gonadotropin (hCG). This hormone is structurally similar to LH. With that, hCG can maintain the corpus luteum and prevent its breakdown. The corpus luteum will then continue to produce progesterone for up to 12 weeks, until the placenta functionally takes over.

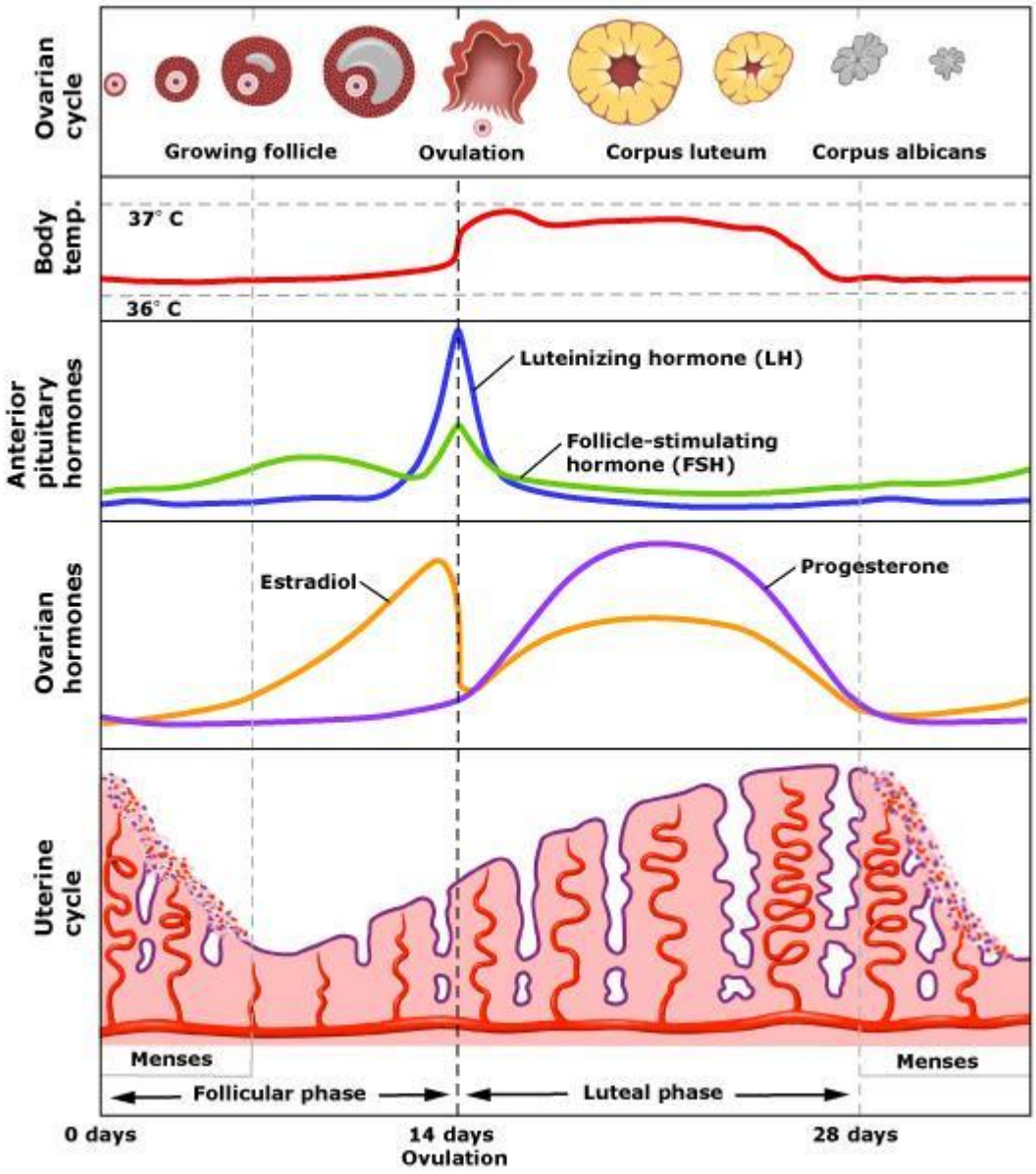
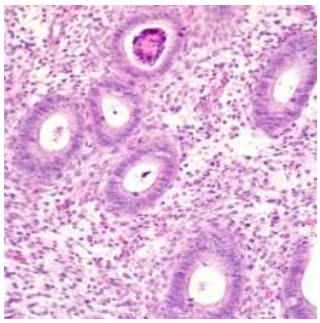


Figure 1-4 - The Human Menstrual Cycle[22]

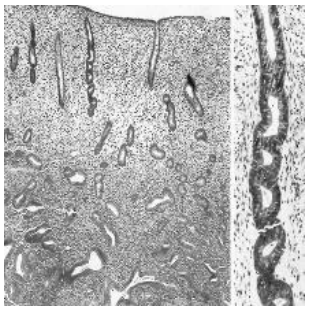
1.1.5.2 Histological Change throughout the Menstrual Cycle

It is known that the endometrium undergoes typical histological changes throughout the menstrual cycle. In the early proliferative phase, endometrial glands are small and straight. During the late proliferative phase; the glands of the endometrium are described as adopting a "tortuous" appearance owing to the fact that they are undergoing considerable proliferation. The functional endometrial layer is typically thin, glands are short and stroma is tight and compact. Towards

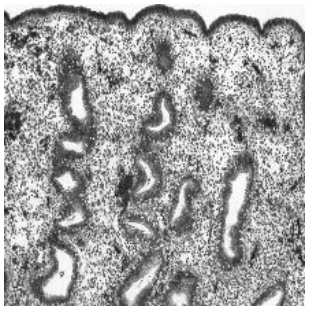
the end of the proliferative phase the glands become longer with pseudostratification and the functionalis layer increases in size.



Around the ovulatory phase it has been calculated that 50% of glands contain subnuclear vacuoles.



During the secretory phase the functionalis layer ceases to proliferate and begins its secretory activity. This layer is thick and full of long coiled glands. There are greater than 50% subnuclear vacuoles seen during this phase and arterioles thicken. As the menstrual phase approaches, shedding of the functional layer occurs. Micrographic images show glandular and stromal breakdown with necrosis and haemorrhage.



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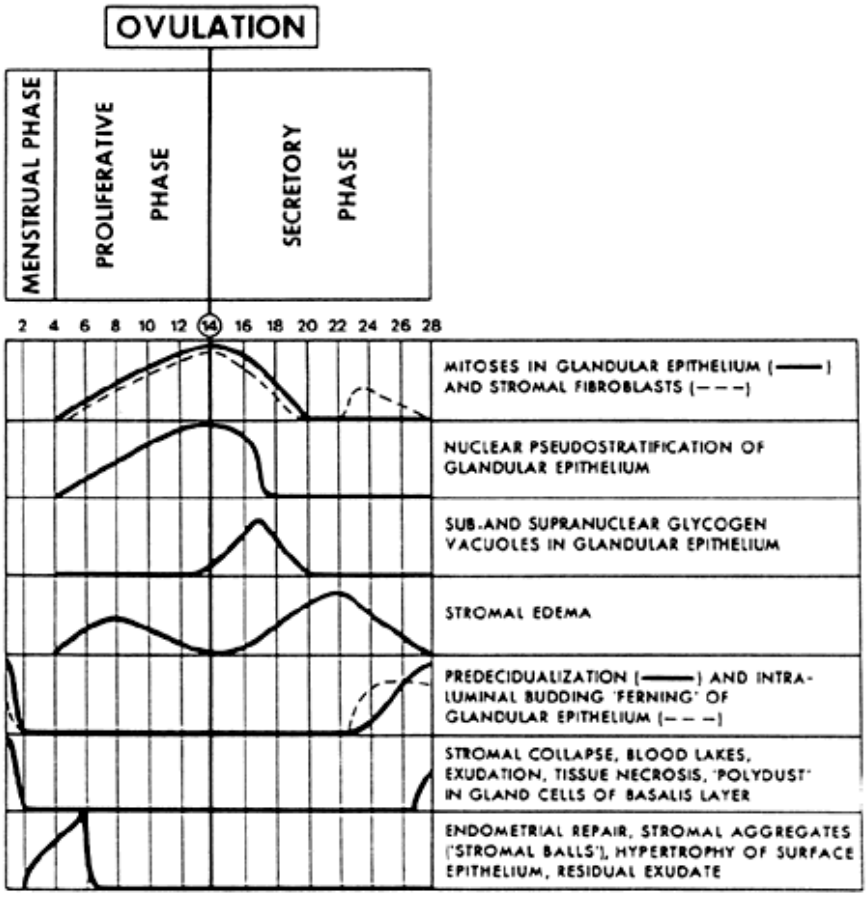


Figure 1-5 - Structural changes throughout the menstrual cycle[24]

1.1.6 The Human Menopause

In women approximately aged 51 years, their menstrual periods typically become irregular and eventually cease. This is due to ovarian shutdown. This phase of a female's life is commonly called the menopause. Ovulation stops and mature oocytes are no longer released from developing follicles. Concentrations of ovarian steroid hormones fall causing a rise in pituitary hormones, LH and FSH due to the loss of negative feedback. Post-menopause, androgens continue to be produced by the adrenal gland at low levels. Oestrogens are subsequently synthesised by the aromatisation of androgens by adipocytes.[25] The histological pattern of the normal postmenopausal endometrium has been described as atrophic. This indicates that there is a thin endometrium with scarce glands and an inactive phenotype. The blood supply is low, with capillaries bearing no resemblance to spiral arteries. It is also very common for postmenopausal females to have endometrium that appears

to have a cystic glandular appearance. The glands are dilated and the epithelial cells are flattened with loose surrounding stroma. In a study by McBride J, Histological examination of 206 postmenopausal endometrial specimens showed that the largest proportion of females had an inactive cystic glandular pattern with a single polyp; closely followed by atrophic-type endometrium.[26] In a study on post-menopausal fallopian tubes transmission and scanning electron microscopy evaluation described fallopian tube epithelial cell structure and change over the peri-menopausal and post-menopausal period. They reported fused microvilli, microplicae-like structures developing over time and low mitotic activity of cells. They also concluded that the epithelium becomes more non-secretory in nature.[27]

1.2 The Reproductive Steroid Receptors

The steroid hormone receptors are transcription factors affecting cellular proliferation and differentiation in specific tissues.[28] Steroid receptors have been studied in great detail in human endometrial tissue but there is only a small amount of evidence about the presence of these in human fallopian tubes; the functional relevance of the presence of these receptors remains unclear.

1.2.1 Androgen Receptor

The androgen receptor (or AR) mediates the physiological effects of androgens. The AR gene is located on chromosome Xq 11-12. By binding to certain DNA sequences; it activates transcription of androgen-responsive genes. The androgen hormone usually binds in the cytoplasm and translocates to the nucleus. It has been said to be most closely related to the progesterone receptor.[29]

1.2.1.1 Androgen Receptor in the Endometrium

Expression of AR is present in normal human endometrium. Although there has been detection of this receptor there is conflicting evidence. Some studies suggest that there is cyclical changes throughout showing a particularly high stromal expression during the proliferative phase. Other studies suggest that androgen receptor expression remains relatively constant throughout.[30] Various studies have reported that the androgen receptor is localised predominantly to the stromal cells of the endometrium (barely detectable in the glands), with an abundance of receptor expression during the proliferative, early and mid-secretory phases.[31] After the menopause, AR persists in the stromal nuclei.[32] In females total and free testosterone and androstenedione have a mid-cycle peak and are relatively higher during the luteal phase.[33] The high AR expression in the secretory phase reflects the circulating levels of androgens suggesting that the androgen receptor is up-regulated in the presence of free androgens.

1.2.1.2 Androgen Receptor in the Fallopian Tube

There is very little evidence on the expression of this receptor in human fallopian tubes, an article in 2010 mentions the expression of this receptor in benign and

malignant fallopian tube samples and states that it is mostly nuclear. Normal fallopian tube samples were analysed to compare to a population with epithelial ovarian carcinoma (EOC). The authors claimed that a reduction in AR expression was observed in EOC cases. [34] This however, was not compared to endometrium. Cyclical changes in expression in premenopausal women were not evaluated and neither was postmenopausal expression of AR.

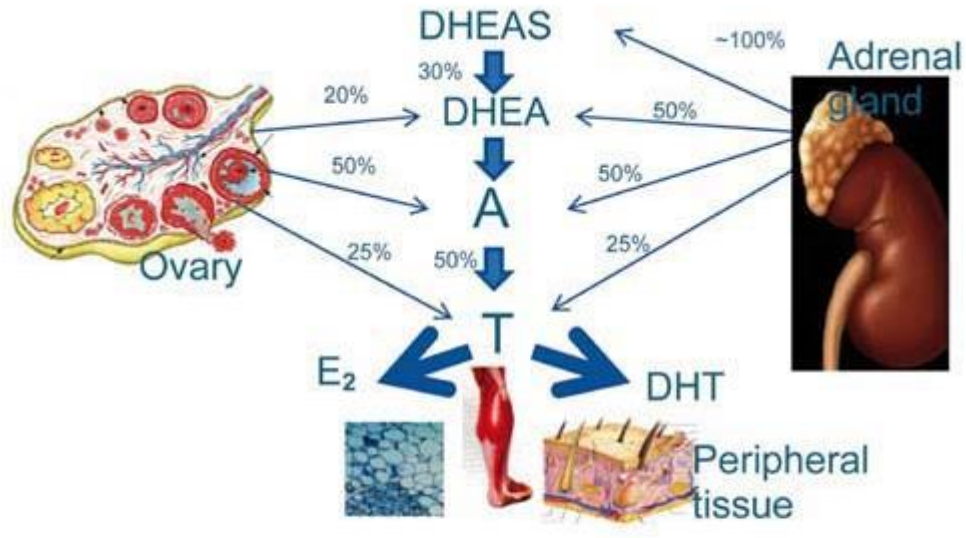


Figure 1-6 - Steroidogenesis in the ovaries, adrenal glands and peripheral tissue related to female sexual function.[35]

1.2.2 Progesterone Receptor

The physiological effects of progesterone are mediated through its interaction with the progesterone receptor. The progesterone receptor has two isoforms progesterone receptor-A (PR-A) and progesterone receptor-B (PR-B). They are both derived from the same gene. The genes controlled by PR have a role in cell cycle control. They moderate transcription of genes that regulate the cell cycle.[36] Since their discovery, extensive controversy exists around the contribution of each of the receptors to the physiological function of progesterone.[37] Progesterone receptors are known to be present in the endometrium. Evidence exists that shows that there are indirect (paracrine) and direct (endocrine) routes of action of progesterone on the endometrium.[38] Progesterone plays a vital role in the human endometrium. The endometrium is most receptive to embryo implantation around days 20-24 – this is normally the time when serum levels of progesterone are at a peak. If the corpus luteum does not detect chorionic gonadotrophin (CG) from an embryo after fertilisation; programmed cell death occurs of the corpus luteum and eventually the endometrium, leading to menstruation. It is also known that the removal of the corpus luteum less than 5 weeks after implantation causes loss of pregnancy due to early decline of progesterone levels.[39]

1.2.2.1 Progesterone Receptor in the endometrium

A review in 2003 by Lessey, looks at the two pathways of progesterone in human endometrium. The author comments on the evidence that progesterone receptors vary throughout the menstrual cycle. The receptor can be found in the glandular epithelia and stromal compartments of the endometrium. Epithelial expression tends to be higher in the proliferative phase and reduced around the mid-secretory phase – which is usually the optimum time of implantation. Stromal expression remains constant throughout however.[40] The two different pathways involve a direct epithelial endocrine pathway or an indirect paracrine pathway involving the stromal compartment inducing specific gene products. This evidence comes from a study that suggests that heparin-binding EGF could be one of the paracrine factors induced by progesterone in stroma; other proteins in the

endometrium rely on HB-EGF for their stimulation, like the beta 3 integrin subunit. They concluded that progesterone has two functions on the endometrium; one of stimulating stromal paracrine factors and the other of inhibiting oestrogen and progesterone receptors.[41]

1.2.2.2 Progesterone Receptor in the Fallopian Tube

More recently, evidence has shown the presence of this receptor in human fallopian tube. In a study by Shao et al., using immunofluorescence imaging, they revealed the presence of both PR A and B in the nuclei of fallopian tubal epithelia. The authors also discovered that both receptors were low in the mid-secretory phase of the menstrual cycle consistent with studies of the endometrium.[42] Another study by Horne et al. also described the presence of both PR A and B in the nuclei of the fallopian tube cells and showed reduced mRNA expression of PR A and B in the secretory phase – consistent with the previous study. In addition, they found that in vitro treatment with progesterone caused down regulation of both receptors.[43]

1.2.3 Oestrogen Receptor

Oestrogen controls many cellular processes in the female reproductive system. Its main targets are ovaries, uterus, vagina and mammary glands. It is also responsible for the growth and maintenance of the skeleton. Most of oestrogen's actions are carried out through binding to the oestrogen receptors. These receptors are known to be ligand-binding transcription factors. To date, two oestrogen receptors are known; oestrogen receptor alpha and beta:

1.2.3.1 Oestrogen Receptor Alpha

This receptor is a nuclear hormone receptor. Oestradiol (E2) and its receptors are involved in gene expression which have an effect on proliferation and differentiation in tissues. Nuclear transactivation is ligand- dependent with either direct homodimer bonding to a palindromic oestrogen response element (ERE) sequence or interactions with other DNA-binding transcription factors.[44] The ESR1 gene encodes for this protein.

1.2.3.2 Oestrogen Receptor Beta

This receptor has a similar affinity to oestrogen receptor alpha and can form a heterodimer with oestrogen receptor alpha. This receptor is similar in the DNA and ligand binding domains however differs in the N-terminal transactivation domain. The ESR2 gene encodes for this receptor. Isoforms Beta-1 and 2 are expressed in the ovary and at a lower level in the uterus. Beta-3 is not present in the female reproductive tract. Beta-4 is expressed at a low level in the ovary and uterus and Beta-5 has low expression in the uterus.[45]

1.2.3.3 Oestrogen Receptors in the endometrium

After the discovery of oestrogen receptor beta (ER Beta) studies looked into the presence of this and the ER alpha in human endometrium and any cyclical changes in its expression to indicate its functional relevance. A study in 1999 by Matsuzaki et al. looked at mRNA expression particularly. They discovered that there was mRNA expression in all uterine cell types including the glandular epithelial cells and stromal cells of the endometrium. They found that ER alpha was expressed more prominently throughout the menstrual cycle than ER beta. They also found that ER beta protein expression is predominantly glandular in the proliferative phase whereas ER alpha is expressed in both glands and stroma. They also noted that both receptors are weaker during the secretory phase. The conclusions drawn from the findings suggest that oestrogenic effects occur mostly through ER alpha; however ER beta will play a role in this particularly in glandular epithelial cells throughout the menstrual cycle.[46] Another study by Brandenburger et al. found that these receptors upregulate the progesterone receptor. The oestrogen receptors are also present in endometrioma tissue.[47] A study in 2000 by Taylor et al. attempted to immunolocalise ER beta in many human tissues. The authors reported the presence of ER alpha and beta in endometrial tissue and fallopian tubal epithelia. Interestingly they reported a very low if not negligible expression of ER beta in the nuclei of glandular epithelia but presence within the cytoplasm of some of these cells. Stromal and luminal nuclei were positive for ER beta. This lack of ER Beta in the glandular epithelia could have been as a result of the samples

being in late secretory phase of the menstrual cycle, which would then support the previous evidence. ER Beta expression in the fallopian tube seemed to be particularly strong in the nuclei of epithelial cells[48] Moderate oestrogen receptor expression was observed in an immunohistochemical study in 1992 in the glandular epithelia and stroma of postmenopausal women. This study did not distinguish between ER-alpha and beta, however.[49]

1.2.3.4 Oestrogen Receptors in the Fallopian Tube

The presence of ER alpha and beta in the fallopian tube has been confirmed by Shao et al. Both receptors are most abundant during the secretory phase of the menstrual cycle, according to this study. ER alpha was found in the lamina propria whereas beta was not.[42] Horne et al. Found that ER alpha was down regulated in vitro with treatment of progesterone. This conflicts with evidence from the former study because the author claims that the ER are most abundant during the secretory phase when progesterone is higher.[43] More research is necessary to confirm the ER status throughout the menstrual cycle. Another study by Shao et al. found that ER beta was mainly found in the ciliated cells of the fallopian tube in rodents – confirmation in humans would need human tissue samples to be tested.[50]

1.3 Stem Cells

Stem cells are unspecialised cells that are capable of self-renewal even after long periods of quiescence. There are two umbrella terms for stem cells: embryonic stem cells and somatic (adult) stem cells. Embryonic stem cells are derived from the embryo from a fertilised ovum. A somatic stem cell is an undifferentiated cell found amongst differentiated cells in an organ. Somatic stem cells have the ability to divide (by mitosis) and differentiate into mature cell types that have specialised functions in tissues and organs. They provide a repair mechanism for the body and replenish tissues.[51] The differentiation capacity of a stem cell is called its potency:

Totipotency – a stem cell that can differentiate into embryonic and extra-embryonic cell types. These have the ability to create an organism. These cells are created after the fusion of a sperm and an egg up until the first few divisions.[52]

Pluripotency- these stem cells have the ability to divide into almost all cells i.e. these are derived from the three germ layers.

Multipotency – stem cells that can differentiate into a closely related cell types.

Oligopotency – can differentiate into a few cell types.

Unipotency – can produce their own one cell type and self-renew.[53]

1.3.1 Identifying the side population cells and Fluorescence Activated Cell Sorting (FACS)

As described previously, it has been suggested that progenitor cells can be identified in the basalis layer of human endometrium and distal fallopian tube but a specific marker for identifying these is yet to be found. One way of identifying potential progenitor cells from differentiated somatic cells (i.e. the undifferentiated proportion of cells) in a tissue is to perform side population analysis.[54] The side population is a proportion of cells that that show different characteristics to the main population for example; stem-like properties. They are identified by particular markers that demonstrate their phenotype.

Hoechst side population analysis is a flow cytometry technique to identify undifferentiated cells in a variety of tissues. After the tissue is digested, cells are

sorted and suspended in a single cell suspension; Hoechst 33342 DNA dye is added to this to be taken up by live cells. ATP-Binding Cassette (ABC) transporters are present in stem and early progenitor cells and they are able to pump this DNA dye out of the cell. This is unlike differentiated cells which incorporate the dye into their DNA. This can be analysed using a flow cytometer with a UV laser. A minor cell population is observed with low fluorescence (the side population) due to the ABCG2 transporters pumping the dye out of the cell and a large proportion of differentiated cells retain the dye, resulting in a bright fluorescent signal. Confirmation of identification of the side population can be achieved by incubation of an inhibitor of the ABC pump (e.g. verapamil). After Hoechst is introduced, the side population phenotype is lost due to pump inhibition and this confirms the presence of these cells – this can be used as the control for this technique.[55]

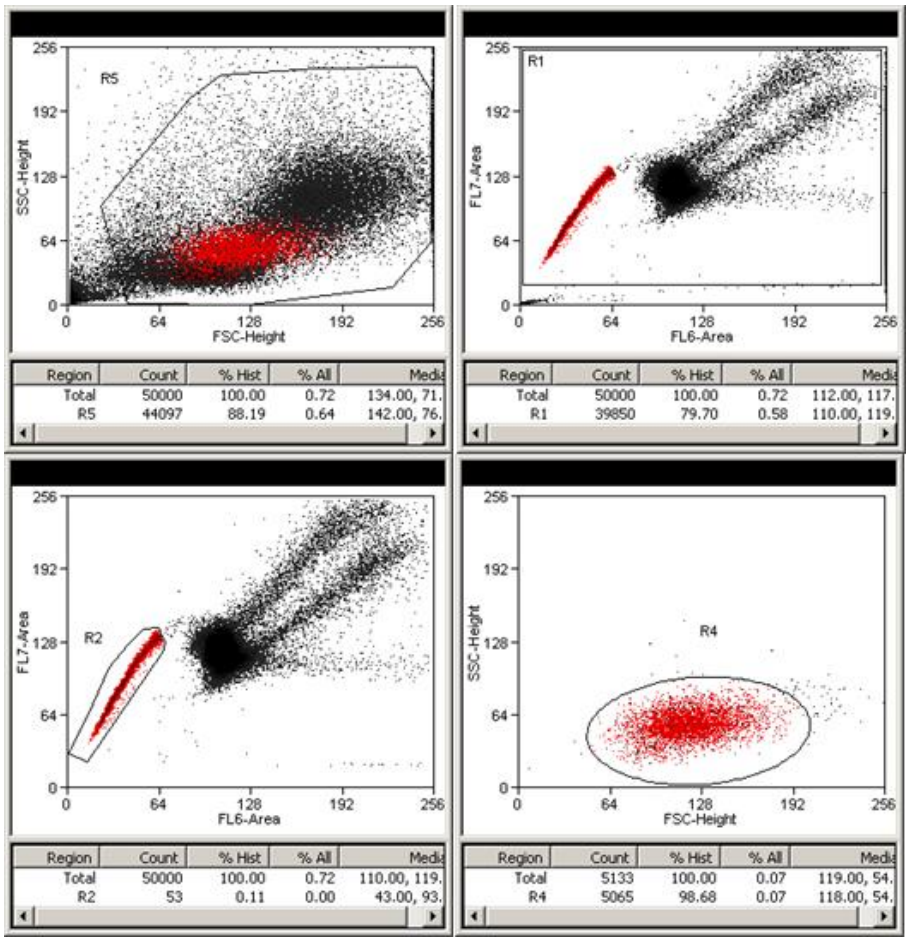


Figure 1-7 - Side Population Analysis and Sorting[55]

Once the side population is identified immunocytochemistry can be performed to analyse the phenotype of these cells in comparison to the non-side population cells.

1.4 The Fallopian Tube and The Endometrium

1.4.1 Stem-like Cells in the distal reproductive tract

It is known that the embryological origin of the fallopian tube and the endometrium is the same.[1] Like the basalis endometrium, recent evidence in the last few years has revealed the presence of stem-like cells in the distal fallopian tube, with pluripotent stem cell properties.

Paik et al. In 2012 revealed that, fallopian tube stem cells are more concentrated at the distal end of the fallopian tube and after histological observation after Fluorescence Activated Cell sorting (FACS); the authors identified that the so-called peg cells in the tube are basally located and undifferentiated. These cells were positive for CD44, a known marker for stem cells with migratory properties.[4]

Up until very recently little has been known about the existence of this cell-type in the reproductive system of females and it has been speculated that stem cells could be implicated in many pathological, gynaecological conditions such as endometriosis and malignancies. In 2012 a study performed by Wang et al. searched for quiescent stem-like cells in the female reproductive system using a mouse model.[56] They did this by performing an in vivo pulse-chase experiment using histone 2B-green fluorescent protein (2B-GFP). This was to try to identify long-term label retaining cells (LT-LRCs) which would suggest that these were quiescent stem-like cells. Using transgenic 2B-GFP mice they administered doxycycline in the drinking water of the mice, to express the 2B-GFP protein and then withdrew this after a specified amount of time. Actively cycling cells would reduce the expression of GFP over time, whereas quiescent cells would retain the GFP for longer. The expression was monitored for up to 47 weeks after the pulse (doxycycline). Methods used for analysis in the study were immunohistochemistry, flow cytometry for sorting of cells, *in vitro* cell culture with a self-renewal assay and differentiation assay of the sorted cells.

Using IHC techniques and immunofluorescence imaging; uterine cells were found to express H2B-GFP labelling particularly in the epithelial compartment. Proximal and distal oviductal cells also had positive labelling. During the chase period it was

found that uterine cells and proximal oviductal cells lost the GFP signal early on (<12 weeks). An interesting finding was that cells in the distal oviduct retained their GFP signal for at least 12 weeks and some cells were labelled after 47 weeks. Cells were termed long-term label retaining cells if they persisted for more than 12 weeks after labelling. LT-LRCs were isolated from the distal oviduct ex-vivo and were found to be able to form spheroids at a higher rate than GFP negative cells. Specific analysis of individual LT-LRCs showed initial formation of an unstructured organoid and subsequent growth of an epithelial-like outer layer. If the spheroids were exposed to serum morphological changes were observed. The spheroids were observed to spread out and budding of hollow tube-like structures occurred. IHC staining of the spheroids to determine any differentiation into specific cell lineages showed that, if allowed to differentiate, the spheroids stained positive for ER alpha and CD44 – similar to the distal oviductal cells. If allowed to differentiate even further the spheroids stained positive for ER alpha, CD44, PR and PAEP; an expression pattern similar to cells of the proximal oviduct and endometrium. A conclusion was made that upon differentiation, spheroids derived from LT-LRCs express markers that are characteristic of cells found in the proximal oviduct and endometrium.

The author thus hypothesised that quiescent stem-like LT-LRCs in the distal oviduct lead to the production of progenitor cells for the epithelial layer of the proximal oviduct and endometrium. The hypothesis suggests that there is migration of cells from the distal oviduct towards the endometrium. The author speculated that the only way to confirm this would be lineage tracing studies, however. This hypothesis creates a link between distal oviduct endometrial epithelium. Studies on the expression of other markers typical of endometrial epithelia need to be tested on fallopian tubal epithelia to confirm this link.

Identification of LT-LRCs in the distal oviduct could open up suggestions into the pathogenesis of ovarian cancer, due to recent evidence that serous ovarian carcinoma originates in the fallopian tube. The author also hypothesises that tissue injury in the distal oviduct could activate otherwise quiescent cells and cause compartmental expansion and differentiation.

In 2013 a study performed by Indumathi et al. compared the biomarkers of stem cells in the endometrium and fallopian tubes to bone marrow due to their special remodelling and regenerative properties. The authors noticed however biomarkers to identify these stem cells were poorly understood. Isolated cells from the endometrium and fallopian tubes were characterised for a wide range of biomarkers by flowcytometry and many of these were positive, like bone marrow. The stem cells were collectively positive for markers of pluripotency (SSEA4 and OCT4), cell adhesion molecules (CD 13, 29, 44, 54 and 166) perivascular (CD140b and 146) and mesenchymal stem cells (CD 73, 90 and 105). The positivity of these particular markers indicated that these stem cells were involved in tissue regeneration and repair.[1]

1.4.2 Stem cell presence in the endometrium

Unlike tube, there is much evidence around suggesting there are progenitor cells in the endometrium. Back in 2007, a study by Kato et al. Was the first to Identify side-population cells in human endometrium. They studied the phenotype of these cells and found that they could repopulate and produce gland and stroma-like cells.[3] This supports the theory that progenitor cells in the endometrium have the capacity to differentiate and regenerate as described in the study above. A study then came out in 2008 by Tsuji et al. Describing this side-population of cells but followed on by revealing that the side-population cells expressed endothelial cell markers, epithelial cell markers and mesenchymal cell markers. The researchers also confirmed that these cells were side-population cells by staining for ABCG2 in the endometrium. Interestingly, they found that these cells were strongly stained in the vascular endothelial and basal layer compartments.[57] With the knowledge that the basalis layer regenerates the functionalis layer every month the accumulation of these cells in the basalis suggests that they could be the reason for this regeneration. Supporting this, a study in 2010 revealed that the side population cells were enriched with stem cells that cause physiological regeneration of the endometrium every month and also claimed that they could be implicated in the pathogenesis of endometriosis due to their capability to migrate and cause angiogenesis in a mouse kidney model.[58]

In 2012, Nguyen et al. studied the Wnt pathway in pre and postmenopausal endometrial cells. The Wnt pathway has been associated with endometrial development and stem cell regulation. With expression of Wnt associated genes in premenopausal and postmenopausal endometrium and a similar gene profile of premenopausal basalis endometrium and postmenopausal endometrium suggests that progenitor cells reside mainly in the basalis layer. These findings show that under the correct hormonal signal (i.e. premenopausal state) these cells could be the source of monthly regeneration for the functionalis layer.[59] A recent study in 2013 suggested that mesenchymal cells play an important role in endometrial regeneration through mesenchymal to epithelial transition. This was due to transitional cells being found expressing epithelial cell and stromal cell markers predominantly in the regeneration zone; the mesometrial part of the endometrium. This finding could also implicate the proliferative diseases of the endometrium.[60]

1.4.3 Endometrial Ablation and Regrowth of the Endometrium

Women with the common problem of menorrhagia (loss of > 80 ml of blood per menstrual cycle) undergo the procedure of endometrial ablation to alleviate this issue. Endometrial ablation is the process of destroying the whole endometrial layer of the uterine cavity leaving behind myometrium only. Since the early 90's global endometrial ablation devices (GEA's) have been introduced, which eliminate the endometrium in all areas of the uterine cavity.[61] With the basalis layer of the endometrium destroyed, the monthly regeneration and shedding of the functionalis endometrium cannot occur, thus supporting the recent evidence that any regenerative cells in the basalis would also theoretically be eliminated.

Although endometrial ablation has a high success rate short term, it has frequently been reported all over the world that after five years the success rates drop dramatically with at least one in four women undergoing hysterectomy. A prospective randomised control trial by Silva-Filho et al. compared women who received a levonorgestrel-releasing intrauterine system (LR-IUS) with women who underwent thermal balloon ablation (TBA) five years post-insertion/operation.

Hysterectomy rates and menstrual blood loss were significantly higher in patients undergoing TBA compared with the LR-IUS revealing that the IUS had higher overall satisfaction rates.[62] This leads on to the question – where is the endometrium coming from to cause this blood loss? Re-growth from the cornua of the uterus has previously been described; however with the newer devices today, this should reduce if not stop this risk. Failure rates have still remained high. A theory could be that the stem cells in the distal fallopian tube migrate towards the uterus and differentiate into endometrial-like cells, eventually repopulating the endometrial cavity. A photo taken of a patient's endometrial cavity with symptoms of menstrual blood loss following ablation shows this re-growth coming from the fundus bilaterally near the tubal junctions (see below). This theory is supported by the evidence from the study of the label retaining cells in the mouse model described in section 1.4.1.

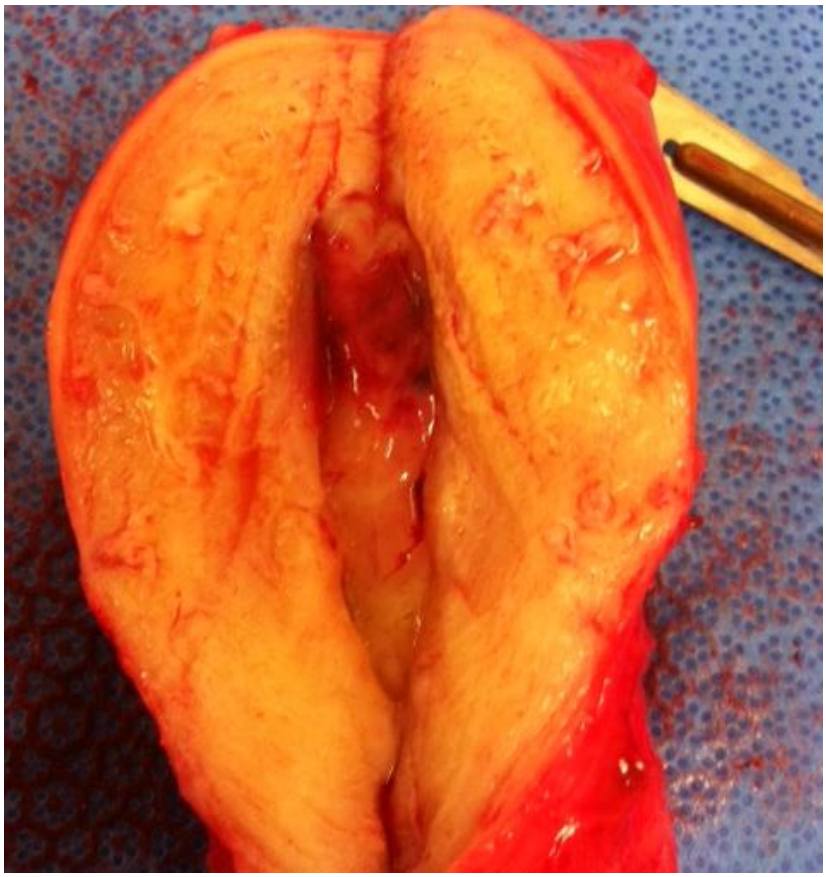


Figure 1-8 – Photo of uterine cavity. Patient had hysterectomy due to ablation failure. Fundal area of cavity lined with endometrium (red) and inferior portion atrophic (yellow).

1.4.4 Characterisation of the Basalis Endometrium

As mentioned in part 1.4.2 it is thought that endometrial progenitor cells reside in the basalis compartment of the endometrium. Even though side population cells have been located – it seems that markers for these cells are yet to be identified. Schwab in 2004 investigated the side population in stromal tissue of the endometrium. They identified that CD90 differentiates basalis stroma from functionalis stroma, however potential stem cell markers were unidentifiable.[63] Before understanding the stem cell component of the endometrium, it is important to characterise the compartments of endometrium to be able to understand the phenotypic differences between the glandular and stromal basalis and glandular, stromal and luminal functionalis.

Recently, epithelial cell markers have been discovered distinguishing the basalis from the functionalis layer of the endometrium. In 2013 it was hypothesised that the epithelial cell markers SOX9 and nuclear beta catenin may distinguish the basalis layer of endometrium from the functionalis with the rationale that these markers are found in abundance in the regenerative intestinal crypts where the progenitor cells reside. Authors found that the basalis endometrium expressed SOX9 and a subpopulation of cells were positive for nuclear beta catenin. They also found that the embryonic stem cell-surface marker SSEA-1 was positive in the basalis region more abundantly than the functionalis. Isolated SSEA-1^{+} cells showed higher telomerase activity and gland-like spheroid growth. With these findings and markers distinguishing the basalis from the functionalis, further studies into the analysis of stem cell activity can be made.[64] With this in mind, if the basalis endometrium exhibits particular phenotypic features and the tubal epithelium is hypothesised to contribute to this mucosa – are there phenotypic similarities between fallopian tubal epithelium and endometrial epithelium?

1.5 Markers to Characterise and Compare The Fallopian Tube and Endometrium

1.5.1 Basalis Markers

1.5.1.1 BCAM

Basal cell adhesion molecule has a wide tissue distribution being highest in intensity in the pancreas and lowest in the brain. It is a marker for the basal surface of epithelial cells and also for the endothelium of blood vessel walls. Its gene encodes for Lutheran blood group antigen which is known to be a member of the immunoglobulin superfamily. BCAM is a receptor for laminin.[65] Laminins are an integral component of all basement membranes, [66] and have many cellular functions including roles in: adhesion, motility, growth, differentiation and apoptosis.[67] BCAM has been found to be present in some epithelial cells in human tissue. Positive expression of this molecule has been found in hepatic epithelial cells, haematopoietic cells, chorionic villi of the placenta and the glomeruli, arterial cells and tubules of the kidney.[68] BCAM has also been found on hair follicles and in areas of inflammatory epidermis.[69] Presence of BCAM is found solely in erythrocytes of the blood.[70] BCAM has also shown up-regulation in some epithelial cell cancers.[71] On immunostaining; BCAM is localised to the basal surfaces of epithelial cells of tissues. Strong cytoplasmic and membranous staining has been observed in certain tissues of which fallopian tube was one – no studies have been published fully describing BCAM expression in the human fallopian tube and its functional relevance remains unclear in this tissue.[72]

1.5.1.2 SOX9

SOX9 or SRY (sex determining region Y)-Box 9 is a transcription factor. Its gene has been located in 17q24. The protein recognises the sequence CCTTGAG and various other HMG-box class DNA binding proteins. [73] SOX9 insufficiency has been associated with campomelic dyplasia, skeletal malformations and XY sex reversal (Bi W et al). With steroidogenic factor-1, SOX9 regulates/stimulates levels of anti Mullerian hormone (AMH) expression[74] thus playing an essential role in male sexual development. Previous studies have shown rich SOX9 presence in basal epithelial

cells. The transcription factor SOX9 has been located in basal epithelia of the prostate, the intestinal crypts and the outer root sheath of hair follicles. Progenitor cells are all thought to reside in these particular regions.[75] As mentioned in section 1.4.4 SOX9 has been strongly detected in the basal glandular epithelia of the endometrium. This is also a region in which progenitor cells are thought to reside.[64] This has yet to be investigated in tubal epithelia and could give an indication as to whether these cells exhibit a basal epithelial cell phenotype.

1.5.1.3 SSEA-1

Stage specific embryonic antigen-1, also known as Lewis X and CD 15 - has a significant role in the adhesion and migration of the preimplantation embryo in murine pluripotent stem cells.[76] It is synthesised by fucosyltransferase 4 (FUT4) and also by FUT9. It is a cell-surface marker that has previously been found to be expressed in the human basalis endometrium and in ectopic endometrial lesions. Studies in our lab discovered that SSEA-1 marked the glandular epithelia in the stratum basalis of the endometrium. The study used magnetic activated cell sorting to isolate SSEA-1$^+$ cells and these were grown in 3D culture. The SSEA-1$^+$ cells were found to produce a higher number of endometrial gland-like spheroids in comparison to the untreated group.[2] Immunohistochemical staining for SSEA-1 in adult murine oviducts shows positivity, however human fallopian tube expression of SSEA-1 is yet to be studied.[77]

1.5.1.4 Beta Catenin

Beta catenin protein or cadherin-associated protein with gene name CTNNB1 has many roles. Some of the key functions of this protein are; cell adhesion with bridges formed between cadherins in the cell junction and also in the cytoskeleton with actin. It is also believed that beta catenin plays a role in gene expression in the canonical Wnt signalling pathway.[78] In the absence of Wnt, ubiquitination of beta catenin occurs - signalling its degradation via the proteasome. In the presence of Wnt however, beta catenin accumulates in the nucleus where it then acts as a co-activator for transcription factors belonging to the TCF/LEF family, leading to the subsequent activation of Wnt-responsive target genes.[79] Studies have shown that

the Wnts activate intracellular cascades that regulate cellular proliferation, differentiation, migration, and polarity.[80]

1.5.2 Luminal Surface Marker

1.5.2.1 Sialylated SSEA-1

The sialylated SSEA-1 or sialylated Lewis X antigen is a tetrasaccharide carbohydrate molecule on the surface of cells. It attaches to the endothelial adhesion molecule E-selectin. This antigen plays a significant role in inflammatory response, being that it is expressed on activated leucocytes and causes attraction of these to local sites of inflammation or injury. It is also involved in cell to cell recognition and recent evidence has shown that it plays a vital role in the fertilisation process. It has been shown to be the means by which the oocyte attracts the sperm; with subsequent binding and fertilisation.[81]

Sialylated SSEA-1 has been identified on human endometrial luminal epithelial cells. It is associated with markers that are known to promote adhesion for implantation of an embryo.[82] A study claimed that sialylated SSEA-1 is a specific tumour marker and reported a high percentage of antigen positivity in patients with ovarian cancer (72.3%) and endometriosis (75 %). There was also low positive expression in other gynaecological malignancies such as endometrial cancer and cancer of fallopian tubes. This study suggested that sialylated SSEA-1 is a specific tumour marker for gynaecological malignancy, particularly ovarian cancer.[83]

1.5.3 Marker of Proliferation

1.5.3.1 KI67

Expression of KI67 protein in cells indicates that they are proliferating. KI67 is therefore considered a marker of proliferation in a cell. The antigen has been detected in the nucleus of cells and has been proven to disappear during the resting phase (G0) period of the cell cycle. KI67 has also been studied in diagnosis and prediction of prognosis in tumour cells.[84] Studies measuring the so-called "KI67 Index" in breast cancer have found a significant increase in index score in

higher grade malignancies and lower overall survival rates.[85] KI67 protein expression has been detected in endometrial glandular epithelia and has been shown to significantly increase in the proliferative phase of the menstrual cycle and diminish dramatically during the secretory phase, when the cells are in a more quiescent state.[86]

Chapter 2 Aims and Hypotheses

2.1 Primary Hypothesis

Markers preferentially found in the endometrial basalis layer are also found in the distal tubal epithelium. This region of the fallopian tube may be phenotypically similar to the basalis layer of the endometrium.

Rationale: As described in the introduction the tubal mucosa exists as a continuum with endometrial mucosa, although endometrial and tubal epithelium have separately been characterised. There is yet to be a comparison of tubal and endometrial mucosa. Recent novel findings suggest a pooling of progenitor cells exist in the distal fallopian tube. Side-population analysis has revealed that the basalis endometrium contains the majority of cells phenotypically suggestive of progenitor cells in this region. It is commonly thought that these undifferentiated cells have the capacity to differentiate under particular conditions and are the source of the basalis layer's monthly regeneration of the functionalis layer. Latest characterisation of the basalis layer of the endometrium has revealed the presence of particular markers that are usually found in abundance in stem cell-rich areas. Comparison of the tubes with the endometrium by testing for the presence of the markers previously found in the basalis compartment is necessary to directly compare the phenotype of the tubal epithelium with the basal glandular epithelium.

2.2 Secondary Hypotheses

2.2.1 The endometrium undergoes cyclical changes every month by shedding and regenerating the functionalis layer under the influence of steroids. The fallopian tube has a distinct hormonal response and cyclical pattern of receptor expression reflects this.

Rationale: This hypothesis aims to investigate whether the tube undergoes the monthly changes that the endometrium experiences. This is to be determined by studying the changes in expression of steroid receptors throughout the menstrual

cycle in the tube and comparing this with matched endometrium. Changes in proliferation will also be determined throughout the menstrual cycle to consider whether the fallopian tubal mucosa undergoes phenotypic changes similar to the character of the endometrium which sheds and regenerates each month.

2.2.2 Fallopian tubal epithelia respond to androgens in a unique way.

Rationale: Androgens have been shown to up-regulate their receptors in the endometrial glandular epithelium and receptors are strongly expressed in the endometrial stroma but no such investigation has been carried out in the fallopian tubes. Both tissues have shown changes in receptor expression under the influence of oestrogen and progesterone treatment in explant culture. There is no evidence to date, looking at the effects of treatment of fallopian tubal mucosa with androgens in explant culture. Investigating the influence of this steroid on the androgen receptor, its cellular location and markers of proliferation could give an insight into the function of this steroid in the fallopian tubes.

2.2.3 The endometrial and fallopian tubal mucosa are in an atrophic state in postmenopausal females.

Rationale: The postmenopausal state of the endometrium is when the cells are of a basalis phenotype with a lack of the monthly changing functional layer. Concentrations of progenitor cells are found in this mucosal layer. Is the tubal epithelium expressing/ not expressing markers that suggest that it is in a quiescent state similar to that found in the endometrium? After the menopause – most pathological change occurs in the female reproductive tract including diseases such as endometrial and ovarian cancer. If expression of markers in the fallopian tube and endometrium are similar; this would suggest a continuum between the two tissues. Recent theories suggest that there may be migration and differentiation of cells from the tube towards the uterus. With this and the suggestion that the tissues are in a continuum – it could be quite possible that the fallopian tubal cells play a part in the development of diseases throughout the reproductive tract. In particular, the diseases that involve change in vulnerable cells for example; progenitor cells undergoing neoplastic formation. Not only would this support the evidence

suggesting that high grade serous ovarian carcinoma begins in the distal fallopian tube, but also tubal cells could be implicated in endometrial cancer and benign, migratory diseases such as endometriosis.

Chapter 3 Materials and Methods

3.1 Ethics

Ethical approval for the collection of fresh human endometrial and fallopian tube samples was obtained by the Local Research Ethics Committee (Collection of human endometrium was approved by Liverpool Adult Ethics committee (REC references; 09/H1005/55 and 11/H1005/4). Full written and informed consent was taken prior to the collection of these samples using a standard consent form for the collection of specimens appropriate for the study (see appendix).

3.2 Sample Collection and Processing

Patients were carefully selected according to the inclusion and exclusion criteria specified during this study - on theatre lists performed in the Liverpool Women's Hospital. Researchers who consented and collected samples were required to attend the Good Clinical Practice course (GCP).

3.2.1 Inclusion and Exclusion Criteria

Table 3-1 - Inclusion and Exclusion Criteria

Premenopausal Population	**Postmenopausal Population**
Inclusion Criteria Normal cycling female. Undergoing hysterectomy for non-endometrial pathology. Bilateral salpingoophorectomy. Hormone treatment-free for at least 3 months prior to operation. Must be able to give valid informed written consent	**Inclusion Criteria** Female. Patient's last menstrual period at least one year previous to operation. Hysterectomy for non-cancerous pathology. Bilateral salpingoophorectomy. Hormone treatment-free. Must be able to give valid informed written consent
Exclusion Criteria Any female on hormone treatment or in the past 3 months. Known endometriosis. Cancer of any type. Hyperplasia. Pregnancy. Salpingitis/other fallopian tubal pathology. Mirena intrauterine system (IUS).	**Exclusion Criteria** Females on HRT. Abnormal postmenopausal bleeding. Cancer. Hyperplasia. Fallopian tubal pathology. Proliferating endometrium on histological examination.

Full thickness sample sections (all endometrial layers through to myometrium) were collected and small sections of fallopian tube were dissected after the uterus was extracted during hysterectomy and bilateral salpingoophorectomy. If the patients were undergoing salpingectomy operations, pipelle samples were taken to obtain endometrial tissue. The acquirement of tissue did not affect the patient's treatment during surgery and no additional procedures were performed.

For full thickness endometrial sections obtained after hysterectomy: the uterus was transferred to a clean trolley, opened anteriorly and a small slice of full thickness uterine tissue was dissected using a sterile scalpel.

A small section of the tissue was transferred into 15ml neutral-buffered formalin (NBF) for paraffin embedding for immunohistochemistry. A small section was also transferred into 500 microliters RNA later, stored at 4^{o}C overnight, the solution was then removed and tissue was frozen at -70^{o}C – it is ready to be RNA extracted. Another small section was transferred into collection media for explant culture on the day of collection:

Table 3-2 - Ingredients in Collection Media

Collection Media for Fallopian Tube	Collection Media for Endometrium	Supplier
Dulbecco's Modified Eagle Medium (DMEM/F12) (Phenol-Red Free)	DMEM	DMEM/F12 – Life Technologies DMEM - SIGMA
Primocin (Antibiotic)	Primocin (Antibiotic)	InvivoGen, though Source BioScience
	1% Fetal Bovine Serum (FBS)	Biosera Ltd

Fallopian tube sections were dissected from the isthmic and fimbrial ends of the tube in a cross-section so that the lumen would be left intact. These were transferred into the same solutions as the endometrium described above and transported back to the lab for processing.

A pipelle is a single use, sterile suction curette for a biopsy of the endometrial tissue in the uterine cavity. The pipelle is passed through the cervix into the uterine cavity and the piston within the sheath should be withdrawn towards the proximal end to create a negative pressure so that mucosal tissue is drawn into the opening at the distal end of the sheath. By rotating and twirling the sheath several times within the cavity the maximal amount of tissue can be retrieved in the pipelle.[87] The collected specimen was dispensed into the appropriate solutions for processing – as mentioned earlier NBF, RNA later and collection media.

