Miss Kanchan Kiran Palial
August 2011
Master of Philosophy

Alterations of Endometrial Basement Membrane Integrity and Stem/Progenitor Cell Markers in Endometriosis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By Kanchan Kiran Palial

August 2011

For Mum and Dad.

Abstract

Alterations of Endometrial Basement Membrane Integrity and Stem/Progenitor Cell Markers in Endometriosis

Palial, K; Drury, J, Heathcote, L, Valentijin, A.J, Gazvani, R, Rudland, P.S. and Hapangama, D.K.

[1]Department of Women's and Children's Health, University of Liverpool, Liverpool, United Kingdom, L8 7SS. [2]School of Biological Sciences, University of Liverpool, Liverpool, United Kingdom, L69 7ZB

Degradation of basement membrane (BM) is reported to be involved in the metastatic process of cancers, and has been shown to be aberrantly expressed in the benign metastatic disease of endometriosis. Emerging evidence show endometrial stem/progenitor cells are involved in endometrial physiology and in certain endometrial pathological conditions, however markers for these cells are lacking. We tested a panel of stem cell markers including cytokeratin 5/6 (CK5/6) and podocalyxin-like protein (PODXL) and stage specific embryonic antigen 1 (SSEA1) in endometrial tissue. Using immunohistochemistry, we aim to investigate if an alteration of BM integrity is associated with differential expression of these stem cell markers in eutopic endometrium collected from fertile controls, post-menopausal (PM) women and patients with endometriosis and endometrial cancer.

1. In the fertile control group, expression of BM components, Collagen IV and laminin, changed across the menstrual cycle and some of these changes agree with previously reported findings. Expression of stem cell markers SSEA1 and PODXL also showed similar changes across the menstrual cycle with both having maximal expression during the ProlP. Expression of BM components appeared to be correlated with stem cell markers. No cyclical changes in CK5/6 were reported in fertile controls.
2. In the PM group a few differences were seen in BM integrity compared with the fertile control group, especially in BM supporting endometrial vessels. Nonetheless, PODXL and SSEA1 expression was similar to the fertile control group.
3. Complete disruption of BM in the endometrial cancer group provided confirmatory results that BM is disruption in this invasive metastatic disease.
4. In the endometriosis group, differences were seen in the expression of the key BM components in eutopic endometrium sampled from women with endometriosis when compared with fertile controls, and this was similar to previous reported findings. Whereas SSEA1 showed a phase dependent expression in the fertile control group, this was lost in the endometriosis group.

Acknowledgements

I would like to thank my supervisors Dr Dharani Hapangama, Professor Philip S Rudland, Josephine Drury, Anthony Valentijin, Lisa Heathcote and Rafet Gazwani for their guidance and encouragement throughout the course of my intercalating year.

I would also like to give special thanks to my family and close friends who gave me continual moral and financial support throughout the year.

Abbreviations

ABCG2 – ATP Binding Cassette Transporter G2

ASCs – Adult Stem Cells

BM – Basement Membrane

CD – Cluster of Differentiation

CK5/6 – Cytokeratin 5/6

COLIV – Collagen type IV

CSCs – Cancer Stem Cells

EC – Embryonal Carcinoma

ECM – Extra-cellular Matrix

EG – Embryonal Germ

EGF – Epidermal Growth Factor

ER – Oestrogen Receptor

ES – Embryonic Stem

ESCs – Endometrial Stromal Cells

ESP – Early Secretory Phase

FACS – Fluorescence Activated Cell Sorting

FIGO – International Federation of Gynaecology and Obstetrics

FSH – Follicular Stimulating Hormone

FUT - Fucosytransferase

GnRH – Gonadotrophin Releasing Hormone

IHC - Immunohistochemistry

LCC – Helical Coiled Coil Domain

LeX – Lewis X

LH – Luteinising Hormone

LN – Laminin N-Terminal

LSP – Late Secretory Phase

MMPs – Matrix Metalloproteinases

MSP – Mid Secretory Phase

NC1 – Non Collagenous Domain

PM – Post-menopausal

PODXL – Podocalyxin-like protein 1

PR – Progesterone Receptor

ProlP – Proliferative Phase

SP – Side Population

SSEA1 – Stage Specific Embryonic Antigen 1

TA – Transit Ampliflying

Table of Contents

Chapter Two: Methods

Appendices

Tables

Figures

Chapter One:

INTRODUCTION

The endometrium is a highly dynamic tissue lining the uterine cavity. Under the influence of ovarian hormones, significant morphological changes occur in the endometrial epithelium and in the underlying stroma and vasculature. These structural changes are precisely timed in the menstrual cycle in order to prepare the uterus for implantation. Correlated with these events, an array of biochemical changes occurs in the organisation of the extracellular matrix (ECM) of this specialised tissue. Basement membrane (BM) is an important part of this matrix that underlies all epithelial sheets, and separates them from the surrounding connective tissue. Degradation of BM has been reported to be involved in the metastatic process of endometrial cancer and has been proposed to play a part in the pathogenesis of endometriosis(1, 2).

The endometrium is a site of repetitive physiological injury and repair. For this reason it has also been hypothesised that the endometrium contains stem cells that may account for its distinct ability to regenerate. Alongside with the disruption of BM reported in the disease process of endometriosis and endometrial cancer, these stem cells are thought to be involved in the pathogenesis of both diseases(3, 4). Additionally, there is some evidence that disruption of BM may cause activation of stem cells(5). Therefore we explored the integrity of BM and associated expression of stem cell markers in the endometrium sampled from normal fertile controls, and from patients with endometriosis and endometrial cancer. In order to make accurate comparisons across these different subject groups, a thorough literature review on the structure and function of the endometrium, endometrial BM and stem cell activity will be discussed.