3.3 Immunohistochemistry

The technique used throughout this project was a two-step immunohistochemical staining technique; using The Vector immPRESS ™ reagent kit, horseradish peroxidase (HRP).

In the body; a set of proteins called antibodies are produced by B cells to attach to and destroy antigens that have entered body tissues and cause cell damage. This technique can be applied, in the lab on tissue samples to detect certain protein expression properties in particular cells. This procedure is called immunohistochemistry.

If a human antigen is injected into a particular animal (for example – a rabbit) the animal produces antibodies in defence against that specific antigen. If the antibodies are subsequently collected and applied to human tissue – these will attach to the antigen of interest. This allows precise location of the antigen within the cell.[88]

During this project the indirect method of immunohistochemistry was followed. This is where a primary, unlabelled antibody is added to the tissue and then a secondary labelled antibody is added to react with the primary antibody. The secondary antibody is produced by adding immunoglobulin of the host of the primary antibody to another animal. A number of the secondary antibodies will detect and build on the primary, attached antibody therefore amplifying the signal detection. The second antibody is labelled with a peroxidase enzyme.[89] Finally,

Diaminobenzidine (DAB) is added – in the presence of peroxidase, an insoluble substrate is formed producing a brown-coloured stain that can be seen under a light microscope.

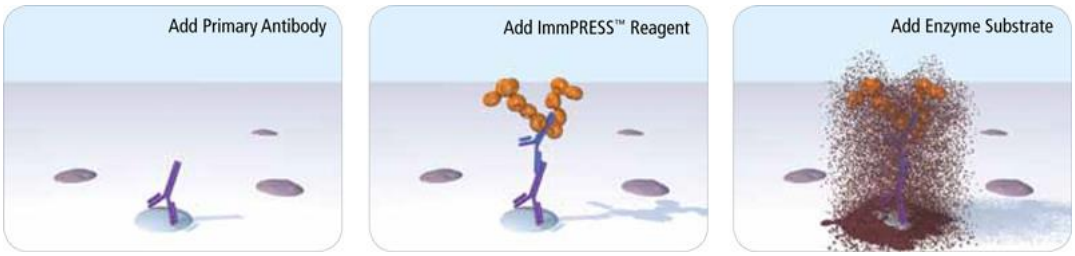


Figure 3-1 - Indirect method, using ImmPRESS system immunohistochemistry [90]

3.3.1 Tissue Processing and Paraffin Embedding

Samples must be preserved for long-term maintenance of tissue and cutting fine sections so that they can be stained easily. Fresh tissue samples were stored in neutral-buffered formalin for 24 hours before processing. The processing procedure took place using the Shandon Citadel 1000 processing machine and involved formalin fixed tissue undergoing dehydration, clearing then impregnation with paraffin wax. The processing schedule was as follows:

Table 3-3 - Processing Procedure

4% Formalin in neutral buffer	45 minutes
60% Ethanol	1 hour
70% Ethanol	1 hour
90% Ethanol	1 hour
100% Ethanol	1 hour
100% Ethanol	1 ½ hours
100% Ethanol	2 hours
Xylene 1	1 hour
Xylene 2	1 ½ hours
Xylene 3	2 hours
Wax 1	2 ½ hours
Wax 2	3 ½ hours

Embedding of samples into paraffin blocks took place in the Shandon Histocentre 3. Here processed samples were transferred into wax-filled moulds to create a paraffin block for cutting.

3.3.2 Cutting Paraffin Sections

Once the samples were embedded they needed to be cut into 3 micrometre thick sections and transferred onto glass slides for undergoing immunohistochemistry. The sections were cut using the Microm HM335 rotary microtome. Excess wax was trimmed to expose the tissue then the microtome blade was fed through the wax block cutting a ribbon of 3 micrometre thick sections. This ribbon was floated onto a warm water bath using forceps. The water bath was warmed to 41^{o}C. Once the ribbon was separated, individual sections were floated onto aminopropyl triethoxy silane (APES) coated slides and left to dry for several hours.

3.3.3 Preparation of Slides

To ensure that tissue sections adhered properly to the glass slides they were heated prior to the immunohistochemical procedure.

Tubes and matched eutopic endometrium were prepared for the immunohistochemical procedure by baking at 37^{o}C overnight or at 60^{o}C for one hour.

3.3.4 De-waxing

For the sections to be ready to stain the tissue needed to be deparaffinised with xylene and subsequently rehydrated with alcohol.

Using a slide rack; the tissue samples were placed in a staining dish containing xylene for 10 minutes. They were then progressively transferred through another dish of xylene, two containers of 100% ethanol, 90% ethanol, 70% ethanol and finally into a dish of distilled water. The tissue samples were ready for antigen retrieval.

Table 3-4 - Dewaxing procedure

Dewaxing Chemical	Time
Xylene 1	10 minutes
Xylene 2	10 minutes
100% Ethanol 1	5 minutes
100% Ethanol 2	5 minutes
90% Ethanol	1 minute
70% Ethanol	1 minute

3.3.5 Antigen Retrieval

The tissue sample underwent formalin-fixation to stabilise the tissue proteins. Whilst stabilising the tissue for processing; this modified the antigen's epitopes and electrostatic charges. Antigen retrieval is a process that enables the restoration of the tissue sample's epitopes. By restoring these; the antigen can react with the antibody and produce a stronger, more visible stain.

This was achieved by preparing a 10mM, pH 6.0 solution of citrate buffer for heating in a pressure cooker. For SSEA-1 antibody, Tris-EDTA buffer was used in antigen retrieval. 10mM of Tris, in 1mM of EDTA, at a pH of 8.0 was required. The citrate/Tris was brought to a rolling boil and the slides placed into this solution, with the lid tightly sealed, for the optimum time according to the antibody being tested (see later for details of each antibody used). Once retrieved; the slides were transferred back into distilled water for the immunohistochemical procedure.

3.3.6 Immunohistochemistry (IHC)

The tissue samples were bathed in Tris Buffered Saline (TBS) for 5 minutes and transferred into 0.3% Hydrogen Peroxide/TBS solution for 10 minutes. The tissue was then washed in TBS twice. Tissue was encircled using a hydrophobic marker to allow more than one section with different antibodies to be stained per slide and to keep all reagents on/around the tissue.

For some IHC procedures another technique to bathe the tissue was adopted. This was the "coverslip" method. A glass coverslip was placed on top of the

primary/secondary antibody to create a thin film over the tissue and ensure that the whole section was completely covered to avoid the edges drying-out.

Some tissue samples (depending on the species that the antibody is raised in) needed blocking with horse serum to prevent none specific binding to cell receptors. If this was required at this stage – the tissue samples were transferred into a humidified chamber, 2 drops of horse serum applied, mixed and left for 20 minutes.

The tissue was subsequently incubated with the desired primary antibody (see table 4). The primary antibody was diluted to the recommended concentration and incubated for the optimum time. After blocking with the horse serum (if applicable) this was removed and the slides were transferred back into the humidified chamber. All antibodies were diluted using BSA diluent (0.5% BSA/TBS) and applied to each tissue sample.

Table 3-5 - Antibody data

Antibody	Concentration	Host Species	Antigen Retrieval Time	Horse serum	Incubation Time	Incubation Temperature	Supplier	Supplier Code	Clone
Beta Catenin	1:200	Rabbit MAb	2 minutes, Citrate	Y	Overnight	4°C	Cell Signalling Technology Herts, UK	#95825	6B3
BCAM	1:100	Rabbit MAb	2 minutes, Citrate	Y	Overnight	4°C	Abcam Cambridge, UK	Ab111181	EPR4164
KI67	1:200	Mouse MAb	4 minutes, Citrate	N	Overnight	4°C	Novocastra, Newcastle, UK	NCL-Ki67-MM1	MM1
SOX-9	1:400	Goat Polyclonal	4 minutes, Citrate	Y	Overnight	4°C	R&D Systems Abingdon,UK	AF 3075	Met1-Lys151
SSEA-1	1:800	Mouse MAb	3 minutes, Tris	N	Overnight	4°C	Biolegend, San Diego, CA 92121	125601/2	MC-480
Sialylated SSEA-1	1:400	Mouse MAb	3 minutes, Citrate	N	Overnight	4°C	Aviva Systems Biology, San Diego, CA 92121	OAMA01510	258-12767
Androgen Receptor	1:75	Mouse MAb	3 minutes, Citrate	N	Overnight	4°C	Dako, Glostrup, Denmark	M3562	AR441
Oestrogen Receptor Alpha	1:50	Rabbit Polyclonal	2 minutes, Citrate	Y	2 hours	Room Temperature	Abcam, Cambridge, UK	Ab137738	
Oestrogen Receptor Beta	1:50	MouseMAb	2 minutes, Citrate	N	Overnight	4°C	Serotec, Kidlington, UK	MCA1974S	PPG5/10
Progesterone Receptor	1:1000	Mouse MAb	2 minutes, Citrate	N	30 minutes	Room Temperature	Dako, Glostrup, Denmark	M3569	PgR 636

3.3.7 Controls for Antibodies

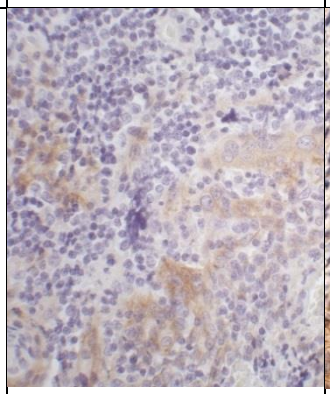
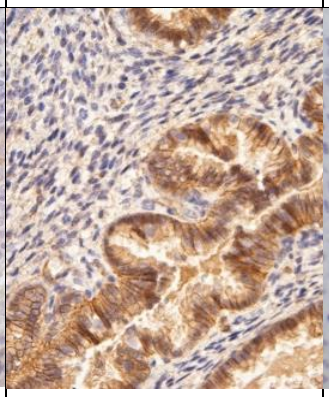
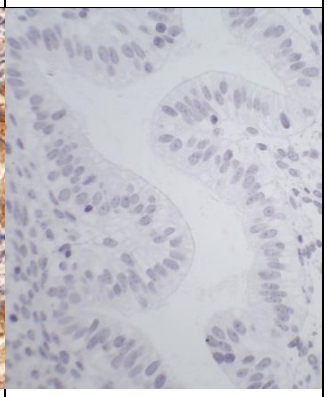
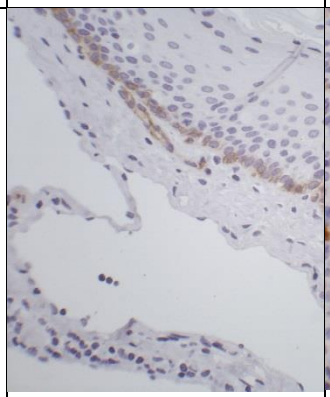
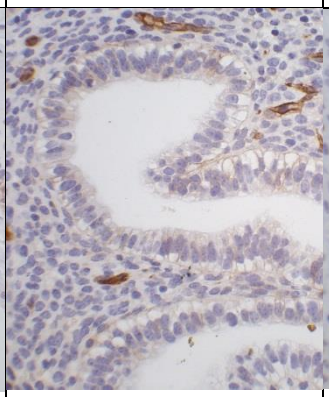
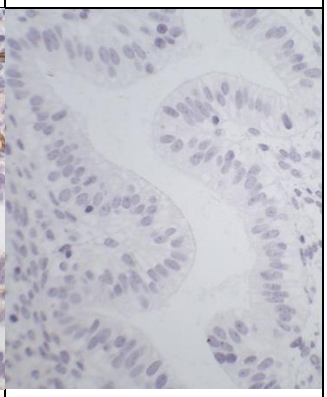
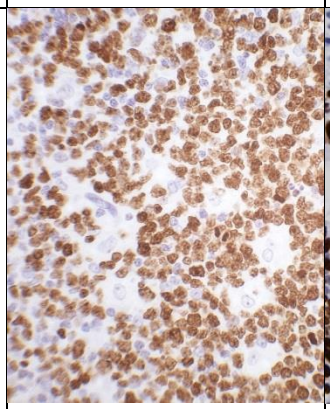
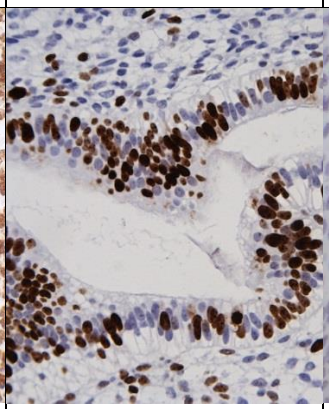
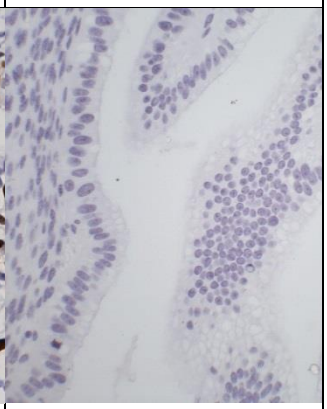
To check the quality of the antibody staining and the protocol carried out - it was important to stain a tissue that is positive for this antibody so that appropriate staining intensity is determined as a benchmark for the samples being tested. This is termed a positive control.

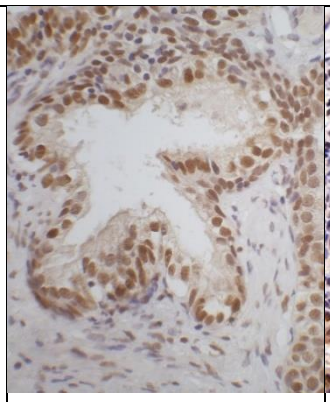
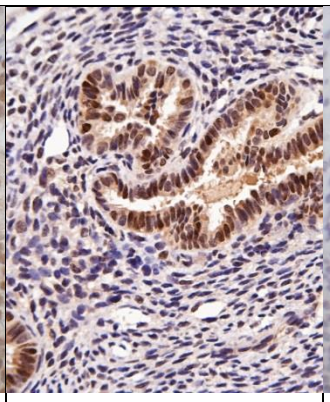
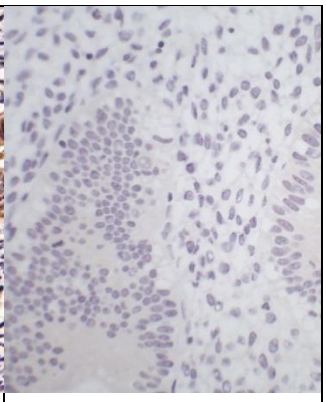
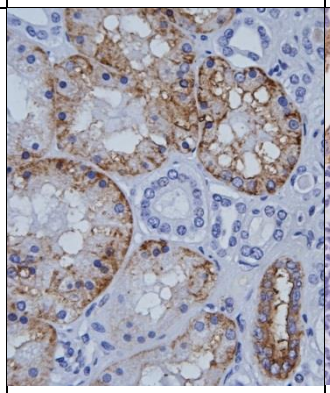
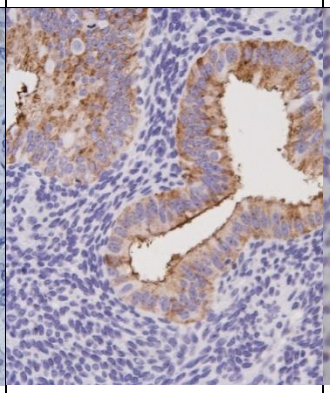
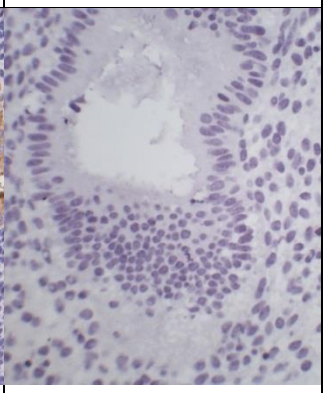
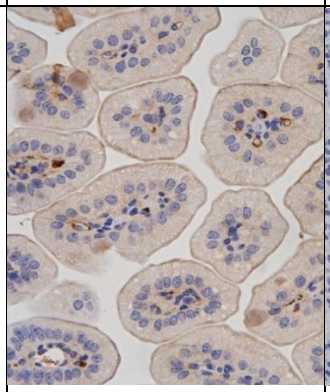
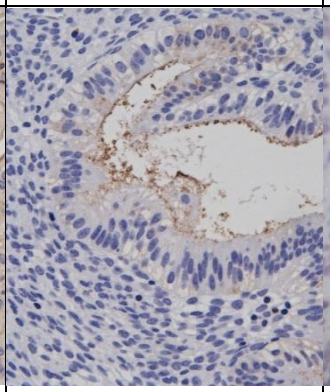
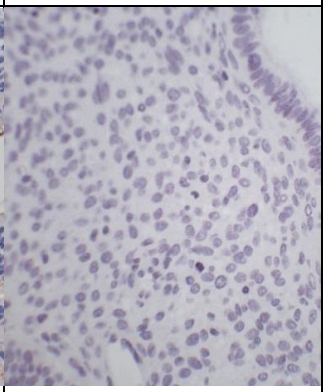
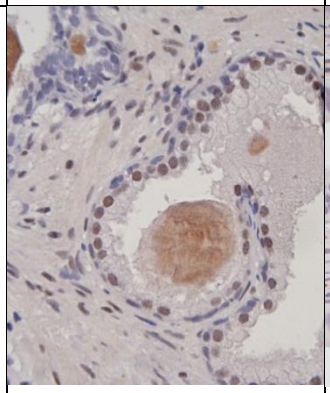
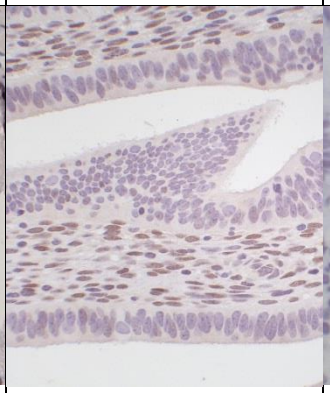
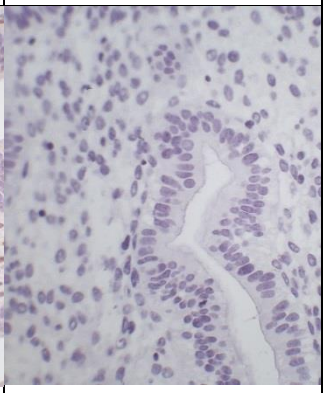
An internal control was placed in each run when performing multiple runs at different times to make sure there was consistency between each experiment. This was usually a sample that was stained in the first IHC run and was thus repeated in all subsequent runs with the same primary antibody. A negative control was one that - instead of the primary antibody being added to the tissue; it was replaced by the IgG of the animal in which the antibody was raised in. This produces a negative stain with no DAB detected and confirms the specificity of the antibody being tested.

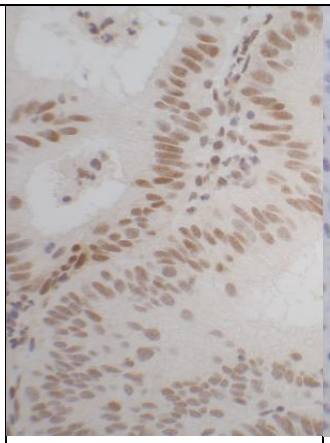
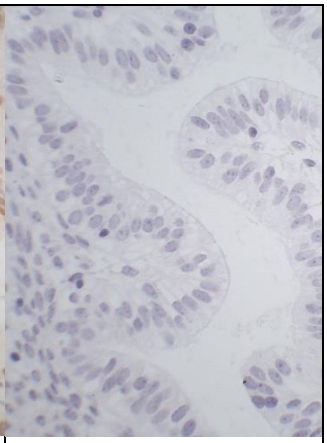
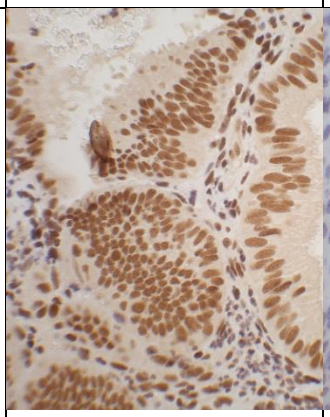
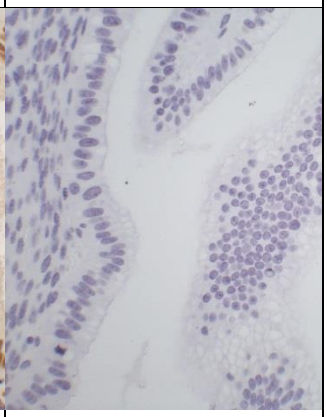
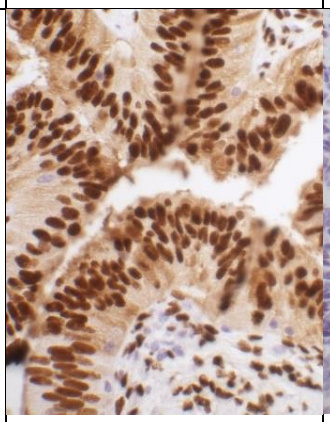
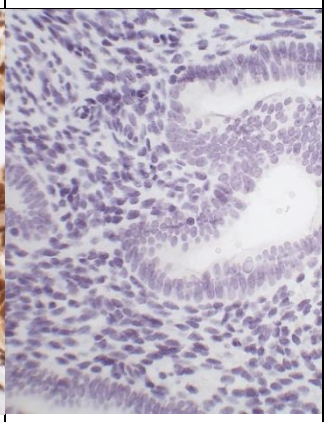
Table 3-6 - Controls in experiment

Antibody	Positive Control	Internal Control	Negative Control
Beta Catenin	Liver	SPCN 129-2	Rabbit IgG
BCAM	Tonsil	SPCN 126-2	Rabbit IgG
KI67	Tonsil	SPCN 126-2	Mouse IgG
SOX-9	Prostate	SPCN 129-2	Goat IgG
SSEA-1	Kidney	SPCN 41-1	Mouse IgG
Sialylated SSEA-1	Mouse Gut	SPCN 126-2	Mouse IgM
Androgen Receptor	Prostate	SPCN 118	Mouse IgG
Oestrogen Receptor Alpha	Endometrium – same as internal	EndoCa 72	Rabbit IgG
Oestrogen Receptor Beta	Endometrium – same as internal	EndoCa 72	Mouse IgG
Progesterone Receptor	Endometrium – same as internal	SPCN 118 Tube	Mouse IgG

Table 3-7 - Picture at x400 Magnification of Controls for each Antibody

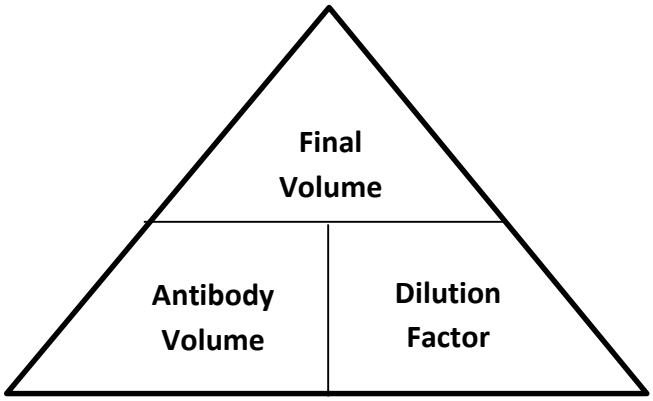
Antibody	External Positive Control	Internal Control	Negative Control
Beta Catenin			
BCAM			
KI67			

SOX-9			
SSEA-1			
Sialylated SSEA-1			
Androgen Receptor			

Oestrogen Receptor Alpha	SAME AS INTERNAL		
Oestrogen Receptor Beta	SAME AS INTERNAL		
Progester-one Receptor	SAME AS INTERNAL		

A formula for antibody calculations:

Final Volume = (Number of sections + 10%) x Amount applied to each section



After incubation the tissue samples were washed twice In TBS for five minutes each. The samples were dried and placed back into the humidified chamber and the Horseradish Peroxidase (HRP) labelled secondary antibody (chosen according to animal the antibody was raised in) was placed onto the tissue sample and incubated for 30 minutes. Two washes for five minutes of TBS were required after incubation.

The slides were then dried and placed into the humidified chamber and Diaminobenzidine (DAB) + substrate solution of dilution 30 µl DAB: 1000 µl diluent was placed onto the tissue sample for 10 minutes. Afterwards, the DAB solution was tapped off and the slide was immersed in water.

The next step was to make sure that the unstained/negative nuclei were stained with haematoxylin for visualisation under a light microscope. The samples were placed into haematoxylin solution for 1 minute 30 seconds. They were then washed under cold water. The samples were then dipped into a solution of 1% acid alcohol. This is because the haematoxylin used, is a “regressive” stain therefore the sample is over-stained and then reduced in intensity by 1% acid alcohol solution. This solution also reduces background blue staining to produce clear, light-blue nuclei that can be observed under light microscopy. The tissue was washed under cold water for five minutes for optimum staining intensity.

3.3.8 Dehydration

Before the slides were mounted they underwent the dehydration procedure. They were transferred through 70% ethanol, 90% ethanol, two dishes of 100% ethanol and two dishes of xylene (see below for details).

Table 3-8 - Dehydration procedure

Dehydration solution	Immersion Time
70% Ethanol	1 minute
90% Ethanol	1 minute
100% Ethanol 1	3 minutes
100% Ethanol 2	3 minutes
Xylene 1	5 minutes
Xylene 2	10 minutes

The final step was to prepare the slides for observation under the microscope. Cover slips were placed over the tissue using consul mount glue and left to dry.

3.4 Reverse Transcriptase - Polymerase Chain Reaction

3.4.1 Ribonucleic Acid (RNA) Extraction

This is the purification of RNA from biological samples. It is important to purify intact RNA from biological samples for the success of downstream applications.[91] For worthwhile results from RT-PCR the best purification methods must be used. RNA is prepared using multi-component kits. The kit used for the samples under study was: Pure Link RNA mini kit (Ambion, Life Technologies, Paisley, UK).

Samples were collected and stored in "RNA Later" solution, in the freezer at -20^{o}C (short-term) and -80^{o}C (long-term). During this procedure it was important to prevent contamination by wearing gloves sprayed with ethanol and cleaning all equipment before use with "RNA ZAP".

Once the fume hood and all equipment inside it was cleaned, frozen sections were removed from the freezer to thaw. Microfuge tubes were prepared corresponding

to the number of samples being extracted. Using appropriate barrier protection, 1000 µl of TRIzol was pipetted into a bijoux. The tissue sample was placed into a bijoux container with TRIzol. This solution broke down the cellular components of the tissue. Care was taken to label each sample to avoid experimental error.

3.4.1.1 Homogenising

A bijoux container was prepared with alcohol and TRIzol for the cleaning of the probe of the homogeniser between each sample to avoid RNA contamination between samples. The tissue was then broken down into a cell suspension. Once the probe had been cleaned, the procedure was repeated for each tissue sample until all tissue was homogenised.

3.4.1.2 Phase Separation

Each cell suspension was then transferred into a microtubule for the next phase of the procedure. The tissue/TRIzol solution was allowed to sit for 5 minutes to allow complete dissociation of nucleoprotein complexes. 200 µl of chloroform was added to each sample and mixed thoroughly. The tissue solution was subsequently placed into a centrifuge and spun for 15 minutes at 4^{o}C. After spinning, the clear liquid (this contains the RNA) was pipetted off each sample, being careful not to get any of the pink coloured protein-mixture into the solution – as this would contaminate. Usually, approximately 500 microlitres of clear liquid could be pipetted off at a time. The liquid was then mixed with the equivalent amount of 70% ethanol.

3.4.1.3 Binding, washing and elution

500 µl of each sample was pipetted into a spin cartridge for filtering the RNA out of the solution. The sample was centrifuged for 30 seconds at room temperature to enable this to happen. The remaining un-spun sample was added to the cartridge and centrifuged to ensure all of the RNA had been filtered and was bound to the membrane inside the spin cartridge. The next step was to wash the sample. 700 µl of Wash Buffer 1 (from the kit) was added to the cartridge and centrifuged for 30 seconds. To complete the wash; 500 µl of Wash Buffer 2 (from the kit) was added to the spin cartridge and centrifuged for 30 seconds, twice. The spin cartridge then

needed to be centrifuged for 1 minute to dry off the membrane. The cartridge was placed into a recovery tube (from the kit) and 30 µl of RNase-free water was pipetted onto its membrane. To ensure the water detached the RNA from the membrane, the tubes were left for 1-2 minutes. For final filtering of the RNA/H_20, the recovery tubes were centrifuged for 2 minutes at room temperature. The cartridge was removed and the RNA-filled solution remained in the tube ready to be stored at -70^{o}C. Care was taken to ensure all equipment was cleaned, particularly the probe of the homogeniser for future use.

3.4.2 Deoxyribonuclease (DNase) Treatment

Treatment with the enzyme DNase destroys any genomic Deoxyribonucleic acid (DNA) contamination in the RNA sample. Reverse transcriptase-PCR (RT-PCR) uses the technique of converting RNA back into DNA. After this, target genes are amplified. This could potentially include any genomic DNA present in a sample. If the sample is contaminated with genomic DNA, one could not decide whether the RT-PCR results are due to the complementary-DNA generated or the genomic DNA in the sample. To prevent this – we add DNase to the RNA samples (an enzyme which breaks down DNA).[92] The solutions used were: 10x DNase1 reaction buffer - B 03035, DNase1 enzyme - B 03036 (New England BioLabs Inc, Hitchin, Herts, UK) and RQ1 DNase STOP solution - M 199A (Promega Corp, Madison, WI 53711-5399 USA).

Before beginning; “RNA ZAP” was used to clean the lab bench and all equipment used during the procedure. With samples of total RNA thawing in an ice-bath, microfuge tubes were prepared corresponding to the number of samples that needed Dnase treatment. 8 µl of each total RNA sample was pipetted into the microfuge tubes. 1 µl of Dnase 1 was pipetted into the RNA solution. 1 µl of 10x buffer was then to the mixture. The samples with the Dnase/buffer/RNA mixture were inserted to a 37°C incubator for 30 minutes. 1 µl of “DNase STOP” solution was added to each sample. The samples were inserted back into the incubator and heated at 65°C for 10 minutes. They were then stored at -70°C before complementary-DNA synthesis.

Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher). Absorbance ratios were also recorded; the 260/280nm ratio demonstrates the RNA purity. The concentration of each sample was 1000ng/µl.

Table 3-9 – RNA Spectrophotometry Values for matched fallopian tube and endometrium in Postmenopausal and Secretory Phase Samples.

Sample (EP: Postmenopausal, SPCN: Secretory Phase)	**RNA ng/µl**	**RNA A260/280**
EP 26 Tube	316.10	2.02
EP 26 Eutopic Endometrium	934.22	2.08
EP 27 Tube	403.87	2.07
EP 27 Eutopic Endometrium	572.58	1.99
EP 30 Tube	344.79	2.00
EP 30 Eutopic Endometrium	431.44	1.98
SPCN 143 Tube	318.08	1.91
SPCN 143 Eutopic Endometrium	997.84	2.02
SPCN 152 Tube	165.20	2.05
SPCN 152 Eutopic Endometrium	1021.90	2.06
SPCN 159 Tube	359.44	2.01
SPCN 159 Eutopic Endometrium	838.84	2.05

Table 3-10 - RNA Spectrophotometry Values for matched fallopian tube and endometrial explants in the Secretory Phase +DHT (Treated with DHT) or –DHT (untreated.)

Sample	RNA ng/µl	RNA A260/280
SPCN 163 Endometrium +DHT	63.24	1.78
SPCN 163 Endometrium -DHT	19.28	1.92
SPCN 163 Tube +DHT	51.64	1.88
SPCN 163 Tube -DHT	48.54	1.78
SPCN 172 Endometrium +DHT	1063.61	2.12
SPCN 172 Endometrium -DHT	1316.40	2.11
SPCN 172 Tube +DHT	551.39	2.06
SPCN 172 Tube -DHT	594.36	2.03
SPCN 175 Endometrium +DHT	679.78	2.06
SPCN 175 Endometrium -DHT	602.78	2.06
SPCN 175 Tube +DHT	789.17	2.02
SPCN 175 Tube -DHT	781.84	1.89

3.4.3 Complementary – DNA (cDNA) Synthesis

cDNA is a double stranded version of messenger RNA (mRNA). A technique for making cDNA is to use the enzyme reverse transcriptase. A template of mRNA is added to the enzyme so that it makes a single strand of cDNA. With this single stranded cDNA; double stranded DNA can be synthesised.[93] By generating cDNA and adding a primer sequence, the positive sequences can subsequently be amplified using polymerase chain reaction (PCR). [94] The kit used for this procedure was (AMV) First Strand cDNA Synthesis Kit E65505 (New England BioLabs Inc, Hitchin, Herts, UK). All equipment was sterilised under UV light before beginning the procedure.

For each sample: 2 µl of dT23UN with a variable amount of nuclease-free H_2O and a variable amount of sample were combined together. The total mixture volume was 8 µl. After RNA spectrophotometry of each sample (using a NanoDrop machine); the approximate concentration of RNA was quantified in each sample. The RNA concentration was expressed in nanograms/76icroliter (ng/µl). We needed to

convert this into a microgram (1000 nanograms). Not every sample's RNA concentration was the same; therefore 1000 ng/µl of each sample was needed to have the same concentration of RNA for each sample.

For example: If the concentration of RNA was found to be 500 ng/µl in order to make this 1000 ng, we multiplied this by 2. Therefore in this instance, we needed 2 microliters of sample to be added to the mixture to make 1000 ng.

Once the quantity of sample was determined, nuclease-free H_2O was added to the mixture to make up a total volume of 8 µl. Referring to the example above; if 2 µl of dT23UN and 2µl of RNA were needed, then 4 µl of NF-H_2O was added to make an 8 µl mix.

The sample-mix was denatured at 70^{o}C for five minutes. After this, 12 µl of "multimix" was added to each sample. The "multimix" consisted of a solution made up of 10 µl of "AMV reaction mix" and 2 µl of "AMV enzyme mix." Samples were subsequently incubated at 42^{o}C for 1 hour for the enzyme mixture to bind to the RNA in the sample and begin cDNA synthesis.

After one hour incubation; the samples were heated to 80°C for five minutes to arrest the synthesis reaction. 30 µl of NF-H_2O was then pipetted into to each sample to make a total volume of 50 µl for each sample. Samples were stored at -80°C for future PCR.

3.4.4 Polymerase Chain Reaction and Electrophoresis

Polymerase chain reaction is based on the ability of a substance called DNA polymerase to produce and amplify DNA sequences complimentary to the template strand. A pair of primers are added (one on each strand) and bind specifically to the 3' ends of the target sequence after heating the DNA to separate the strands. The primers then promote DNA polymerase enzyme called Taq to use the free nucleotides to bind to these sequences and synthesise a complimentary DNA sequence. The polymerase can only bind to molecules with a primer attached making this a very specific way of identifying a DNA sequence. The process above is

called a cycle and is repeated many times on the copied DNA strands to amplify the sequence; eventually forming billions of target sequence molecules.[95]

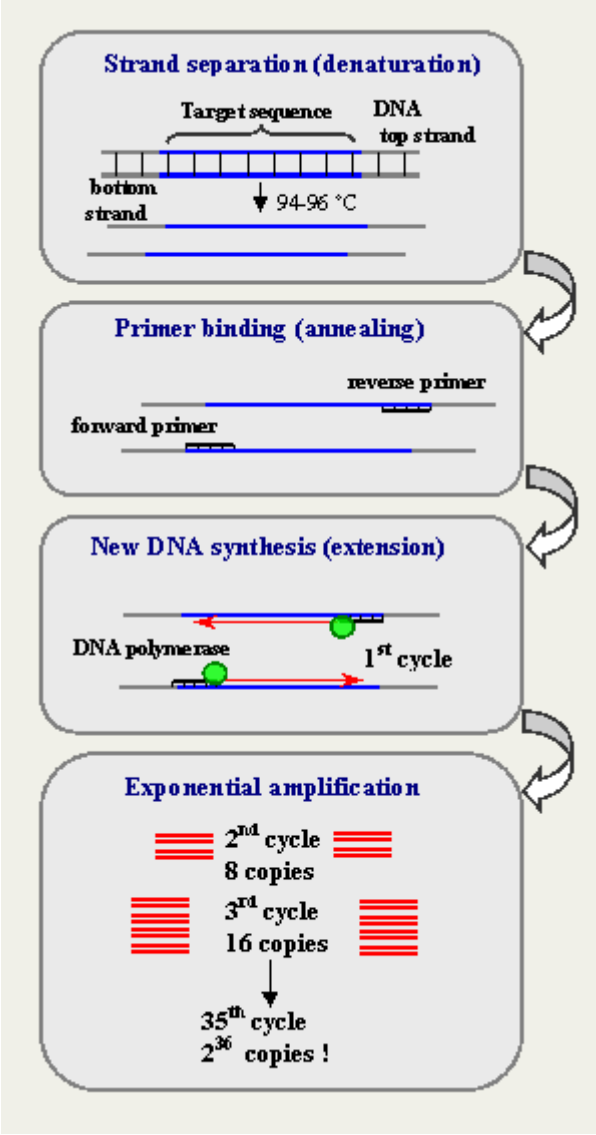


Figure 3-2 - How PCR works[8]

For preparation of the primer with cDNA it was essential to carry out this procedure in a sterile ultraviolet hood to prevent contamination. All equipment used in this experiment was UV sterilised for 10 minutes.

A combination of HotStart Taq 2x master mix (New England Biolabs Inc, Hitchin, Herts, UK), the primer mix and nuclease-free water was prepared. A total volume of 9 µl "mastermix" per sample was used in this experiment. 5 µl of which was Taq Hotstart, the amount of primer mix depending whether it was AR, SOX9 or BCAM

gene expression being tested (either 0.5 µl or 1 µl) and nuclease-free water was added to make the solution up to 9 µl. All primer concentrations were 100µM. The amount of "mastermix" was prepared according to the quantity of samples used for example; for a sample size of 15, the "mastermix" needed was 15 x the quantity of each substance in the mix. 1 µl of the cDNA sample was pipetted into a microtubule with the 9 µl of "mastermix." For the PCR reaction, the internal control of the cDNA experiment that did not contain reverse transcriptase (NoRT) was added to the sample set, a negative control for the PCR experiment was also needed. This was achieved by adding 1 µl of nuclease-free water instead of cDNA. A positive control was also needed for the validation of the target gene in the PCR which was dependent on the primer used. Sample mixtures were placed in a PCR machine and the appropriate program selected - as temperatures and times varied at each stage depending on the primer sequence. In some instances, primers investigating particular target sequences needed more PCR cycles than others. The samples were held in a 4°C environment after the PCR reactions had taken place (See below for details for each primer).

Table 3-11 - Primer Details, Controls and Programs

Primer Name	Sequence (5'-3')	Company	Amount per sample for PCR	Positive Control	Program for PCR
YWHAZ	Forward CGTTACTTGGCTGAGGTTGCC Reverse GTATGCTTGTTGTGACTGATCGAC	**SIGMA**	1 µl	N/A	**Stage 1:** 95°C - 30 seconds. **Stage 2:** (30 cycles) 95°C – 20 seconds. 56°C – 20 seconds. 68°C – 30 seconds. **Stage 3:** 68°C – 5 minutes. 4°C Hold.
GAPDH	Forward ACCACAGTCCATGCCATCAC Reverse TCCACCACCCTGTTGCTG	**SIGMA**	0.5 µl	N/A	**Stage 1:** 95°C - 30 seconds. **Stage 2:** (25 cycles) 95°C – 20 seconds. 61°C – 20 seconds. 68°C – 30 seconds. **Stage 3:** 68°C – 5 minutes. 4°C Hold.
SOX9	Forward GTACCCGCACTTGCACAAC Reverse TCTCGCTCTCGTTCAGAAGTC	**SIGMA**	0.5 µl	LNCAP	**Stage 1:** 95°C - 30 seconds. **Stage 2:** (35 cycles) 95°C – 20 seconds.

					56°C – 20 seconds. 68°C – 30 seconds. **Stage 3:** 68°C – 5 minutes. 4°C Hold.
Androgen Receptor (AR)	Forward AGGATGCTC TACTTCCGCCC Reverse CTGGCTGTACATCCGGGAC	**SIGMA**	0.5 µl	LNCAP	**Stage 1:** 95°C – 2 minutes. **Stage 2:** (30 cycles) 95°C – 20 seconds. 58°C – 20 seconds. 68°C – 30 seconds. **Stage 3:** 68°C – 5 minutes. 4°C Hold.
BCAM	Forward CCTCGTCGTTGCTGTCTTCT Reverse TTGCAGATAGCAGGCCACT	**SIGMA**	0.5 µl	HEK293	**Stage 1:** 95°C – 30 seconds. **Stage 2:** (30 cycles) 95°C – 20 seconds. 56°C – 20 seconds. 68°C – 30 seconds. **Stage 3:** 68°C – 5 minutes. 4°C Hold.

After the PCR procedure was finished, the samples were loaded into an agarose gel and a current passed through the samples to show the expression of the target sequence in each sample. To make the gel, a casting tray was prepared in an electrophoresis chamber. To make a 2% agarose gel (standard for all primers except GAPDH) - 2g of agarose powder was added to 100 millilitres of TBE buffer. GAPDH required a 1.5% agarose gel - 1.5g agarose powder in 100mls of TBE buffer. The solution was microwaved for a minimum of 4 minutes gently swirling the mixture half-way through. The powder was completely dissolved and a clear solution remained. The gel was then cooled to a warm temperature without it setting.

10 microliters of "Sybr safe" liquid was pipetted into the gel solution. "Sybr safe" binds to the base pairs in the DNA so that when UV light is applied, the DNA is exposed and an image can be taken. The solution was mixed gently by swirling so that the "Sybr safe" incorporated itself into the solution.

The gel was poured into an electrophoresis casting tray, making sure no large air bubbles remained in the mixture. Electrophoresis combs were inserted into the tray to create wells needed for the addition of the samples. The liquid, gel mixture was left to stand for at least 20 minutes so that it set into a solid gel. Making sure the gel was orientated so that the wells were horizontally spread across the casting tray once the gel has set; the combs were pulled out gently, to leave the wells ready for sample-insertion. 2 microliters of loading dye was added to each sample so that it appeared on the gel and because it contained the heavy substance, glycerol. This made sure that the sample fell accurately into the well without spreading across the gel and contaminating adjacent samples. 10 microliters of each sample were pipetted into the wells and a 50 base pair DNA ladder marked the beginning of each set. The electrophoresis machine was set to 110 volts for 45 minutes. Care was taken to ensure the current was rising from the top of the chamber and moving towards the samples in a way that they would move away from the wells and down the gel.

After 45 minutes the gel was ready for photographing. This was performed in a UV Chamber. The gel was unloaded onto a UV plate and UV light was passed through it to create an image of the gene expression in the samples onto the computer. After adjusting the brightness of the sample a picture was saved onto the computer or printed indicating the varying expression between each of the samples.

3.5 Tissue Explant Culture

The principle of explant culture is to isolate cells from a tissue or small pieces of the tissue itself; that have been collected from a donor (in this case human fallopian tube and eutopic endometrium). In growth media, small pieces of tissue adhere to a growth surface and migrate outwards – this is known as *outgrowth.* The cells selected have the ability to migrate.[96]

This lab procedure took place in a class II microbiological safety cabinet – this was to take care to prevent contamination with live tissue specimens and maintain a sterile environment for preparing the tissue samples for culture. The hood was sterilised before and after use with 70% ethanol.

In this study matched eutopic endometrial samples and fimbrial fallopian tube samples were collected and incubated for 24 hours with Dihydrotestosterone (DHT – SIGMA). A small amount of DMEM/F12 media was poured into a petri-dish assigned to holding tube tissue and another for eutopic endometrial tissue. The tissue was placed into the corresponding petri-dish so as to wash the cells before incubation. A 1mM DHT solution was prepared by mixing with methanol. A 5% mixture of 10% charcoal-stripped foetal bovine serum with DMEM/F12 media was then prepared for culture of the cells. DHT was added to the serum/media combination at a concentration of 1ul DHT in 1000 µl of serum/media. A vehicle was included for comparison with the DHT by addition of 1 µl of methanol instead of DHT. A 24-well dish was labelled clearly for incubation of: fallopian tubes with DHT, fallopian tubes without DHT, eutopic endometrium with DHT and endometrium without DHT. 1000 µl of DHT mixture was pipetted into wells labelled "DHT" and 1000 µl of vehicle was added into each well labelled "vehicle." Tissue samples were cut into 3-4 mm sections and equal quantities of tissue sections were

added into each well making sure that the fallopian tube tissue was separate from the endometrial tissue. Contamination was prevented by submerging the scalpel and sterile forceps into 70% ethanol after tissue was dissected and placed into each mixture. Ensuring that the lid was on the well plate; the samples were placed into a 36.9^{o}C CO_2 incubator for 24 hours. After 24 hours each cultured tissue sample was either immersed in RNA later and frozen for future PCR or placed in NBF and refrigerated for paraffin embedding and IHC (all explained above).

3.6 Sample Analysis

3.6.1 Immunohistochemistry

Once samples had dried in the fume hood after mounting, they were analysed and scored according to the DAB staining intensity of the tissue. The method of scoring changed according to the antibody that was used in the procedure:

Table 3-12 - Scoring Techniques according to antibody.

Antibody	Scoring Method
Beta Catenin	Nuclear: Percentage Positive Cytoplasmic: Quickscore Junctional: Quickscore
BCAM	Quickscore
KI67	Percentage Positive
SOX9	Threshold analysis on image J
SSEA-1	Quickscore
Sialylated SSEA-1	Quickscore
Androgen Receptor	Modified Quickscore
Oestrogen Receptor Alpha	Modified Quickscore
Oestrogen Receptor Beta	Modified Quickscore
Progesterone Receptor	Modified Quickscore

Tissue was analysed using the **Nikon Eclipse 50 microscope** and images were analysed, captured and saved using computer software called **NIS elements 4.00.**

The camera used with this software and connected to the microscope was called **Nikon Digital Sight camera.**

3.6.1.1 Quickscore

Quickscore analysis is a way of scoring sections that differ in intensity throughout the tissue. This method takes into account the percentage of tissue that is stained and also the changing intensity throughout the sample.

To begin, the slide was placed under the microscope and the quality of the stain was observed. Some nuclei were stained with blue haematoxylin – these were negative. Some cells were stained with brown DAB – these were positive. When looking at a cytoplasmic staining the DAB concentrated inside the cell with no defined edge. If junctional staining was present the DAB outlined the cell. It either outlined the whole cell, the lateral parts of the cell junction or the basal layer of the cell. A nuclear stain had brown DAB staining in the round nuclei inside the cell and it was confined to this organelle. Some antibodies like Beta Catenin had staining in all three parts of a cell. In this case, each part stained had to be scored separately as function of this antibody changes if it's junctional compared to if its nuclear.

Samples were divided into compartments; basal glands, basal stroma (if applicable), superficial glands, superficial stroma (if applicable) and luminal epithelium for endometrial tissue. For tube the epithelial compartment and stromal compartment were analysed.

The intensity of the staining was observed in each compartment. The scores were divided into:

0 = none, 1 = weak staining, 2 = moderate staining, 3 = strong staining.

The overall percentage of staining was evaluated throughout the compartment:

If 25% is stained – a score of 1 is given.

If 50% is stained – a score of 2 is given.

If 75% is stained – a score of 3 is given.

If 100% is stained – a score of 4 is given.

The highest a compartment could score is 12. This meant that 100% of the compartment is strongly stained. The calculation is:

3 (strong staining observed) X 4 (100% stained) = 12

If this was not the case, the percentage scores could be spread across each intensity-score and each score given was multiplied and the total was added together:

Table 3-13 - Example of Quickscore

Sample	0 (None)	1 (Weak)	2 (Moderate)	3 (Strong)	Total /12
Basal Glands	2	1	1		3
Superficial Glands		1	2	1	8

The scores were recorded in excel then processed to form a graph and statistically analysed using GraphPad Prism software (see below).

3.6.1.2 Threshold Analysis

For SOX-9 scoring it was necessary to use this method of analysis. It involved capturing images on image J software, taking away the stromal compartment to leave the glands or epithelium, splitting the image into DAB and haematoxylin stains separately and recording the percentage threshold of each stain. This used the principle that haematoxylin is a background stain and should be the baseline for calculating the amount of DAB that had stained the sample. Using this principle, the DAB staining did not exceed the haematoxylin staining.

10 images were captured of each layer to represent the compartment or the whole tissue was captured in the case of some isthmic tubes. These images were saved as Tiff files and opened in imageJ. Selecting the free-hand selection tool – an outline

was drawn carefully around the edge of the gland and the surrounding stroma was cleared, leaving just the glandular epithelia.

Once the glands were cut out, “colour deconvolution” was selected. This separated the DAB from the haematoxylin staining and created separate pictures in order to calculate the threshold of the colour. The threshold tool was selected, the haematoxylin image was highlighted and area of staining calculated by imageJ. By selecting the measure tool the area that was highlighted, was measured. The same process was repeated on the DAB stained picture.

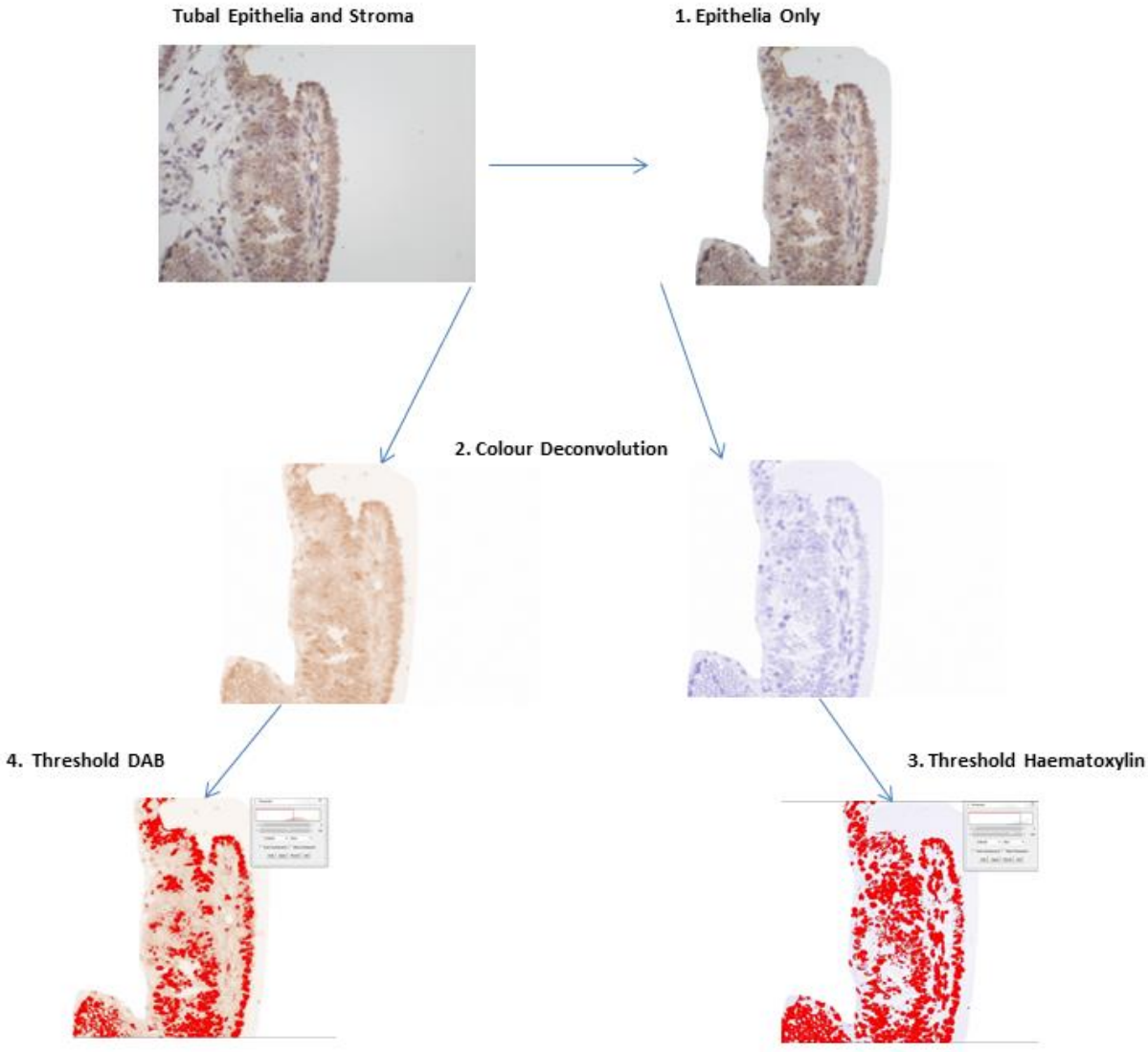


Figure 3-3 - Threshold analysis of epithelia using imageJ. Guide to analysis in order from 1-4.

The following calculation was then made to obtain the overall percentage threshold of this image:

$$\textbf{Area stained} = \frac{\textbf{Percentage area of DAB stain}}{\textbf{Percentage area of haematoxylin stain}}$$

This analysis and measurement was made for all 10 pictures taken for each compartment. An average of each of the measurements was made to represent an overall score of the tissue compartment:

Picture scores for 1+2+3+4+5+6+7+8+9+10/10 = overall score for compartment

3.6.1.3 Percentage Positive Staining

This method of analysing a stained section is an estimate of the area of stained cells expressed as an overall percentage. For some antibodies the DAB staining did not vary in intensity throughout the tissue sample – it was either positive or negative. In this case the most appropriate method of quantifying the staining of the antibody was to estimate the percentage of positive cells. Each compartment was analysed and a close approximation of the positively stained cells were calculated. The percentages were recorded in Excel and graphically represented on GraphPad.

3.6.1.4 Modified Quickscore

Steroid receptor antibody staining changed in intensity throughout and so the most appropriate way of analysing these is to use a quickscore method. The percentage area stained was usually divided into 25% increments however a modified quickscore method narrowed the percentage bands. This modification refined the scoring of each sample. The principle being; if >40% of a section was stained at a certain intensity then it was assumed that the majority of the compartment had that particular staining intensity. If a lower percentage of the section was stained the scoring was spread over the bands to show the variation within the tissue sample. This essentially narrowed the percentage staining bands. They were divided as follows (the intensity scoring was the same as section 3.6.1.1):

0 -19% = 1

20 - 39% = 2

40% = 3

>40% = 4

The highest score a compartment could get was 12 therefore the percentages were divided and spread over the intensities but could never exceed a quickscore of 12.

3.6.2 Polymerase Chain Reaction

3.6.2.1 Densitometry

After the images were taken of the electrophoresis gel, densitometry was performed to determine the relative transcript levels of AR, SOX9 and BCAM. This could be achieved using the imageJ software. The image was rotated to ensure that the wells were straight so that a band of expression could be plotted. Using the rectangular selection tool a tight frame was created around the bands (each band corresponding to the relative transcript level in the sample). This software enabled the user to create a graph in the form of a histogram representing the relative mRNA expression with each peak corresponding to each sample. The peaks were then connected using the straight line selection tool. The wand tool was then selected to click on each graph, identifying the area under the peak. A table was created to numerically quantify the area selected and this represented the amount of signal in each sample.

The methods above were performed in triplicate. The ratio of each gene transcript was normalized to YWHAZ and GAPDH independently. YWHAZ and GAPDH were the housekeeping genes used in this study. These arbitrary values were used to compare the relative RNA levels between the different samples.

3.6.3 Statistical Analysis of Data

3.6.3.1 Organisation of Data in Excel

The scores of the IHC samples were split into compartments. All 17 premenopausal matched samples were dated and re-organised into their cycle stages. Further analysis was always cycle-stage specific to analyse any cycle-dependent variation in the eutopic endometrium and the fallopian tube. The postmenopausal samples were also grouped together.

3.6.3.2 GraphPad Prism

GraphPad Prism is a piece of software for Windows and Mac computers that enables a scientist to produce 2D scientific graphs, curves (nonlinear regression,) statistics and organise data. It is normally used by biologists, social and physical scientists.[97]

Once data was collected in an excel spreadsheet – that being scoring or densitometry data; It was copied into GraphPad and a graph produced. GraphPad enables the user to select the graph necessary for representing the data appropriately. The graph selected for analysis of the trends between each group, in each compartment was a bar chart. GraphPad automatically created a spreadsheet in which to place data. The data was organised as below:

	A	B	C	D	E	F
	BG	SG	LE	Fimbrial Tube	Isthmic Tube	Cycle stage
	Y	Y	Y	Y	Y	Y
1	5	4	4		1	0
2	4	3	3	2		0
3	3	3	4	4		0
4	4	2		3	5	0

Figure 3-4 - Example of GraphPad Spreadsheet data for BCAM in menstrual phase. BG – Basal glandular epiethalia, SG - Superficial Glandular Epithelia, LE – Luminal Epithelia, Fimbrial fallopian tube and Isthmic Fallopian Tube.

Once the data was pasted into the spreadsheet a bar chart graph was automatically plotted of this data. It was then labelled and formatted further so that it looks like below:

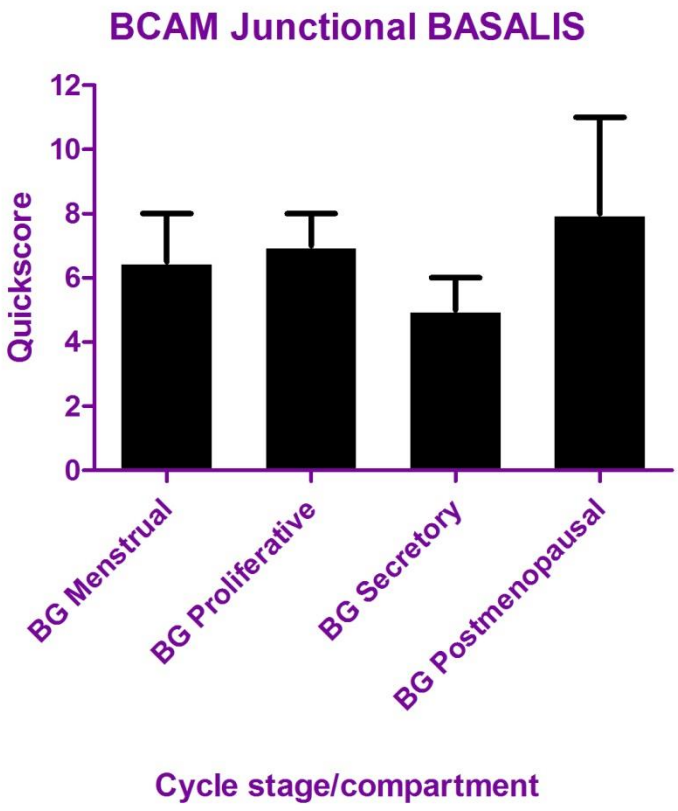


Figure 3-5 - Example of GraphPad Spreadsheet data for BCAM in menstrual phase. BG – Basal glandular epiethalia, SG - Superficial Glandular Epithelia, LE – Luminal Epithelia, Fimbrial fallopian tube and Isthmic Fallopian Tube.

On the X axis, the data was split into cycle stages. On the Y axis, the score for each sample in that phase was plotted.

Bar charts were created for each compartment across the menstrual cycle stages. Layouts were created within GraphPad prism to analyse the trends across all of the compartments and compare fallopian tubal mucosa with endometrial mucosa. These can be found in chapters 4, 5 and 6.

For the PCR relative mRNA expression graphs a whisker graph with minimum to maximum values to show spread of expression values within each sample group was used. Columns for tissue and phase were plotted for example; fallopian tube in the secretory phase or postmenopausal fallopian tube. The normalised ratio/relative expression values were plotted along the Y axis. A representative graph is below:

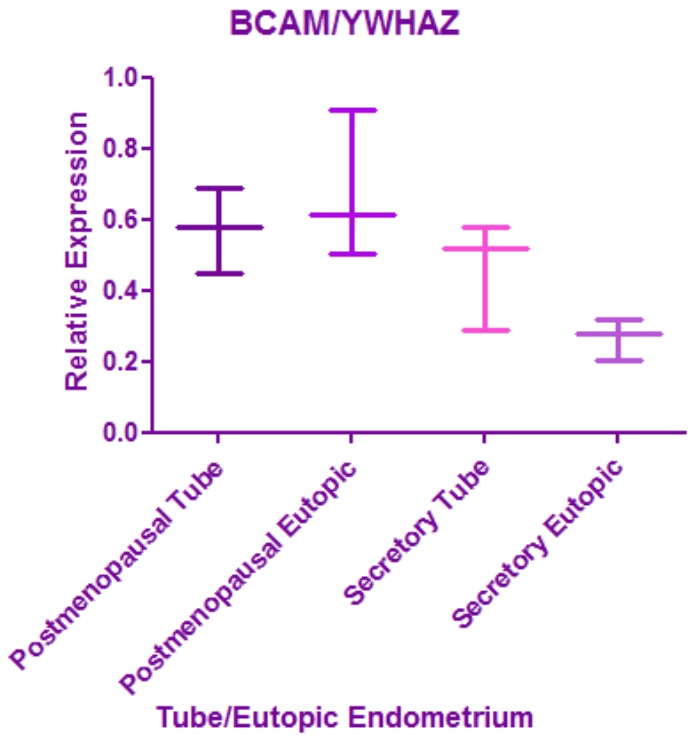


Figure 3-6 - Example of a whisker graph for BCAM PCR

3.6.3.3 Statistical Analysis

Descriptive statistics were calculated as each graph was produced. For the sample groups analysed, median with range was plotted on the graphs and commented on throughout the results section. Non-parametric T-Tests (Mann-Whitney and Wilcoxon tests) were performed where appropriate on the IHC and PCR data.

Chapter 4 Investigating Phenotypic Similarities between Fallopian Tube and Endometrium

4.1 Introduction

The fallopian tube and endometrium have the same embryological origin[1], arising from the Mullerian ducts.[18] Recent studies investigating the phenotypical differences in endometrial epithelial cells have revealed for the first time a set of markers that distinguish the epithelial cells from the stratum basalis of the endometrium. The markers identified were nuclear SOX9, cell surface marker SSEA-1 and nuclear beta catenin.[2] Since the basalis is the germinal zone of the endometrium, the progenitor cells with regenerative abilities are expected to reside in this region. It is thought that the abundance of progenitor cells residing in the basalis region give the endometrium its huge capacity to regenerate every month.[3] Agreeing with this notion, Valentijn et al demonstrated that basalis epithelial cells sorted on the expression of surface marker SSEA1, contain the cells with the ability to generate endometrial gland-like phenotype in 3D culture in vivo.[2] Recently, it has also been thought that the distal fallopian tubal mucosa may also contain a stem population,[4] an increase in cells expressing markers that were shown to be stem cell markers in other tissues, were identified here.[1] This suggests that in the fallopian tube, the distal mucosa may be the equivalent to progenitor cell-rich endometrial basalis. Definite stem or progenitor cells for either of these epithelial regions, are yet to be identified. The characterisation of the epithelial layer of different parts of the fallopian tube in the context of the basalis markers that have recently been described in the endometrium, is yet to be done.

Therefore, this chapter describes the work characterising the epithelial layer of the fallopian tube in the context of a panel of markers that were expressed by basalis epithelial cells with the germinal capacity in the endometrium - SOX9, SSEA-1 and beta catenin. Expression pattern of two new potential markers of basalis epithelium (sialylated SSEA-1 & BCAM) were also assessed.

BCAM is a marker that is localised to the basal layer of epithelial cells and is a receptor for laminin.[65] Laminins have many functions including; adhesion, motility, growth, differentiation and apoptosis. Basolateral BCAM staining on the epithelial cell surface indicates that there is an interaction with laminin which is thought to be important to maintain epithelial basolateral polarity. [98]

SOX9 is a transcription factor and has been located in basal epithelia of the prostate, the intestinal crypts and the outer root sheath of hair follicles. Progenitor cells are all thought to reside in these particular regions.[75]

SSEA-1 is an embryonic stem-cell surface marker and has recently been immuno-localised to the glandular epithelial cells of the stratum basalis, confirming that this region is phenotypically distinct from the stratum functionalis.[2]

Beta catenin is a downstream regulator of the canonical Wnt pathway and its accumulation in the nucleus leads to the activation of Wnt-responsive target genes.[28] Studies have shown that the Wnts activate intracellular cascades that regulate cellular proliferation, differentiation, migration, and polarity.[80] Nuclear beta catenin has been identified in a sub-population of epithelial cells in the basalis layer of the endometrium, whilst the nuclei of the stratum functionalis epithelia rarely expressed this marker.[64]

The cell surface carbohydrate molecule, sialylated SSEA-1 (Sialyl SSEA1) is a marker that is involved in adhesion and cell to cell recognition. It has been identified as the key marker by which the oocyte attracts sperm in order to facilitate fertilisation.[99] It is also abundantly present on the surface of epithelial cells in the endometrium and increases around the secretory phase of the menstrual cycle (the implantation period), along with MUC1. It is thus thought to play a role in facilitating the implantation of an embryo.[82]

A study comparing the markers that characterise the endometrium and in particular, the basalis layer has been conducted in order to gain an insight into whether the fallopian tubal epithelia is phenotypically similar to the endometrial glandular epithelia in this region. It was also necessary to investigate whether

postmenopausal epithelia of the endometrium and fallopian tube changed expression of these markers in this period.

4.1.1 Patient Demographics and Methods

This study was conducted over a 12 month period from August 2013-August 2014.

For this procedure N=17 premenopausal samples were split into groups according to the menstrual cycle phase (see: Table 4-1- Number of samples in each group for immunohistochemistry) were stained from matched full thickness eutopic endometrium and fallopian tube taken from the isthmic and fimbrial ends of the tube.

N=5 matched full thickness eutopic endometrium and fallopian tube postmenopausal sections were also collected.

Table 4-1- Number of samples in each group for immunohistochemistry

Study Group	Number Eutopic Endometrium	Number Isthmic Tube	Number Fimbrial Tube
Menstrual phase	4	3	3
Proliferative phase	6	6	3
Secretory phase	7	5	4
Postmenopausal	5	2	5

Table 4-2 – Table of Demographics for Patients in Immunohistochemical Study

Menstrual Cycle Phase	Sample number		Age	BMI	Smoker	Parity
Menstrual Phase	4	*Mean (± SD)* *Median* *Range*	41 (±4) 40.5 37-47	29.3 (±5.0) 30.8 21.2-34.4	1 (25%)	2 (±1) 2 1-3
Proliferative Phase	6	*Mean (± SD)* *Median* *Range*	43 (±5) 43 37-53	29.3 (±6.7) 26.1 23.1-39.2	0 (0%)	2 (±1) 2 0-3
Secretory Phase	7	*Mean (± SD)* *Median* *Range*	43 (±4) 44 37-48	27.7 (±6.6) 23.8 21.7-40.1	3 (43%)	2 (±1) 2 1-4
Postmenopausal	5	*Mean (± SD)* *Median* *Range*	57 (±7) 55 48-66	25.6 (±1.8) 24.9 23.6-28.1	2 (40%)	2 (±1) 2 1-3

For polymerase chain reaction 3 matched secretory phase samples and 3 matched postmenopausal samples were examined; the tubal tissue was fimbrial. Inclusion and exclusion criteria can be found in chapter 3.

Table 4-3 - Table of Demographics for Patients in RT-PCR Study

Menstrual Cycle Phase	Sample number		Age	BMI	Smoker	Parity
Secretory Phase	3	*Mean (± SD)* *Median* *Range*	41 (±7) 45 32-47	26.9 (±3.7) 26.6 22.6-31.6	0 (0%)	1 (±1) 0 0-2
Postmenopausal	3	*Mean (± SD)* *Median* *Range*	57 (±7) 52 52-66	29.5 (±7) 24.9 24-39.6	1 (33%)	3 (±0.5) 3 2-3

4.1.2 Reference Genes for Polymerase Chain Reaction

Two reference genes were used in the reverse transcriptase-PCR reactions. YWHAZ was suggested to be one of the best housekeeping genes for normal/diseased endometrium for qPCR, whilst GAPDH was shown not to perform so well. GAPDH has been shown to be a better housekeeping gene in the fallopian tube than YWHAZ for qPCR. In this study matched endometrium and fallopian tube were investigated and therefore GAPDH and YWHAZ were chosen to produce an efficient normalised ratio of both tissues. Relative expression of the target sequence under study was determined by using two reference genes, resulting in the production of two graphical representations of gene expression. As a result of having two reference genes instead of one, the validity of each of these reference genes could be examined. Observing the expression of BCAM, SOX9 and AR in the samples with both YWHAZ and GAPDH as housekeeping genes – the graphical patterns for all genes were relatively similar. With BCAM it could be seen that after being normalised to both GAPDH and YWHAZ, similar patterns of relative expression were produced – thus proving to be effective reference genes for both fallopian tube and endometrial tissue.

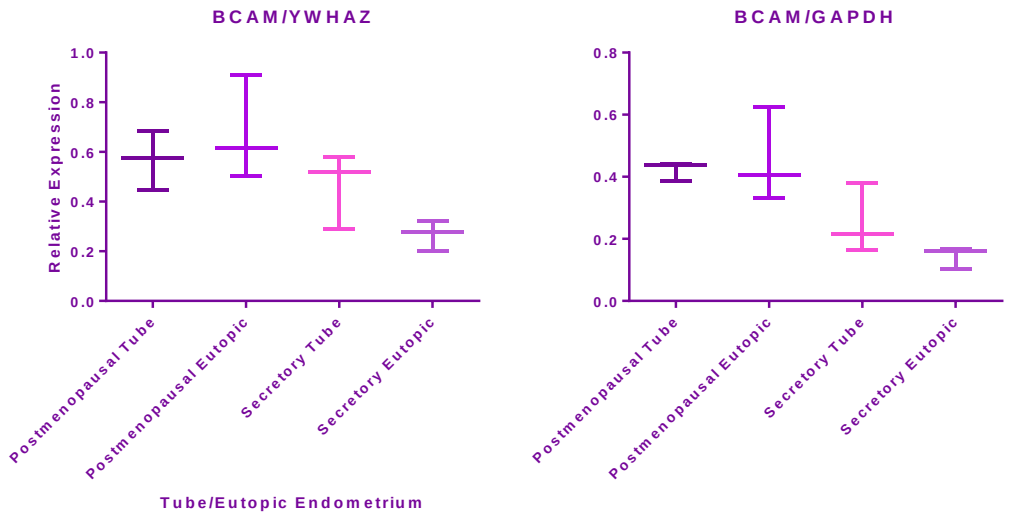


Figure 4-1 - Whisker plot graphs with median, minimum and maximum values for BCAM/ YWHAZ and GAPDH as reference genes. Relative expression values for Postmenopausal fallopian tube (N=3), Postmenopausal eutopic endometrium (N=3), Secretory phase fallopian tube (N=3) and Secretory phase eutopic endometrium (N=3).

Differences between GAPDH and YWHAZ were observed in the SOX9 PCR results. The general trend was similar with lower endometrial secretory phase expression of SOX9 in comparison to the postmenopausal samples and secretory tube however; with YWHAZ as the reference gene it appeared that the median SOX9 mRNA expression in the secretory phase fallopian tube was higher than all other sample sets. Whilst this is strikingly clear on observation; the maximal and minimal expression scale on the box and whisker diagram overlapped with the other sample sets which meant that the relatively higher tubal expression was not statistically significant (P=>0.05). The protein expression of SOX9 shows a stronger expression in the postmenopausal tube in comparison to the secretory tube. With this in mind, the PCR - graph that most depicts the protein expression pattern, is the relative expression of SOX9 with GAPDH as the housekeeping gene. This is because postmenopausal fallopian tube had relatively higher SOX9 expression than the fallopian tube in secretory phase. As a consequence, the GAPDH data for normalisation of RT-PCR for AR, SOX9 and BCAM has been chosen to represent the mRNA expression in fallopian tube and endometrium in the results.

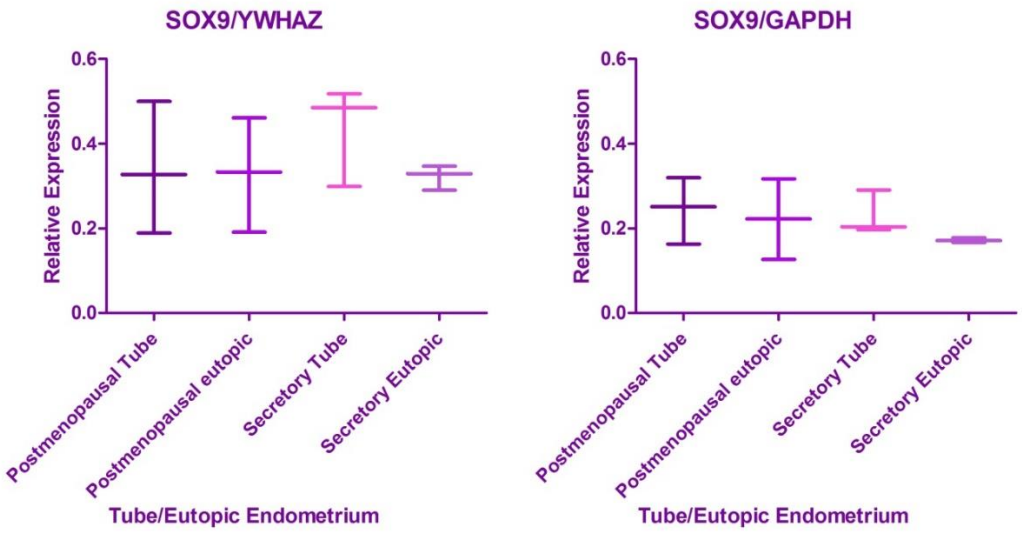


Figure 4-2 - Whisker plot graphs with median, minimum and maximum values for SOX9/ YWHAZ and GAPDH as reference genes. Relative expression values for Postmenopausal fallopian tube (N=3), Postmenopausal eutopic endometrium (N=3), Secretory phase fallopian tube (N=3) and Secretory phase eutopic endometrium (N=3).

4.2 Basalis Markers

4.2.1 Basal Cell Adhesion Molecule (BCAM)

4.2.1.1 Immunohistochemistry

It has been found previously that BCAM is present in the cell junctions; either basolaterally or covering the whole cell surface of epithelial cells using immunohistochemical staining techniques. The study findings regarding this molecule are described below:

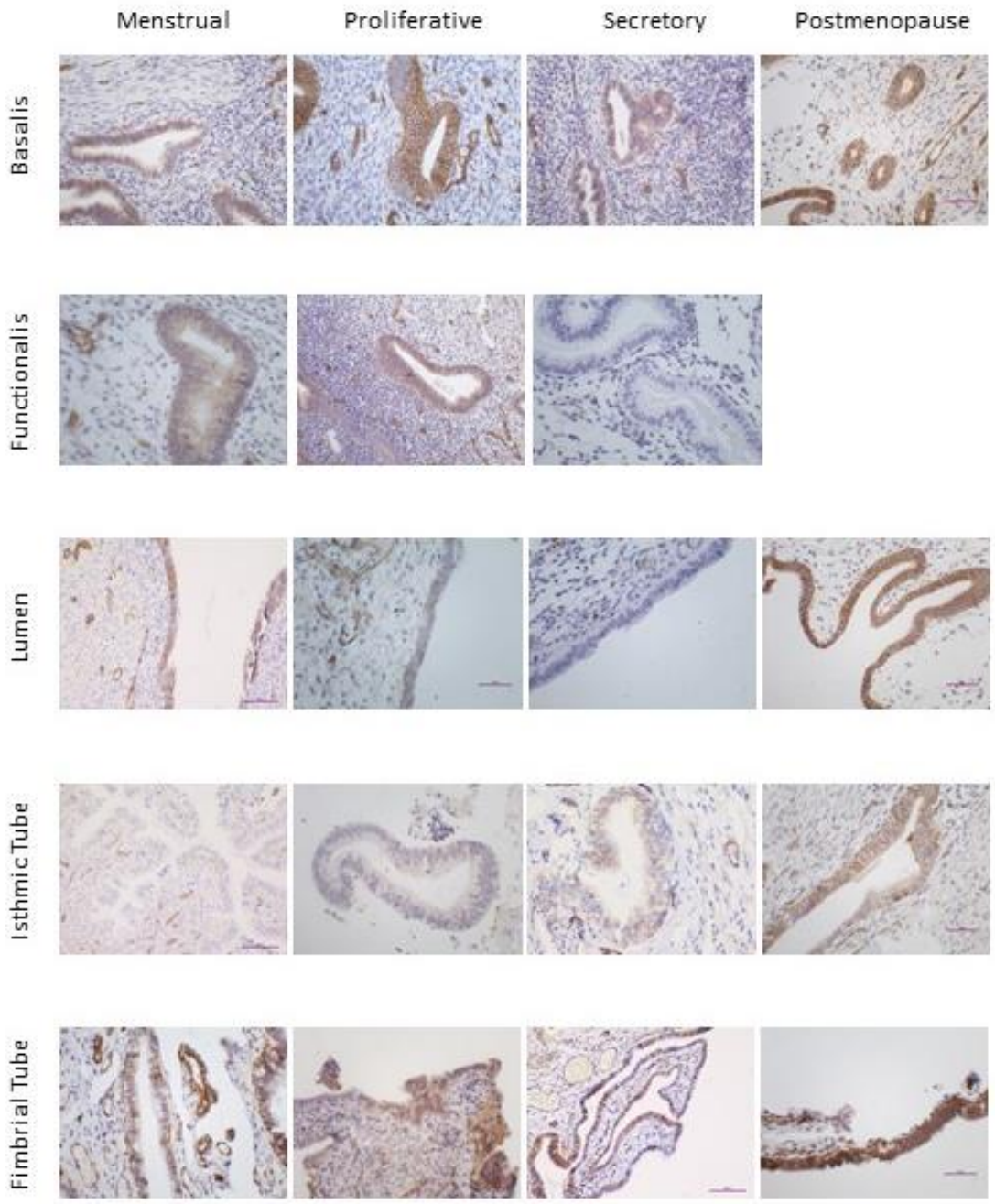


Figure 4-3 – Images for BCAM IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia.

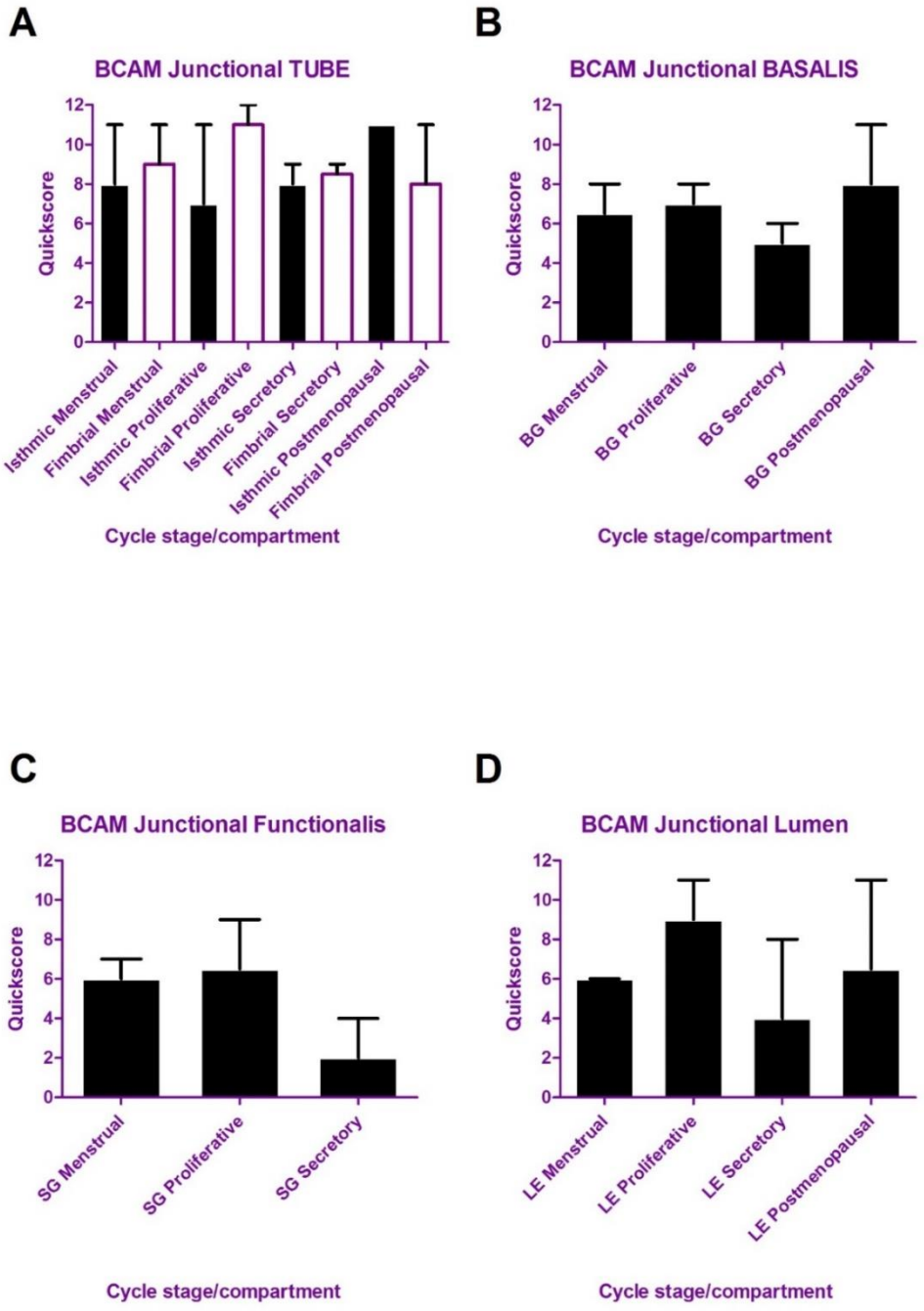


Figure 4-4 – Bar graphs showing median quickscores with range for the immunohistochemical staining of BCAM divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of fallopian tube quickscores with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=3), Isthmic fallopian tube in the secretory phase (N=5), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=1), postmenopausal fimbrial fallopian tube (N=5). B) Bar graph of basal glandular epithelia quickscores with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=5). C) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=6) and secretory phase (N=7). D) Bar graph of luminal epithelia in menstrual phase (N=3), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=4).

Starting with junctional/basal expression of BCAM in the premenopausal fimbrial end of the tube; interestingly the highest expression lies in the proliferative phase of the cycle and there is a drop in junctional staining during the secretory phase (median = 11 and 8.5, respectively). Junctional staining is seen in the postmenopausal fimbrial epithelial cells with expression comparable to that in the secretory phase of the cycle (median = 9 and 8.5 respectively). The isthmic section of the fallopian tube shows a different pattern of BCAM junctional staining across the menstrual cycle. It appears that this molecule is present consistently across the cycle with little variation of expression therefore does not appear to be cycle dependent. There is a sharp increase in junctional/basal BCAM expression however in the postmenopausal isthmic region.

In the basalis layer of the endometrium, epithelial glands showed positivity for BCAM, this was however subject to change throughout the phases of the menstrual cycle. Junctional BCAM expression in the basal glandular compartment was highest in the proliferative phase of the cycling endometrium (MQS=7) and lowest in the secretory phase (MQS=5), a rise in this molecule in the junctions of the endometrial epithelia is seen in the postmenopausal population (MQS=8). Functional glandular epithelia also express BCAM with the same pattern as the basalis but with much weaker expression in the secretory phase (MQS=2). Luminal epithelia show very high junctional levels of BCAM in the proliferative phase (MQS=9) with the same overall pattern as the rest of the endometrium. Postmenopausal lumen has moderate junctional expression reflective of the menstrual phase of cycling endometrium (MQS=6.5).

It appeared that junctional staining of BCAM in the fimbrial end of the fallopian tube had the same pattern of expression throughout the menstrual cycle as the endometrium due to the fact that BCAM increased in the proliferative phase and was lowest in the secretory phase. Also notably, the isthmic region did not vary during the menstrual cycle showing that this region is potentially distinctive from the rest of the distal reproductive tract. High expression of BCAM in the junction is present in both the fimbrial tube and basal glands in postmenopausal women.

Luminal junctional staining is weaker than the tubes and basalis endometrium (MQS=6.5).

4.2.1.2 Distribution of Junctional Staining Between Tissue Compartments

In the endometrial basalis compartment throughout the menstrual cycle the distribution of junctional staining varied greatly. Most samples seem to have a mixture of epithelial cells with basal, basolateral and whole cell staining. A minority of tissue samples had only basolateral staining in the menstrual and proliferative phase. More samples in secretory phase were found to have only basolateral staining (3/6). In the functionalis it was clear that the vast majority of the sample population had whole cell junctional epithelial staining in abundance with only some basal stained epithelia. Interestingly, the luminal epithelium had mostly basolateral staining, particularly the samples in the secretory phase of the menstrual cycle. Although the vast majority of the fallopian tube (isthmic and fimbrial ends) showed basal and basolateral staining regardless of menstrual phase; it is to be noted that the fimbrial end of the fallopian showed a number of samples with whole cell BCAM staining in comparison to 100% of isthmic samples having basal staining of BCAM.

In the postmenopausal basal endometrium all samples in the population showed glands with whole cell junctional staining. The lumen had whole cell staining however; some basolateral staining remained. In contrast to the premenopausal fallopian tube the postmenopausal tube contained more whole cell tubal junctional staining with some basolateral BCAM remaining.

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4.2.1.3 Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR).

Using RT-PCR techniques; BCAM primer was added to each of the samples to investigate the mRNA expression of this molecule.

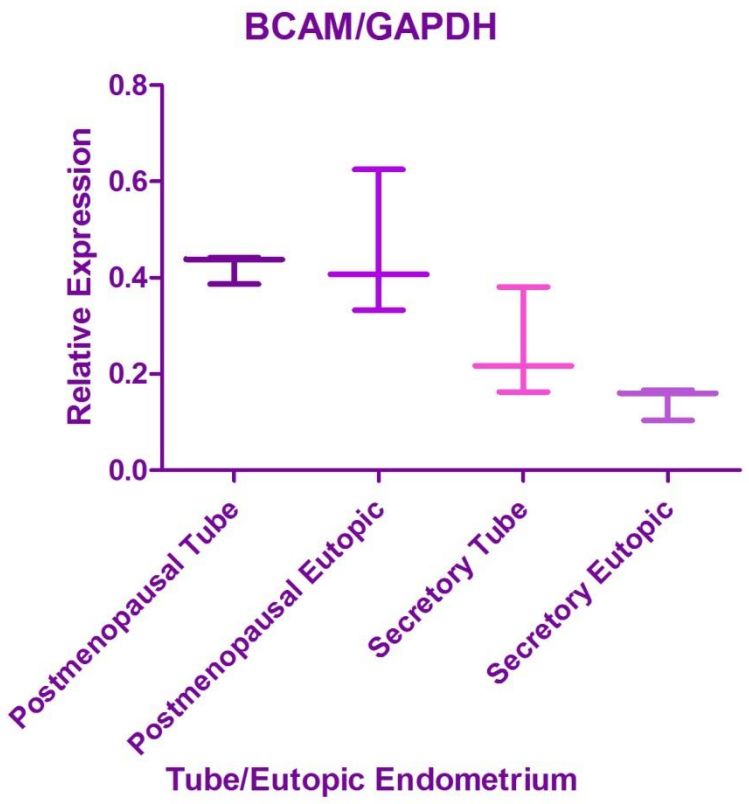


Figure 4-5 – Whisker graph with median and minimum to maximum values showing relative expression of BCAM in N=3 matched postmenopausal eutopic endometrium and fallopian tube samples and N=3 matched secretory phase eutopic endometrium and fallopian tube samples normalised to GAPDH, the reference gene.

BCAM mRNA was expressed most abundantly in the postmenopausal eutopic endometrium closely followed by postmenopausal distal tubal tissue. Secretory phase eutopic endometrium was found to have the lowest mRNA expression overall and secretory phase tube had slightly higher relative expression, but lower than the postmenopausal samples.

After observing the graphical results for both RT-PCR and IHC – it is clear that both expression patterns support each other. Postmenopausal protein and mRNA expression of BCAM in eutopic endometrium was higher than that of the secretory phase protein and mRNA.

4.2.2 (Sex Determining Region Y)-Box 9 (SOX9)

4.2.2.1 Immunohistochemistry

Threshold analysis of expression of SOX9 looked at the percentage of positive nuclear DAB staining over negative haematoxylin staining.

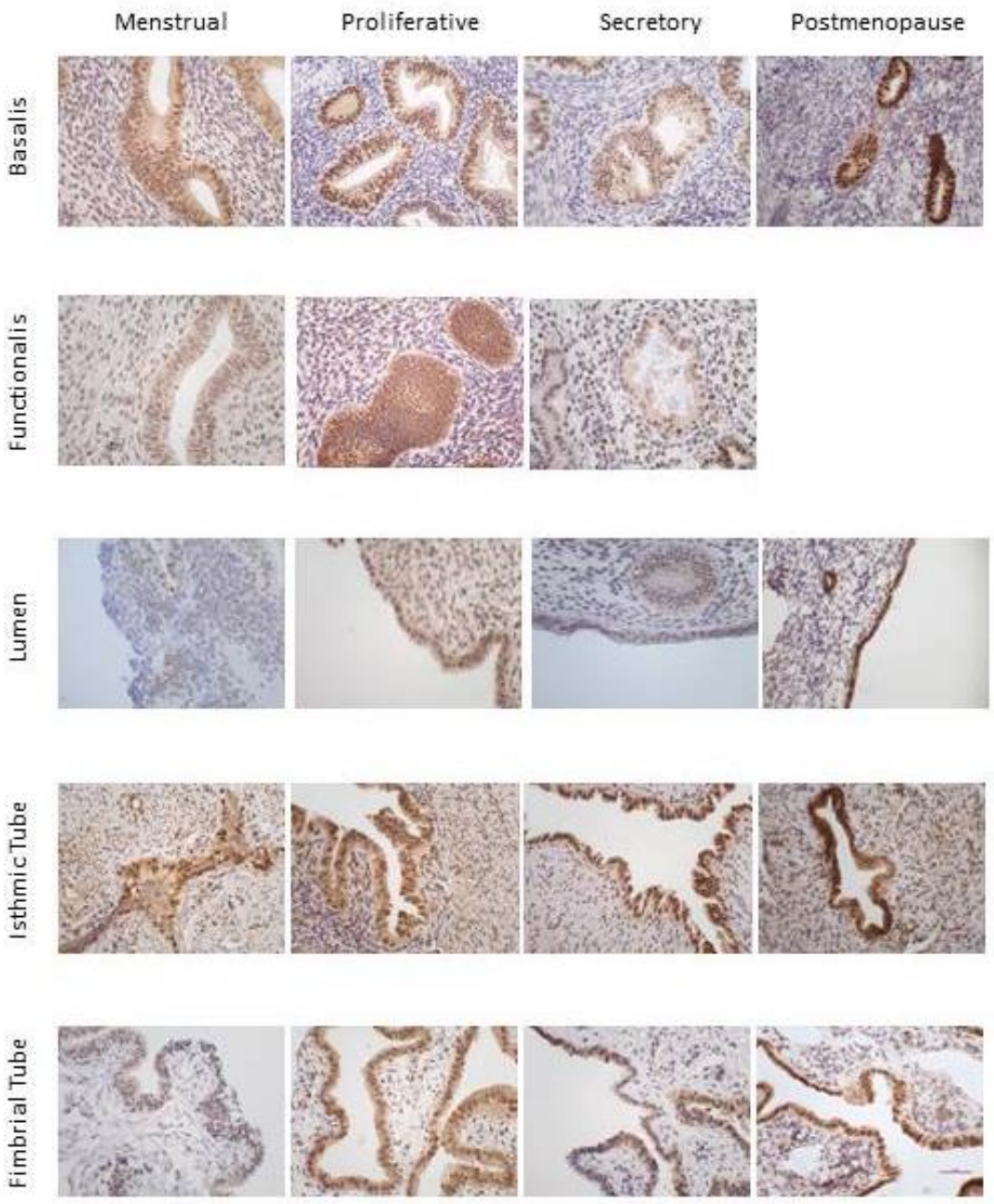


Figure 4-6 - Images for SOX9 IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia

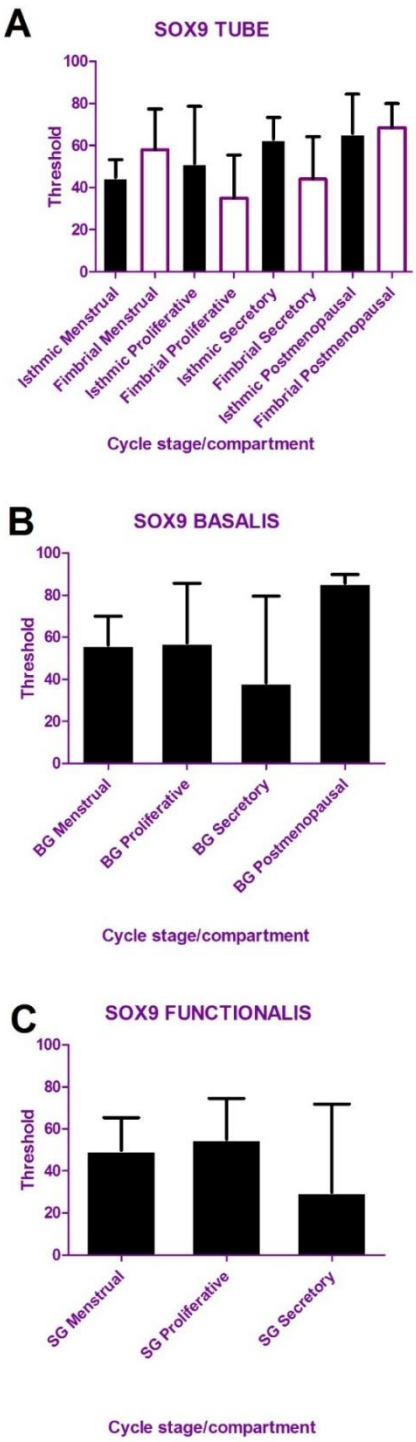


Figure 4-7 – Bar graphs showing median threshold percentage with range for the immunohistochemical staining of SOX9 divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of fallopian tube threshold percentage with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=3), Isthmic fallopian tube in the secretory phase (N=5), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=2), postmenopausal fimbrial fallopian tube (N=5). B) Bar graph of basal glandular epithelia threshold percentage with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=5). C) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=5) and secretory phase (N=6).

Beginning in the fallopian tube, the fimbrial end had varying patterns of expression throughout the menstrual cycle. The highest median threshold percentage (MTP) was found to be during the menstrual phase (58 %). The proliferative phase saw a decrease in nuclear SOX9 (MTP 35%) and a subsequent small increase in expression in the secretory phase (MTP 44 %). An increase in SOX9 is seen to occur in the postmenopausal distal tubal epithelium (MTP 76 %). Interestingly it seems as though the expression of SOX9 increases in a step-wise manner in the isthmic end of the fallopian tube from the beginning to the end of the menstrual cycle with the most abundantly positive staining occurring in samples collected during the female's secretory phase (MTP 63%). A small increase in SOX9 expression is apparent after the menopause (MTP 66 %).

The endometrial basal glands have positive SOX9 staining throughout the menstrual cycle however during the secretory phase – there is a decrease in MTP in comparison to the other two phases (MTP 38 %). No difference in nuclear SOX9 was observed between the menstrual and proliferative phases. Postmenopausal endometrium had very strong positive SOX9 expression in the basalis (MTP 86 %). The functionalis' superficial glands had a very similar pattern of expression throughout the menstrual cycle to the basalis. A small 5% increase in MTP was seen to occur during the proliferative phase in functional glands, however. A dramatic diminution of SOX9 was observed during the secretory phase (MTP 29 %).

On comparison of all compartments the isthmic end of the tube seemed to have its own unique variation of expression of SOX9 throughout the menstrual cycle. Both tubal regions however did see an increase in expression of SOX9 protein expression in the secretory phase; an observation that is the opposite of what was revealed in the endometrium. As stated above - the superficial and basal endometrial compartments mirror each other, with the functionalis compartment's SOX9 expression appearing to be at a lower level if displayed graphically. Even though the infundibular end of the tube was seen to increase its SOX9 protein expression after proliferative phase the MTP was similar to that of the endometrial layers. The most unanimous finding between the endometrium and fallopian tubal regions was

that - postmenopausal SOX9 protein expression increased to high MTP levels; exceeding that of cycling endometrium and fallopian tube.

4.2.2.2 RT-PCR mRNA expression of SOX9

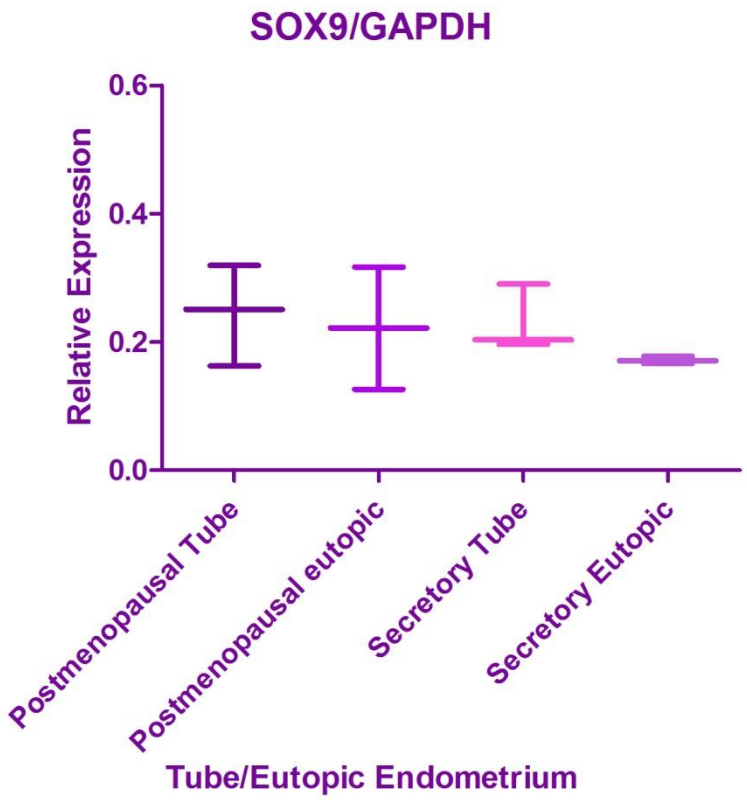


Figure 4-8 - Whisker graph with median and minimum to maximum values showing relative expression of SOX9 in N=3 matched postmenopausal eutopic endometrium and fallopian tube samples and N=3 matched secretory phase eutopic endometrium and fallopian tube samples normalised to GAPDH, the reference gene.

The densitometry data collected for the PCR mRNA expression of SOX9 displays evidence that postmenopausal expression of SOX9 in the eutopic endometrial and fimbrial tubal samples are the same. Secretory phase distal fallopian tube had stronger mRNA levels of SOX9. The secretory endometrium showed expression comparative to that of the postmenopausal tissue.

Although subtle, the expression of SOX9 relative to GAPDH is higher in the postmenopausal eutopic endometrium and fimbrial fallopian tube than the secretory phase tissue. This reflects the IHC results and supports the conclusion drawn from the IHC data that SOX9 is higher in endometrium and fallopian tube when in the postmenopausal state.

4.2.3 Stage Specific Embryonic Antigen-1 (SSEA-1)

4.2.3.1 Immunohistochemistry

As described in previous chapters, SSEA-1 is a cluster of differentiation molecule. It is present on the surface of cells and its expression can be measured using the quickscore method taking into account the overall percentage of positive staining and the distribution of intensity of this staining throughout the tissue compartment.

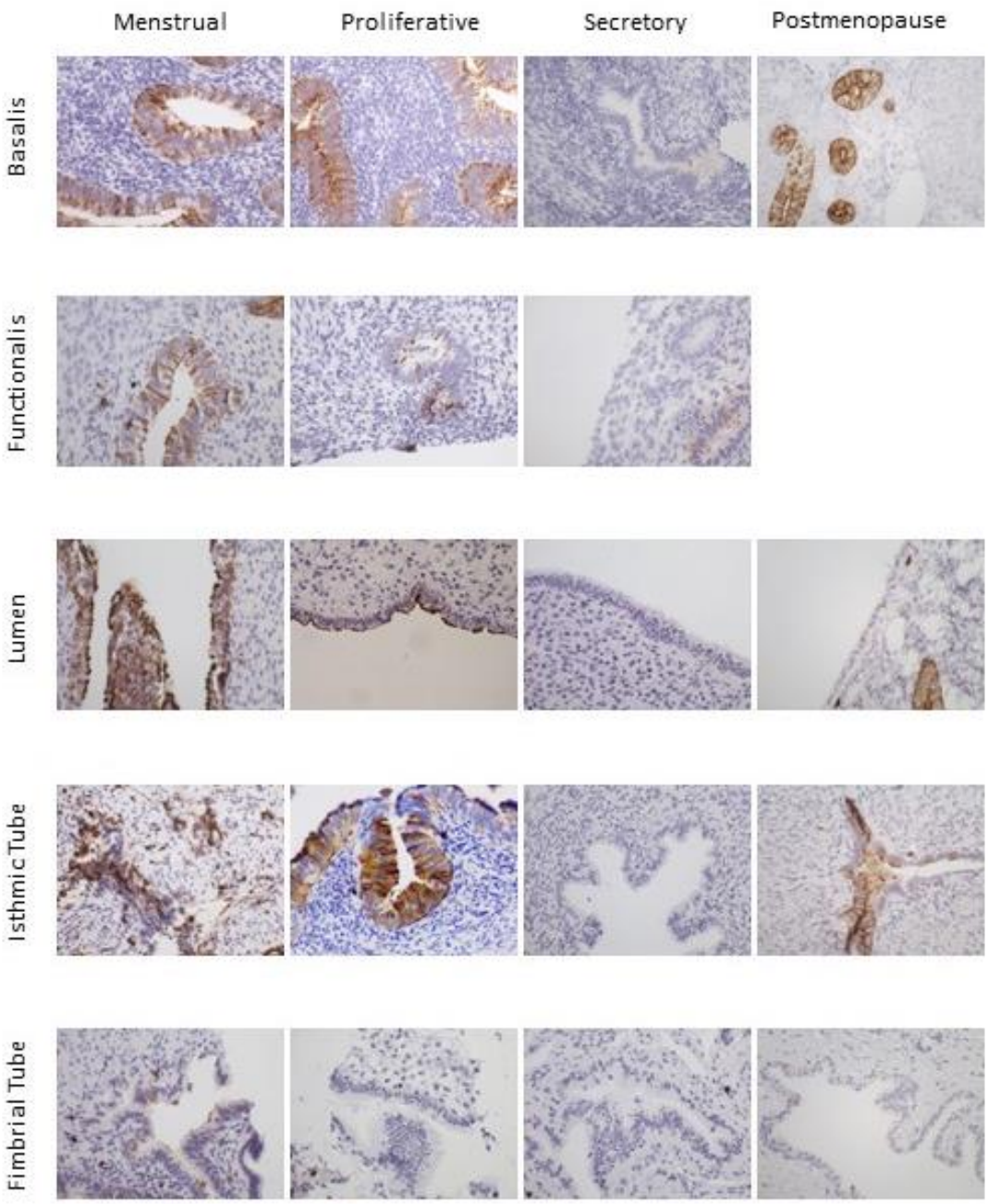


Figure 4-9 - Images for SSEA-1 IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia.

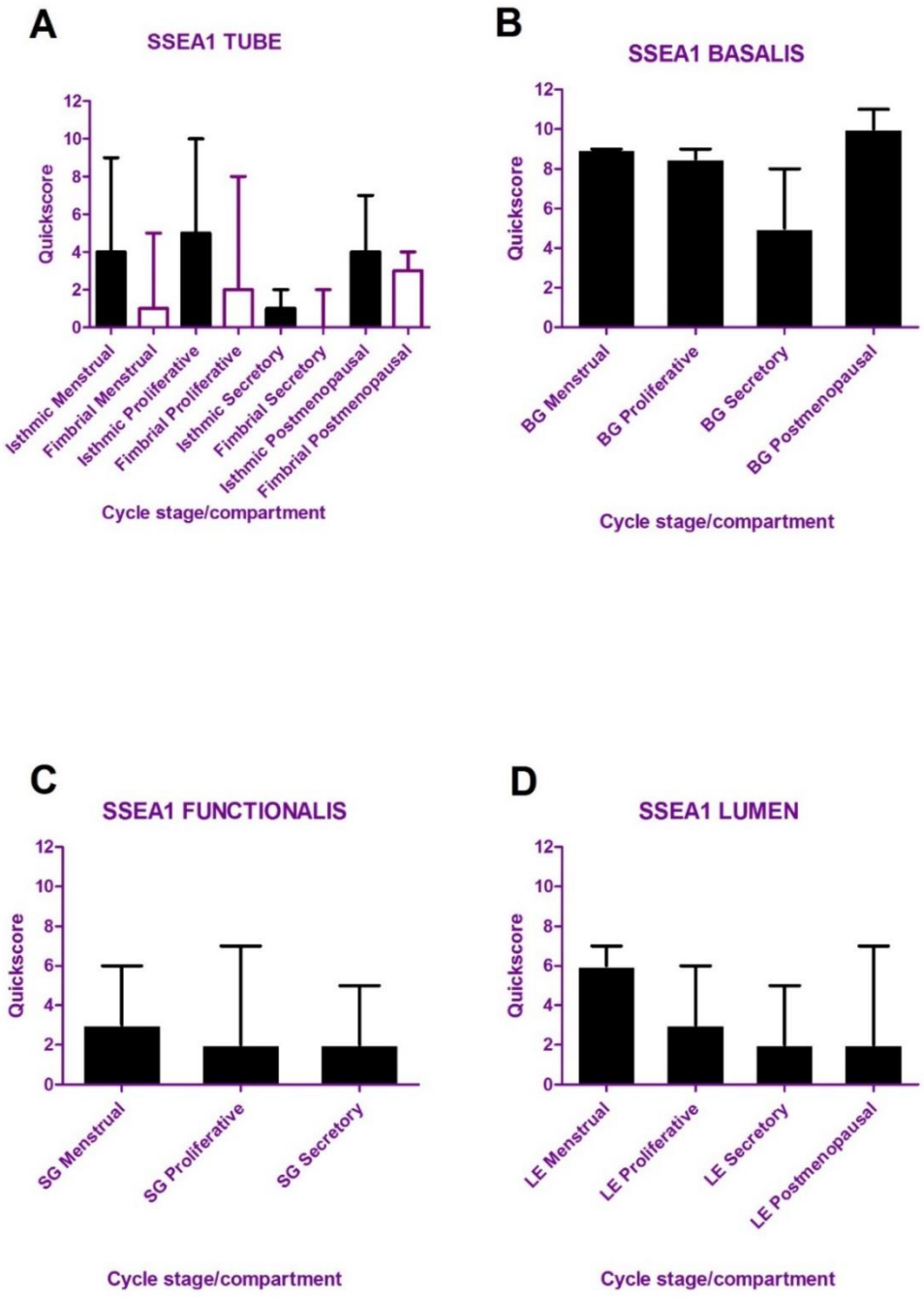


Figure 4-10 - Bar graphs showing median quickscores with range for the immunohistochemical staining of SSEA-1 divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of fallopian tube quickscores with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=3), Isthmic fallopian tube in the secretory phase (N=5), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=2), postmenopausal fimbrial fallopian tube (N=5). B) Bar graph of basal glandular epithelia quickscores with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=5). C) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=5) and secretory phase (N=7). D) Bar graph of luminal epithelia in menstrual phase (N=3), proliferative phase (N=5), secretory phase (N=7) and postmenopause (N=4).

Beginning at the fimbrial end of the fallopian tube, SSEA-1 is present but with low expression. Cyclical variation was seen to occur in this region with low expression in the menstrual phase and an increase during the proliferative phase. No SSEA-1 was seen to be expressed during the secretory phase. In comparison to the cycling endometrium, the SSEA-1 expression in the postmenopausal population increased. The isthmic region of the fallopian tube was observed to have exactly the same pattern as described above but at a higher overall expression level than the fimbrial tube Median Quickscores (MQ) of 4,5,1,4 and 1,2,0,3 respectively.

The endometrium was seen to vary according to tissue compartment greatly. High expression of SSEA-1 existed within the basal epithelia although there was cyclical variation. The menstrual and proliferative phases have high expression of this molecule and a decrease is then seen in the secretory phase (MQ = 5). Postmenopausal SSEA-1 in basal epithelia is very high (MQ = 10). The Functionalis and Luminal layers showed a different pattern of expression with lower expression reflected in the quickscore analysis. Although little variation between the menstrual phases existed there seemed to be a slightly higher expression of this molecule in the menstrual population and lower expressions of SSEA-1 in the proliferative and secretory phases. The lumen had low expression of SSEA-1 in the postmenopausal period (MQ = 2.5)

On comparison of the expression patterns in the different tissue compartments, the tubes had a similar expression pattern throughout the menstrual phases to the basalis compartment of the endometrium however, at a much lower level than the basal glands. The quickscores observed in this tissue where in the same region as the functionalis layer of the endometrium - but with a pattern that reflected the changes seen in the basalis. The superficial glandular epithelia and the luminal epithelia had a similar downward trend throughout the cycling endometrium and the luminal epithelia maintained this low expression after the menopause; unlike the basal glands which demonstrated a high SSEA-1 expression (MQ = 2.5 and 10, respectively).

4.2.4 Beta Catenin

Beta catenin, a key part of the canonical Wnt pathway (as explained previously) can be found in the nuclei, cytoplasm and junctions of cells.

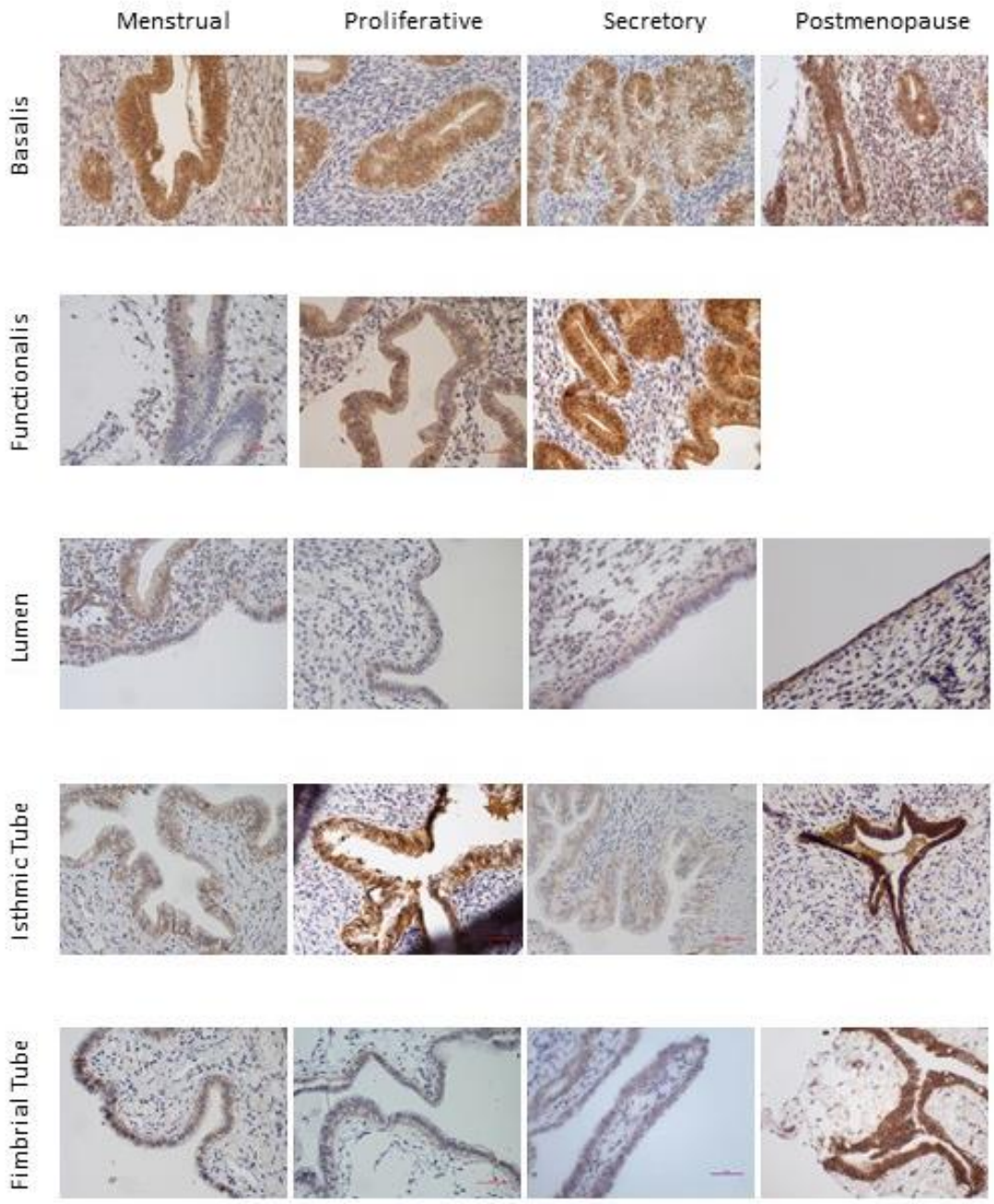


Figure 4-11 - Images for Beta Catenin IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia.

4.2.4.1 Immunohistochemistry (Nuclear)

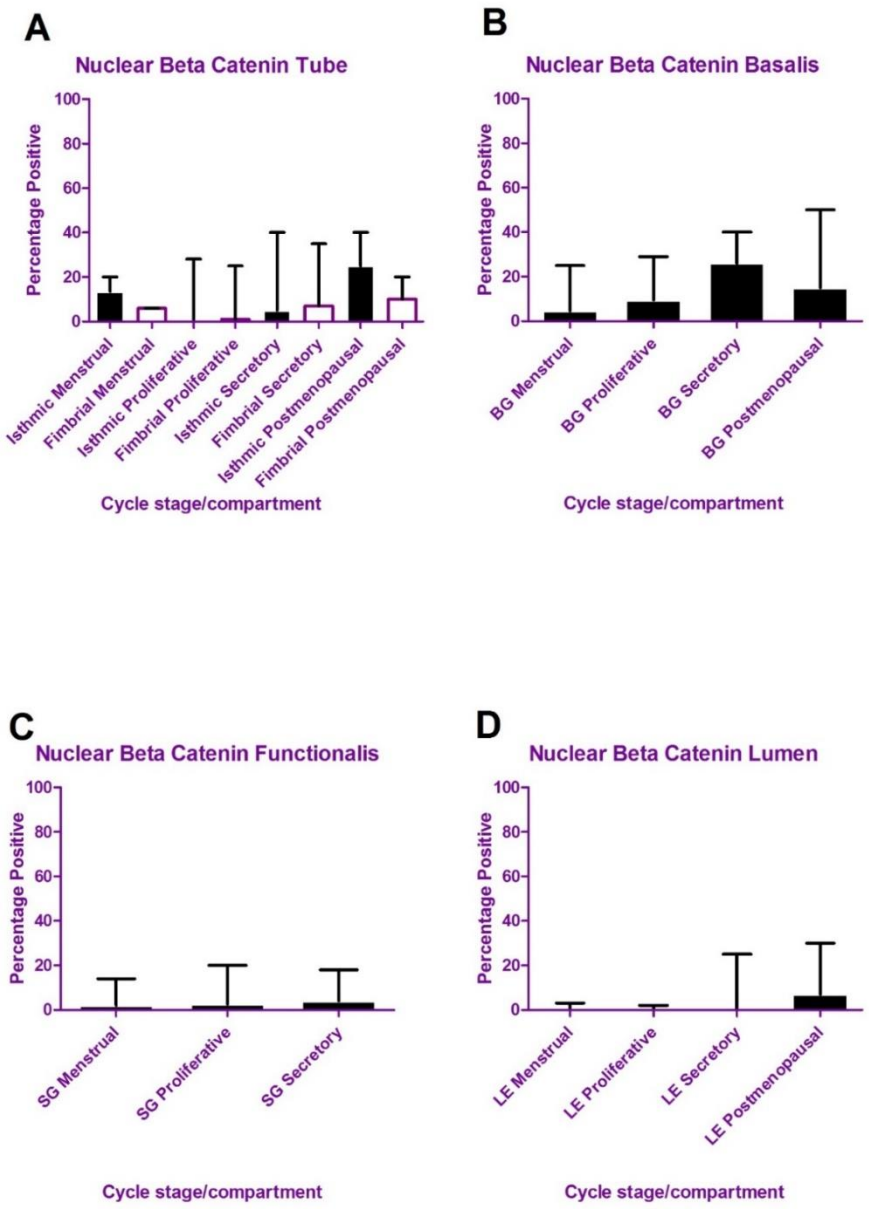


Figure 4-12 - Bar graphs showing median percentage positive with range for the immunohistochemical staining of Nuclear Beta Catenin divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of fallopian tube percentage positive nuclei with: Isthmic fallopian tube in the menstrual phase (N=2), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=3), Isthmic fallopian tube in the secretory phase (N=5), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=2), postmenopausal fimbrial fallopian tube (N=5). B) Bar graph of basal glandular epithelia percentage positive nuclei with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=5). C) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=6) and secretory phase (N=7). D) Bar graph of luminal epithelia in menstrual phase (N=3), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=4).

The nuclear positive staining for beta catenin was very low throughout both the tubes and endometrium however; there was some positivity and some variation between cycle stages that can be commented on. The distal fallopian tube had the lowest percentage positive nuclear beta catenin in the proliferative phase of the cycling population with a slightly higher expression in the secretory phase. The postmenopausal population had a median positive beta catenin of 10% - this being higher than the premenopausal population. More proximally at the isthmic end of the tube greater variability could be seen. The lowest expression of beta catenin also occurred during the proliferative phase and the highest expression was seen in the menstrual phase population. Secretory phase of the menstrual cycle saw a slight increase in expression of nuclear beta catenin but by no means as abundant as the menstrual phase population. Like the fimbrial end, increased percentage positivity was seen in the postmenopausal isthmic tube (Median = 25 %).

The endometrium has great compartmental variability. Starting in the basalis endometrial glands from median percentage positive scores it seems as though nuclear beta catenin increases in a step-wise manner throughout the menstrual cycle; with the lowest expression in the menstrual phase and the highest expression in the secretory phase (median 26 %). Postmenopausal basal epithelia also expressed nuclear beta catenin but at a low level (median 15%). The superficial glandular epithelia had consistently low nuclear beta catenin throughout the menstrual cycle with little variability. The luminal epithelia had negative nuclear beta catenin staining in the menstrual, proliferative and secretory phases of the cycle (median 0 %). The postmenopausal lumen had more nuclear positivity (median 7%) than the cycling endometrium but lower than the basalis region (median 15 %).

On comparison of the tissues and compartments there is some variability seen. The tubes share a similar pattern of beta catenin expression throughout the menstrual cycle however; the basalis epithelial glands express nuclear beta catenin in a different pattern. As has been described the glands increase their nuclear beta catenin in a step-wise manner throughout the cycle with the highest positivity being in the secretory phase. The tubes show their highest expression in the menstrual

and secretory phases and the lowest nuclear beta catenin in the proliferative phase. The basalis glandular epithelia also had an overall higher expression of nuclear beta catenin than the tubes and other compartments of the endometrium. The postmenopausal expression of nuclear beta catenin in the basalis was also slightly higher than the fimbrial tube (15 and 10 %, respectively). The highest beta catenin expression in the postmenopausal population was seen in the isthmic tube (median 25 %). The superficial epithelia had very low expression of nuclear beta catenin and in contrast to the tubes and basalis, showed little variability through the cycle. Luminal epithelia had absent nuclear beta catenin throughout the whole menstrual cycle with no variability. The negligible percentage positive nuclei in the lumen are similar to the superficial glandular epithelia.

4.2.4.2 Immunohistochemistry (Cytoplasmic)

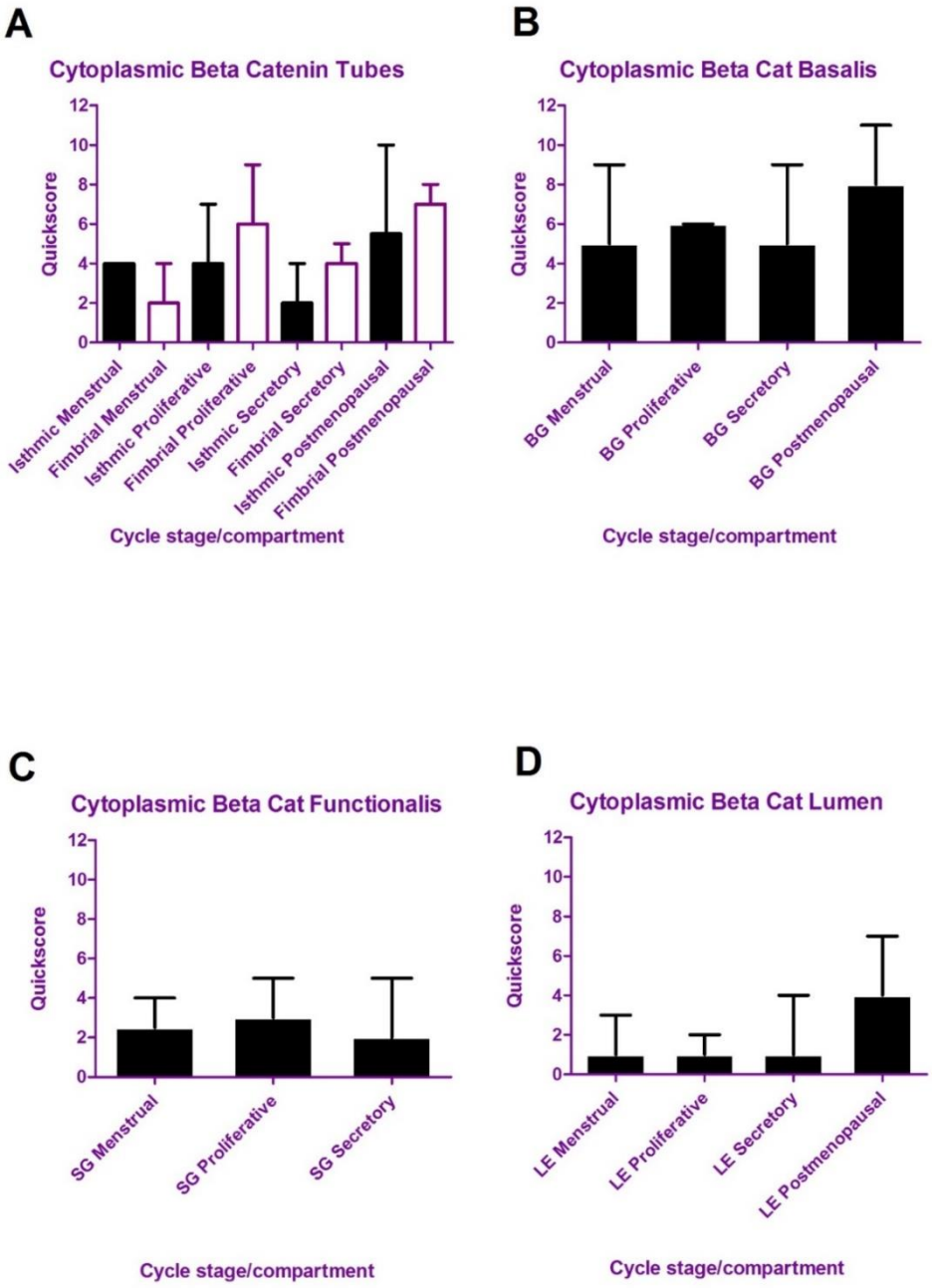


Figure 4-13 - Bar graphs showing median quickscores with range for the immunohistochemical staining of Cytoplasmic Beta Catenin divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of fallopian tube quickscores with: Isthmic fallopian tube in the menstrual phase (N=2), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=5), Fimbrial fallopian tube in the proliferative phase (N=3), Isthmic fallopian tube in the secretory phase (N=5), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=2), postmenopausal fimbrial fallopian tube (N=5). B) Bar graph of basal glandular epithelia quickscores with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=5). C) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=6) and secretory phase (N=7). D) Bar graph of luminal epithelia in menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=4).

The cytoplasmic presence of beta catenin was moderately expressed in fallopian tubes and eutopic endometrium. Cyclical variation occurred in all tissues. The fimbrial fallopian tube expressed cytoplasmic beta catenin most abundantly during the proliferative phase of the menstrual cycle (MQ = 6). The weakest presence of beta catenin in the cytoplasm occurred during the menstrual phase (MQ = 2). Isthmic fallopian tube (proximal) had lowest expression of beta catenin during the secretory phase of the menstrual cycle (MQ = 2). Both the menstrual and proliferative phases had moderate cytoplasmic beta catenin presence. Postmenopausal cytoplasmic beta catenin is moderate in both the proximal and distal ends (MQ = 5.5 and 7, respectively).

In matched eutopic endometrium; the basal epithelial cells also had moderate expression of cytoplasmic beta catenin. Cyclical variation was subtle but a slight increase during the proliferative phase was observed (MQ = 6). The functional glandular epithelia were noticeably lower than the other compartments with median quickscores of 3 or lower. Little cyclical variation occurred however; proliferating glands had a slight increase in expression of beta catenin (MQ = 3). The luminal epithelial cytoplasmic presence of beta catenin was low with no cyclical variation (MQ = 1). Postmenopausal cytoplasmic beta catenin was high in basal glandular epithelia (MQ = 8). Moderate luminal expression of beta catenin was observed (MQ = 4).

On comparison between the two tissues and compartments both basal glandular epithelia and tubal epithelia showed moderate expression of cytoplasmic beta catenin throughout. The fimbrial fallopian tube and basal glandular epithelia had the same expression of beta catenin during the proliferative phase. The isthmic tube had lowest expression during the secretory phase whereas fimbrial tube had weaker expression during the menstrual phase. Low expression of beta catenin was observed in the functional epithelial glands and luminal epithelia with little cyclical variation. Overall cyclical variation of beta catenin throughout the menstrual cycle was less obvious than seen in the tubal epithelia. Postmenopausal expression was higher in all tissues and compartments. Highest expression of cytoplasmic beta catenin was observed in the basal glands, closely followed by the fimbrial epithelia

(MQ = 8 and 7, respectively). Both proximal tubal epithelia and lumen had moderate expression of this molecule in the postmenopausal sample population.

4.3 Luminal Surface marker

4.3.1 Sialylated SSEA-1 (Sialyl SSEA-1)

4.3.1.1 Immunohistochemistry

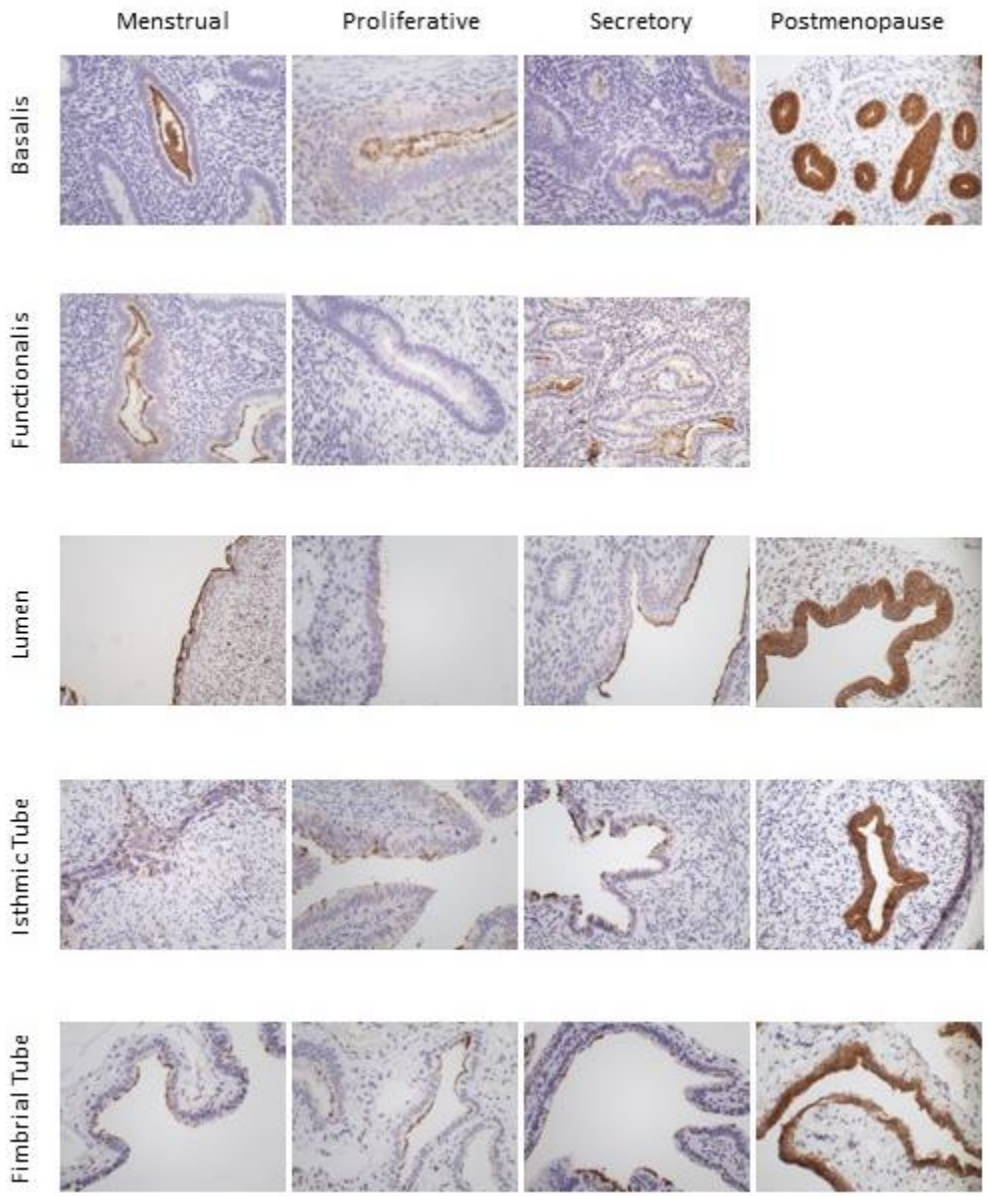


Figure 4-14 - Images for Sialylated SSEA-1 IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia.

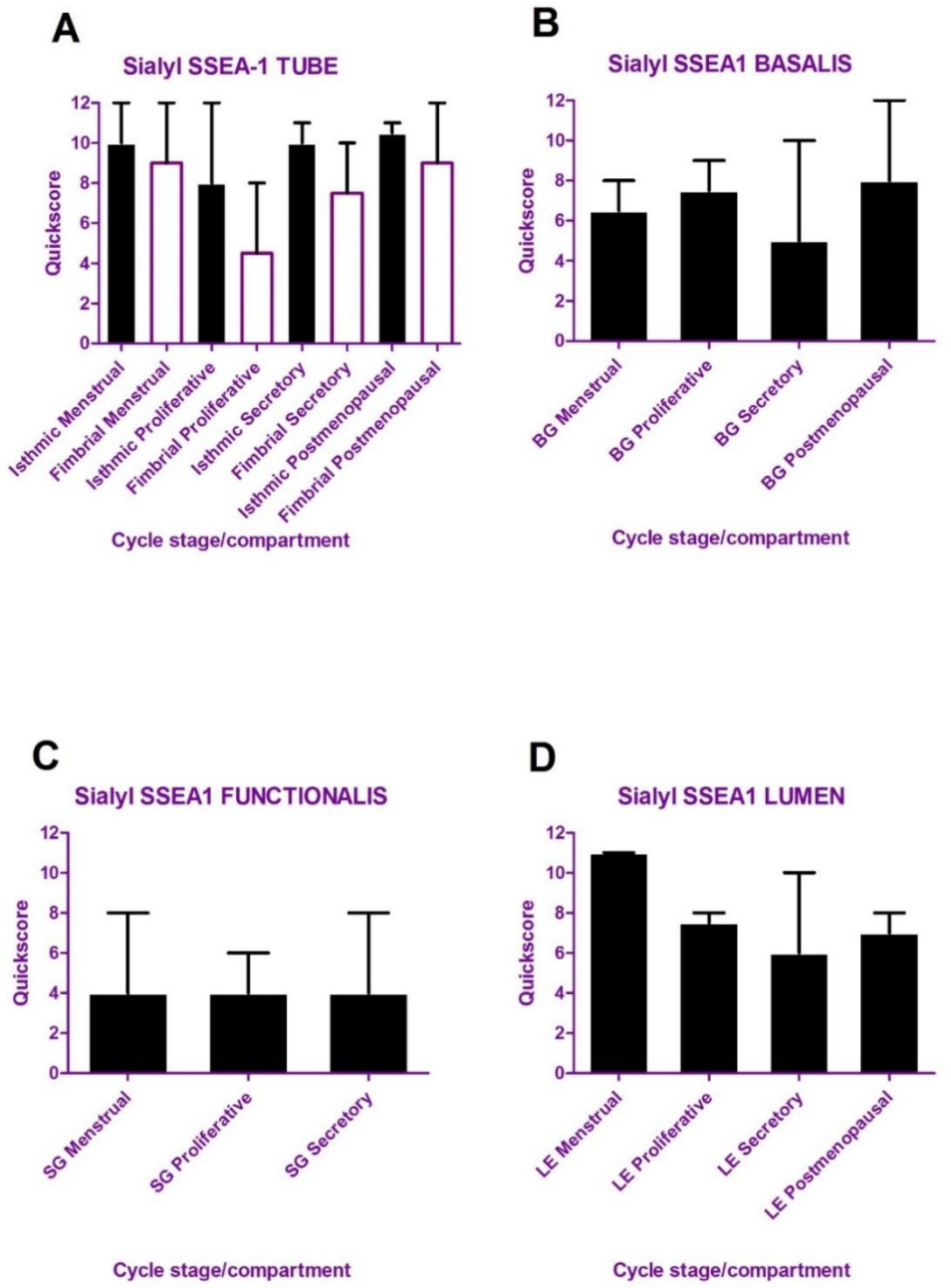


Figure 4-15 - Bar graphs showing median quickscores with range for the immunohistochemical staining of Sialylated SSEA-1 divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of fallopian tube quickscores with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=2), Isthmic fallopian tube in the secretory phase (N=5), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=2), postmenopausal fimbrial fallopian tube (N=5). B) Bar graph of basal glandular epithelia quickscores with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=5). C) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=5) and secretory phase (N=7). D) Bar graph of luminal epithelia in menstrual phase (N=3), proliferative phase (N=4), secretory phase (N=7) and postmenopause (N=4).

The sialylated form of SSEA-1 is abundantly expressed in the human fallopian tubes. Starting with the fimbrial epithelia, strong expression of Sialyl SSEA-1 existed on the surface of the cells. The expression of this molecule goes down in the proliferative phase with the highest levels being in the menstrual phase and the lowest in the proliferative phase (MQ = 9 and 4.5, respectively). This surface marker is expressed very strongly at the isthmic end of the fallopian tube. It seems to follow the same expression pattern as the fimbrial tube but at a higher expression level. Lower expression is during the proliferative phase (MQ = 8) and higher expression is seen in the menstrual and secretory phases (MQ = 10). Postmenopausal expression of Sialyl SSEA-1 is very high with the MQ of the fimbrial epithelia being 9 and the MQ at the isthmic end being 10.5 therefore increasing more proximally. The overall expression at the isthmic end of the tube was generally higher in the premenopausal population.

The basalis endometrial glands have a moderate expression of Sialyl SSEA-1 with the highest expression being in the proliferative phase and lowest expression in the secretory phase of the menstrual cycle (MQ = 7.5 and 5, respectively). The postmenopausal levels of Sialyl SSEA-1 reflect that of the proliferative phase of the cycling endometrium i.e. there is relatively strong expression in this population group (MQ = 8). The expression of Sialyl SSEA-1 is more weakly expressed in the superficial glandular epithelia. The lowest expression is during the proliferative phase and slightly higher expression can be seen in the menstrual and secretory phases but the quickscores remain low throughout (MQ = 3 and 4, respectively). Interestingly the luminal epithelia strongly express this molecule. The expression pattern varies greatly with the strongest surface staining being in the menstrual phase and then decreasing until the secretory phase where the expression is lowest (MQ = 11 and 6, respectively). The postmenopausal lumen had moderate expression of this molecule with an MQ of 7; a median quickscore that is close to the basalis endometrium therefore suggesting a lack of phenotypic variability between the two layers during this time.

The comparison of the tissues shows that - whilst expression is apparent in the basalis compartment of the endometrium, the strongest Sialyl SSEA-1 was present

in the luminal epithelia. The high quickscore values given across the menstrual phases and postmenopausal tissue of the luminal epithelia are very similar to that of the isthmic and fimbrial tube. Although the isthmic tubal epithelia had a strong presence of Sialyl SSEA-1 – the pattern of expression seemed to negatively correlate throughout the cycle with the fimbrial and luminal epithelia. The fimbrial tube had the same pattern of variable expression throughout the menstrual cycle as the lumen. The difference in expression between the phases however; was more subtle in the fimbrial tube than the lumen. The weakest expression of Sialyl SSEA-1 was on the surface of the superficial glandular epithelia. Basal glandular epithelia has the same strong expression of Sialyl SSEA-1 in the proliferative phase as the luminal and fimbrial epithelia (MQ = 8). In the postmenopausal population the fallopian tube seems to have stronger expression of Sialyl SSEA-1 than the endometrium overall.

4.2 Summary of Results and Discussion Points

4.2.1 Comparison between the endometrial basalis epithelium and fallopian tubal epithelium

The results have been explained in detail above however, there are some salient points that will be summarised.

An observation from the results was that the distal tubal epithelia and endometrial basalis epithelia have the same cyclical pattern of BCAM expression. Notably, more whole cell surface staining was observed in these regions suggesting less engagement with laminin.

Table 4-4 – Table demonstrating the quickscore levels of BCAM represented by the symbol + in distal fallopian tube and basal glandular epithelia. The key below explains the corresponding expression levels according to the results shown in graphs in figure 4-4.

KEY	
Quickscore Value/Percentage Positive	Representative Symbol for each value (+)
>0 ≤3 OR >0% ≤25%	+
>3 ≤6 OR >25% ≤50%	++
>6 ≤9 OR >50% ≤75%	+++
>9 ≤12 OR >75% ≤100%	++++

BCAM	Fimbrial Fallopian Tubal Epithelia	Endometrial Basalis Epithelia
Menstrual Phase	+++	+++
Proliferative Phase	++++	+++
Secretory Phase	+++	++
Postmenopausal	+++	+++

It is apparent from the results that SSEA-1, SOX9 and cytoplasmic/ nuclear beta catenin were expressed throughout the menstrual cycle in the endometrial basalis epithelium. Highest expression was seen in the postmenopausal samples. It can also be seen that the same is true for both the isthmic and fimbrial portions of the fallopian tubal epithelium for the expression of SOX9 and cytoplasmic beta catenin. Generally however, SSEA-1 expression was lower than the endometrial basalis.

Table 4-5 – Tables demonstrating the quickscore and percentage positive levels of SSEA-1, cytoplasmic beta catenin and SOX9 represented by the symbol + in fallopian tube and basal glandular epithelia. The key above explains the corresponding expression levels according to the results shown in graphs in figures 4-7, 4-10 and 4-13.

SSEA-1			
	Isthmic Fallopian Tubal Epithelia	Fimbrial Fallopian Tubal Epithelia	Endometrial Basalis Epithelia
Menstrual Phase	++	+	+++
Proliferative Phase	++	+	+++
Secretory Phase	+	-	++
Postmenopausal	++	+	+++

Cytoplasmic Beta Catenin			
	Isthmic Fallopian Tubal Epithelia	Fimbrial Fallopian Tubal Epithelia	Endometrial Basalis Epithelia
Menstrual Phase	++	+	++
Proliferative Phase	++	++	++
Secretory Phase	+	++	++
Postmenopausal	++	+++	+++

SOX9			
	Isthmic Fallopian Tubal Epithelia	Fimbrial Fallopian Tubal Epithelia	Endometrial Basalis Epithelia
Menstrual Phase	++	+++	+++
Proliferative Phase	++	++	+++

Secretory Phase	+++	++	++
Postmenopausal	+++	+++	++++

The results above suggest that the postmenopausal endometrium retains a basalis phenotype and fallopian tubal epithelia show an increase in some basalis markers, postmenopause.

Sialylated SSEA-1 was high in the fallopian tubal epithelia, particularly in the isthmic portion of the fallopian tube. This is a discussion point for chapter 7. It may suggest that there is a unique functional role for the carbohydrate ligand on the antigen in the epithelial cells of the proximal fallopian tube, possibly playing a part in the creation of a sperm reservoir.

Table 4-6 – Table demonstrating the quickscore levels of Sialylated SSEA-1 represented by the symbol + in isthmic and fimbrial fallopian tubal epithelia. The key above explains the corresponding expression levels according to the results shown in figure 4-15.

Sialylated SSEA-1	Isthmic Fallopian Tubal Epithelia	Fimbrial Fallopian Epithelia
Menstrual Phase	++++	+++
Proliferative Phase	+++	++
Secretory Phase	++++	+++
Postmenopausal	++++	+++

Chapter 5 Investigating Sex Steroid Hormone Receptor Expression and Proliferation in Human Fallopian Tube and Endometrium

5.1 Introduction

Endometrium is the main target organ for ovarian steroid hormones. These hormones induce their effects on the endometrium via similar receptors. The steroid hormone receptors are: Oestrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR). Endometrial expression of the steroid hormone receptors has been investigated in some detail. Expression of the steroid receptors vary throughout the menstrual cycle in response to the fluctuating steroid hormones in the circulation.

There are 2 main isoforms of oestrogen receptors, namely ER alpha and ER beta – the effect of E2 via ER alpha particularly affects proliferation of the endometrium. The ESR1 gene encodes for ER alpha and the ESR2 gene encodes for ER beta and they are located on different chromosomes.[45] ER alpha is more highly expressed in glandular and stromal cells of the endometrium than ER beta. ER beta is thought to play a direct inhibitory role in the glandular epithelia of the endometrium.[46] Both receptors were found to decrease during the secretory phase of the menstrual cycle. In all cells of the endometrium, ER expression reaches its maximum expression in the late proliferative phase of the menstrual cycle suggesting the maximum E2 effect at that particular time in the cycle, which also correlates with high proliferative activity. Brandenburger et al. Found that E2 working via ER alpha up-regulates PR expression.[47] Moderate expression of ER is present in glands and stroma in the postmenopausal endometrium.[49]

PR has two isoforms originating from the same gene – PR-A and PR-B. There are both direct and indirect routes of action of progesterone on the endometrial epithelium. Progesterone may act directly on the glandular epithelia via epithelial PR and indirectly through the action on stromal PR thereby inducing a variety of

growth factors that act on the epithelial cells of the endometrium. Expression of PR in the epithelium tends to increase during the proliferative phase and decrease in the mid-secretory phase, when progesterone is highest. Progesterone down-regulates its own receptor. Stromal PR expression remains consistently high throughout the menstrual cycle.[40] After menopause, it has been reported that moderate glandular expression of PR is present but weak expression is evident in the stromal compartment.[49] The particular antibody clone used in this study recognised both PR-A and PR-B isoforms.

Studies have shown that androgen receptor is predominantly located in the stroma of the endometrium and the receptor responds to the cyclical variation of the circulating hormone showing an increase AR expression in the proliferative and secretory phases of the menstrual cycle.[31] This receptor has been reported to persist in the stroma of the endometrium, after menopause.[32]

Although the general consensus is that the steroid receptor expression in the endometrium is fully characterised, the existing evidence is very limited for the details of steroid receptor expression in functionally very different stratum basalis and functionalis. There are also no conclusive studies on comparisons of healthy pre and post-menopausal endometrium. For these reasons, from the existing data, it is difficult to conclude the normal pattern of steroid receptor expression in healthy human endometrium. Therefore, this study also examined the different zones of the full thickness endometrium across the cycle in pre-menopausal women and in a group of healthy postmenopausal women.

Some evidence exists on steroid receptor presence in human fallopian tube, which showed that the tubal epithelial expression of ER alpha and beta were present and more prominent in the secretory phase of the menstrual cycle.[42] Another study claimed that epithelial expression of ER alpha was down-regulated after treatment with progesterone.[43] The latter study conflicts with the evidence above that stated that ER alpha increased during the secretory phase, when progesterone was highest. PR A and B has been reported to be expressed in both the epithelia and stroma of human fallopian tubal mucosa. There have been reports that PR is

reduced during the secretory phase, in the fallopian tube. Progesterone treatment down-regulates PR in the tube. This is consistent with endometrial response of this receptor during the menstrual cycle.[42, 43] Very little evidence surrounds the presence of AR in the fallopian tubes except that it is abundantly expressed in the nuclei of the epithelia.[34]

Detailed examination of steroid hormone expression in both distal and proximal parts of the tube, across the menstrual cycle of premenopausal women and in postmenopausal women is yet to be compared with the matched normal endometrial steroid receptor expression. This chapter describes the examination of matched full thickness eutopic endometrium and proximal (isthmic) and distal (fimbrial) parts of the fallopian tube in pre and postmenopausal women for the expression of steroid receptors. Furthermore, correlations were made with the steroid receptor expression with the co-expression of the proliferative marker KI67 to understand if the endometrial cell proliferation is particularly associated with alteration in the steroid receptor expression.

5.1.1 Summary of Methods and Demographics

For this investigation 17 premenopausal matched eutopic endometrial and fallopian tube samples (Isthmic and fimbrial region) were collected and stained for KI67, AR, ER Alpha, ER Beta and PR using immunohistochemistry techniques, grouped throughout the menstrual phases (See Table 5-1 - Number of samples in each group for immunohistochemistry). N=5 matched full thickness eutopic endometrium and fallopian tube postmenopausal sections were also collected.

Table 5-1 - Number of samples in each group for immunohistochemistry

Study Group	Number Eutopic Endometrium	Number Isthmic Tube	Number Fimbrial Tube
Menstrual phase	4	3	3
Proliferative phase	6	6	3
Secretory phase	7	5	4
Postmenopausal	5	2	5

Table 5-2 - Table of Demographics for Patients in Immunohistochemical Study

Menstrual Cycle Phase	Sample number		Age	BMI	Smoker	Parity
Menstrual Phase	4	*Mean (± SD)* *Median* *Range*	41 (±4) 40.5 37-47	29.3 (±5.0) 30.8 21.2-34.4	1 (25%)	2 (±1) 2 1-3
Proliferative Phase	6	*Mean (± SD)* *Median* *Range*	43 (±5) 43 37-53	29.3 (±6.7) 26.1 23.1-39.2	0 (0%)	2 (±1) 2 0-3
Secretory Phase	7	*Mean (± SD)* *Median* *Range*	43 (±4) 44 37-48	27.7 (±6.6) 23.8 21.7-40.1	3 (43%)	2 (±1) 2 1-4
Postmenopausal	5	*Mean (± SD)* *Median* *Range*	57 (±7) 55 48-66	25.6 (±1.8) 24.9 23.6-28.1	2 (40%)	2 (±1) 2 1-3

Messenger-RNA (mRNA) expression of Androgen Receptor was also investigated using reverse transcriptase –polymerase chain reaction (RT-PCR) on 3 secretory phase and 3 postmenopausal matched eutopic and fimbrial fallopian tube tissue samples. Immuno-staining for steroid receptors of the tissue samples were scored using a modified quickscore method (see chapter 3 for detailed description) and percentage positive staining was used to analyse the KI67 Staining. Densitometry was performed on the PCR samples to evaluate relative mRNA expression of each sample using YWHAZ and GAPDH as the reference genes for endometrial and fallopian tube samples.

Table 5-3 - Table of Demographics for Patients in RT-PCR Study

Menstrual Cycle Phase	Sample number		Age	BMI	Smoker	Parity
Secretory Phase	3	*Mean (± SD)* *Median* *Range*	41 (±7) 45 32-47	26.9 (±3.7) 26.6 22.6-31.6	0 (0%)	1 (±1) 0 0-2
Postmenopausal	3	*Mean (± SD)* *Median* *Range*	57 (±7) 52 52-66	29.5 (±7) 24.9 24-39.6	1 (33%)	3 (±0.5) 3 2-3

5.2 Marker of Proliferation: KI67

KI67 was present in the nuclei of epithelial cells and stroma in the endometrium; which has been shown to be a good indicator of the proliferative activity of a cell.[86]

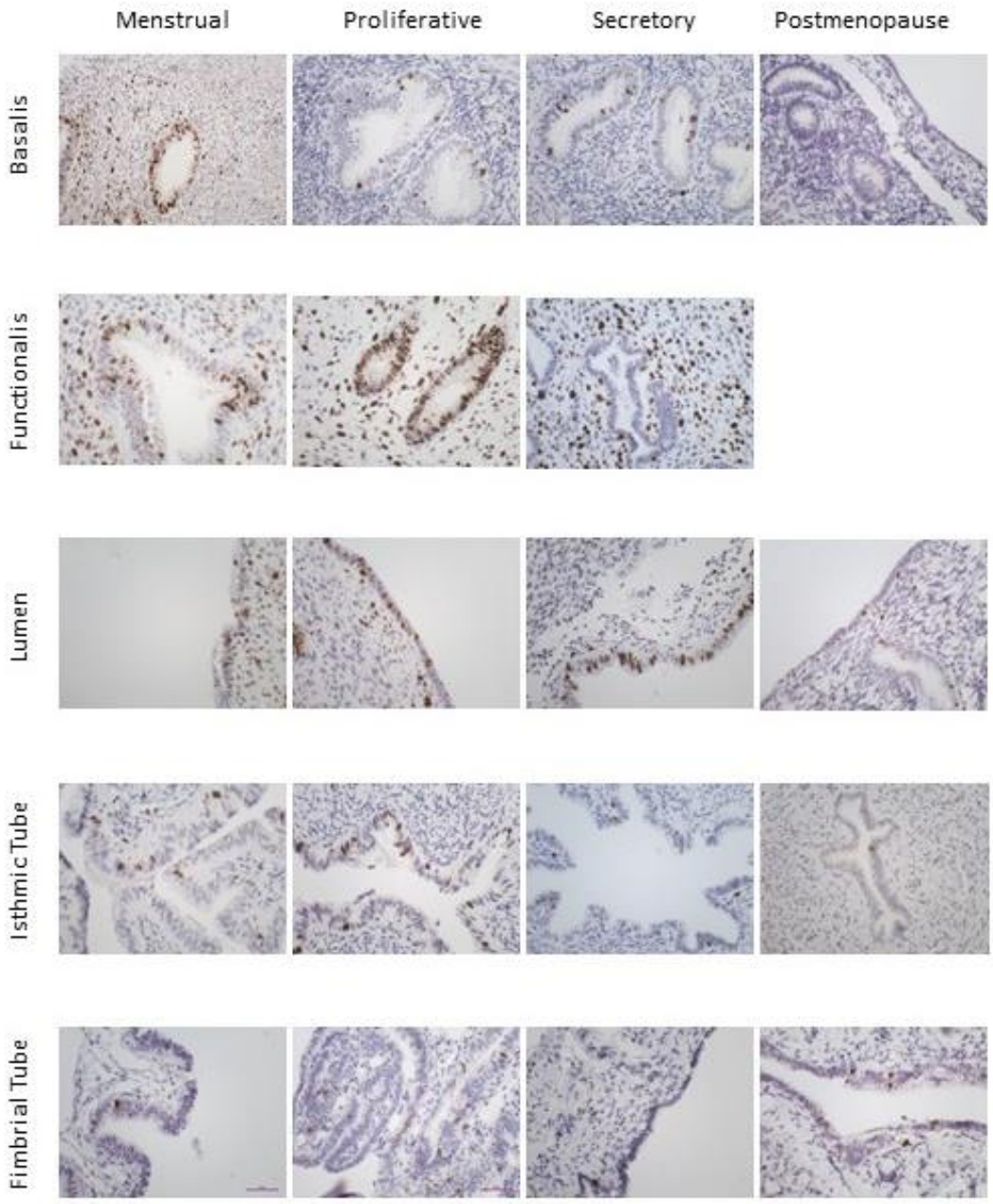


Figure 5-1 - Images for KI67 IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia.

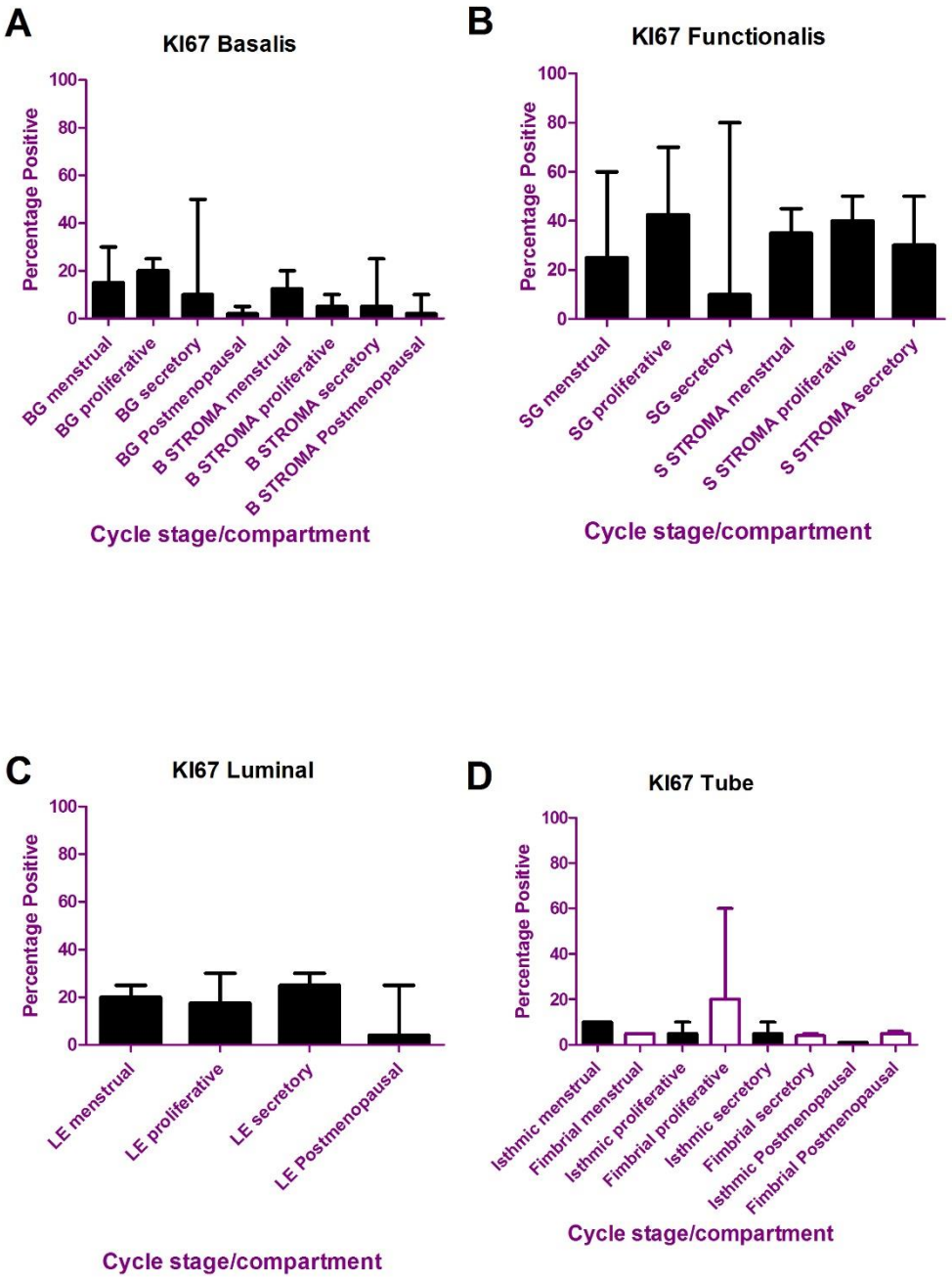


Figure 5-2 - Bar graphs showing median percentage positive nuclei with range for the immunohistochemical staining of KI67 divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of basal glandular epithelia percentage positive nuclei with: basal glands in the menstrual phase (N=4), proliferative phase (N=5), secretory phase (N=7) and postmenopause (N=5.) B) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=4) and secretory phase (N=7.) C) Bar graph of luminal epithelia in menstrual phase (N=3), proliferative phase (N=4), secretory phase (N=7) and postmenopause (N=4.) D) Bar graph of fallopian tube percentage positive nuclei with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=3), Isthmic fallopian tube in the secretory phase (N=3), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=2), postmenopausal fimbrial fallopian tube (N=5.)

5.2.1 Immunohistochemistry

Epithelial cells in the basalis layer of the endometrium showed variability in ki67 expression throughout the menstrual cycle. As expected, highest expression was seen in the proliferative phase of the menstrual cycle with 20% **Median Percentage Positive (MPP)** staining. Lowest percentage positivity in the cycling endometrium was seen in the population in secretory phase of the menstrual cycle (MPP 10%). The postmenopausal epithelia (remaining basalis) showed very low positivity (MPP 2 %). It was of note that the stromal and epithelial compartments of the basalis layer of the endometrium had a different expression pattern of KI67. Highest percentage positivity of stromal KI67 expression was seen in the menstrual phase of the cycle (MPP 12.5 %) whilst lower expression (MPP 5 %) was seen during the rest of the cycle phases studied. Very low KI67 expression was seen in the postmenopausal endometrium (MPP 2 %). The functional epithelial cells had the same pattern of KI67 expression as the basal epithelial cells. The highest epithelial expression of KI67 was in the proliferative phase had a MPP of 42.5 %. In the secretory phase, the lowest KI67 expression was observed in epithelial cells - with an MPP of 10 %. Functional stromal KI67 staining had the same pattern of expression as epithelial cells with less variability between the cycle phases. The luminal epithelium had higher secretory phase KI67 expression than in the rest of the phases (MPP 25 %). Interestingly, in the luminal epithelium, the lowest KI67 positivity was seen in the proliferative phase (MPP 17.5 %).

The epithelial cells of the fimbrial/infundibular end of the fallopian tube showed a clear difference in Ki67 staining throughout the menstrual cycle. In the menstrual phase, low KI67 expression was apparent with MPP staining at 5%. There was then an increase in expression of KI67 in the proliferative phase (MPP 20%). This was followed by a dramatic decrease in expression in the secretory phase of the menstrual cycle (MPP 4%). This Low expression of KI67 seen in secretory phase was almost similar to that seen in the postmenopausal fallopian tube (MPP 5%). The isthmic fallopian tube had very low KI67 expression throughout the menstrual cycle with little variability except for an increase in expression during the menstrual

phase of the menstrual cycle (MPP 10%). Postmenopausal isthmic tube had negligible positive KI67 expression (MPP 1 %).

Over all it can be seen that the functionalis compartment of the endometrium has higher expression of KI67 than the stratum basalis compartment and fallopian tubes. The most dramatic difference in expression could be seen in the fimbrial end of the fallopian tube in the proliferative phase of the menstrual cycle with a median percentage increase of 15 % between the menstrual and proliferative phase. The same pattern of expression throughout the menstrual cycle was seen between the basal glands, the functional glands and stroma and the fimbrial fallopian tube where the highest expression was in the proliferative phase population and lowest expression was in the secretory phase. The isthmic tubal epithelia and basal stromal nuclei had the same pattern of expression with higher expression in the menstrual phase. Very low percentages of positive KI67 nuclei were seen in the postmenopausal population. It seemed that some samples had no presence of this molecule at all, whereas other samples had some KI67 expression therefore revealing a median low positive expression within the postmenopausal tissue samples.

5.3 Results: Sex Steroid Receptors

5.3.1 Androgen Receptor

5.3.1.1 Immunohistochemistry for Fallopian Tube and Endometrial Mucosa

Using a modified quickscore to evaluate the staining for AR in the fallopian tube and endometrial epithelia and stroma; large differences in staining were seen between the tissues and the compartments.

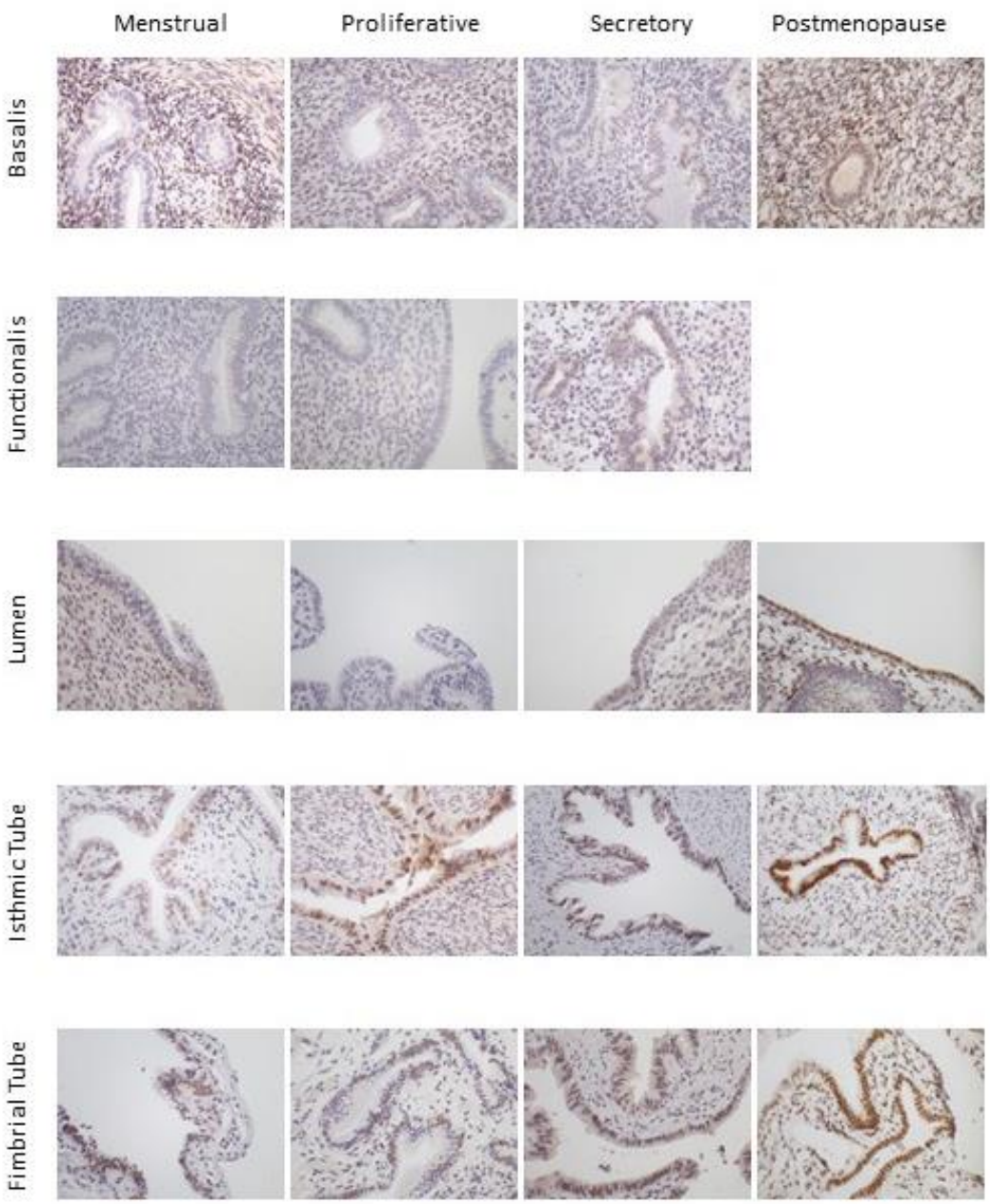


Figure 5-3 - Images for Androgen Receptor IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia

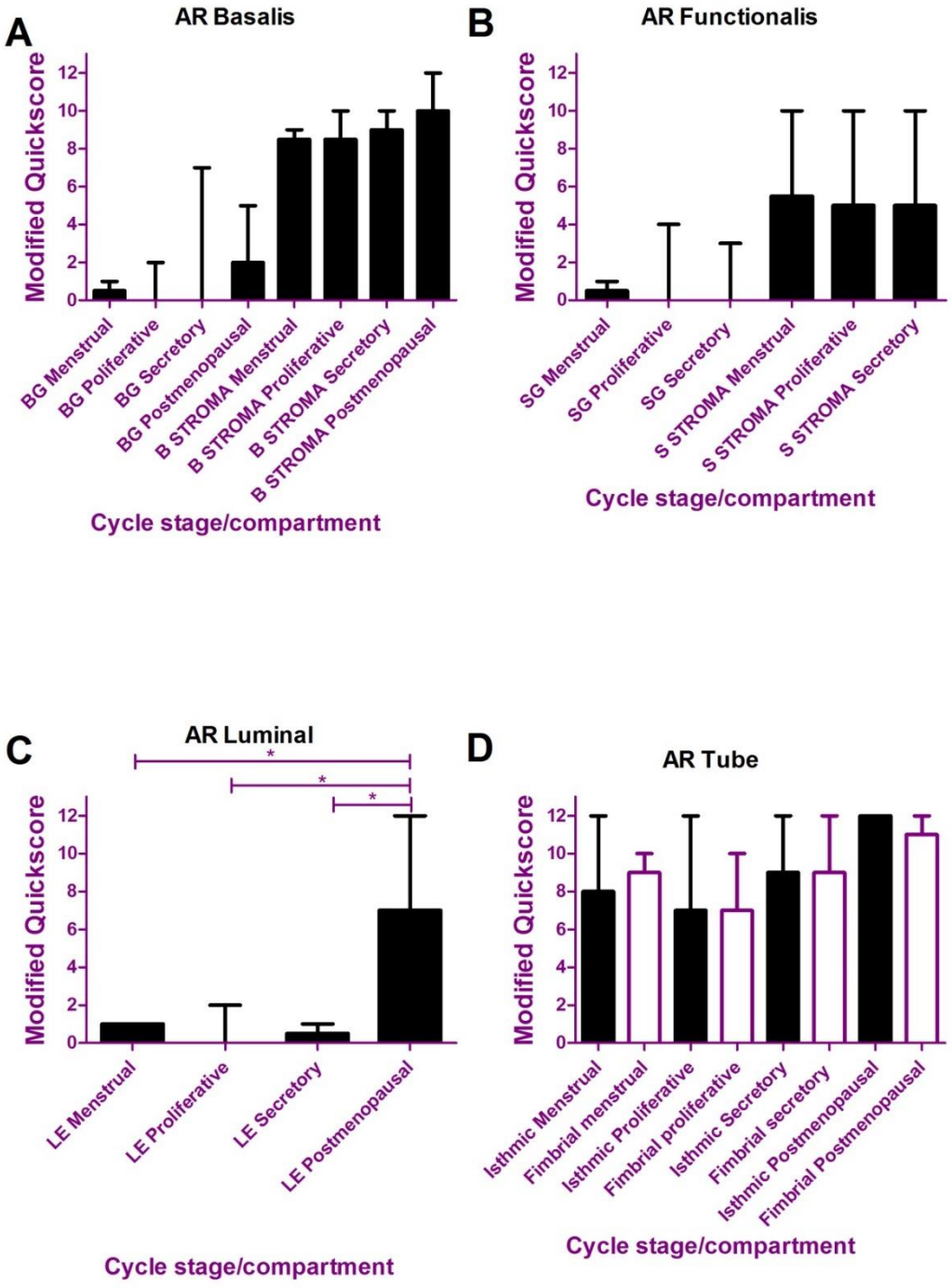


Figure 5-4 - Bar graphs showing median modified quickscores with range for the immunohistochemical staining of Androgen Receptor divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of basal glandular epithelia quickscores with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=6) and postmenopause (N=5.) B) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=5) and secretory phase (N=6.) C) Bar graph of luminal epithelia in menstrual phase (N=3), proliferative phase (N=5), secretory phase (N=6) and postmenopause (N=4). Postmenopausal modified quickscore significantly different (P<0.05) to the secretory, proliferative and menstrual phase luminal epithelia after analysis with the Mann-Whitney U, non-parametric test. D) Bar graph of fallopian tube quickscores with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=5), Fimbrial fallopian tube in the proliferative phase (N=2), Isthmic fallopian tube in the secretory phase (N=3), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=1), postmenopausal fimbrial fallopian tube (N=5.)

In the basalis region of the matched endometrial samples, the basal glandular epithelial cells had low/negligible AR expression. The menstrual phase had very low positive staining with a **Median Quickscore** (MQ) of 0.5 and the proliferative and secretory phase samples were found to be negative for AR. In the postmenopausal endometrium, basal epithelial cells showed low positivity (MQ = 2). The stroma showed a different picture. This compartment had strong positive staining for AR with median quickscores of 8.5/12 and above. There was little variation between cycle phases however; the highest presence of AR was in the secretory phase of the menstrual cycle and slightly lower in the proliferative phase and menstrual phase (MQ = 9 and 8.5, respectively). High stromal expression of AR was present in the postmenopausal population (MQ = 10). The superficial glandular epithelia in the functionalis had the same expression of AR as the basalis glands, a very low positive expression in the menstrual phase population and no AR presence in the proliferative and secretory samples. Superficial stromal expression of AR was moderate; with median quickscore values of 5 and above. Slight phasic variability was shown to occur with a minor increase in AR staining in the endometrium in the menstrual phase (MQ = 5.5). The luminal epithelium, like the glands, had very low AR presence. All median quickscore values were 1 or below. The menstrual phase had the higher level of expression and the proliferative phase was negative for AR. In contrast, postmenopausal luminal epithelia showed a statistically significant increase in expression of AR. ($P<0.05$).

The immediate finding within the distal fallopian tubal population was that there was strong AR presence. MQ of 7/12 and above were scored for this region of the fallopian tube - indicating strong staining with high percentage of positive nuclei for AR. Cyclical variation was present with the highest expression of AR being in the menstrual and secretory phase of the menstrual cycle (MQ = 9). Even higher expression was noted in the postmenopausal sample population (MQ = 11). The proximal end of the cycling fallopian tube had a slightly lower median quickscore levels than the fimbrial end (7 and above). Like the distal end, the lowest expression of AR fell within the population in the proliferative phase of the cycle and the highest expression of AR was found to be in the secretory phase of the

menstrual cycle. In the postmenopausal state, this region had very strong, abundant AR staining (MQ = 12). Stromal staining was very weakly observed in all of the samples with little variation so was omitted from scoring. The epithelial staining was the most prominent.

Comparing the endometrial tissue with fallopian tubes it was clearly evident that tubal epithelial cells had high AR expression comparable to; the stromal compartment of the endometrium. With the phenotypic differences between these cells however; they cannot be functionally comparable to each other. The glandular epithelial cells in all compartments of the endometrium had a contrasting low expression of AR. The pattern of expression of AR throughout the premenopausal cycling population was high in the fallopian tubal epithelia. This was also the case for the basal stromal compartment however; less cyclical variation was seen. The functional stroma also had relatively lower AR expression in the proliferative phase like the basal stromal compartment. In the postmenopausal state the tubal epithelia appeared to have relatively higher AR expression than in the cycling endometrium in all tissues and compartments. Highest AR presence seemed to be in postmenopausal isthmic tube closely followed by fimbrial tube and basal stroma (MQ = 12, 11 and 10, respectively). Lowest postmenopausal expression of AR was seen in glandular epithelium of the endometrium.

5.3.1.2 Reverse Transcriptase – PCR for Androgen Receptor

mRNA detection of androgen receptor (AR) was present in both endometrium and fallopian tubes. Relative expression of AR differed between tissues and between pre and post-menopausal women.

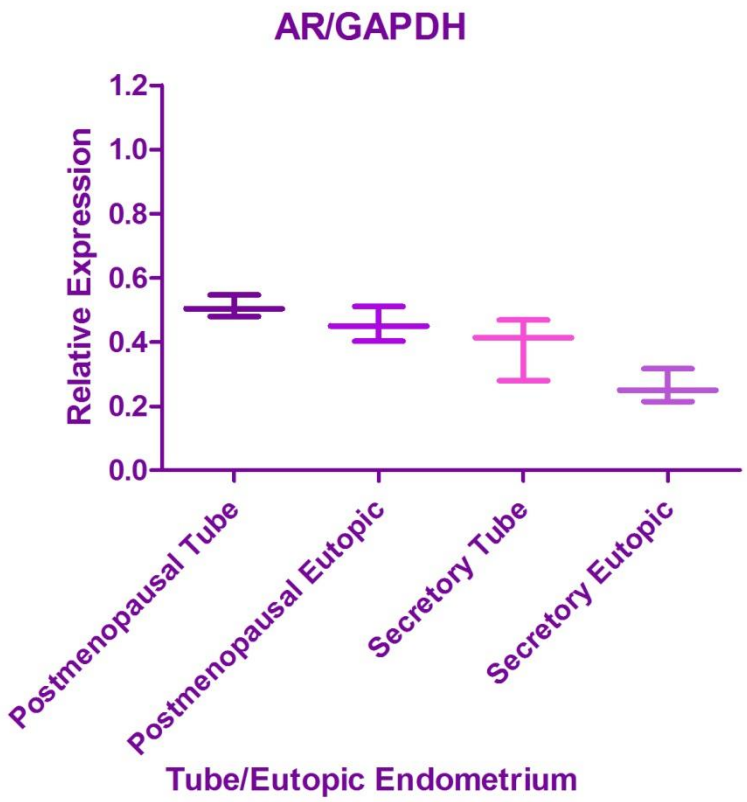


Figure 5-5 - Whisker graph with median and minimum to maximum values showing relative expression of Androgen Receptor in N=3 matched postmenopausal eutopic endometrium and fallopian tube samples and N=3 matched secretory phase eutopic endometrium and fallopian tube samples normalised to GAPDH, the reference gene.

The highest relative expression of AR was detected in postmenopausal fallopian tubes, whilst the lowest mRNA expression of AR was found to be located in the secretory phase eutopic endometrium. On comparing tissues tubal expression of AR was consistently higher in both cycling females and postmenopausal females than eutopic endometrium. Postmenopausal expression of AR in both the fallopian tube and eutopic endometrium was relatively higher than the premenopausal, secretory phase population.

After comparing protein expression of AR to mRNA expression - the data sets are consistent with each other. Postmenopausal expression of AR is relatively higher in comparison to the secretory phase population in both endometrium and fallopian tubes, like the PCR outcome. Fallopian tubal protein expression of AR is also consistently higher than endometrial expression in both pre and postmenopausal sample sets.

5.3.2 Progesterone Receptor

In the presence of Progesterone Receptor (PR), positive DAB staining is detected in epithelial and stromal cell nuclei.

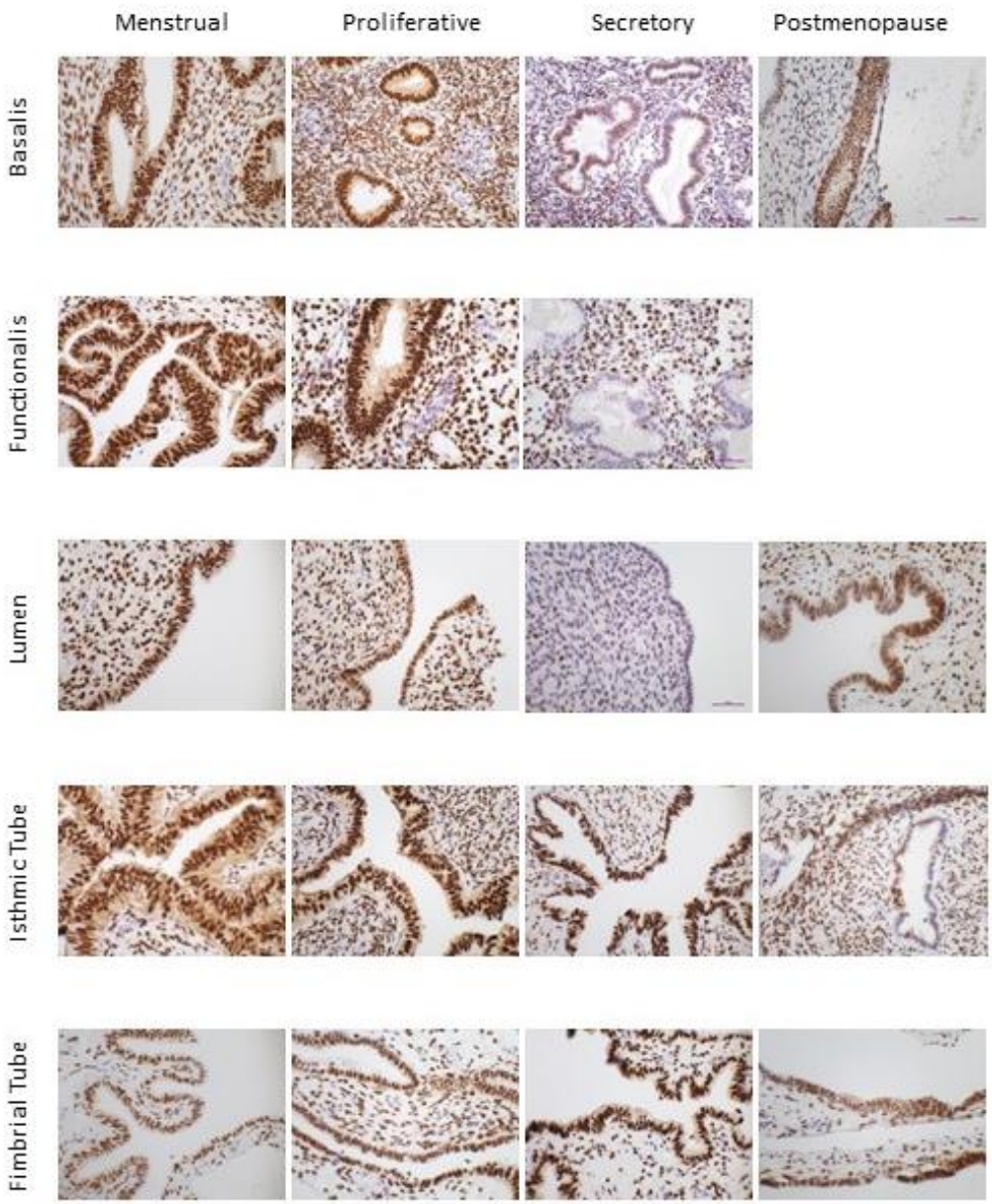


Figure 5-6 - Images for Progesterone Receptor IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia

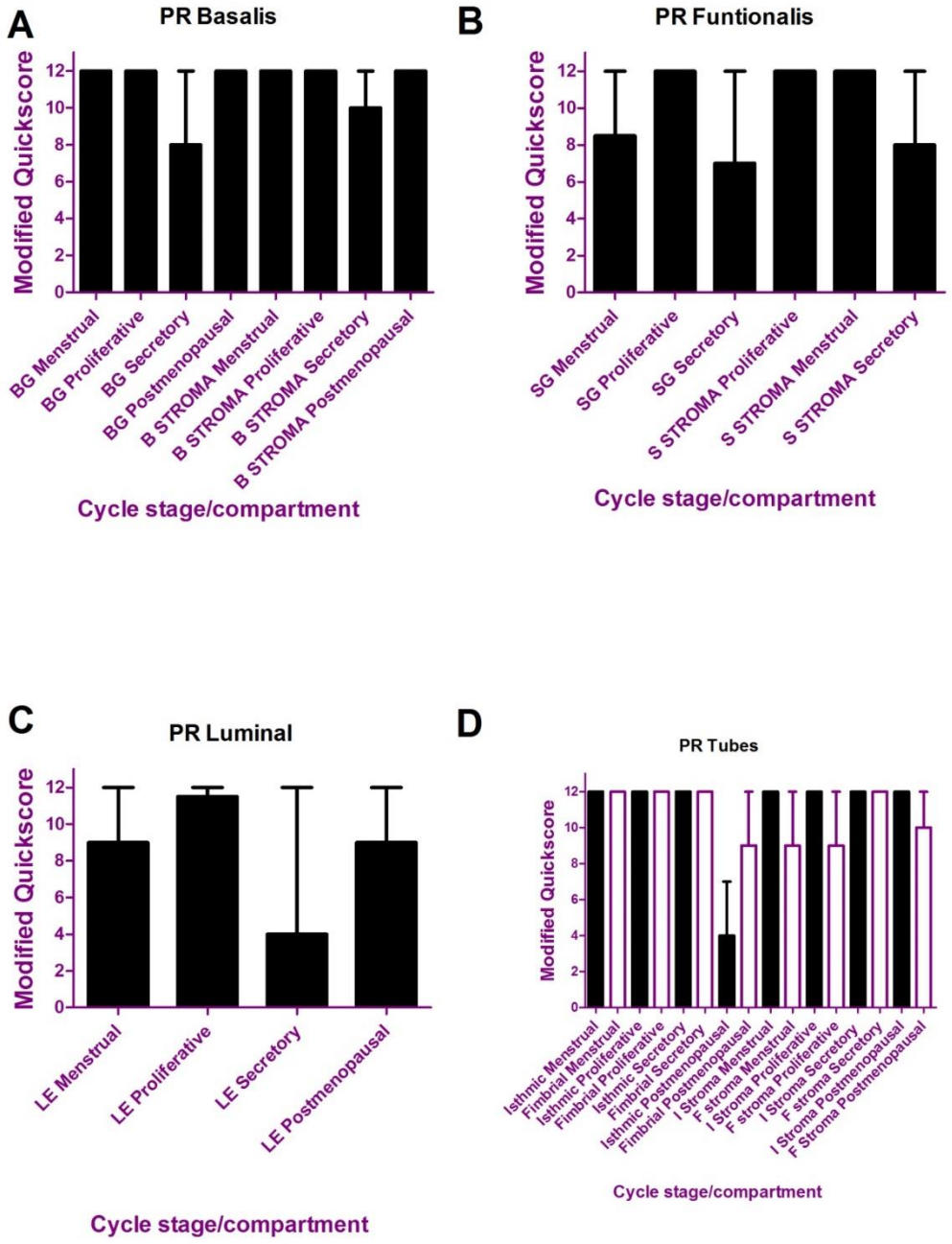


Figure 5-7 - Bar graphs showing median modified quickscores with range for the immunohistochemical staining of Progesterone Receptor divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of basal glandular epithelia quickscores with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=4). B) Bar graph of functional glands in the menstrual phase (N=4), proliferative phase (N=5) and secretory phase (N=7). C) Bar graph of luminal epithelia in menstrual phase (N=2), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=4). D) Bar graph of fallopian tube quickscores with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=3), Isthmic fallopian tube in the secretory phase (N=5), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=2), postmenopausal fimbrial fallopian tube (N=5).

Endometrial expression of progesterone receptor in the basal epithelial cells varied throughout the menstrual cycle. At the beginning of the cycle progesterone receptor was very strongly expressed in the epithelial nuclei (MQ = 12/12). As the female enters the secretory phase of the cycle, a reduction in expression of this receptor appeared to occur (MQ = 8/12). The basalis stromal nuclei also expressed progesterone receptor abundantly (MQ = 12/12). A slight reduction in expression of PR was also seen to occur in the secretory phase population (MQ = 10). Postmenopausal expression of PR was strong in both the epithelial and stromal compartment (MQ = 12/12). In the functionalis, the highest PR expression in both epithelia and stroma was seen to occur in the proliferative phase of the menstrual cycle (MQ = 12/12). Lowest presence of this receptor was evident in the secretory phase of the menstrual cycle in the epithelial and stromal cell nuclei (MQ = 7 and 8/12, respectively). Luminal expression of PR had the same pattern of expression as the functionalis layer of the endometrium, with relatively strong PR staining in the postmenopausal population, equivalent to fimbrial epithelial PR expression.

Progesterone receptor was strongly expressed in fallopian tubes. The fimbrial epithelial cells were consistently strongly expressing this receptor throughout the menstrual cycle. No cyclical variation was seen. The postmenopausal fimbrial epithelial cells however show a diminished expression of this receptor falling to a median quickscore (MQ) of 9/12 from 12/12 that was seen in the premenopausal population. Tubal stroma also expressed this receptor; at the fimbrial end the receptor was highly expressed in both the pre and postmenopausal population (MQ of 9/12 and above). The highest expression of PR seemed to be during the secretory phase of the menstrual cycle (MQ 12/12). Stromal expression of PR fell slightly in the postmenopausal fimbrial tube (10/12). The isthmic epithelia of the fallopian tube also had constant strong expression of PR with no variation between phases in the premenopausal population (MQ = 12/12). Postmenopausal expression of this receptor decreased dramatically (MQ = 4/12). Isthmic stroma showed very strong expression of PR in both the pre and postmenopausal population.

On comparing the tissues and regions; the tube appeared to maintain a high level of PR expression throughout the menstrual cycle with no variation between phases of the cycle and region of the tube. The difference found between the proximal and distal tube, occurred within the postmenopausal population. The isthmic epithelia had a lower expression of this receptor than the fimbrial epithelia (MQ = 4 and 9, respectively). The isthmic stroma had comparatively lower expression of PR in the menstrual and proliferative phases of the cycling population and a lower expression of PR in the postmenopausal population than the distal end. The tube differed from the endometrium because it did not vary its expression of PR throughout the menstrual cycle. Unlike the fallopian tube, the basal glandular epithelia and stroma both showed a reduction in PR during the secretory phase. The postmenopausal basalis had high levels of this receptor whilst tubal epithelial expression diminished. Stroma at the fimbrial end of the tube maintained high levels of PR in the postmenopausal population, in the same way that the postmenopausal endometrial basal epithelia and stroma does. The functionalis showed distinctively high PR expression in the epithelia during the proliferative phase of the cycle, with relatively lower levels of PR in the menstrual and secretory phases. The basalis did not show variation between the menstrual and proliferative phases in this sample set however also found a reduction in expression of PR during the secretory phase of the menstrual cycle. Functional stroma had exactly the same pattern of PR expression as basal stroma.

5.3.3 Oestrogen Receptor Alpha

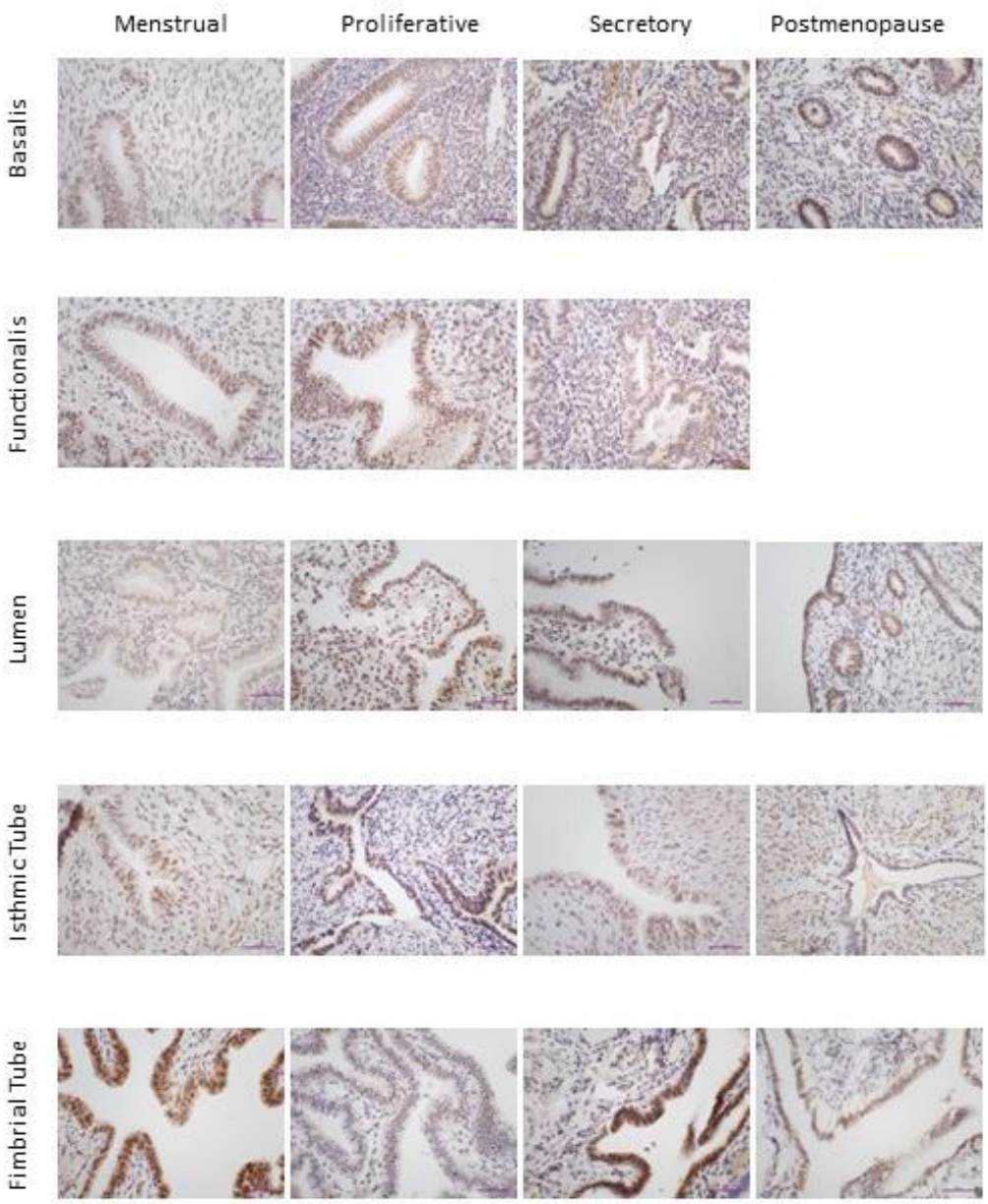


Figure 5-8 - Images for oestrogen receptor alpha IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia.

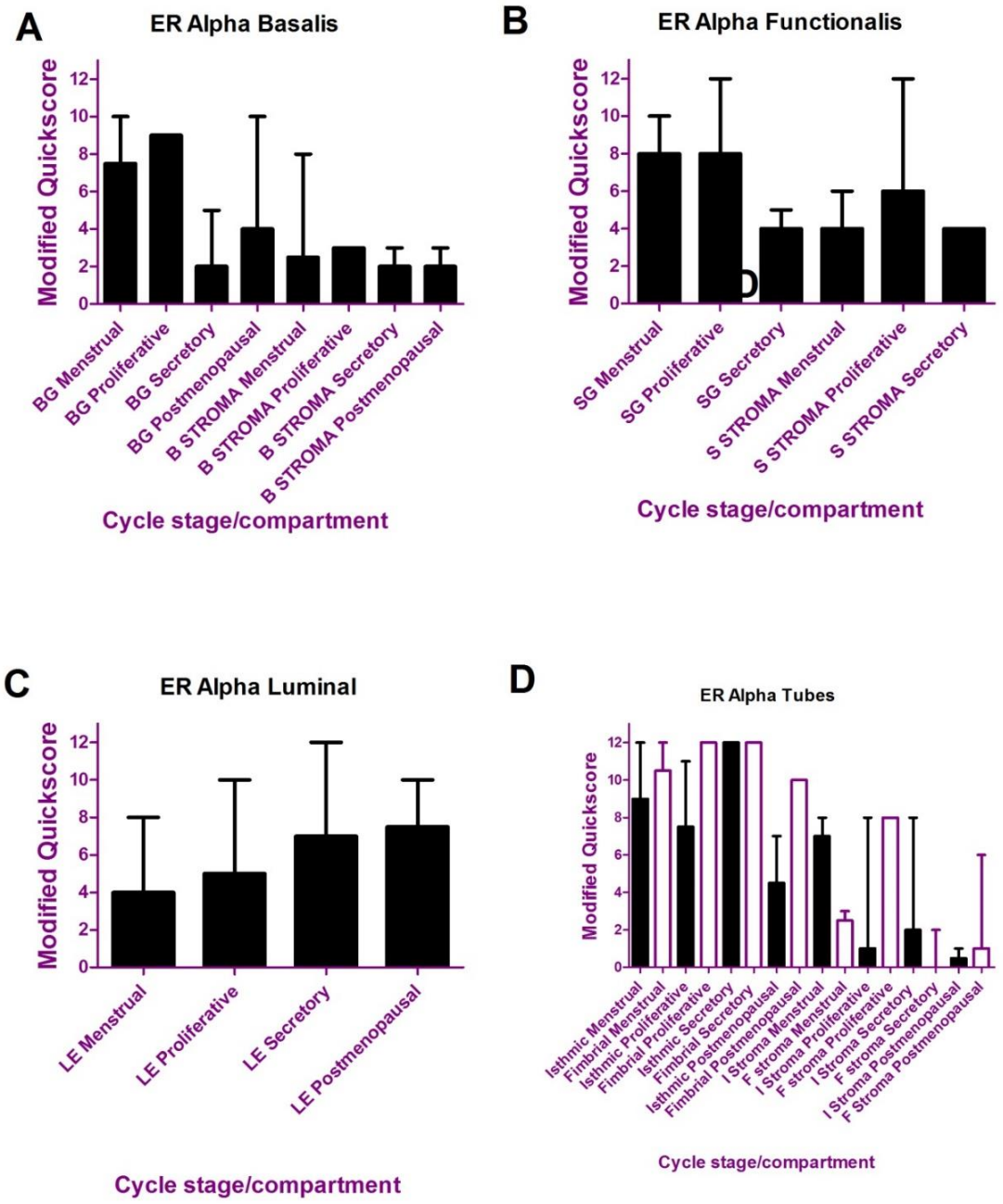


Figure 5-9 - Bar graphs showing median modified quickscores with range for the immunohistochemical staining of Oestrogen Receptor Alpha divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of basal glandular epithelia quickscores with: basal glands in the menstrual phase (N=4), proliferative phase (N=5), secretory phase (N=7) and postmenopause (N=5). B) Bar graph of functional glands in the menstrual phase (N=3), proliferative phase (N=4) and secretory phase (N=7). C) Bar graph of luminal epithelia in menstrual phase (N=2), proliferative phase (N=2), secretory phase (N=7) and postmenopause (N=4). D) Bar graph of fallopian tube quickscores with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=2), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=1), Isthmic fallopian tube in the secretory phase (N=5), fimbrial fallopian tube in the secretory phase (N=3), postmenopausal isthmic fallopian tube (N=2), postmenopausal fimbrial fallopian tube (N=5).

The endometrium showed positivity for ER alpha in both the epithelial cells and stromal cells. Beginning in the epithelial nuclei of the basalis region of the endometrium; the highest expression of ER alpha appeared to be during the proliferative phase of the menstrual cycle and lowest presence of ER alpha in the epithelia was during the secretory phase of the menstrual cycle (MQ = 9 and 2, respectively). Stromal expression of this receptor was lower in the premenopausal population however follows the same pattern as the epithelia, though to a lesser degree. Postmenopausal epithelia of the endometrium had low- moderate expression of this receptor (MQ = 4,) whilst stromal expression was very weak. The functionalis layer of the endometrium has both stromal and epithelial expression of ER alpha. Highest expression lies within both the menstrual and proliferative phases of the menstrual cycle, leaving the lowest expression of epithelial ER alpha within the secretory phase population (MQ = 8, 8 and 4 respectively). Stromal ER alpha found the highest concentration of ER alpha to be in the proliferative phase (MQ = 6). The luminal ER alpha expression was highest during the secretory phase of the menstrual cycle (MQ = 7), differing from the glandular epithelia. High luminal epithelial expression was apparent in the postmenopausal endometrium (MQ = 7.5).

Fimbrial epithelia had high ER alpha expression in the proliferative phase. This also reflects the expression seen in the basal and superficial glands of the endometrium. ER alpha remained high in the secretory phase in the fimbrial fallopian tubal epithelia which is not seen in the endometrium, where a reduction of ER alpha is seen during the secretory phase in both compartments.

Nuclear expression of ER alpha in the fimbrial tubal epithelia was high. Slight cyclical variation could be seen in this region. The highest presence of this receptor appeared to occur in the proliferative phase and remained high during the secretory phase (MQ = 12/12). Therefore the lowest expression of ER alpha was seen during the menstrual phase of the cycling, premenopausal fallopian tube. Expression of ER alpha was moderate-high in the postmenopausal population but lower than the premenopausal population (10/12). Wide variation between cycle stages was

observed in the fimbrial tubal stroma. High ER alpha expression occurred during the proliferative phase of the menstrual cycle with lower ER alpha in the menstrual and secretory phases (MQ = 8, 2.5 and 0, respectively). Postmenopausal stromal expression of this receptor was very low (MQ = 1). More proximally, at the isthmic end of the fallopian tube high epithelial expression of ER alpha was evident. Cyclical change in the epithelia occurred, but in a different way to the more distal end of the tube. Highest epithelial expression occurred during the secretory phase of the menstrual cycle and diminished expression occurred in the proliferative phase (MQ = 12 and 7.5, respectively). Postmenopausal expression of ER alpha in the epithelia is low-moderate (MQ = 4.5). The isthmic stroma also had wide cyclical variation of ER alpha but this region showed the lowest expression of this receptor to occur during the proliferative phase and highest expression during the menstrual phase. As with the distal tubal stroma, postmenopausal presence of ER alpha was weak (MQ = 0.5).

Larger variation in fimbrial tubal stromal expression of ER alpha was seen in comparison to the consistently low median levels in the basalis endometrial stroma. Like the fimbrial end of the tube, the functional stroma had higher expression of ER alpha in the proliferative phase of the cycle therefore following the same pattern of expression. The isthmic epithelial portion of the fallopian tube had a different pattern of expression than all other regions or tissues' being that ER alpha expression was higher during the secretory phase of the menstrual cycle. On the contrary to the endometrium and distal fallopian tube; the isthmic stromal nuclei also seemed to show a low expression of ER alpha in the proliferative phase of the cycle. Highest postmenopausal epithelial expression of ER alpha was within the fimbrial epithelia (MQ = 10). Remaining epithelial compartments had low-moderate expression of the receptor. In contrast, stromal expression of ER alpha was very weak throughout all tissues.

5.3.4 Oestrogen Receptor Beta

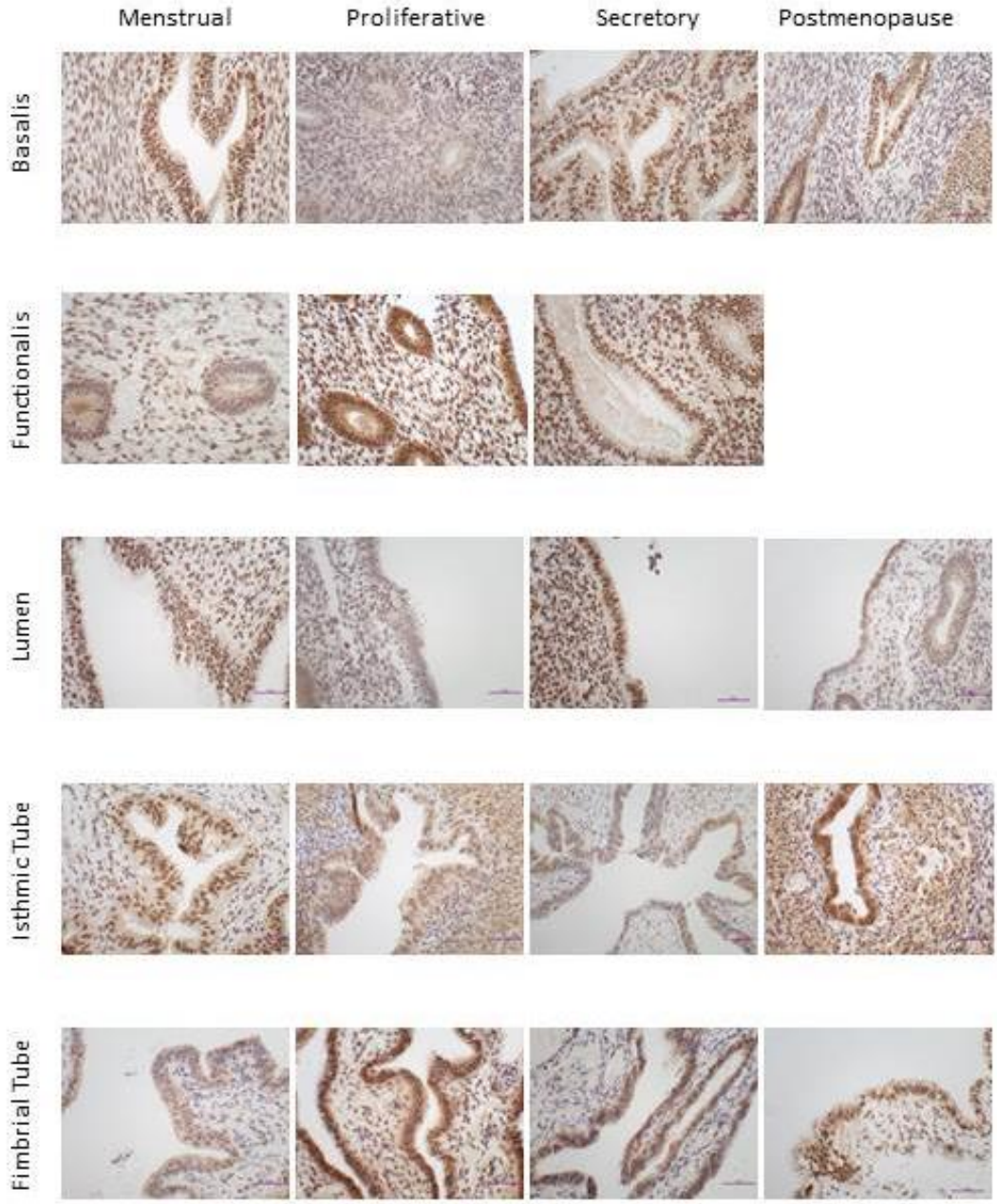


Figure 5-10 - Images for oestrogen receptor beta IHC staining at 400x magnification across the menstrual phase, proliferative phase, secretory phase and postmenopause. In the stratum basalis, stratum functionalis, luminal epithelia, isthmic fallopian tubal epithelia and fimbrial fallopian tubal epithelia.

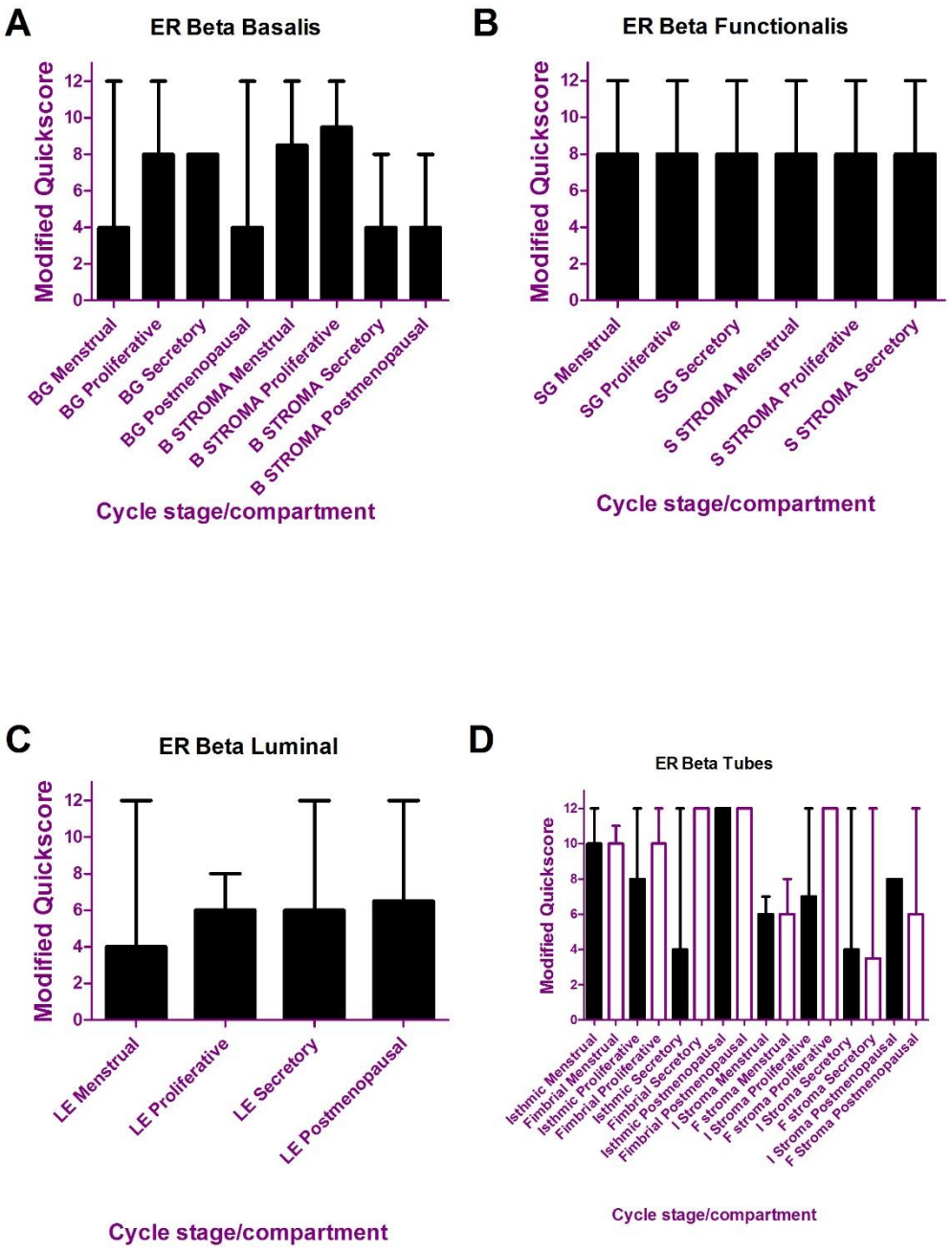


Figure 5-11 - Bar graphs showing median modified quickscores with range for the immunohistochemical staining of Oestrogen Receptor Beta divided by tissue and compartments throughout the menstrual cycle phases and postmenopause. A) Bar graph of basal glandular epithelia quickscores with: basal glands in the menstrual phase (N=4), proliferative phase (N=6), secretory phase (N=7) and postmenopause (N=5). B) Bar graph of functional glands in the menstrual phase (N=3), proliferative phase (N=5) and secretory phase (N=7). C) Bar graph of luminal epithelia in menstrual phase (N=3), proliferative phase (N=3), secretory phase (N=7) and postmenopause (N=4). D) Bar graph of fallopian tube quickscores with: Isthmic fallopian tube in the menstrual phase (N=3), fimbrial fallopian tube in the menstrual phase (N=3), Isthmic fallopian tube in the proliferative phase (N=6), Fimbrial fallopian tube in the proliferative phase (N=2), Isthmic fallopian tube in the secretory phase (N=3), fimbrial fallopian tube in the secretory phase (N=4), postmenopausal isthmic fallopian tube (N=1), postmenopausal fimbrial fallopian tube (N=5).

The basalis glandular epithelia expressed ER-B. Weaker expression occurred in the menstrual phase with an increase in ER-B during the proliferative phase and remained high into the secretory phase of the cycle (MQ = 4, 8 and 8). Weaker ER-B expression was observed in the postmenopausal basal epithelia (MQ = 4). Stromal cells of the basalis also expressed ER-B. An increase in this receptor was seen to occur in the proliferative phase (MQ = 9.5). A subsequent reduction in presence of ER-B arose in the secretory phase of the cycling population (MQ = 4). Postmenopausal expression of ER-B remained low in stromal nuclei. Consistently moderate-high expression of ER-B existed in the functionalis compartment of the endometrium, in both stroma and epithelial cell nuclei. No cyclical variation occurred with average median quickscores of 8 throughout. Luminal epithelial presence of ER-B had an increase in ER-B expression during the proliferative phase increasing to a moderate quickscore of 6, this moderate expression was maintained during the secretory phase. Postmenopausal expression of ER-B in the lumen was moderate (MQ =6.5).

Oestrogen receptor beta (ER-B) is present in the nuclei of epithelial and stromal cells. Starting at the distal end of the fallopian tube, epithelial expression was highest in the secretory phase of the menstrual cycle however in both the menstrual and proliferative phases, ER-B expression was strongly expressed (MQ = 12, 10 and 10/12 respectively). The isthmic fallopian tube had a different pattern of expression. ER-B decreased in a step-wise manner throughout the menstrual cycle with the highest expression in the menstrual phase (MQ = 10). Weakest expression of ER-B was noted in the secretory phase, with a median quickscore of 4. Epithelial expression of ER-B in the postmenopausal population was strong at the fimbrial and isthmic end of the fallopian tube (MQ = 12). Wide variation between stages of the menstrual cycle was seen in the fimbrial stroma of the fallopian tube. Highest stromal expression of ER-B was seen in the proliferative phase and lowest in the secretory phase (MQ = 12 and 3.5). Isthmic stroma had lower overall expression and less variation but the same pattern of expression as the distal end of the tube.

Postmenopausal expression of ER-B was moderate in the fimbrial fallopian tube (MQ = 6). Isthmic tubal stroma had moderate – high expression (MQ = 8).

After comparing the tissues, the epithelia of the distal fallopian tube had highest expression of ER-B in the secretory phase of the menstrual cycle, this is also the case in basal glandular epithelial cells however the overall strength of ER-B expression was higher in the distal fallopian tubes than the endometrium as a whole. The isthmic end of the fallopian tube was seen to have a different pattern of expression with less ER-B during the secretory phase – the expression was more reflective of the luminal compartment of the endometrium (MQ = 4 and 3, respectively). In the postmenopausal population higher receptor expression was observed in the fallopian tube than the endometrium. Lowest ER-B expression occurred in the postmenopausal basal glandular epithelia. The tubal stroma and the stroma of the basalis endometrium had the same pattern of ER-B expression with abundant ER-B during the proliferative phase and reduced receptor expression in the secretory phase population. Functional stromal presence remained constantly moderate-high therefore showing no cyclical variation. Postmenopausally, the isthmic tubal stroma had a higher presence of ER-B in comparison to the endometrial stromal compartment.

5.4 Comparing proliferation with sex steroid receptor expression.

5.4.1 KI67 and Androgen Receptor

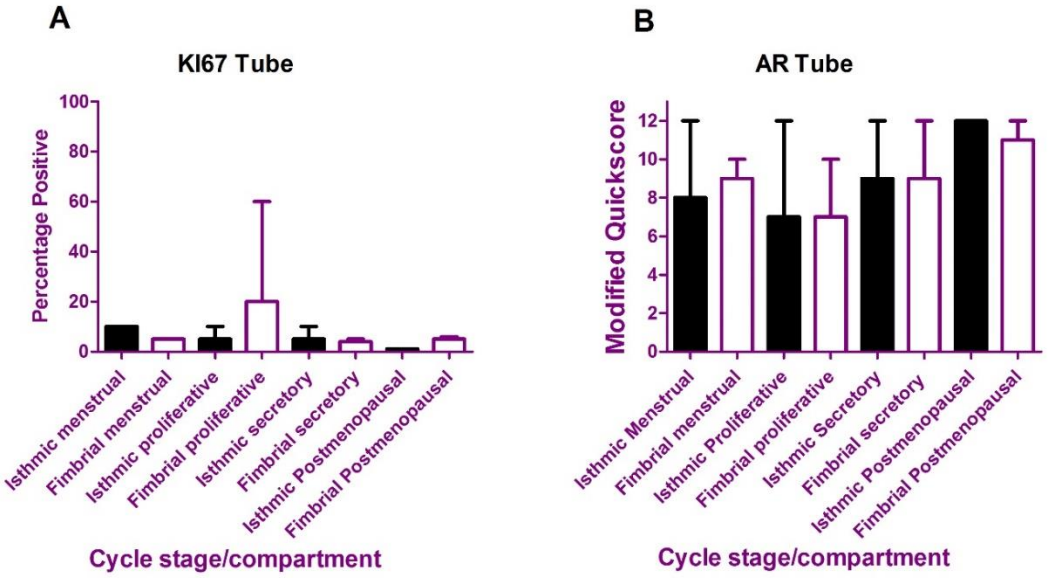
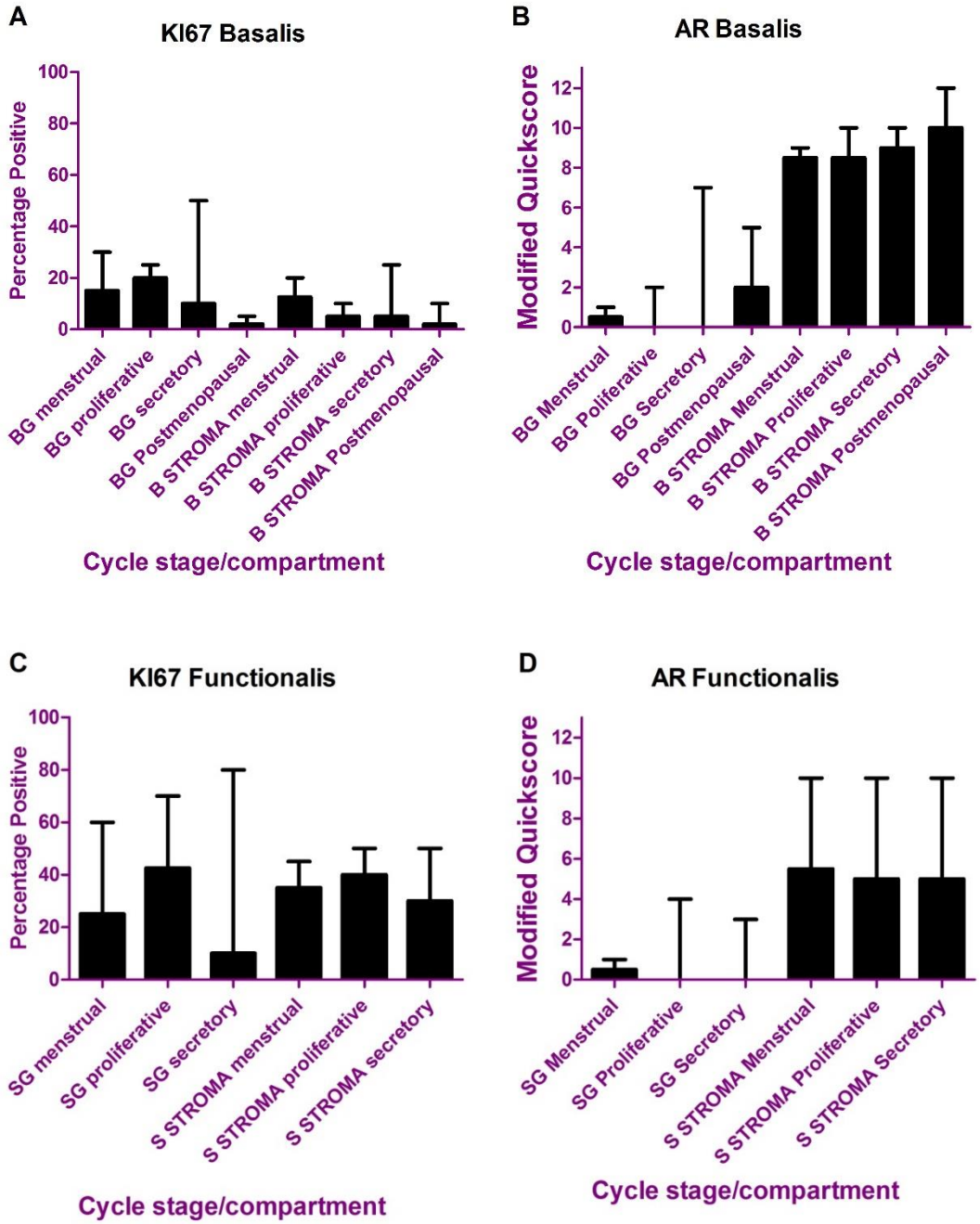


Figure 5-12 - Bar graphs showing median percentage positive and median quickscore values with range for the immunohistochemical staining of KI67 and AR in isthmic and fimbrial fallopian tube throughout the menstrual cycle phases and postmenopause. For details of individual graphs see figures 5-2 and 5-4, respectively.

Observing the pattern of expression of KI67 and androgen receptor in the distal fallopian tubes it was noticeable that KI67 expression was generally low except during the proliferative phase, where there was a sharp increase in percentage of positive cells. This was not the case with AR; the receptor was abundant in all phases of the menstrual cycle. The decrease in AR expression during the proliferative phase opposes the increase of KI67 activity in the fimbrial fallopian tube during this phase. Not only was this the case, high expression of AR was apparent in the postmenopausal population whereas very low/negligible positivity of KI67 is evident. The same applies to the proximal tube; high AR expression is throughout the pre and postmenopausal population whereas low KI67 is shown in this region.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KI67 Basalis
Percentage Positive
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BG proliferative
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Cycle stage/compartment
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AR Basalis
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BG Poliferative
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BG Postmenopausal
B STROMA Menstrual
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B STROMA Secretory
B STROMA Postmenopausal
Cycle stage/compartment
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KI67 Functionalis
Percentage Positive
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SG proliferative
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Cycle stage/compartment
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Modified Quickscore
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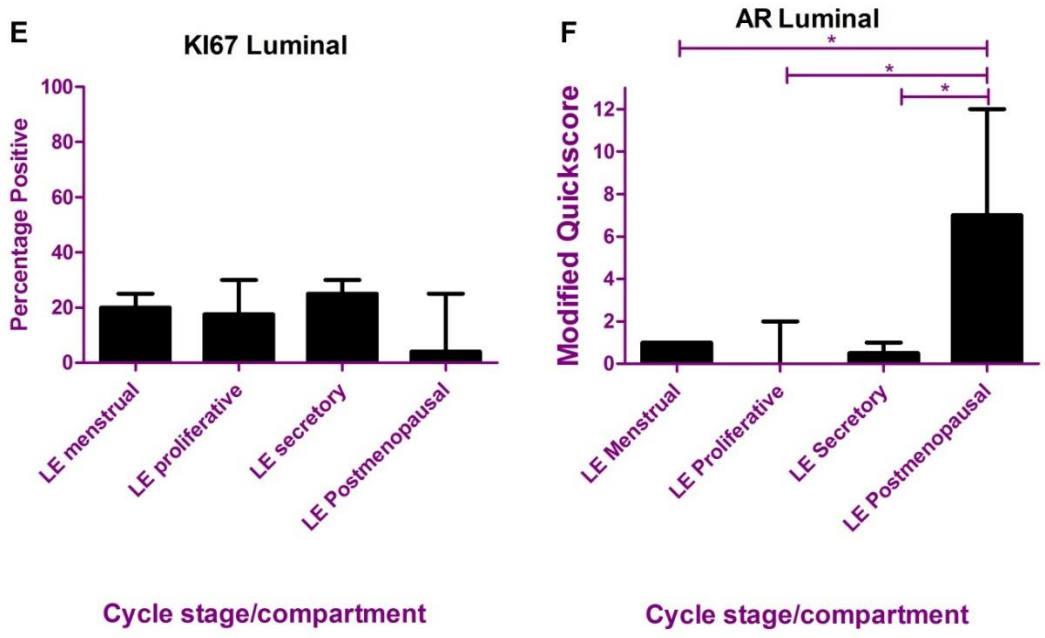


Figure 5-13 - Bar graphs showing median percentage positive and median quickscores with range for the immunohistochemical staining of KI67 and AR in endometrial compartments throughout the menstrual cycle phases and postmenopause. For details of individual graphs see figures 5-2 and 5-4, respectively.

In the endometrium, the basalis glandular epithelium had low or negligible expression of AR in the cycling population. Ki67 was variable in this population with an increase in positivity around the proliferative phase of the menstrual cycle. An increase in AR was seen to occur in the postmenopausal basal glands whereas this population sample had the opposite effect on KI67 expression being that it was low. Androgen receptor was high in the basal stroma, particularly in the postmenopausal endometrium and interestingly KI67 positivity was lowest in this population. In the functionalis layer of the endometrium when KI67 was highest, during the proliferative phase, AR was low. The stroma had both moderate expression of KI67 and AR however, a slight decrease in AR was observed during the proliferative phase and a slight increase in KI67 was seen during this phase. Low luminal epithelial expression of KI67 was observed in postmenopausal lumen whereas high AR expression was seen in this population.

5.4.2 KI67 and Progesterone Receptor

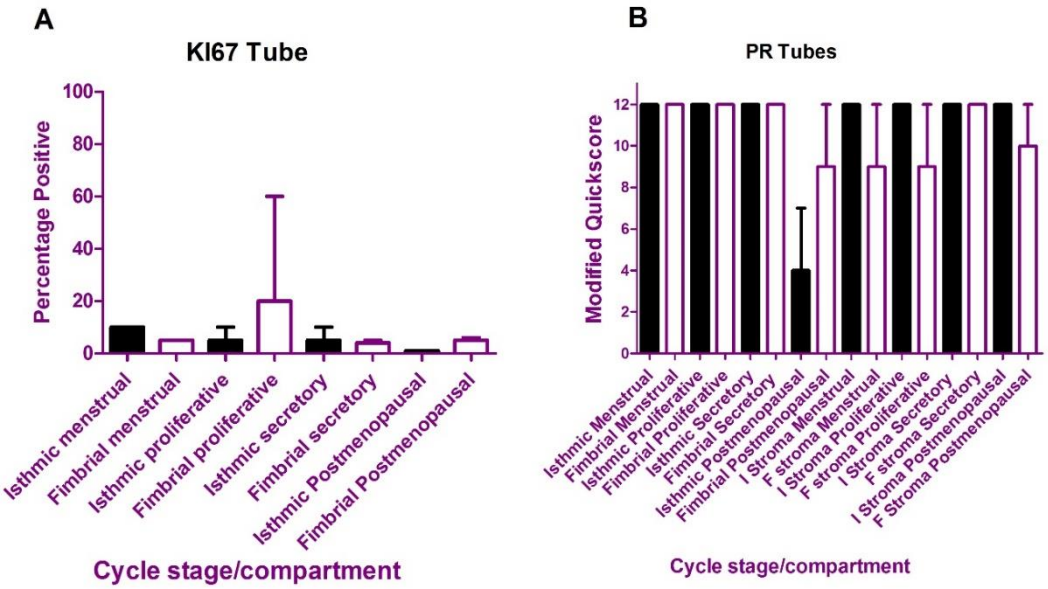
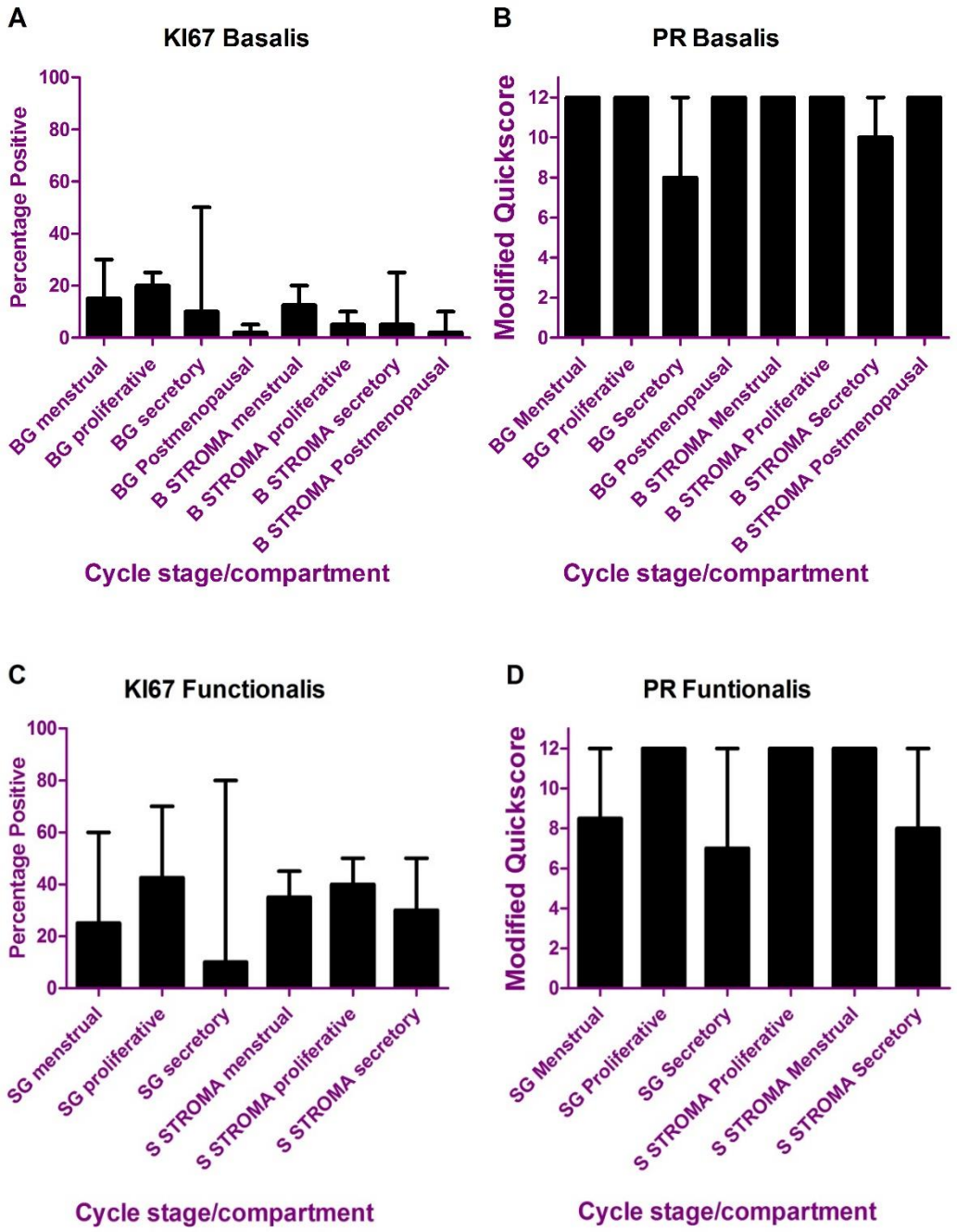


Figure 5-14 - Bar graphs showing median percentage positive and median quickscores with range for the immunohistochemical staining of KI67 and PR in isthmic and fimbrial fallopian tube throughout the menstrual cycle phases and postmenopause. For details of individual graphs see figures 5-2 and 5-7, respectively.

Progesterone receptor was highly expressed in the fallopian tubes; a decrease in expression of this receptor was observed in the postmenopausal samples and this also occurred with KI67. This was the case for both proximal and distal ends of the tube.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Percentage Positive
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BG proliferative
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BG Postmenopausal
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Cycle stage/compartment
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Cycle stage/compartment
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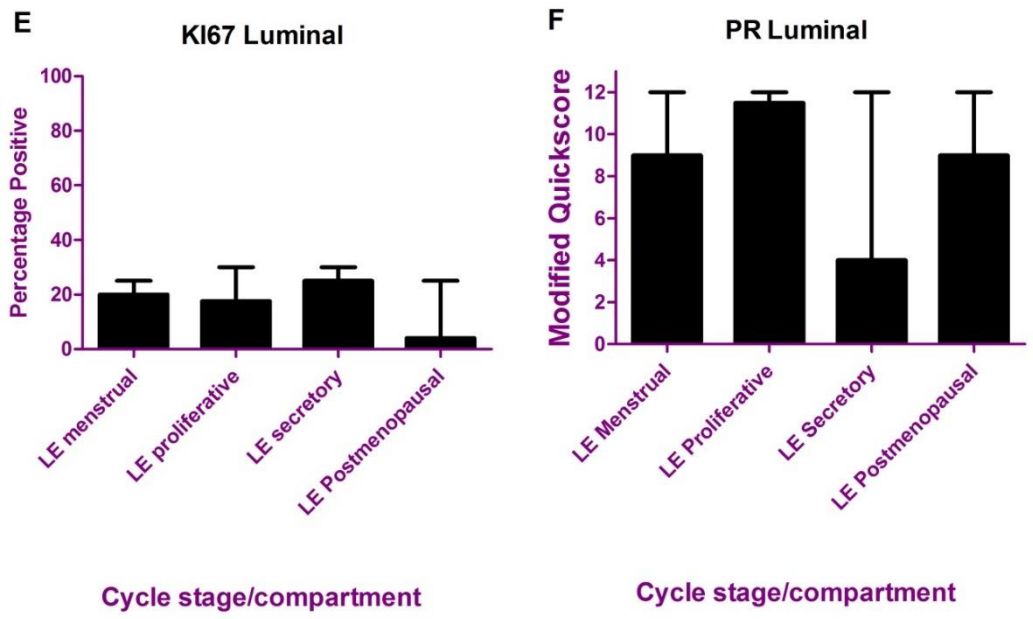


Figure 5-15 - Bar graphs showing median percentage positive and median quickscores with range for the immunohistochemical staining of KI67 and PR in endometrial compartments throughout the menstrual cycle phases and postmenopause. For details of individual graphs see figures 5-2 and 5-7, respectively.

Correlation of KI67 and PR in the basalis endometrium showed a decrease in expression of both molecules during the secretory phase in both the glandular epithelial cells and stromal cells of this layer. In the functional endometrium, the same cyclical pattern of expression exists between KI67 and PR. There is an increase in expression during the proliferative phase and a decrease in expression during the secretory phase of the cycling functional glands. Functional stromal expression of KI67 has subtle variation with a decrease in expression during the secretory phase which correlates with PR expression during this phase. The KI67 expression was low in the luminal epithelia whereas the PR expression dynamically changed throughout the menstrual cycle – comparable to the glandular epithelia.

5.4.3 KI67 and Oestrogen Receptor Alpha

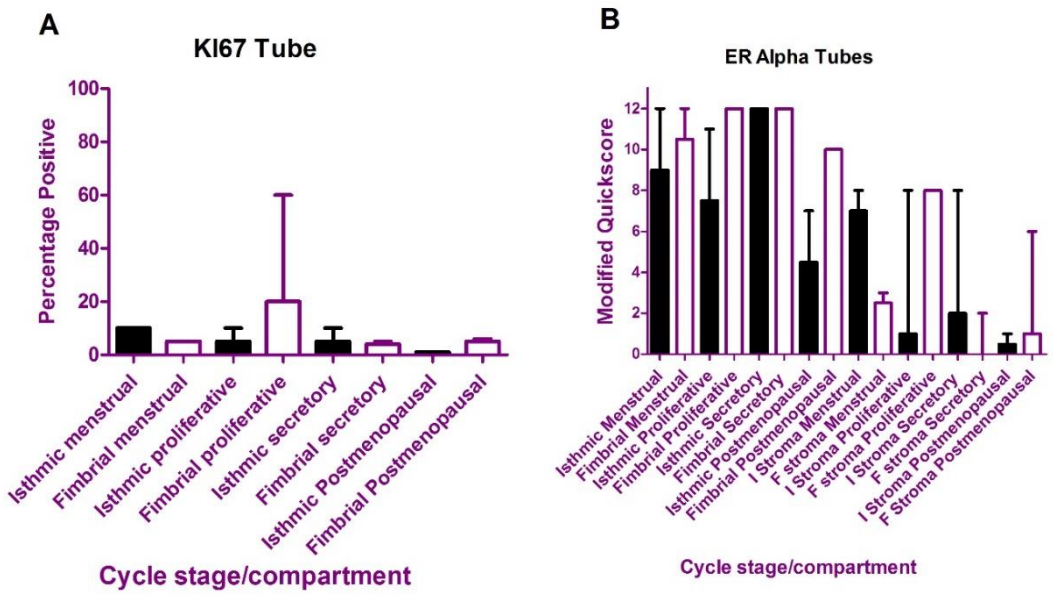
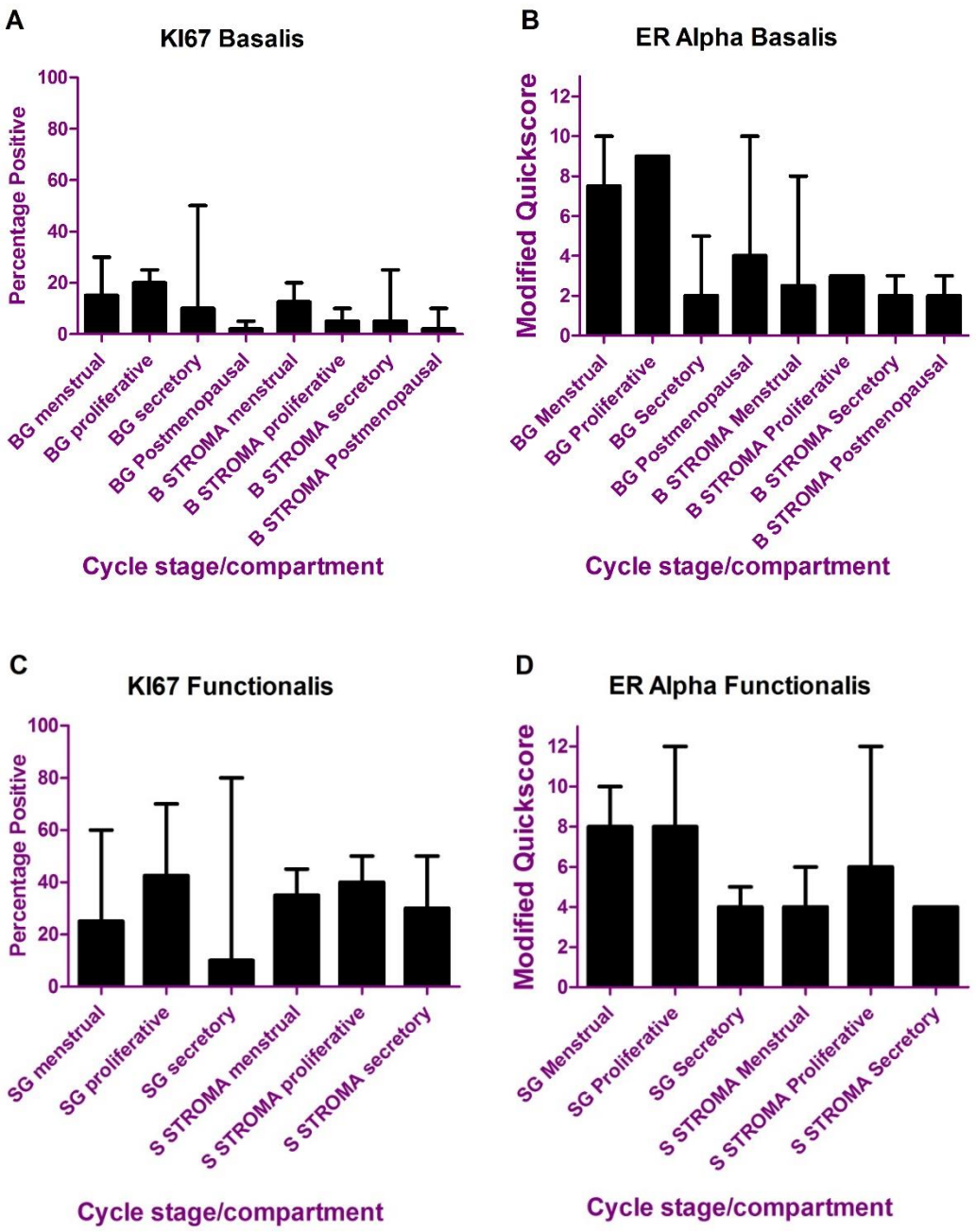


Figure 5-16 - Bar graphs showing median percentage positive and median quickscores with range for the immunohistochemical staining of KI67 and ER Alpha in isthmic and fimbrial fallopian tube throughout the menstrual cycle phases and postmenopause. For details of individual graphs see figures 5-2 and 5-9, respectively.

Starting in the distal tube, the expression of ER ALPHA was high throughout. The cyclical pattern of expression had some correlation with KI67 positivity. Expression of ER ALPHA increased during the proliferative phase in the same way that KI67 did. Postmenopausal decrease in KI67 correlated with a decrease seen in ER ALPHA expression in this region. The same pattern also occurred in the postmenopausal isthmic tube. In the fallopian tubes however, KI67 was relatively low throughout whereas ER ALPHA expression was high. KI67 was not expressed in tubal stroma which was different to ER ALPHA therefore could not be correlated.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Percentage Positive
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BG proliferative
BG secretory
BG Postmenopausal
B STROMA menstrual
B STROMA proliferative
B STROMA secretory
B STROMA Postmenopausal
Cycle stage/compartment
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ER Alpha Basalis
Modified Quickscore
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BG Proliferative
BG Secretory
BG Postmenopausal
B STROMA Menstrual
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B STROMA Secretory
B STROMA Postmenopausal
Cycle stage/compartment
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KI67 Functionalis
Percentage Positive
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SG menstrual
SG proliferative
SG secretory
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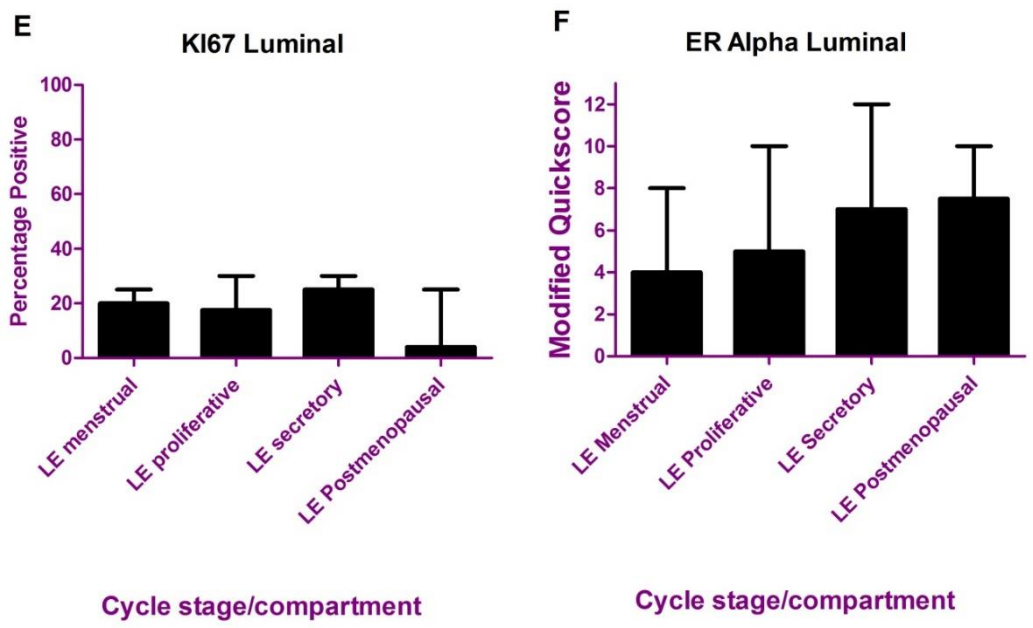


Figure 5-17 - Bar graphs showing median percentage positive and median quickscores with range for the immunohistochemical staining of KI67 and ER Alpha in endometrial compartments throughout the menstrual cycle phases and postmenopause. For details of individual graphs see figures 5-2 and 5-9, respectively.

The basal glands of the endometrium had the same pattern of expression after staining for KI67 and ER ALPHA. There was an increase during the proliferative phase and a decrease seen during the secretory phase. In the stroma of the basalis the low KI67 expression in both the secretory and postmenopausal population was also expressed with ER ALPHA staining. KI67 and ER ALPHA correlated in the functionalis layer of the endometrium in both the glandular epithelia and stromal cells with a decrease in expression during the secretory phase of the menstrual cycle. The luminal proliferative index was low and the ER ALPHA expression was moderate with a minor increase towards the secretory phase of the menstrual cycle. The postmenopausal expression was also moderate.

5.4.4 KI67 and Oestrogen Receptor Beta

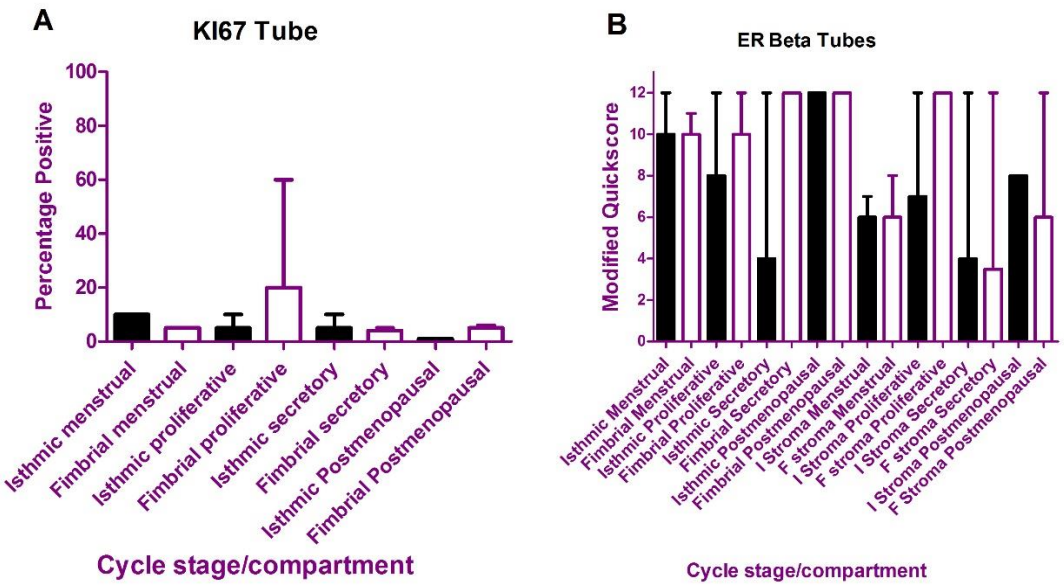
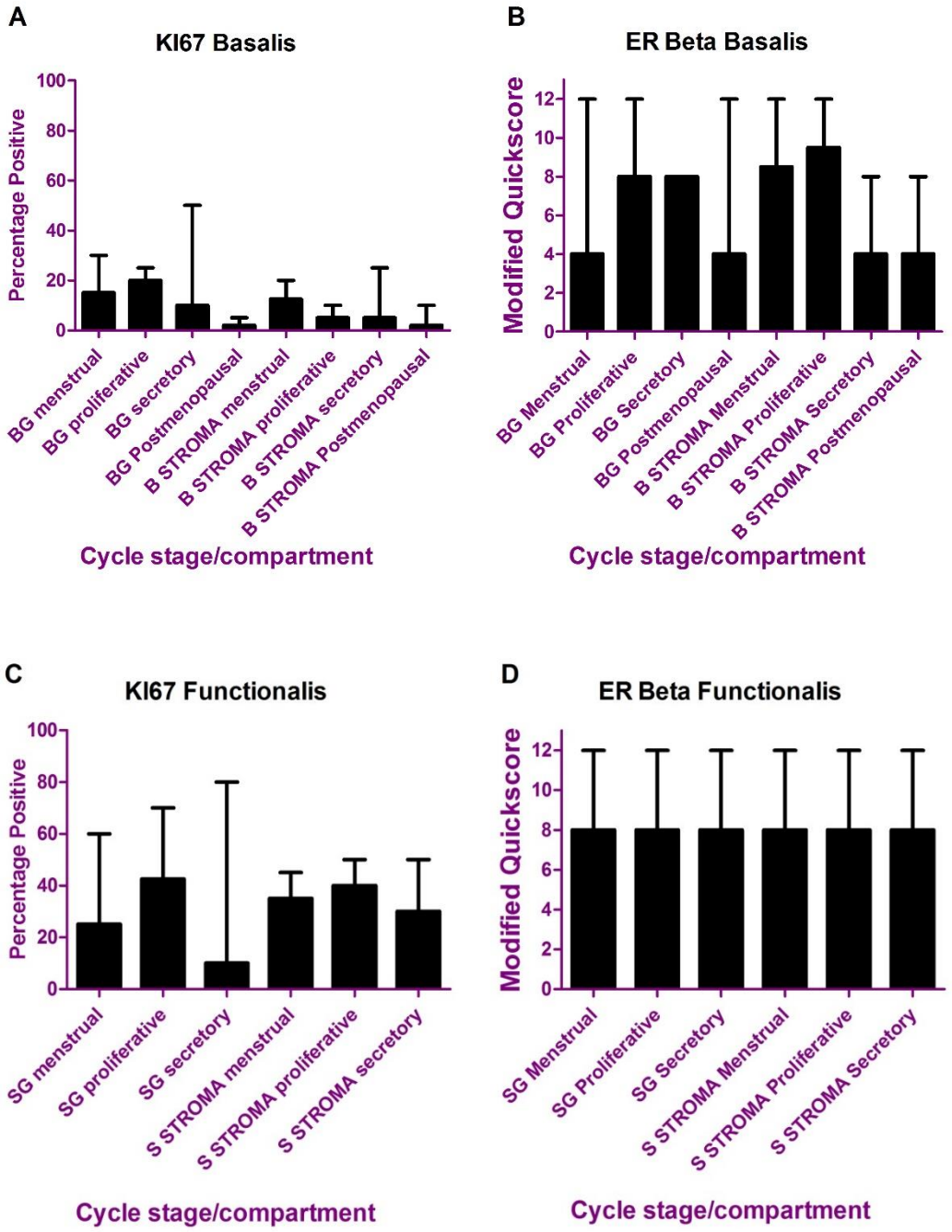


Figure 5-18 - Bar graphs showing median percentage positive and median quickscores with range for the immunohistochemical staining of KI67 and ER-Beta in isthmic and fimbrial fallopian tube throughout the menstrual cycle phases and postmenopause. For details of individual graphs see figures 5-2 and 5-11, respectively.

KI67 expression did not correlate with ER-B in the tubal epithelia except for the decrease in ER-B seen during the secretory phase in the isthmic tube. Again stromal expression of ER-B varied during the cycle however KI67 expression was not evident in the tubal stroma.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KI67 Basalis
Percentage Positive
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BG secretory
BG Postmenopausal
B STROMA menstrual
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B STROMA secretory
B STROMA Postmenopausal
Cycle stage/compartment
B
ER Beta Basalis
Modified Quickscore
12
10
8
6
4
2
0
BG Menstrual
BG Proliferative
BG Secretory
BG Postmenopausal
B STROMA Menstrual
B STROMA Proliferative
B STROMA Secretory
B STROMA Postmenopausal
Cycle stage/compartment
C
KI67 Functionalis
Percentage Positive
100
80
60
40
20
0
SG menstrual
SG proliferative
SG secretory
S STROMA menstrual
S STROMA proliferative
S STROMA secretory
Cycle stage/compartment
D
ER Beta Functionalis
Modified Quickscore
12
10
8
6
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SG Menstrual
SG Proliferative
SG Secretory
S STROMA Menstrual
S STROMA Proliferative
S STROMA Secretory
Cycle stage/compartment

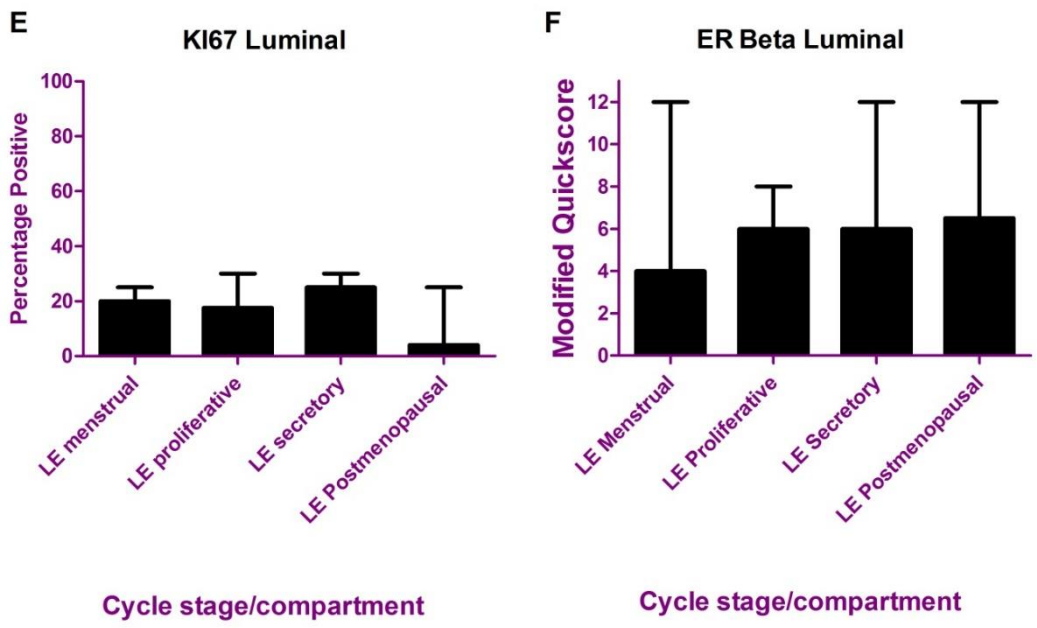

Figure 5-19 - Bar graphs showing median percentage positive and median quickscores with range for the immunohistochemical staining of KI67 and ER-Beta in endometrial compartments throughout the menstrual cycle phases and postmenopause. For details of individual graphs see figures 5-2 and 5-11, respectively.

In the basalis endometrial epithelial cells the increased positivity of KI67 during the proliferative phase and reduced positivity in postmenopausal samples also occurred when the tissue was stained for ER-B. The basal stroma had low expression of ER-B in the secretory cycling population and postmenopausal population therefore correlating with KI67 expression. Moderate expression was seen with both KI67 and ER-B expression in functional glandular epithelia however the cyclical variation with KI67 was not apparent in this population after staining with ER-B. The same expression of KI67 and ER-B occurred in the functional stroma however, with moderate staining that remained constant throughout the menstrual cycle. The luminal epithelial proliferative index had little cyclical variability and remained low in all population groups. The ER beta expression was moderate with little cyclical variability. ER beta could be influencing the normal function of the luminal epithelia, maintaining the cells in a low proliferative state.

5.5 Summary of Results and Discussion Points

5.5.1 Comparing the proliferative index and sex steroid hormone expression patterns of endometrial epithelia with fallopian tubal epithelia

Sex steroid hormones and their receptors play a major role in the proliferation and regeneration of the endometrium every month. We are still unsure as to whether the steroid hormones have the same effect on the fallopian tubal mucosa. The results in this thesis help to gain an insight into the possible proliferative abilities of the tubal and endometrial mucosa. Expression patterns of the sex steroid hormones and their association with proliferation has also been analysed in both tissues.

According to the marker of proliferation KI67, the most proliferative compartment is the functionalis layer where cell proliferation varies widely according to the menstrual cycle phase. Generally, proliferation is low in the premenopausal tubal epithelium and basalis epithelium of the endometrium. Postmenopausal expression of KI67 was very low in both fallopian tube and endometrial mucosa.

Table 5-4 – Table demonstrating the percentage positive levels of KI67 represented by the symbol + in fallopian tube, basalis endometrial epithelia and functionalis endometrial epithelia. The key below explains the corresponding expression levels according to the results shown in figure 5-2.

KEY	
Quickscore Value/Percentage Positive	Representative Symbol for each value (+)
>0 ≤3 OR >0% ≤25%	+
>3 ≤6 OR >25% ≤50%	++
>6 ≤9 OR >50% ≤75%	+++
>9 ≤12 OR >75% ≤100%	++++

KI67 - Epithelial				
	Isthmic Fallopian Tubal Epithelia	Fimbrial Fallopian Tubal Epithelia	Endometrial Basalis Epithelia	Endometrial Functionalis Epithelia
Menstrual Phase	+	+	+	+
Proliferative Phase	+	+	+	++
Secretory Phase	+	+	+	+
Postmenopausal	+	+	+	NA

The data in this chapter suggests that steroid receptor expression has a unique pattern in fallopian tubal epithelial cells. The most striking feature was the AR expression in the epithelia of the fallopian tube. Epithelial AR was highly expressed in fallopian tubes in the premenopausal population. AR was either absent or low in the epithelial cells of the endometrium, however. AR is mainly limited to the stroma of the premenopausal endometrium. In the

postmenopausal population, tubal epithelial AR expression remained high and some positivity was observed in the epithelial cells of the endometrium.

Table 5-5 – Table demonstrating the modified quickscore levels of AR represented by the symbol + in fallopian tube, basalis endometrial epithelia and basalis endometrial stroma. The key above explains the corresponding expression levels according to the results shown in figure 5-4.

AR				
	Isthmic Fallopian Tubal Epithelia	Fimbrial Fallopian Tubal Epithelia	Endometrial Basalis Epithelia	Endometrial Basalis Stroma
Menstrual Phase	+++	+++	+	+++
Proliferative Phase	+++	+++	-	+++
Secretory Phase	+++	+++	-	+++
Postmenopausal	++++	++++	+	++++

From the results and the pattern of AR expression in both mucosae, it could be suggested that stromal and epithelial androgen action may be different. AR may also influence epithelial cell proliferation.

Another salient feature from the results in this particular chapter is that after observing the expression patterns of ER alpha and beta, the proximal and distal portions of the fallopian tube had varying patterns of expression suggesting that the two regions respond in a different way to oestrogen.

Table 5-6 – Table demonstrating the modified quickscore levels of ER α and ER β represented by the symbol + in isthmic and fimbrial regions of the fallopian tube. The key above explains the corresponding expression levels according to the results shown in figures 5-9 and 5-11, respectively.

ER α	Isthmic Fallopian Tubal Stroma	Fimbrial Fallopian Stroma
Menstrual Phase	+++	+
Proliferative Phase	+	+++
Secretory Phase	+	-
Postmenopausal	+	+

ER β	Isthmic Fallopian Tubal Epithelia	Fimbrial Fallopian Epithelia
Menstrual Phase	++++	++++
Proliferative Phase	+++	++++
Secretory Phase	++	++++
Postmenopausal	++++	++++

The data suggests that proximal and distal fallopian tubal epithelia have unique patterns of expression for the steroid receptors, it may be suggested that there is a possible barrier created by the isthmus thereby separating the endometrial and fallopian tubal mucosa whilst in the premenopausal state.

Chapter 6 Effect of Androgens on Matched Fallopian Tubal and Endometrial Mucosa

6.1 Introduction

The effect of androgen has been investigated on endometrial tissue however; the authors stated that they used androstenedione (A4), a pro-hormone (Bukulmez et al). In this study - dihydrotestosterone (DHT), an active androgen that binds to the androgen receptor, was used to treat the cells *in vitro.* Other articles have published the use of DHT as a treatment in explant culture, including the creation of an *in vitro* model for human prostate.[100] The effect of androgen on endometrial tissue has not been studied in great detail even though it has been speculated that this steroid hormone is important for the normal function of the cycling endometrium.[101] Androgen receptor expression was shown to be differentially expressed in the tubal mucosa compared to the endometrium (Chapter 5). Therefore we hypothesised that the effect of androgen in the two tissues will be different.

Epithelial and stromal cells as well as endothelial cells and leucocytes are included in complex solid tissue architecture in both the endometrium and tubal mucosa. Correct cellular orientation within this complex architectural organisation and the cell-cell interaction is particularly important for the normal function including the hormonal responsiveness. For example, this fact is highlighted by the observation that the inhibitory effect on endometrial epithelial cell proliferation by progesterone is via an indirect effect on stromal cells that increase hand2 and supress stromal cell production of FGFs that act on epithelial cells.[102] Therefore, examining hormonal responsiveness of endometrial cells in vitro requires the presence of at least stromal and epithelial cells. Furthermore, the growth in the usual 2D environment also may alter the functional ability and gene expression profile of cells in comparison to the 3D growth, which is more relevant to the existence *in vivo*. There are previous publications where small tissue samples (1-

2mm) were grown in short-term (24-72) culture to assess the effect of a variety of hormones and drugs where authors have reported preservation of tissue architecture and similar data to that observed *in vivo*.[103] Therefore, explant culture method - that keeps the normal tissue architecture in 3D and also retains the normal cellular components of the endometrium enabling them to exist and interact; was chosen as the preferred technique to study the effect of androgens on the tubal and endometrial mucosa. This was the first study to investigate the effect of androgens on matched fallopian tube and endometrial mucosa, in explant culture. Previous studies have looked at the effects of androgens on various tissues *in vitro.*

It is thought that androgens up-regulate their own receptor. AR was up-regulated by DHT treatment in human skeletal muscle *in vitro*. [104] Testosterone treatment on leydig cells of rats also found an increase in AR expression. [105] This needs to be investigated in endometrial and fallopian tube tissue *in vitro.*

The treatment of fallopian tubes with androgens has not been studied to date. Fallopian tube tissue has been treated with progesterone *in vitro*. The study found that ER alpha and PR mRNAs were down-regulated after treatment.[43] This indicates that progesterone causes transcriptional changes by down-regulating its receptors. This treatment was in isolation and it is important to investigate the function of all of the steroid hormone receptors together in order to re-construct an environment that is reflective of the *in vivo* state.

In this chapter, matched fallopian tube and endometrial tissue has been treated with DHT, in explant culture to study the immediate, direct effects that active androgens have on these tissues. The effect that androgen has on proliferation was investigated by immuno-staining for Ki67. The effect of androgen on its own receptor, AR has been explored through the use of IHC and RT-PCR techniques and the effect of DHT on the marker SOX9 was also studied.

6.1.1 Samples and Demographics

Methods of analysis investigating the effect of androgens on matched tubal and endometrial tissue included; treating explant samples with dihydrotestosterone (DHT)/ Methanol (Vehicle) for 24 hours and either: embedding the tissue for immunohistochemistry (IHC) or RNA extracting the samples for reverse transcriptase-PCR (RT-PCR) analysis. Methods can be referred to in chapter 3.

Samples included in this analysis were; 4 matched eutopic endometrial samples with fimbrial fallopian tube tissue explants. This sample set underwent IHC staining for Androgen Receptor (AR), SOX9 and KI67. 3 matched eutopic endometrial and fimbrial fallopian tube explant samples, in the proliferative phase of the menstrual cycle were RNA extracted for RT-PCR. SOX9 and AR gene expression was analysed.

Table 6-1 – Patient Demographics for immunohistochemistry and reverse transcriptase-polymerase chain reaction explant study

IHC/RT-PCR	Sample number		Age	BMI	Smoker	Parity
IHC	4	*Mean (± SD)* *Median* *Range*	43.5 (±7) 47 31-49	30.9 (±8) 28.1 23.3-43.9	1	2 (±1) 1.5 1-3
RT-PCR	3	*Mean (± SD)* *Median* *Range*	42 (±2) 42 39-45	32.9 (±6) 36.9 24.6-37.2	0	3 (±1) 2 2-4

6.2 IHC Staining of Explants Treated with DHT

6.2.1 KI67

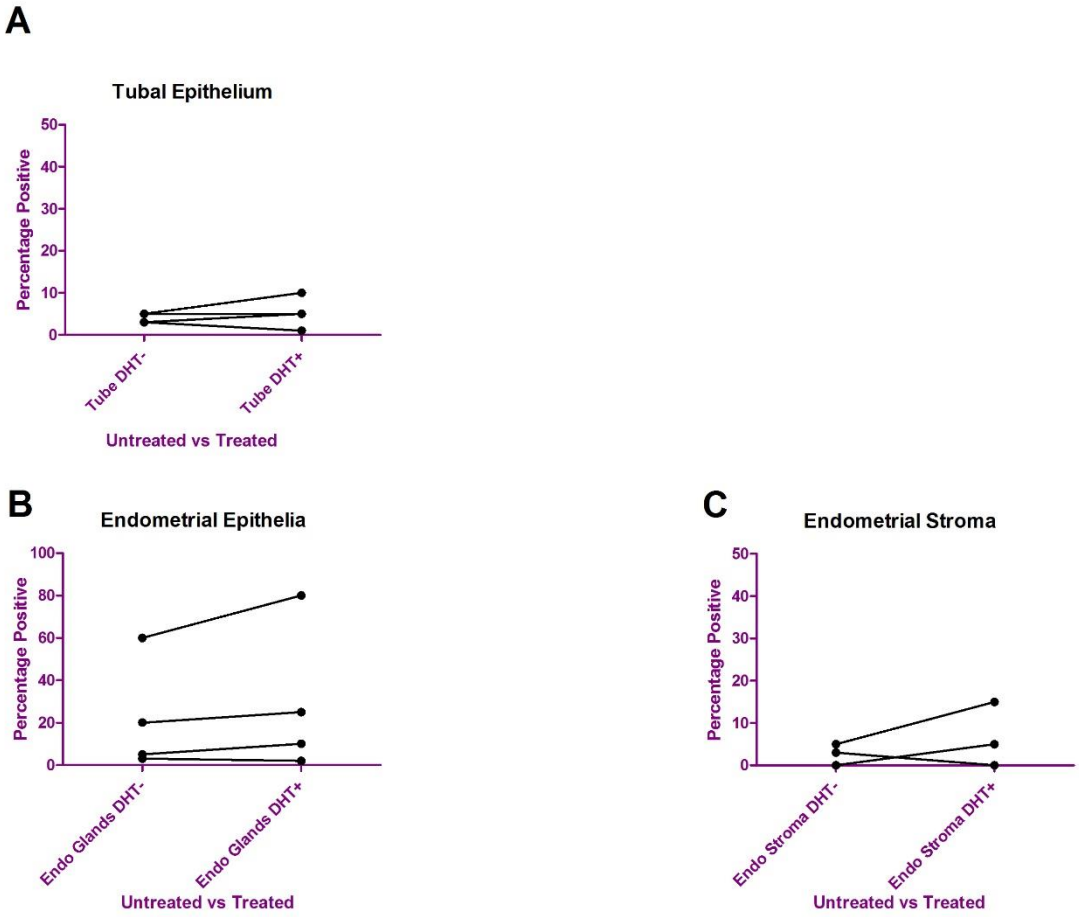

Figure 6-1 – Line graph representing the percentage positive KI67 values for N=4 matched eutopic endometrium and fallopian tube (fimbrial) samples grown in explant culture for 24 hours with DHT or vehicle (methanol). A) Fallopian tubal epithelial staining for KI67 in the untreated mucosa (-DHT) vs the treated mucosa (+DHT). B) Endometrial epithelial staining for KI67 in untreated mucosa (-DHT) vs treated mucosa (+DHT). C) Endometrial stromal staining for KI67 in untreated mucosa (-DHT) vs treated mucosa (+DHT).

Explants showed some positivity for KI67. Beginning with the fallopian tube explants; low positive staining was evident. For samples that were not treated with DHT, positivity of <10% was observed. The treated tissue with DHT shows a 2/4 increase in KI67 positivity (3-5% and 5-10% increase). One sample remained the same and another decreased very slightly (3-1 %). This therefore showed an overall

trend towards increasing expression of KI67 after DHT treatment. Endometrial epithelial positivity varied greatly between samples. In general after DHT treatment, KI67 increased in percentage positivity. Endometrial stroma also increased KI67 expression after DHT treatment in 2/4 tissue samples. One treated sample's KI67 expression decreased and another stayed the same. The samples that had a decrease in KI67 expression after DHT treatment were different for each compartment therefore no consistent decrease was seen throughout the same sample. Generally however, an increase in KI67 expression can be seen in the treated group of explants in comparison to the untreated sample set. The sample size however; makes this impossible to conclude and would need further investigation with larger sample sizes.

6.2.2 SOX-9

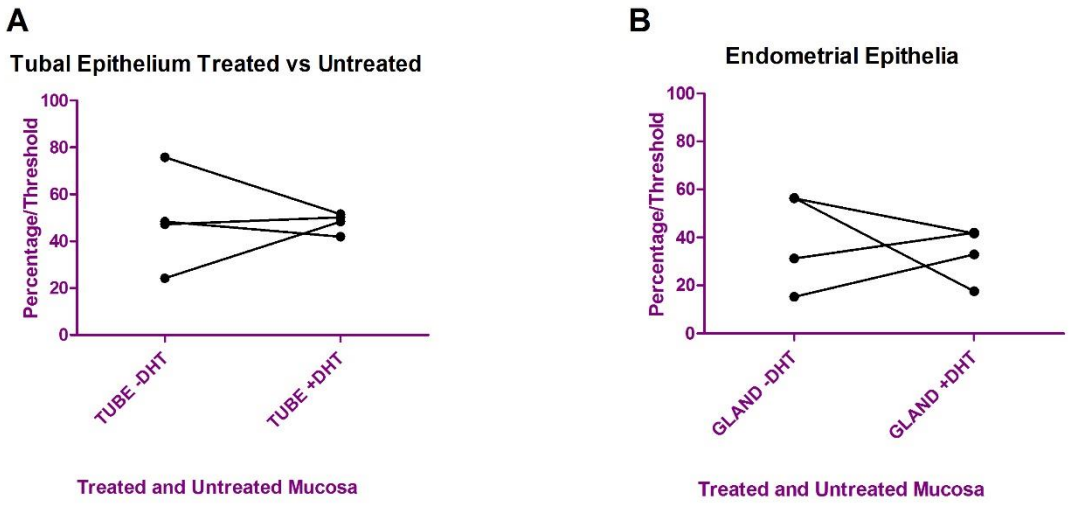

Figure 6-2 - Line graph representing the percentage positive SOX9 values for N=4 matched eutopic endometrium and fallopian tube (fimbrial) samples grown in explant culture for 24 hours with DHT or vehicle (methanol). A) Fallopian tubal epithelial staining for SOX9 in the untreated mucosa (-DHT) vs the treated mucosa (+DHT). B) Endometrial epithelial staining for SOX9 in untreated mucosa (-DHT) vs treated mucosa (+DHT).

After threshold analysis of each explant stained for SOX9, the fallopian tubal epithelia show positivity for SOX9 in both treated and untreated explant tissue samples. 2/4 samples showed a decrease in SOX9 expression after treatment with DHT, one sample stayed virtually the same and the other increased SOX9 concentration. The endometrial epithelia showed half of the samples increasing SOX9 expression after DHT treatment, and half decreasing SOX9 expression after

DHT treatment. A prominent trend in both tissue sets was a comparatively lower SOX9 expression in the DHT treated tissue to the untreated samples. Expression levels of SOX9 were similar for both fallopian tubal epithelia and endometrial epithelia. Again, the sample size was small however.

6.2.3 Androgen Receptor (AR)

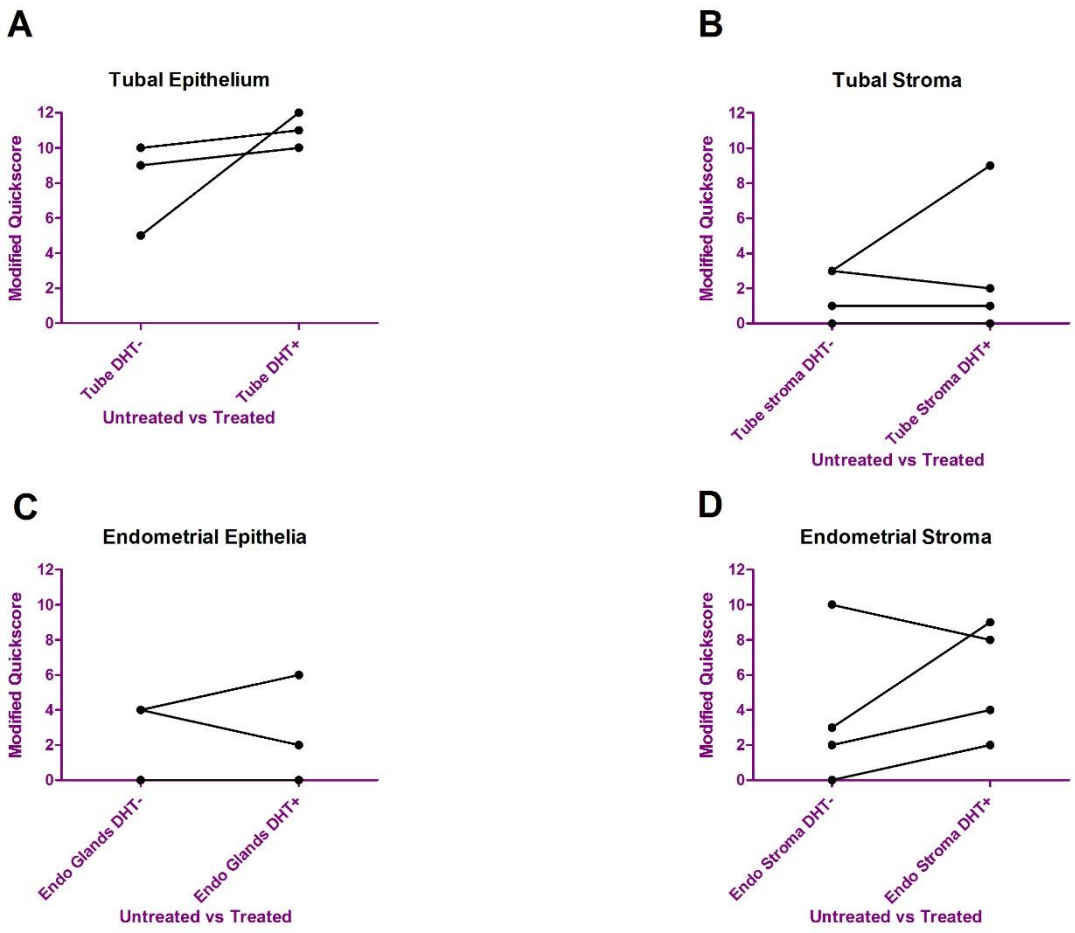

Figure 6-3 - Line graph representing the modified quickscore AR values for N=4 matched eutopic endometrium and fallopian tube (fimbrial) samples grown in explant culture for 24 hours with DHT or vehicle (methanol). A) Fallopian tubal epithelial staining for AR in the untreated mucosa (-DHT) vs the treated mucosa (+DHT). B) Fallopian tubal stromal staining for AR in the untreated mucosa (-DHT) vs the treated mucosa (+DHT). C) Endometrial epithelial staining for AR in untreated mucosa (-DHT) vs treated mucosa (+DHT). D) Endometrial stromal staining for AR in untreated mucosa (-DHT) vs treated mucosa (+DHT).

After fallopian tubes were treated with DHT the androgen receptor expression in the epithelia increased in all samples in the set in comparison to the vehicles. Endometrial epithelia did not change greatly overall. One sample increased its receptor expression, one stayed the same and one decreased its expression.

Therefore a trend could not be seen. Endometrial stroma increased its receptor expression after DHT treatment in comparison to the untreated tissue overall; with 3/4 of the samples showing higher expression after DHT treatment. In general, it seemed as though DHT treatment in tubal epithelia and endometrial stroma caused a potential increase in AR expression. Therefore it could be suggested that fimbrial tubal epithelial response is comparable to endometrial stroma. Looking at expression of AR in both fallopian tube and endometrial tissues; it seems as though the strength of expression is higher in the tubes overall.

6.3 RT-PCR

Samples were normalised to GAPDH. This was the reference gene chosen to represent the m RNA expression of the markers in this thesis. See chapter 4 for explanation.

6.3.1 Androgen Receptor

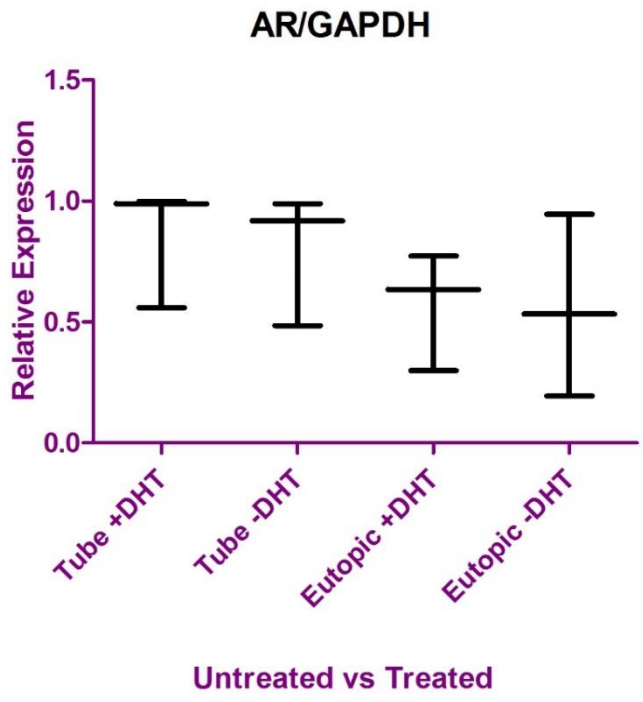

Figure 6-4 - Whisker plot graphs with median, minimum and maximum values for AR/GAPDH In N=3, secretory phase matched fallopian tube explants +DHT, fallopian tube explants –DHT, eutopic endometrial explants +DHT and eutopic endometrial explants –DHT.

Reverse transcriptase-PCR (RT-PCR) on fallopian tube and endometrial explants showed positive expression of AR relative to YWHAZ and GAPDH. First, the expression pattern was the same with both reference genes, confirming their validity as housekeeping genes for endometrial and fallopian tubal mucosa. Fimbrial fallopian tube that had been treated with DHT expressed AR at a comparatively higher level than the untreated group. Treated eutopic endometrium also expressed AR more highly than the vehicle. Also to note; the fallopian tube showed a stronger expression of AR overall than eutopic endometrium. Sample numbers were small however, with n=3. A larger sample size would be needed to confirm these results.

6.3.2 SOX9

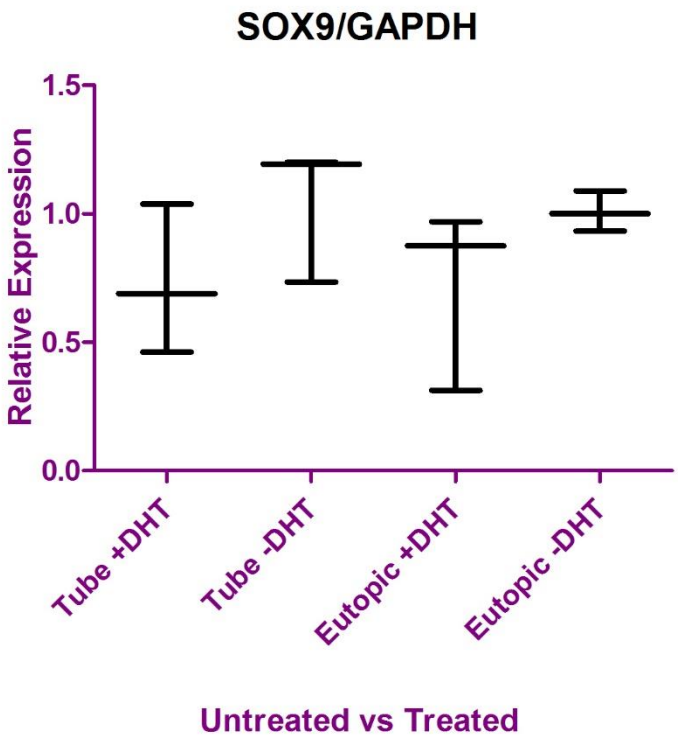

Figure 6-5 - Whisker plot graphs with median, minimum and maximum values for SOX9/GAPDH In N=3, secretory phase, matched fallopian tube explants +DHT, fallopian tube explants –DHT, eutopic endometrial explants +DHT and eutopic endometrial explants –DHT.

RT-PCR of SOX9 showed positive mRNA expression in both endometrial and fallopian tube tissue. Again, the same expression pattern could be seen with both YWHAZ and GAPDH. In fimbrial fallopian tube, SOX9 was expressed more strongly

in the untreated group thus being comparatively less strong after treatment with DHT. The same difference in SOX9 expression was evident in the eutopic endometrial samples. The treated endometrium had less strong SOX9 expression in comparison to the untreated endometrium. Eutopic endometrium had stronger SOX9 expression overall compared with the fimbrial fallopian tube. Again the sample size was small and would need increasing for more robust results and statistical significance.

6.4 Comparison of IHC and RT-PCR data

6.4.1 AR

A comparison between IHC and PCR data is necessary to see whether protein and mRNA expression reflect each other. In summary, the protein expression of AR seems to increase after 24 hours of DHT treatment in both fallopian tubal epithelia and endometrial stroma. The endometrial epithelia remained low and did not show a significant change after treatment. The mRNA expression of AR was comparatively higher in the DHT treated group in comparison to the untreated group in both fallopian tube and endometrial mucosal samples. These findings seem to be the same as each other; both showing an increase in AR expression after DHT treatment in endometrium and fallopian tube. The protein and mRNA expression of AR also seemed to be higher in the fallopian tubal mucosa than the endometrium. The RT-PCR results show expression of endometrial tissue including glands and stroma. The fimbrial fallopian tube tissue is also a mixture of epithelia, stroma and some muscularis. The individual layers cannot be distinguished in this experiment however; a consistent trend could be seen in both IHC and PCR data suggesting that DHT treatment could have an effect on AR expression.

6.4.2 SOX9

On comparison of SOX9 protein and mRNA expression the general trends were the same. In summary; fallopian tubal epithelia expressed higher protein levels of SOX9 in the untreated sample set. The endometrium showed an overall reduced protein expression in the DHT treated samples due to the larger difference in threshold in the samples that had lower expression of SOX9 in the treated group compared with

the vehicle tissue. The PCR data showed higher mRNA expression of SOX9 in the untreated tissue compared with the treated tissue in both endometrium and fallopian tube. The PCR data suggests that after 24 hours of DHT treatment a decrease in SOX9 expression occurs in comparison to the same tissue that is untreated. The IHC results also showed an overall decrease in SOX9 levels in the treated sample group however the data was less conclusive because some tubal and endometrial samples were showing an increase in SOX9 expression post-treatment. This meant that the overall decrease was very subtle and would need a larger sample size and further investigation to produce more conclusive results.

6.5 Summary of Results and Discussion Points

6.5.1 The effect of androgen on endometrial and fallopian tubal mucosal proliferation and gene expression using a tissue explant culture *in vitro*.

The data obtained from this particular study suggested that DHT treatment for 24 hours increased KI67 expression and up-regulated AR expression in fallopian tubal epithelia and endometrial stroma in particular. RT-PCR mRNA expression of AR confirmed the IHC findings.

Data obtained for the expression of SOX9 showed that DHT treated samples had lower mRNA expression of SOX9 in comparison to the untreated controls.

From the data, it could be suggested that androgens increase the proliferative index of fallopian tubal and endometrial mucosa. Androgens up-regulate their own receptor targeting the epithelia of the fallopian tube and stroma of the endometrium, specifically. Finally, androgen reduces/ inhibits SOX9 expression in tubal and endometrial epithelia and this could possibly influence proliferation. SOX9 is thought to be a suppressor of the Wnt/beta catenin pathway.[115] An active Wnt/beta catenin pathway may influence proliferation. Suppression of SOX9 may activate this pathway thus increasing proliferation.

Chapter 7 Discussion

7.1 Investigating Phenotypic Similarities between Fallopian Tube and Eutopic Endometrium

Tubal epithelium exists as a continuum with the endometrial epithelium and these tissues share the same embryonic origin. Both epithelia are of columnar phenotype. This is different to the cervical epithelium, which also adjoins the endometrium at the distal end of the reproductive tract in that it is a stratified squamous epithelium and has a well characterised boundary, in the transformation zone.[106] As described in the introduction and hypotheses of this thesis, although both the endometrium and fallopian tubal mucosa have been characterised to some extent individually; little direct comparison has been made between both tissues with no exploration on a possible boundary between these epithelia, which has seemingly very different functions. When considering the endometrium, although the two functional zones are well established both clinically and histologically, there is little data on the differences between exact cell types that are located in these zones. Recent evidence from our group in Liverpool has revealed some markers that characterise the basalis endometrial epithelial cells. This provides a start by which further markers can be identified with the possibility to explore functional relevance in the different endometrial zones.[64, 2]

The work presented in this thesis examines the expression patterns of a variety of phenotypical markers that have been proposed to be, or shown to be differentially expressed in stratum basalis and functionalis. The expression of the same markers has been examined in both proximal and distal fallopian tubal epithelium to identify a similarity between the two epithelia. Discussion below explains the data presented in this thesis in the context of the available evidence.

7.1.1 Basal Cell Adhesion Molecule (BCAM) in Fallopian Tube and Endometrial Epithelia

To my knowledge, this is the first description and comparison of the presence of BCAM in the endometrium and tubal epithelium, to date. The results section of this

thesis revealed that this molecule was abundantly present in the epithelial cell junctions of the fallopian tube and the endometrium. Similar high expression was seen between the fimbrial fallopian tubal epithelia and basal glandular epithelia and moderate levels of expression were observed in the functional and luminal epithelia of the endometrium. Proximal tube (isthmic area) showed moderate BCAM expression with little cyclical variability. During the premenopausal menstrual cycle; a universal increase of BCAM expression occurred during the proliferative phase. Lower expression was observed in the secretory phase. The postmenopausal tissue (typically non-proliferative) also revealed higher expression scores for BCAM staining in the fallopian tube and in the glandular epithelium.

Basolateral BCAM staining on the epithelial cell surface indicates that there is an interaction with laminin which is thought to be important to maintain the typical epithelial basolateral polarity associated with differentiated tall-columnar epithelial phenotype.[98] The observed BCAM staining on the whole surface of the epithelial cells may suggest that these cells do not fully engage with laminin in the basement membrane, may be less polarised, more mobile and have a less differentiated phenotype. As was described in the results, the tissues tended to have a mixture of basolateral and whole cell surface positive BCAM expression. More whole cell surface expression of BCAM could be observed in the basal glandular epithelia, suggesting less engagement with laminin than the other compartments. Basolateral junctional staining predominated in the secretory phase in the basal glands. Staining was strongly expressed in the basal glandular epithelia during the proliferative phase but this was predominantly whole cell surface staining. This suggests that less engagement with laminin occurs during this phase. Postmenopausal endometrial BCAM showed a substantial amount of whole cell surface and some basolateral expression.

During the postmenopausal period, both endometrial glands (which are atrophic, with a basalis phenotype)[59] and tubal epithelia showed intense staining for BCAM indicating that; the epithelial cells in this region are similarly engaged with laminin in the postmenopausal period - which is typically in a hypo-oestrogenic state. Furthermore, this may also suggest that fallopian tube and endometrial epithelial

cellular engagement with laminin (in the basement membrane) exists as a continuum and are therefore, similar. Messenger RNA expression for BCAM in the postmenopausal fallopian tube and matched endometrium was similarly higher in comparison to the same of the pre-menopausal secretory phase samples. This may support the hypothesis that, the epithelia of fallopian tube and endometrial basalis have a similar phenotype in the postmenopausal state. The limiting factor in PCR is that the mRNA expression reflects the whole endometrium and tubes and compartments cannot be split and may contain a different proportion of other cell types than the epithelial cells.

BCAM is also known as Lutheran blood group glycoprotein. Many refer to this molecule as Lutheran/Basal Cell Adhesion Molecule (LU/BCAM). This is because even though Lutheran is a known splice variant of BCAM in immunohistochemistry, antibodies cannot distinguish between LU and BCAM because both epitopes are in the extracellular domain.[107] BCAM is a receptor for laminin.[65] Laminins are an integral component of all basement membranes, [108] and have many cellular functions including roles in: adhesion, motility, growth, differentiation and apoptosis.[109] BCAM has been found to be present in some epithelial cells in human tissue. Positive expression of this molecule has been found in hepatic epithelial cells, haematopoietic cells, chorionic villi of the placenta and the glomeruli, arterial cells and tubules of the kidney.[110] BCAM has also been found on hair follicles and in areas of inflammatory epidermis.[111] Presence of BCAM is found solely in erythrocytes of the blood.[112] Further research would be required to confirm these findings in the endometrium and in tubes and to examine if similar functional roles of BCAM described in other organs are relevant to the function of BCAM in endometrial and tubal epithelium.

7.1.2 (Sex Determining Region Y)-Box 9 (SOX9)

The presence SOX9 in matched eutopic endometrial and fallopian tube samples in both pre and post-menopausal women was determined using immunohistochemistry (IHC) and reverse transcriptase- polymerase chain reaction (RT-PCR) laboratory procedures. SOX9 is a high-mobility-group (HMG) domain transcription factor.[113] Its nuclear location would therefore support that SOX9 was

functional in the cells of expression, which was observed in both eutopic endometrial and fallopian tubal epithelia. Threshold analysis using imageJ software was used to calculate the percentage of DAB/haematoxylin staining, to quantify the expression of this molecule. SOX9 has been located in many human tissues including those in the female and male reproductive tract.[2, 59] These include; sertoli cells of the testes in males and more recently, in the female endometrial epithelia.[114, 64]

From the results in this thesis, it can be seen that strong positive staining of SOX9 is present in the basalis throughout the menstrual cycle with very strong positive staining in the postmenopausal state. In previous studies, the endometrial epithelia were found to express SOX9 abundantly in the basalis layer of the endometrium. SOX9 expression was also very high in the postmenopausal endometrium.[64, 2] Postmenopausal endometrium retains the basalis phenotype, which subsequently stores the progenitor cells in this region.[2, 59] With this in mind, the high expression of SOX9 in the basalis and postmenopausal epithelia suggests that there may be a tissue specific role for SOX9 in this region. It has also been suggested that SOX9 influences the proliferation capacity of epithelial cells. There is some evidence surrounding this. An article by *Akiyama et al* suggested that cross-talk between SOX9 and β Catenin (Wnt pathway) influenced the proliferative abilities of chondrocytes. They found that when SOX9 was overexpressed in mutant mice this resulted in inhibition of proliferation forcing differentiation of chondrocytes. These changes were also evident in beta catenin inactivated mice, thus suggesting that interaction between these two molecules may control a chondrocyte's ability to differentiate and proliferate.[115] This was however an animal study, performed on mice and further research would be necessary to confirm if a similar phenomenon occurs in human tissues. Other studies have supported these findings for example; haploinsufficieny of SOX9 in humans causes premature skeletal mineralisation.[116] Relating this evidence to the results; the high expression of postmenopausal epithelial SOX9 would be expected because the tissue is in a quiescent, non-proliferating state, yet the Wnt/ β catenin pathway is not expected to be active in inactive epithelial cells. The functional epithelial expression of SOX9 was generally

less strong than the basal compartment, which agrees with the possibility that the activated stem cell containing basalis epithelium which is expected to have an active, Wnt/β catenin pathway also has high SOX9 expression likely to regulate and harness the proliferation process.

Relating this evidence to the fallopian tubes, both distal and proximal fallopian tubal epithelia showed high expression of SOX9. Particularly high expression was evident in the postmenopausal epithelia which was comparable to the basal glandular epithelia. If strong presence of SOX9 indicates that epithelial cells are in a more quiescent, non-proliferating state; this would suggest that tubal epithelia are more quiescent or exhibiting a less differentiated phenotype in the postmenopausal period. Similarly, more proliferative pre-menopausal tubal epithelia also showed high nuclear SOX9 expression and theoretically this would be beneficial because this would deter a blastocyst from implanting in the fallopian tube and therefore allow its transport towards the functional endometrium where implantation normally takes place. This also provides evidence to suggest that tubal epithelial cells are phenotypically similar to basal glandular epithelial cells.

The PCR data showing expression patterns of SOX9 relative to GAPDH supports all of the evidence above.

Further research would be necessary to confirm the role of SOX9 in proliferation and differentiation in both endometrial and fallopian tubal cells. Hypothetically, tubal pathology could also be associated with a loss of SOX9 expression. This highlights the direction for research that could investigate this further.

7.1.3 Stage Specific Embryonic Antigen-1 (SSEA-1).

Immunohistochemistry was performed in matched eutopic endometrial and fallopian tubal samples to determine the presence of SSEA-1 (also known as Lewis X or CD15). The staining pattern was observed and quickscore methods of analysis were used to record its presence.

The antigenic epitope SSEA-1 or Lewis X carbohydrate has a role in the adhesion and migration of the preimplantation mouse embryo.[117] It is a cell surface marker

and cell surface staining was observed on the endometrial and fallopian tubal epithelia after IHC.

In this study, human fallopian tubes were compared with endometrium to examine whether any similarities were present between the fallopian tubal epithelia and glandular epithelia of the basalis/functionalis compartment of the endometrium. Basal glandular epithelia strongly expressed SSEA-1 in comparison to the functional epithelia. SSEA-1 has previously been found to accumulate in the basalis compartment of the endometrium - the region where progenitor cells reside. Studies in our lab discovered that SSEA-1 was strongly expressed in human glandular epithelia in the stratum basalis region of the endometrium. Endometrial cells were isolated on their expression of SSEA-1 and grown in 3D culture. The SSEA-1 expressing cells were found to produce a higher number of endometrial gland-like spheroids in comparison to the cells from the same biopsy that did not express SSEA-1.[2] The study also analysed epithelial SSEA-1 staining patterns and menstrual cycle variability in human endometrial samples. From the results in this thesis it can be seen that SSEA-1 was particularly strong in the menstrual and proliferative phases which was similar to the staining reported in the previous publication that claimed that glandular epithelial expression was stronger in the proliferative phase. Another similarity between this study and the article by Valentijn et al. Was the observation in the postmenopausal endometrial glands; where SSEA-1 expression was very strong. It is known already that postmenopausal endometrium retains the basalis phenotype and this evidence further confirms that this marker is specific for cells that have a phenotypical similarity to the basal glandular epithelium of the endometrium. Luminal epithelia had low SSEA-1 expression in the postmenopausal population therefore differentiating the luminal epithelial cell from the basal glandular epithelial cells.

With the similarities between these results and the previously reported results from our labs – this confirms the reproducibility of the data and thus increases the robustness of the findings.

SSEA-1 has not been described in the human tubal epithelium before and my results show the, premenopausal tubal epithelial expression of SSEA-1 to be low in comparison to the basal endometrial glandular epithelia. This indicates that there may be phenotypic differences in endometrial and fallopian tubal epithelia in the premenopausal female. Variability between cycle stages however, was the same in both the glandular epithelia and proximal and distal epithelia of the fallopian tube - with an increase in SSEA-1 expression during the proliferative phase and very low expression during the secretory phase of the menstrual cycle. This shows that even though expression was lower, the expression pattern was similar – indicating similar cellular response between tissues. Another interesting finding was that – a relative increase in expression of SSEA-1 was observed in postmenopausal fallopian tubal epithelial cells. This was relative to the cycling epithelial expression of SSEA-1 in the tubes and was a finding also observed in the postmenopausal glandular endometrium. Similarities in the pattern of the cellular expression of SSEA-1 existed between both mucosal linings in both pre and postmenopausal women. It has briefly been mentioned that SSEA-1 is present in the epithelia of mouse oviducts but there was little conclusive evidence from this. In this thesis, human tissue was tested for the presence of this marker to directly compare cells of the basalis endometrium and fallopian tubal tissue.[118]

This marker was selected because its presence in specific tissue compartments has been postulated to indicate that primitive, progenitor cells may reside in those particular regions. As has been previously stated; epithelial progenitor cells are likely to reside in the basalis compartment of human endometrium.[119] This was considered because of the basalis compartment's remarkable capacity to regenerate the functionalis layer of endometrium every month under steroid hormonal regulation. Evidence for this theory was first provided by Chan et al. who discovered clonogenic epithelial and stromal cells residing in the human endometrial tissue.[120] Further research has expanded on this and has lead researchers to believe that a stem cell population resides primarily in the basalis compartment of human endometrium. Recently, it has also been thought that the distal fallopian tubal mucosa may also contain a stem cell population,[4] an increase

in cells expressing markers that were shown to be stem cell markers in other tissues, were identified here.[1] This suggests that in the fallopian tube, the distal mucosa may be the equivalent to stem cell-rich endometrial basalis. The increase in expression of SSEA-1 in postmenopausal fallopian tubal epithelia provides evidence to suggest that the fallopian tubal mucosa becomes phenotypically more similar to the stem cell-rich endometrium, after menopause.

7.1.4 Beta Catenin

Beta catenin was localised to the cell nucleus, cytoplasm and junctions after IHC staining of endometrial and fallopian tubal tissue. Here we focus on the nuclear and cytoplasmic staining as these cellular locations informs us about beta catenin's involvement in the Wnt pathway.

In this thesis, the results show that there is an increase in cytoplasmic beta catenin expression during the proliferative phase of the menstrual cycle in endometrial and tubal epithelia, especially in the basalis compartment of the endometrium and fimbrial tubal epithelia. It has been found previously that endometrial positivity for beta catenin increases during the proliferative phase.[121] Thereby suggesting that these particular cells have the capacity to proliferate and differentiate. It has also been found that beta catenin is essential for normal endometrial epithelial function and glandular formation. Overexpression has been found to cause hyperplasia in mice.[122] From the results nuclear expression of beta catenin was low – a select population of nuclei were positive for this molecule in the basalis compartment of the endometrium. This supports the results made previously in our labs, studying beta catenin expression in normal endometrium.[64] Cytoplasmic positivity was stronger and more evident therefore quickscore levels were higher throughout. Again, like nuclear staining; cytoplasmic beta catenin was highest in the basalis region suggesting that this molecule resides here in the regenerative layer, like the intestinal crypts. The Wnt pathway is known to be involved in cellular differentiation, proliferation and migration.[123] In the absence of Wnt protein molecules; beta catenin is degraded by its phosphorylation at its N terminus. In the presence of Wnt this does not happen and beta catenin is allowed to accumulate in the cytoplasm and translocate to the nucleus where it can interact with HMG box

family and together, activate target genes. It has been found that activation of beta catenin in chondrocytes results in an increase in cell proliferation and differentiation.[124] The Wnt pathway is important in the organisation of the intestinal epithelium including maintaining cells in their undifferentiated state. It has previously been found that loss of beta catenin results in a loss of intestinal epithelial cells in the intestinal crypts – the site where regeneration takes place.[125]

The presence of beta catenin in fallopian tubal epithelia is yet to be compared to the endometrium. Expression of nuclear beta catenin was low in this study but cytoplasmic accumulation of this molecule was moderate in the fallopian tubes. The distal fallopian tube saw an increase in cytoplasmic accumulation of beta catenin during the proliferative phase, similar to the expression pattern in the basal endometrial glands. Beta catenin was been shown to be expressed for the first time in fallopian tubal tissue in humans in 2013. The article compared normal fallopian tubal epithelia with pathological tubes that had ectopic pregnancy. The study found that beta catenin was located in the epithelial portion of the tube. The study also found that women with salpingitis or ectopic pregnancy had significantly higher beta catenin expression than in the normal fallopian tube samples. They did not evaluate the expression throughout the menstrual cycle nor did they compare it to endometrial glandular epithelia.[126] Supporting evidence has claimed that an increase in beta catenin expression increases the proliferative abilities of an epithelial cell.[115] Presence of this molecule in the fallopian tubes and its relatively large variation between cycle stages in comparison to the endometrial glandular epithelia provides evidence to show that tubal epithelial cells are responsive to the Wnt pathway and have some differentiation and proliferative abilities.

A unanimous finding, in both tissues was that cytoplasmic beta catenin increased in the postmenopausal population. Nuclear beta catenin remained low however; with a small subpopulation of positive cells. Even though the postmenopausal epithelia are in a quiescent state; accumulation of cytoplasmic beta catenin could suggest that the epithelial cells in this population have the capacity to differentiate, thus making them more susceptible to hyperplastic or metaplastic changes. The low nuclear expression suggests that beta catenin may be prohibited from entering the

nucleus in this population. The progenitor cells in the postmenopausal endometrium could be the cells that are strongly positive for this molecule thus more vulnerable to change. It may also be suggested that tubal epithelial cells have a similar phenotype to glandular epithelial cells in the postmenopausal population.

7.1.5 Sialylated SSEA-1 (Sialyl-SSEA-1)

IHC analysis with representative quickscore data showed the expression of this marker on the cell surface of epithelia in matched endometrial and fallopian tube samples.

From the results it can clearly be seen that luminal epithelia of the endometrium were strongly positive for Sialyl-SSEA-1. The embryo attaches to the endometrial luminal epithelia at implantation therefore this would support previous evidence. The pattern of expression throughout the menstrual cycle however was not significantly different between phases therefore a larger sample size would be needed in order to confirm that Sialyl-SSEA-1 was abundant particularly during the implantation period. The glandular epithelia showed some positivity but was generally weaker. Postmenopausal endometrial expression of Sialyl-SSEA-1 was moderate in both the glandular epithelia and lumen suggesting that expression continues. Similar expression was observed therefore showing phenotypical similarities between these cells in the postmenopausal state. Hey et al. Described the presence of Sialyl-SSEA-1 in human endometrium. The authors claimed that there was a link between this and the marker MUC1. MUC1 has been found to be abundantly expressed during the implantation period and is thought to play a role in the promotion of adhesion for implantation. Here, the authors linked this marker with Sialyl-SSEA-1 - claiming that expression patterns throughout the menstrual cycle were similar, in particular the increase shown to occur during the secretory phase.[82] Therefore it was speculated that Sialyl-SSEA-1 is associated with implantation of the embryo in endometrial tissue.

Sialylated SSEA-1 or Sialylated Lewis X is a selectin ligand. It is known to be an essential carbohydrate ligand for human sperm-egg binding. This is because this oligosaccharide is most abundantly present as the terminal sequence on the N- and

O-glycans of the human zona pellucida (extracellular matrix of the oocyte).[99] It has a role in the adhesion of various cancer cell types to endothelial cells[127]

If Sialyl-SSEA-1 was the key component for adhesion during implantation; it would be expected that the tubal epithelial cells would be negative for this marker given that implantation would not normally occur in this site. Interestingly the tubal epithelia, particularly at the isthmic end, strongly expressed this marker in this thesis. There was a reduction of Sialyl-SSEA-1 during the proliferative and increased expression during the secretory phase. It could be speculated that the presence of this marker has a different role in the premenopausal fallopian tube. After ovulation, fertilisation occurs. There has been evidence to believe that in mammals; a sperm reservoir is formed in the isthmic fallopian tube after it has entered the uterus and ascended towards the fallopian tube in order to fertilise the oocyte. The sperm is thought to stick to ciliated epithelial cells through the binding of carbohydrate residues on the surface of these cells. [128] This is thought to be present in order to restrict the amount of sperm entering the ampulla to promote a single sperm entering the oocyte and thus avoid polyspermy.[129] With this in mind, a recent study in goats found that Sialyl-SSEA-1 was present on the oviductal epithelia. It was particularly abundant in the isthmic and ampullary portion of the fallopian tube. The authors suggested that this marker could have a role in the creation of a sperm reservoir.[130] From the results in this thesis; it can be seen that in human tissue - the isthmic region of the fallopian tube abundantly expresses this marker. This data would expand on and support the suggestions made in previous studies in other mammals and would suggest that the proximal end of the fallopian tube is a reservoir for sperm in the premenopausal woman. Further studies would need to confirm this and functional studies could be performed. Postmenopausal expression of Sialyl-SSEA-1 was abundant in the fallopian tube as well as the endometrium. The functional relevance of this is yet to be confirmed.

7.2 Investigating Sex Steroid Hormone Receptor Expression and Proliferation in Human Fallopian Tube and Endometrium

7.2.1 KI67 and Proliferation

KI67 is a protein that indicates the proliferative ability of a cell. It marks the growth fraction of a cell population and is located in the nucleus.[84] When a cell is positive for this protein after IHC staining - the DAB is clearly visible under a light microscope which enables accurate counting of positive cells and subsequent determination of the fraction of proliferating cells in the area of tissue being analysed. Overexpression of this marker has also been implicated in various cancers for example; breast cancer. When cells are in the resting state KI67 is not present.[84]

From the results it can be seen that the glandular epithelia in both the basalis and functionalis layers of the endometrium undergo cyclical variation with the highest KI67 expression in the proliferative phase and lowest during the secretory phase. Higher KI67 expression was detected in the superficial glandular epithelia indicating that this region has higher mitotic activity and cell proliferation. Stromal cells of the functionalis also had proliferative ability and less so in the basal stroma. Every month, under hormonal control, the endometrium undergoes cyclical changes where the mucosa has a period of regeneration and proliferation, secretion and shedding. KI67 positivity has been detected in glandular epithelial cells of the endometrium. A study by Ilie et al. showed that cyclical variation of this marker existed. Higher positivity was observed in the proliferative phase sample population in comparison to the secretory phase population (22.5 % and 2 %, respectively). This indicated that this marker could accurately demonstrate that during the period of increased mitosis and proliferation (the proliferative phase of the menstrual cycle) an increase in KI67 occurs.[131] This was also stated by Konstantinos et al. The authors also correlated KI67 scores with endometrial carcinoma severity.[86] This could support the hypothesis that the basalis region is generally less proliferative and is the site of the undifferentiated, quiescent, progenitor cells. Confirmation that KI67 is a good marker of proliferative activity was seen in the postmenopausal results which showed very little/negligible KI67 expression. This is due to the fact the postmenopausal endometrium is in a more quiescent state.

KI67 expression in the normal fallopian tube was generally low. This would be expected because the endometrium regenerates and proliferates to promote the implantation of an embryo. The normal fallopian tube however; does not function

as a site of implantation thus would be expected to have low proliferation. There was still some cyclical variability at the distal end showing an increase in proliferative activity during the proliferative phase. This could indicate that this region's epithelia has proliferative tendencies under specific hormonal control. The proximal tubal epithelia did not show this cyclical variation, suggesting that it is phenotypically different to the distal end of the tube. KI67 has been identified in the oviducts of mammalian epithelia and its expression has been investigated as a marker of proliferation in fallopian tubes with dysplastic changes – to indicate whether there was an association with ovarian cancer.[132] It has also been analysed in the oviducts of pigs to indicate whether steroid receptors affect proliferative activity, but not in humans.[133] It is necessary to compare this marker with steroid receptor expression in the fallopian tubes to deliberate whether proliferation is affected by cyclical hormonal changes. Like the endometrial epithelia, postmenopausal KI67 expression was very low/negligible indicating that these cells also become more quiescent.

7.2.2 Androgen Receptor (AR)

Immunohistochemical staining with modified quickscore analysis methods and RT-PCR techniques were used to study the nuclear expression of this receptor in both human endometrium and fallopian tube samples.

It can be seen from the results that endometrial expression of AR is confined mainly to the stromal nuclei. This is in agreement with previous literature. Stromal expression was stronger in the basal compartment compared to the functional compartment but both had abundant expression throughout the cycle. Basal stromal expression of AR was highest during the secretory phase. Glandular presence was low which has also been mentioned in previous studies. This would suggest that any effects of androgens on the endometrial glandular epithelia would be through the activation of stromal AR – thus an indirect effect. A statistically significant increase in luminal epithelial AR expression occurred in the postmenopausal population – showing that the receptor is up-regulated; possibly promoting an anti-proliferative state. Androgen levels in the serum undergo cyclical changes. Studies have shown that total and free testosterone and androstenedione

have a mid-cycle peak and are relatively higher during the luteal phase.[33] Androgen receptor activity has been explored in the endometrium.[134] A study on AR knockout mice revealed that these animals had smaller uteri, abnormal uterine growth in response to gonadotrophins, large reductions in the number of pups in each litter and abnormal placentation.[101] Various studies have reported that the androgen receptor is localised predominantly to the stromal cells of the endometrium (barely detectable in the glands), with an abundance of receptor expression during the proliferative, early and mid-secretory phases.[31] After the menopause, AR persists in the stromal nuclei.[32]

From the results, it can be seen that AR is strongly expressed in the epithelia of fallopian tubes. The first striking difference between the endometrium and fallopian tube is that the expression here - is epithelial. This would suggest that androgens have a direct effect on the epithelia of tubal mucosa as opposed to the indirect effect on endometrial epithelia. The strong staining pattern is evident in both proximal and distal tubal epithelia with a unanimous decrease in androgen receptor expression during the proliferative phase and higher expression during the secretory phase. This reflects the high serum androgen levels that are present during the early and mid-secretory phase of the menstrual cycle.[33] Both the endometrial stroma and the tubal epithelia have high AR expression in the postmenopausal population. RT-PCR results also confirmed this. Only one previous study has mentioned the presence of androgen receptor in human tubal epithelia. This was an article written by Nodin et al. in which they identified the abundantly positive expression of this receptor in the epithelia of normal fallopian tubes. They were however; comparing this mucosa to epithelial ovarian carcinoma and did not analyse this expression in conjunction with cyclical changes, nor did they compare the tubal expression with endometrium.[34] Interestingly, this study found that epithelial ovarian carcinoma had dramatically reduced expression of AR in comparison to the normal healthy fallopian tubal tissue. In this thesis it has been found that AR expression is strong in postmenopausal fallopian tubal epithelia. Most epithelial ovarian carcinoma cases occur after menopause.[135] Therefore, we can confirm that that there is a difference in AR expression in normal, healthy

postmenopausal women. The article failed to state whether the normal fallopian tube controls were in the postmenopausal or premenopausal state. Combining the evidence; it may be that changes/loss of AR expression may be implicated in the pathogenesis of epithelial ovarian carcinoma. As suggested in previous articles; AR could have an anti-proliferative function and promote the atrophic state that is apparent in the postmenopausal period. The luminal epithelia up-regulates its receptor in the same way that the tube does. This suggests that there is a continuum between the endometrium and the fallopian tube in this particular population.

Correlations made between KI67 and AR revealed that tubal expression of KI67 appeared to be the opposite of the AR expression for example; during the secretory phase KI67 decreased and AR increased. Both endometrial stromal and fallopian tubal epithelial AR was strongly expressed in the postmenopausal population whereas KI67 expression was negligible. It has also been found that E2 up-regulates AR whereas progesterone has been found to down-regulate AR.[136] An article by Slayden et al. found that anti-progestins up-regulated AR in human endometrium. Anti-progestins are known to inhibit endometrial growth therefore it was speculated that AR may influence this anti-proliferative effect and promote atrophy of the endometrium.[137] These studies further point to the hypothesis that AR has anti-proliferative tendencies.

7.2.3 Progesterone Receptor (PR) in Endometrial and Fallopian Tubal Mucosa

Progesterone is known to down-regulate its own receptor. High concentrations of E2 induce PR synthesis.[138] Given that the receptor is responsible for gene transcription - its presence is detected in the nucleus using immunohistochemistry techniques. The particular clone used in this study recognised both PR-A and PR-B isoforms.

In the present study, the results are in agreement with previous studies. A decline in PR occurred in both compartments of the endometrium during the secretory phase of the menstrual cycle. The expression was high during the proliferative phase of the cycle. When E2 levels are particularly high.[138] This occurred in both the stromal

and epithelial cells of the endometrium. The stromal decline of PR in the basalis, during the secretory phase was less evident. It has been speculated in previous studies that there is a need for PR during this phase in the stroma for continued growth and differentiation.[139] Interestingly, PR expression was high in the postmenopausal population in both the stromal and epithelial compartments in the current study. A previous study by Mylonas et al. revealed that PR-A expression was high in the postmenopausal population while PR-B expression was low. [140] Both PR-A and PR-B was detected with this antibody therefore separate isoforms could not be distinguished.

The steroid hormone progesterone is essential for successful pregnancy to be maintained. It is has essential roles in glandular differentiation and glycogenesis, proliferation of stroma and development of pre-decidual cells.[138] Progesterone receptor has been identified in the human endometrium. It is known that PR varies throughout the menstrual cycle.[141] From the results shown in this thesis it is also evident that PR varies in both the stromal and epithelial cells of the endometrium. Due to the high E2 levels during the proliferative phase of the menstrual cycle, PR increases in stromal and epithelial cells of the endometrium.[140] During the mid - late secretory phase a decline in PR expression can be seen – this is due to the fact that during this phase, an increase in serum progesterone occurs.[142]

In the current study, it seems that high progesterone expression was observed in epithelia and stroma of the fallopian tube throughout the menstrual cycle with little/no cyclical variation. This conflicts with evidence from previous studies. Functional relevance of this receptor still remains unclear in this tissue. Postmenopausal localisation of PR has not been reported previously. It seems as though epithelial PR is reduced but stromal PR remains high. The stromal PR is therefore reflective of the stromal endometrial PR expression. This suggests a phenotypic and possibly functional similarity between the stroma. The reduction of PR seen in the epithelial compartment of the fallopian tube suggests a unique phenotype of these cells in the postmenopausal population. In a previous study, progesterone receptor was localised to the nucleus of all cell types in fallopian tubal mucosa.[42] In the same study epithelial cells of the fallopian tubes decreased during

the secretory phase and mRNA levels also detected this pattern. Another study, using PCR techniques saw a decline in the secretory phase in relative expression of PR. Treatment of cells with progesterone caused down-regulation of PR-AB and B *in vitro*. Horne et al. However only had data collected from PCR techniques which would not take into account the individual compartments of the fallopian tube.[43] High exposure of fallopian tubal ciliated epithelial cells to progesterone caused a decrease in motility of the cilia leading to dysfunction and possible risk of ectopic pregnancy.[143] This could explain the high progesterone receptor positivity detected in the premenopausal epithelial cells of the fallopian tube because the presence of the receptor indicates that there is a low interaction with progesterone in the normal fallopian tubal epithelia of the premenopausal female. This could be the optimal environment for ciliary activity to occur and allow smooth transport of the oocyte down the fallopian tube. It has been said that the delicate ratio between both PR isoforms is what makes the endometrium vulnerable to pathological change.[140] This could also be the case in the fallopian tube. Further functional studies are needed to confirm this.

Correlating the KI67 marker with PR in the endometrium. It was observed that PR diminished during the secretory phase and KI67 also decreased. Progesterone is high during the secretory phase and a reduction in proliferation is seen during this time, typically. The high PR during the proliferative phase may indicate that the receptor influences proliferation in the premenopausal endometrium. The discrepancy that is evident is that PR increases in the postmenopausal population. It could be that the particular isoform that dominates (PR-A) in this population may have anti-proliferative tendencies when expressed in isolation. Progesterone receptor was highly expressed in the fallopian tubes whereas KI67 expression was low throughout, suggesting that this mucosa has unique steroid hormone response in comparison to the endometrial mucosa. The postmenopausal tubal epithelia had both reduced PR expression and KI67 expression suggesting an anti-proliferative function of PR in this tissue sample. Studies with immuno-staining for both PR-A and PR-B would be useful on postmenopausal endometrial and fallopian tubal tissue to see whether one isoform dominates. The luminal expression of PR

dynamically changed throughout the menstrual cycle whereas the proliferative index remained low. This suggests that progesterone does not influence proliferation in this type of epithelia. This steroid hormone may have a different function here.

7.2.4 Oestrogen Receptor Alpha and Beta in the endometrial and fallopian tubal mucosa

With the transcriptional effects of ER alpha and beta these receptors are present in the nuclei of cells. IHC techniques can localise these receptors. Oestrogen Receptors alpha and beta have been immunolocalised in the endometrium. They have an essential role in the regulation of endometrial growth.[47]

From the current study it is evident that both ER alpha and beta were present in human endometrium. There also seems to be a lack of evidence surrounding the compartmental differences in ER expression throughout the endometrial zones. ER alpha expression was higher during the proliferative phase of the menstrual cycle and reduced during the secretory phase in glandular epithelia. This supported the evidence by Mylonas et al. Whose results showed that both oestrogen receptors peaked during the proliferative phase of the menstrual cycle and declined in both the glandular and stromal tissue during the secretory phase.[144] Functional stroma had the same pattern of expression as the epithelia. Basal stroma had lower expression of ER alpha but a minor increase during the proliferative phase was observed. In the past it has been suggested that oestrogenic effects occur mainly through ER alpha and ER beta modulates oestrogenic action.[46]

ER beta expression in basal glandular epithelia appeared to increase during the proliferative and secretory phases of the menstrual cycle. The persistent expression of ER beta suggests that it may have a different function to ER alpha. The functional glands had consistently high ER beta expression with no variation between phases, unlike the dynamic changes occurring with ER alpha. The basal stromal ER beta expression increased during the proliferative phase and decreased during the secretory phase. The expression in the basalis was stronger for this receptor, suggesting that the receptor is dominant in this compartment. This indicates that it

may have a different role to the stratum functionalis. These results conflicted with those of a study conducted by Taylor et al. which claimed that their IHC analysis demonstrated that ER beta expression was reduced in the proliferative phase and increased in the secretory phase in endometrial glandular epithelia. Stromal ER beta did not correlate with other steroid receptors.[145] It has been suggested that ER beta could have a different function to ER alpha; as it has been shown that it has opposite transcriptional effects after its binding to oestrogen and anti-oestrogens.[146]

The current results found that reduced expression of ER alpha and beta occurred in the postmenopausal population in the glandular and stromal compartments. This was supported by Mylonas et al. claiming that the postmenopausal endometrium has been shown to express ER alpha and beta at lower levels than the proliferative endometrium.[140] Interestingly, the luminal epithelia had moderate expression of both ER alpha and beta in the postmenopausal population, suggesting that these receptors are active here. The interaction between ER alpha and beta receptors could be influencing the luminal epithelial phenotype in the postmenopausal state.

From the current study it was observed that ER alpha had variations in expression from the proximal to the distal end of the fallopian tube. At the distal end of the tube, ER alpha was high in both epithelia and stroma during the proliferative phase consistent with previous studies on endometrial epithelia. This was also seen in endometrium indicating that the function of this receptor is the same in both tissues. The proximal tube had the opposite trend; a higher secretory phase expression and lower proliferative phase expression was apparent, in keeping with evidence from Shao et al.[42] The proximal and distal ends of the fallopian tube appear to have different cyclical activation patterns of ER alpha. Further studies would need to confirm this.

ER beta had differing expression between the proximal and distal ends of the fallopian tube. Fimbrial epithelia had high secretory expression of the receptor, in keeping with previous evidence.[42] The stroma however had low secretory ER beta expression suggesting that both cells have differing functions during this time. The

proximal tubal stroma and epithelia had low expression of this receptor during the secretory phase. The differing ER expression from the proximal to the distal ends of the fallopian tube suggests that these cells respond in a different way to each other and this may have functional significance. In keeping with endometrial mucosa in the postmenopausal state; the ER alpha expression reduced consistent with the atrophic state that ensues throughout. Interestingly, the ER beta expression increased. This further points to the suggestions that ER beta has a unique function. It may even act to inhibit the activation of ER alpha in epithelia given the high postmenopausal expression of ER beta and lower ER alpha in the fallopian tube and endometrium.

ER alpha and beta have previously been identified in human fallopian tubes. The study by Shao et al. Identified both oestrogen receptors in the epithelia and stroma of the fallopian tube, however little or no ER beta was identified in the stroma. They claimed that a mid-secretory increase in both receptors occurred. ER alpha also increased around the late ovulatory phase. The study however; looked at expression of the steroid hormones in solely the ovulatory and secretory phase of the menstrual cycle.[42] Horne et al. Identified ER alpha and beta in the fallopian tubes and mRNA expression of these receptors were consistently high during the menstrual phase. ER beta decreased during the proliferative phase whereas ER alpha was highly expressed during this phase and moderate expression was observed during the secretory phase of the menstrual cycle for both receptors.[43] Both studies identified that ER was expressed during the secretory phase of the menstrual cycle however; the differing expression during the proliferative phase suggests that ER beta has a distinct function to ER alpha.

This is the first study to make a correlation between KI67 and the oestrogen receptors in the fallopian tubes. The comparison showed that the fimbrial tubal epithelial expression of ER correlated with KI67 showing that oestrogen receptor influenced proliferation in this cell. Relative expression of Ki67 was low however. All tubal epithelia had reduced expression of ER alpha and KI67 postmenopause, further suggesting that this tissue is atrophic in this population. ER beta expression in the distal fallopian tubes did not correlate with KI67. This suggests that ER beta

does not have a proliferative function in this region. Postmenopausal increase of ER beta suggest a non-proliferative or anti-proliferative function for this receptor. ER alpha in the endometrium also correlated with KI67 therefore further confirming that this receptor influences proliferation. It has been stated in previous studies that ER alpha is associated with proliferation.[145] The secretory ER beta expression was high and thus did not correlate with KI67 suggesting that this receptor may have a more secretory role.

7.3 Effect of Androgens on Matched Tubal and Endometrial Mucosa

Females produce and secrete a large amount of androgen, even more so than the female reproductive steroid, oestrogen. Circulating androgens include: dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione (A), testosterone (T) and dihydrotestosterone (DHT). Even though the latter two have the smallest serum concentrations, they are the only two that bind to the androgen receptor.[147] DHT can be used as a treatment in explant cultures to observe the effects of androgens on tissue. Various tissues have been treated *in vitro* with androgens including the endometrium (to look into factors that mediate the induction of aromatase).[148] In the current study it was found that the androgen receptor was highly expressed in fallopian tubal epithelia. This differed from the mainly, stromal expression seen in the endometrium. With this in mind, a study to compare the effects of androgens on the mucosa of fallopian tubes and endometrium enables us to gain an insight into the effects of this steroid on the mucosa of these tissues and elucidate any differences that could be observed to hypothesise further. This was the first study to look at androgen response in human fallopian tubes. This was a small, preliminary, descriptive study and a larger sample size would confirm the findings from this thesis.

7.3.1 Effect of Androgens on Androgen Receptor

The current study found that AR protein expression in fallopian tubal epithelia was higher in the DHT treated group than the untreated group. This suggested an up-regulation of AR under the influence of DHT. The endometrial stroma had higher expression of AR overall, after DHT incubation. The response of androgens on the

androgen receptor in fallopian tubal epithelia demonstrates that the receptor is sensitive to the exposure of androgens – this suggests a functional relevance of androgens and its receptor within fallopian tubal epithelia. RT-PCR results also confirmed that the androgen receptor was at a higher relative expression in the samples that had been incubated with DHT. This has further supported the theory that androgens up-regulate their receptor in endometrial and fallopian tubal mucosa. Stronger AR protein and mRNA expression was evident in the fallopian tubal mucosa in comparison to the endometrium. This was also supported by the high protein and mRNA expression in the original (non-explant) fallopian tube samples. The functional relevance of this remains unclear.

It has been found in previous studies that *In vitro* treatment of tissue with testosterone up-regulated the androgen receptor.[105] It has also been shown that satellite cells from human skeletal muscle increased AR protein expression after DHT treatment *in vitro.* The authors concluded here, that androgens increased muscle mass through mediating differentiation of mesenchymal precursor cells.[104] This further supports the evidence that AR is up-regulated by active androgens. It is known that AR is confined mainly to the stromal compartment of the endometrium.[31] Therefore androgens would respond mainly to the receptors in this compartment. The up-regulation of this receptor after treatment with androgens confirms the previous discussion (see 7.2.2) that androgen receptor varied throughout the menstrual cycle in accordance with the cycling serum androgen levels *in vivo.*[33]

A brief look at the effect of androgens on proliferation and the transcription factor SOX9; may further indicate the role of androgens in endometrial and fallopian tubal mucosa.

7.3.2 Effect of Androgens on Proliferation Marker KI67

In the normal state, the endometrial stroma and fallopian tubal epithelia had low KI67 expression when AR expression appeared to be strongly expressed. Looking at the protein expression of KI67 after being treated with androgens it seemed as though there were some mixed results however; a general trend towards a higher

KI67 expression after treatment with DHT seemed to be the case in both endometrial and tubal mucosa. With the small sample size it would be difficult to make any robust conclusions from this evidence however it may indicate that there is a delicate balance between the steroid hormone receptors and their proliferative abilities *in vivo.* Any change in androgen levels could disturb this balance. The increase in KI67 expression could indicate that under the influence of an excess of androgens in otherwise normal tissue, epithelial and stromal cells could be vulnerable to changes in capacity to differentiate and proliferate. High AR expression and androgens are a key part in the development and progression of prostate cancer even though AR and androgens are required in the normal prostate. This demonstrates that any changes in the receptor and its environment could make a tissue susceptible to pathological change and increase its proliferative index.[149] It is also important to note that *in vivo,* the other reproductive steroid hormones are also functioning alongside this hormone. Therefore further explant treatment with progesterone, E2 and androgens on fallopian tubal and endometrial tissue would be a more accurate way of simulating the *in vivo* environment. Conclusions could then be drawn from observations made after removal/addition of each of the hormones in turn, therefore increasing our knowledge of the functionality of these hormones in fallopian tubal and endometrial mucosa.

7.3.3 Effect of Androgens on SOX9

The effect of androgens on SOX9 expression in normal fallopian tubal and endometrial tissue has not been considered before. From the current study a downward trend was seen in mRNA expression of SOX9 in the samples incubated with DHT in comparison to the untreated group. This suggests that androgens may decrease SOX9 expression. The protein expression of SOX9 was less clear-cut however did also show an overall downward trend after DHT treatment in both fallopian tube and endometrial epithelia. The transcription factor SOX9 has been located in basal epithelia of the prostate, the intestinal crypts and the outer root sheath of hair follicles. Stem and progenitor cells are all thought to reside in these particular regions.[75]

Studies have shown that SOX9 overexpression can down-regulate AR. If androgens up-regulate AR and cause a decline in SOX9 – it may be that the decline in SOX9 is what facilitates the up-regulation of AR. SOX9 has also be implicated in particular pathological events for example prostate cancer. Prostate cancer cells are known to be androgen dependent. Androgens and androgen receptor are normally found in the luminal epithelia of the prostate. This cell type is known to be more quiescent and less proliferative. When prostate cells undergo malignant change, androgen receptor and androgen dependence increases however; their proliferative ability also increases. This could suggest that other pathways and markers could be involved in the maintenance and growth of this cancerous cell-type. This includes interactions between SOX9 and the Wnt/beta catenin pathway.[150] As described previously.[115] Cross-talk exists between SOX9 and the Wnt/beta catenin pathway. Here it was suggested – when SOX9 is overexpressed pathological change is the same as when beta catenin is inactivated. The active Wnt/beta catenin pathway is involved in cellular proliferation and differentiation. If androgens cause a reduction of SOX9 expression, the Wnt pathway may become more active. As a result; target cells could increase their capacity to differentiate and proliferate. This could mean that they are more susceptible to pathological change. Again, the delicate balance between all of the reproductive steroid hormones is necessary and when one hormone dominates this can change the phenotypic and functional abilities of a cell. Cells that are particularly vulnerable to this change are progenitor cells with the capacity to differentiate.

7.4 General Discussion

This study was a descriptive analysis, characterising pre and postmenopausal fallopian tubal mucosa. Normal fallopian tubes have not previously been analysed using markers that characterise the endometrium. One of the aims of the study was to elucidate whether the fallopian tubes exhibited some phenotypic similarities with the basalis layer of the endometrium. This endometrial compartment is of interest because it has the important role of regenerating the functionalis compartment of the endometrium every month.[151] Its remarkable regenerative capability has led to studies to reveal that adult progenitor cells of various

phenotypes reside in the basalis compartment of the endometrium and postmenopausal, atrophic endometrium.[152] As described in the introduction section of this thesis; novel evidence has emerged claiming that side population cells reside in the distal fallopian tubes that have the capacity to differentiate and migrate. The question that came to mind therefore, was – does the fallopian tubal mucosa exhibit phenotypic similarities to the regenerative endometrial mucosa of the female reproductive tract that is also thought to contain adult progenitor cells: the basalis compartment of the endometrium? The markers that are known to phenotypically identify the basalis layer of the endometrium were tested and analysed on matched human fallopian tubal samples.

After having discussed the individual markers it is necessary to consider the tubal and glandular epithelia as a whole. It can clearly be seen that some similarities exist between the mucosae and some variations were also seen from the proximal to the distal end of the fallopian tubes themselves.

Considering the results for BCAM, the receptor for laminin (EI Nemar et al), the fimbrial fallopian tubal epithelia had high expression of this molecule, which was also the case in the basal glandular epithelia. The other compartments and proximal tube had moderate expression of this molecule. This suggested that there was a similarity between the distal fallopian tubal and the basal glandular engagement with laminin in the epithelial cells. This was further supported by the evidence that an increase in expression of BCAM occurred in the postmenopausal samples. This suggested that phenotypical similarities exist between the tubal and endometrial epithelia.

SOX9 is a transcription factor found to be abundantly expressed in the basalis region of the endometrium, it has also been located in tissues that contain a stem cell population and have regenerative capacity.[64, 75] The results confirmed the strong presence of this molecule in the basalis region of the endometrium and interestingly, the fallopian tubes were also strongly positive for this marker. This gives a good indication that the tubal epithelial cells share this phenotypical resemblance to the basalis layer of the endometrium. This was reiterated by the

strong presence of SOX9 in the postmenopausal epithelia of the endometrium and fallopian tube. Strong SOX9 expression in postmenopausal, atrophic tissue (with the capability of regenerating under the correct hormonal stimuli)[153] supports the theory that this marker resides in basal tissue regions that contain quiescent, progenitor cells. The strong presence of this in fallopian tubal epithelia could suggest that cells residing in this region have stem-like properties. Analysis of fallopian tubal epithelia for stem-cell markers would be required to confirm this. Beta catenin is a downstream molecule in the Wnt pathway. There is evidence to suggest that cross-talk exists between this molecule and SOX9.[115] The intestinal crypts, the prostate and the endometrium require this interaction in order to function as a regenerative tissue. An interesting finding was that cytoplasmic beta catenin increased in the postmenopausal population (glands and tubal epithelia) but nuclear beta catenin remained low. SOX9 expression was particularly high in this population suggesting that there may be an interaction between this molecule and beta catenin, interfering with the accumulation of beta catenin in the nucleus. With this in mind, the capacity for the epithelia to differentiate and regenerate may be reduced and a quiescent-type cell could remain.

SSEA-1 a surface marker, varied throughout the menstrual cycle in the same way that the basalis endometrial epithelia did. As previous studies have found, SSEA-1 was particularly strong in the basalis compartment.[2] Tubal epithelial expression was weaker in the cycling endometrium however; a relative increase in expression at both the proximal and distal ends occurred in the postmenopausal samples. This was also the case with the basal glands. Luminal epithelia remained low. As with the markers above, it seems as though the cells become phenotypically more similar to the glandular epithelial cells after menopause. Not only is this the case, SSEA-1 is thought to be present in tissue regions containing progenitor cells. The positivity suggests that a population of cells exist in the tube that phenotypically resemble the basal glandular epithelia.

As described in section 1.1.5, sialylated SSEA-1 is essential for sperm-egg binding.[99] The reason this marker was investigated was because it is thought to play many roles in the adhesion of cells in the body. It is abundantly present on the lumen of

the endometrium because it is believed to play an important role in the adhesion process of the embryo during implantation. An interesting finding was that tubal epithelia (especially at the proximal end) were highly positive for this molecule. Another theory drawn from these findings was that this marker indicated that there was adhesive properties of epithelia particularly in the proximal tube so that the epithelia could create a sperm reservoir for adequate control of sperm entering the ampulla. This has been discussed previously.[128]

Proliferative tendencies in the epithelia of the fallopian tubes were low. A slight increase during proliferative phase, in the distal tubal epithelia indicated that proliferation was cycle-dependent, to some extent. The endometrium had the classical proliferative pattern of an increase during proliferative phase and decrease during secretory phase. Negligible KI67 in the postmenopausal samples confirmed the atrophic state that is adopted in the tissue during this period.

Steroid receptors have been studied in some detail in endometrial tissue. There has been evidence that the receptors also reside in fallopian tubal tissue however; some of this has been conflicting. Most of the normal tissue has been compared with pathological events occurring in the tube for example; ectopic pregnancy. This study has aimed to compare the fallopian tubal mucosa with normal endometrial mucosa in pre and postmenopausal women. This is beneficial to see whether fallopian tubes respond to steroid hormones in the same way that the endometrium does throughout the menstrual cycle.

Combining all of the evidence from section 7.2 - some interesting findings became apparent in the fallopian tubes. Androgen receptor (AR) was strongly expressed throughout the fallopian tubal epithelia. This differed from the stromal expression observed in the endometrium, showing that androgens directly affect the epithelia in fallopian tubes. There was cyclical variation with a reduction in receptor expression during the proliferative phase and stronger expression during the secretory phase. Postmenopausal expression was high. This suggested that when receptor expression is high cells seem to be of a less proliferative type and have a more secretory role. The promotion of proliferation has been linked to the

expression of oestrogen receptor alpha (ER alpha).[47] In the distal fallopian tube, higher expression during the proliferative phase and reduction of expression in postmenopausal samples would support the evidence above. Interestingly, ER alpha and beta seemed to have opposing expressive tendencies throughout the menstrual cycle. Postmenopausal expression of ER beta increased while ER alpha decreased. The distal tubal epithelial expression of ER alpha increased during the proliferative phase. The proximal tubal mucosa seemed to have the opposite receptivity to steroid hormones. ER alpha was higher during the secretory phase and ER beta was low during this phase. This region could experience more growth and differentiation during the secretory phase of the menstrual cycle. In fact, the higher secretory phase expression of ER alpha was shared with the luminal epithelia. A functional similarity may occur in the premenopausal cycling female. Progesterone receptor was high throughout the menstrual cycle in the tubes suggesting that progesterone does not interact with these cells, particularly - which would be beneficial in this region as implantation does not occur here. A decrease in receptor expression occurred in the postmenopausal proximal tubal epithelia indicating that the receptor is being down-regulated here.

A small, preliminary study observing the effects of active androgen (DHT) on endometrial and fallopian tubal mucosa was conducted. Previous studies showed that active androgens up-regulate their receptor.[105] This was also observed in this current study. Although androgen receptor expression suggests a secretory role in a cell, high exposure to active androgens has indicated that proliferation increases. This would suggest that any small increase in exposure to this steroid hormone could change the function of a cell. This is supported by the role of androgens in prostate cancer. Androgens play an important part in promoting cancerous change in this particular tissue (Heinlein CA et al). Exposure to Androgens seems to reduce expression of SOX9. Overexpression of SOX9 has been linked to an inactive Wnt pathway therefore a reduction in expression of SOX9 could activate the Wnt pathway. This could potentially increase the proliferative ability of the target cell. The evidence above would suggest that initial, direct exposure to active androgens could play a role in carcinogenesis.

7.5 Clinical Implications

This study was a descriptive, characterisation study on a small sample population comparing normal fallopian tubal mucosa with endometrial mucosa. The tissues were compared in a normal, healthy state because it is necessary to understand the basic phenotype of normal fallopian tubal cells before studying the tube in its pathological state. If baseline expression of particular markers or steroid receptors is known; then subsequent changes in any of these can be detected in pathological tissue and help to gain an insight into the pathogenesis of gynaecological disease.

To theorise however; in the introduction it was mentioned that high grade serous ovarian carcinoma is now believed to begin in the fallopian tubes. The evidence suggested that the fallopian tubal secretory epithelial cell is the cell of origin of this aggressive cancer.[154] Data suggests that the fimbrial end of the tube is the site of origin and studies looking at particular cancer markers have supported this.[155] Recent studies using Fluorescence Activated Cell sorting (FACS) analysis have revealed that stem cells are more concentrated in the distal fallopian tube.[4] A recent pulse-chase study on mice also revealed that quiescent stem-like cells were particularly concentrated in the distal fallopian tube and had potential migratory abilities.[56] This stem cell population could have the capacity to differentiate under certain environmental changes.

Evidence has suggested that an accumulation of abnormalities in gene expression occurs after menopause, in the previously benign tube which leads to the development of precursor lesions and carcinogenesis.[155] As is already known, most endometrial carcinogenesis occurs in the postmenopausal period. This suggests that cells residing in these regions are more vulnerable to malignant change. From the results and discussions above it can be seen that the basalis markers increase in expression in the postmenopausal endometrial glands. Not only was this the observation, but an increase in the expression of these markers also occurred in the postmenopausal distal fallopian tubal epithelia. This would suggest that the cells of the distal fallopian tube become phenotypically more similar to the glandular

epithelia. Positive expression of SSEA-1 and SOX9 is usually evident in tissue regions where progenitor cells reside. This would support the evidence that the distal fallopian tube is a region where stem-like cells can be found. With strong positive expression of the basalis markers in postmenopausal endometrial and fallopian tubal epithelia, the knowledge that the postmenopausal endometrium is at risk of malignant change and the evidence that both tissues contain cells with stem-like properties; there could be reason to believe that the postmenopausal distal fallopian tubal epithelia is a tissue region that is at risk of carcinogenesis.

After the menopause, ovarian shut-down occurs and steroid hormone production in this organ ceases. This means that circulating levels of oestrogen and progesterone dramatically diminish. Androgens however; are still produced by the adrenal gland and although circulating testosterone eventually diminishes, as the transition from the pre to postmenopausal state occurs, transient increases in circulating levels of this hormone occur.[156] From the discussion above it has been suggested that exposure to active androgens (without other steroid hormonal exposure) causes an up-regulation of its receptor and an increase in the proliferation index of the cell. It could be suggested that the target cell becomes more susceptible to malignant change. With this, and the knowledge that postmenopausal women are exposed to transient increases in circulating androgens it could be hypothesised that the androgens increase the target cell's capacity to undergo malignant change.

The benefits of prophylactic salpingectomy have been considered in order to reduce the risk of females developing ovarian cancer.[155] With the evidence in this thesis linking the postmenopausal tubal epithelia to the endometrial glandular epithelia and the current speculation that a stem cell population resides in the fimbria making the cells more vulnerable to malignant change, it may be clinically useful for females to undergo prophylactic salpingectomy at time of surgery. Another angle to consider is that it could be of long-term benefit for women to have a laparoscopic salpingectomy at the time of endometrial ablation. This could be of benefit for three reasons: one being that this would reduce the patient's long-term risk of ovarian cancer. The other is that removal of the tubes could reduce the risk of ablation failure because one would be removing the other source of progenitor

cells in the reproductive tract (which could contribute to the endometrial mucosal re-growth that occurs after time). The final reason is that patients who may normally have had undergone a hysterectomy for menorrhagia, could have the less invasive procedure of ablation which reduces risk.

7.6 Limitations

This was a novel study on matched fallopian tubal and endometrial epithelia, thus it was not possible to pre-power the sample sizes. A small sample population was selected in order to observe the trends of the selected markers and steroid receptors in both tissue populations. To be thorough in the analysis of the fallopian tubal and endometrial mucosa, the samples were split into whether they were pre or postmenopausal and their menstrual cycle stage. Naturally, this reduced the sample size in each of the groups. Another limitation encountered was that ideally, each sample would have a full thickness section of endometrium, an isthmic tube and a fimbrial tube. On collection of tissue it is not always possible to collect both the isthmic and fimbrial end of a fallopian tube therefore some sections only had one end or the other. This also meant that there were slight discrepancies between the quantities of fimbrial or isthmic tubes in each group. Another limitation to note was that this was a novel study in laboratories of The Women's Hospital which meant that prior to the commencement of this project the collected fallopian tubal sample size was the sample-set available for staining at the time. After the study began, more matched fallopian tubal samples were collected however; and are available for staining at a later date to increase the robustness of the trial.

Due to the small sample number it was only possible to observe the trends occurring throughout the cycle stages and in the postmenopausal samples. The trends were clearly evident but statistical significance could not be determined. Further work would be necessary to increase the sample numbers to determine if the trend observed would yield statistically significant differences between the tissue during each cycle stage and the fallopian tubal and endometrial mucosa. In addition, the use of non-parametric tests on the data collected is more likely to produce non-significant P values.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on the sample population. This technique is not quantitative. It was only possible to determine the relative expression of mRNA between each of the samples in the set (i.e. postmenopausal eutopic endometrium against secretory phase eutopic endometrium). In order to confirm the findings from the results and quantify the mRNA expression of the target gene in eutopic endometrium and fallopian tube it would be necessary to perform quantitative real time-PCR (qPCR).

Two housekeeping genes were used in the RT-PCR experiments: YWHAZ and GAPDH. These were used to normalise the expression of the target genes in the PCR experiment. Both of the reference genes have been shown to be expressed in the endometrium and fallopian tube – but recent evidence suggests that Ubiquitin C (UBC) is the reference gene of choice for both the endometrium and fallopian tube.[157] It could increase the robustness of the study to have UBC as the third reference gene. Future work could include this.

7.7 Future Work

Future work would be needed in order to confirm the trends found during this study and thus prove the theories that have been discussed above as this was a short-term project over a 12 month period.

First, the sample size would ideally be increased. Therefore the markers and steroid receptors could continue to be analysed in these tissues, but on a larger scale to increase the robustness of the findings.

The explant analysis of the fallopian tubal tissue produced novel findings. It would however; be of benefit to treat matched fallopian tubal and endometrial mucosa with all of the steroid hormones. In order to gain an insight into the function of all of the steroids on fallopian tubal mucosa; it could also be of benefit to combine hormonal treatment to see the effect they have on the tissue and then remove one of the hormones to see if any change occurs. This work would help to gain an insight into the overall function of the fallopian tube in its normal state - in order to subsequently hypothesise further about changes that could occur in tubal pathology.

Another possible idea for further research would be to observe the effects of the treatment of androgens on normal and postmenopausal fallopian tubal and endometrial mucosa. Serum levels of androgens could be measured in every patient to quantify the amount of exposure in both pre and postmenopausal women. This would help to increase knowledge about cellular response to androgens in the normal postmenopausal woman and to determine whether the epithelia are vulnerable to changes in proliferative index under direct exposure to this hormone.

7.8 Conclusions

It can be concluded from this study that both eutopic endometrial and fallopian tubal epithelia uniformly express basalis markers after the menopause. Observations included strong SOX9 expression and an increase in SSEA-1 expression in the distal tubal epithelia and glands of the endometrium. The trend indicates that there are phenotypical similarities between the tissues. There is evidence to suggest that positive expression of these markers is seen in tissues that contain stem cell populations. With the strong positive expression of these markers seen in both tubal and endometrial epithelia after menopause, it could be suggested that these regions are where progenitor cells reside.

It is evident from the findings that dynamic changes in steroid hormone receptor expression occur throughout the menstrual cycle in the endometrial and fallopian tubal mucosa. Research has shown that this happens in response to the changes of circulating steroid hormones during the monthly cycle. This is important for the normal function of these organs. There were some fundamental differences in receptor expression however; observed between the fallopian tubes and the endometrium which would indicate that there are differences in steroid hormone response in the premenopausal state. It would appear that this would be beneficial because the endometrium undergoes growth and differentiation in order to promote embryo implantation and this is not the function of fallopian tubal mucosa in its normal state. The fallopian tube functions to promote the transport of the fertilised embryo towards the implantation site: the uterus.

It was apparent that some differences in expression of steroid receptors occurred in the proximal and distal ends of the fallopian tube. ER alpha and beta interaction was distinct at both ends. ER alpha increased during the secretory phase of the menstrual cycle in the isthmic fallopian tubal epithelia whereas this receptor increased earlier, in the proliferative phase at the fimbrial end. The expression at the distal end of the fallopian tube resembled the expression in the glandular epithelia of the endometrium. The isthmic end did not. This may be necessary for its normal function and could serve a purpose in separating the endometrial mucosa from the fallopian tubal mucosa in the premenopausal state. The phenotypic and functional differences could be necessary, particularly at the proximal end of the fallopian tube because an embryo should not implant here. Any changes in receptor expression at the proximal end of the fallopian tubes (from the ampullary region to the isthmus) could increase the likelihood of an ectopic pregnancy therefore this particular portion of the fallopian tube would be recommended for use as a normal control in research of ectopic pregnancy.

It can also be hypothesised that a lack of steroid hormone exposure in the postmenopausal state causes a change in steroid receptor expression indicating that the postmenopausal fallopian tubal and endometrial mucosa undergoes phenotypic change due to the sudden decline in the circulating ovarian steroid hormone concentrations. A sudden exposure to a circulating steroid hormone after the menopause could alter the cell and make it more vulnerable to malignant change.

It can be concluded that treatment of fallopian tubal and endometrial mucosa with the active androgen, DHT, causes an up-regulation of the androgen receptor. High expression of androgen receptor is evident in fallopian tubal epithelia whilst androgen receptor expression is predominantly in the stroma of the endometrium. This indicates that androgens directly affect the epithelia of the tube whereas there appears to be an indirect effect in the endometrium. The direct treatment of endometrial and fallopian tubal mucosa with androgen reduces the SOX9 expression and increases the proliferative index of the tubal epithelia and endometrial stroma. This could be linked to cross-talk between SOX9 and the

Wnt/beta catenin pathway with the reduction of SOX9 promoting the activation of this pathway and thus increasing the proliferative ability of the cells.

Further morphological assessment of the fallopian tube would be necessary to investigate its contribution towards high grade serous ovarian carcinoma and a possible link with endometrial cancer. The study of changes in hormone receptor status would be necessary in order to gain an insight into the pathological involvement of the fallopian tube in ectopic pregnancy. Further investigation into the presence of progenitor cells in the distal fallopian tube and their possible migratory abilities could assess potential tubal involvement in the pathogenesis of endometriosis and regeneration of the endometrium following the endometrial ablation procedure.

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Appendix

Appendix 1: RT-PCR data Normalised to YHWAZ

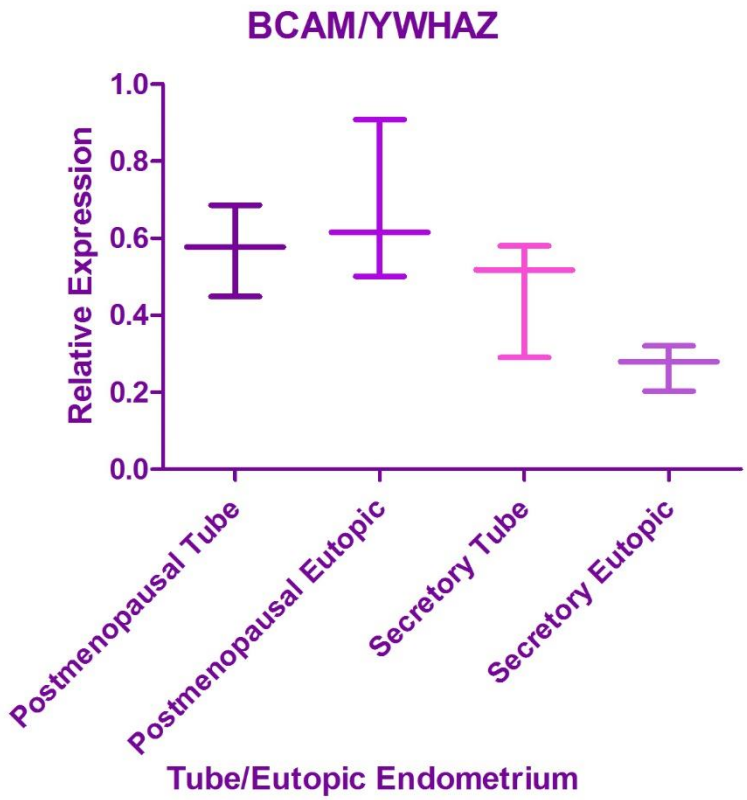

Figure 1 – Whisker graph representing relative expression of BCAM normalised to YWHAZ for N=3 postmenopausal fallopian tube, N=3 postmenopausal eutopic endometrium, N=3 secretory phase fallopian tube and N=3 secretory eutopic endometrium.

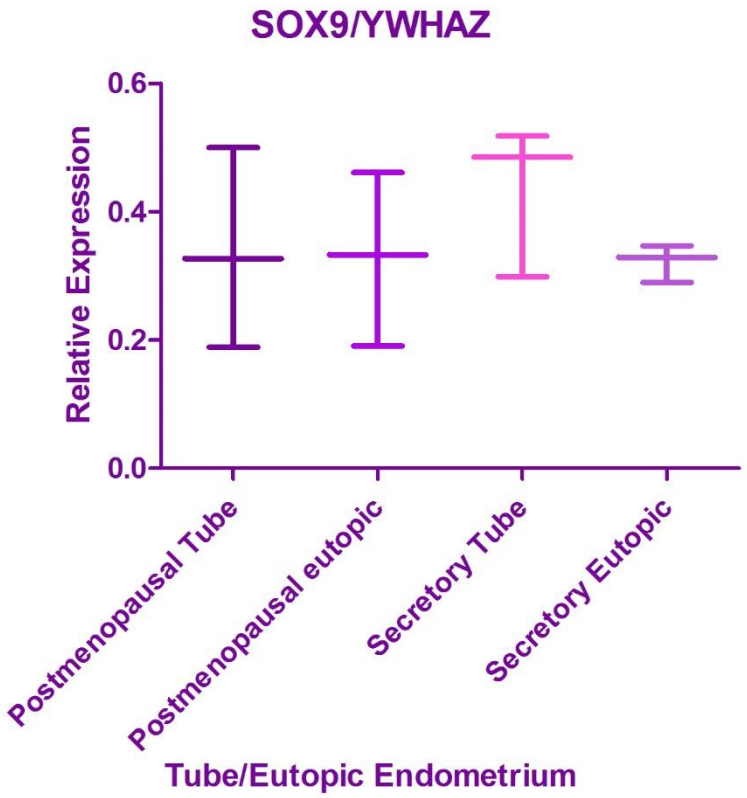

Figure 2 - Whisker graph representing relative expression of SOX9 normalised to YWHAZ for N=3 postmenopausal fallopian tube, N=3 postmenopausal eutopic endometrium, N=3 secretory phase fallopian tube and N=3 secretory eutopic endometrium.

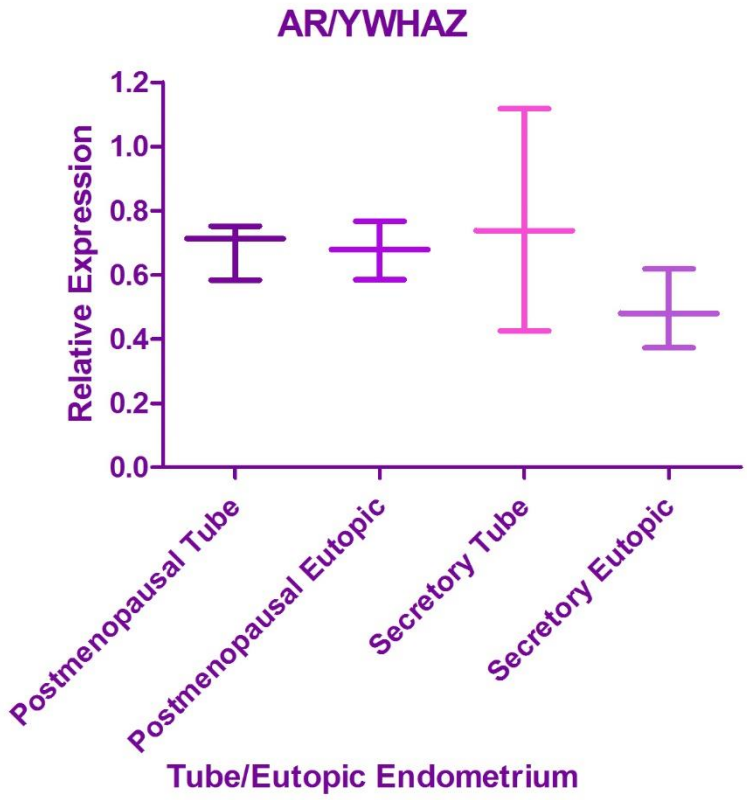

Figure 3 - Whisker graph representing relative expression of AR normalised to YWHAZ for N=3 postmenopausal fallopian tube, N=3 postmenopausal eutopic endometrium, N=3 secretory phase fallopian tube and N=3 secretory eutopic endometrium.

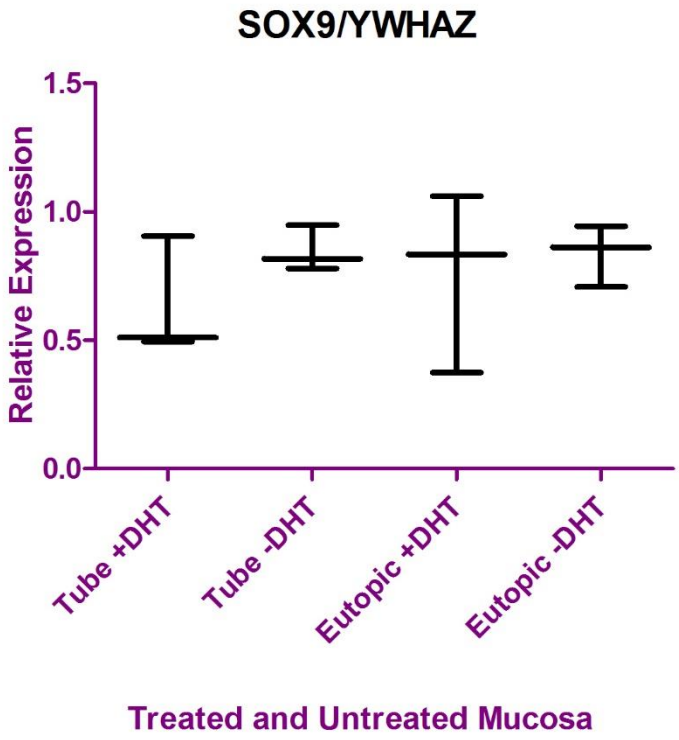

Figure 4 - Whisker graph representing relative expression of SOX9 normalised to YWHAZ for secretory phase N=3 fallopian tube +DHT, N=3 fallopian tube -DHT, N=3 eutopic endometrium +DHT and N=3 eutopic endometrium -DHT.

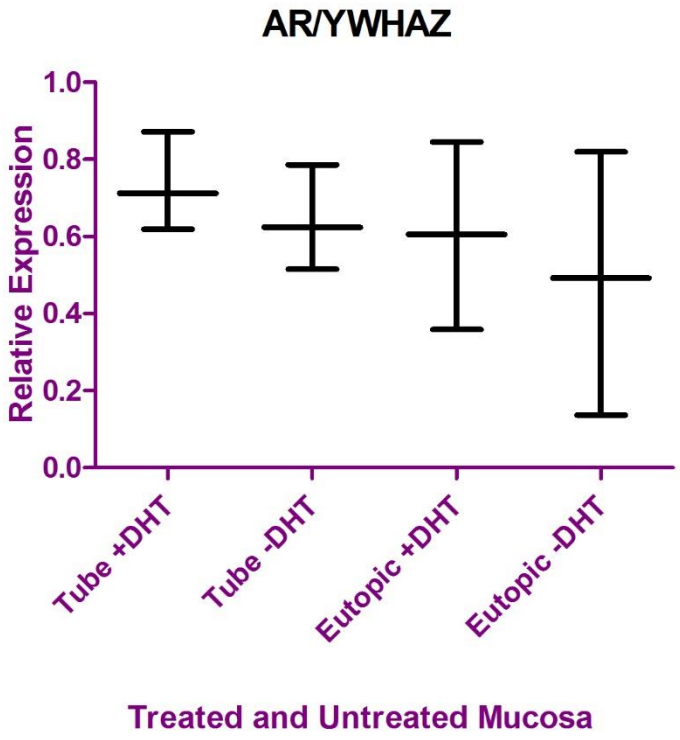

Figure 5 - Whisker graph representing relative expression of AR normalised to YWHAZ for secretory phase N=3 fallopian tube +DHT, N=3 fallopian tube -DHT, N=3 eutopic endometrium +DHT and N=3 eutopic endometrium -DHT.

Appendix 2: Patient Information Sheets and Consent Forms

Liverpool Women's
NHS Foundation Trust

Ethics Submission No: 09/H1005/55
PATIENT INFORMATION SHEET

"Endometrial stem cell Study"

The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.

Version 1: Endometrial biopsy only

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this

Why are we doing the study?

The inner lining of the womb (endometrium) may play an important part in endometriosis. **Endometriosis** is a common condition in which patches of the inner lining of the womb appear in parts of the body other than the cavity of the womb and seen in 1 in 10 women below the age of 50. It can cause painful periods, pelvic pain, pain with sexual intercourse and infertility. It is possible that an abnormality of specials cells in the endometrium called stem cells can cause endometriosis. If so, the information from this study will help us to develop new techniques to diagnose and treat this distressing condition.

What are stem cells?

Stem cells are special cells that can renew themselves (adult stem cells), and their job in the body is not yet determined. The inner-lining of the womb (endometrium) has these stem cells that can become many different types of cells, and they are likely to be responsible for its monthly regeneration. With monthly bleeding these cells are shed and can be expelled into the abdominal cavity. If these cells are implanted in the pelvis they can cause endometriosis as endometriosis occurs when endometrial cells of are found growing outside of the womb. We believe that abnormalities of these stem cells may cause endometriosis.

Why have I been chosen?

We are looking for a total of 160 women (you must have been off all hormonal medicines for at least 3 months), who have regular periods. We are specifically looking for 80 women who have endometriosis and another 80 completely healthy women who have had at least one baby. If you belong to any of these groups we will ask you if you would want to take part in the study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason.

A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

1. *If you are having a hysterectomy:*
   Your operation will continue exactly as planned. However, once the operation is over, a small sample of the womb that has already been removed will be taken for the research.
2. *If you are not having a hysterectomy:*
   Your operation will continue exactly as planned. However, a pipelle sample (see below) will also be taken from you for research.

The sample of endometrium will be processed in the lab to isolate the stem cells from it. Therefore, **NO** extra surgery will be performed for the study. A **blood sample** (5mls = teaspoonful of) will also be taken from your veins.

How is the endometrial pipelle sample done?

Whilst you are in the clinic (without anaesthetic) or whilst you are under anesthetic, the doctor will place a speculum (just like when you have a cervical smear) in the vagina. A plastic instrument (like a blunt drinking straw) will then be introduced through the neck of your womb to gently suck some cells from the inner lining of the womb. These cells will be then sent to the laboratory to be examined. This procedure is routinely done in our Gynaecology clinic and apart from the mild lower abdominal period like discomfort and vaginal spotting, it does not usually cause other problems. If you are going to have the biopsy taken at the time of the operation under anesthetic, you will not have any extra discomfort.

What are the possible benefits of taking part?

We do not expect you to have any extra benefits by taking part in this study. However, you will be helping us to improve our knowledge about endometriosis.

What if something goes wrong?

There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Data management

The information gathered from this study will be entered into a computer database and will be analyzed in a strictly confidential manner, in compliance with the Data Protection Act. Regulatory authorities for approving medicines and the University of Liverpool may wish to look at medical records to check that the study has been performed correctly. All information, which is collected about you during the course of the research will be kept

strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognized from it. Once we carryout the study on the samples you kindly donate, if there is any surplus tissue, it will be stored in the department of obstetrics & Gyanecology and will be used in other ethically approved studies.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988) in the Liverpool Women's Hospital, Crown Street, Liverpool.

If you want to find out more about the study from someone who is not directly involved in it and can give you unbiased advice, please contact Mr Jonathan Herod, Consultant, in Gynaecology Out Patient Clinic, telephone no. 0151 708 9988.

Liverpool Women's

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis.**

Name of Researcher: Dr Dharani Hapangama, Senior Lecturer
University of Liverpool / Liverpool Women's Hospital

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... (version .....) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from [University of Liverpool & Liverpool Women's Hospital] or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study and for my GP to be informed of my part taking. ☐
5. I agree for surplus tissue to be stored in the department of obstetrics & Gyanecology and to be used in other ethically approved studies. ☐

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

SDS0210M P.I.S. (February 2005)

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Endometriosis Studies Clinical Data Collection Form

Sample ID: _____

Date of sample: _____/_____/_____

Sample type: Full thickness/pipelle (circle as appropriate)

Age: _____

Height (m): _____

Weight (kg): _____

BMI: _____

Smoker: yes/no (circle as appropriate)

Endometriosis: yes/no (circle as appropriate)

Endometriosis stage: _____

Adenomyosis: yes/no (circle as appropriate)

Menorrhagia: yes/no (circle as appropriate)

Fibroids: yes/no (circle as appropriate)

Reason for Surgery: _____

Previous gynae history/surgery: _____

Parity: _____ Infertility: _____

Miscarriages: _____ PCOS: yes/no (circle as appropriate)

TOP: _____

Days of bleeding: _____

Cycle Length: _____ Irregular cycle: yes/no (circle as appropriate)

LMP: _____/_____/_____ Menopause: _____/_____/_____

Contraceptive/hormone treatment: _____

Other information: _____

_____

_____

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