1.1 – Structure & Function of the Endometrium

The uterus is a pear shaped organ consisting of a fundus superiorly, body, isthmus and the inferior cervix. It comprises of an outer serosal lining, a thick fibromuscular wall known as the myometrium and a modified mucosal lining known as the endometrium(6). Human endometrium is structurally and functionally divided into two major regions: the functionalis and the basalis regions, see figure 1.1. The functionalis, comprising the upper two thirds of the endometrium, contains a superficial layer with a few glands and abundant stroma and a deeper layer with many glands and less stroma(7). Epithelial cells constitute the luminal and glandular epithelium. The lower basalis layer containing the basal region of the glands, dense stroma and large vessels, is thought to serve as the germinal compartment for generating the new functionalis each month(8). The endometrium responds to ovarian hormones and undergoes a remarkable series and variety of morphological changes during the reproductive years and after menopause. The ovarian and uterine cycle will be discussed in detail outlining these changes.

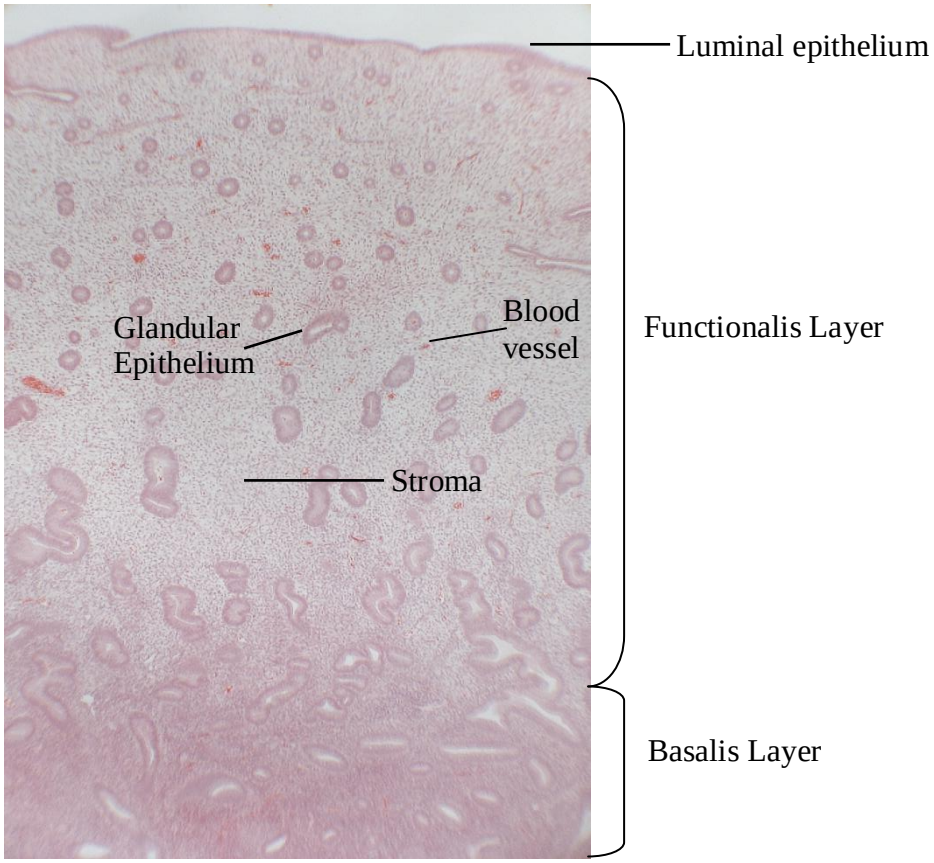


Figure 1.1: Micrograph illustrating the structure of the endometrium.

1.1.1 The Ovarian Cycle

A normal menstrual cycle is mostly a reflection of the ovarian cycle. At birth the human ovaries contain approximately 1,000,000 primordial follicles arrested at prophase of the first meiotic division. Over time, the follicle pool continually depletes, with regular escape of follicles from the "resting state" re-entering meiosis. Of these 'escaped' follicles, only 400 will sequentially mature and ovulate during an average women's reproductive lifetime(9).

Initiation of follicular recruitment and maturation from the primordial pool is a process that continuously occurs over several months and is independent of gonadotrophin control(10). Subsequently the gonadotrophins, follicular stimulating hormone (FSH) and luteinising hormone (LH), play distinct roles in follicle maturation in a cylical manner. Both are released by the anterior pituitary in response to pulsatile stimulation of hypothalamic gonadotropin releasing hormone (GnRH)(11). During the luteal-follicular transition, FSH serum concentrations rises as oestrogen decreases due to the demise of the corpus luteum (10). This intercycle rise in FSH, recruits a cohort of mature FSH responsive pre-antral follicles which synchronously develop into large antral pre-ovulatory follicles(10). From the mid- to late-follicular stages, a critical concentration of FSH opens the 'FSH window' allowing entrance of the most sensitive antral follicle. This 'lead' follicle will acquire significant aromatase enzyme activity within its granulosa cells, leading to increased synthesis and secretion of oestradiol from androgenic precursor. The "two-cell, two gonadotropin" hypothesis specifies the need for both LH, to stimulate production of precursor androgens by theca cells, which are then transferred into granulosa cells to be aromatised into oestrogens under the influence of FSH(11, 12). Such actions of both FSH and LH are influenced by paracrine and autocrine actions of substances produced locally in the ovaries(12)**.** Raised serum oestradiol ensures rapid suppression of FSH production, via a negative feedback system(12). The mid follicular fall of FSH causes atresia of the less mature follicles that are unable to grow without adequate FSH concentrations, ensuring mono-ovulation(10).

A rapid rise in oestradiol, during the latter days of the follicular phase, promotes hypothalamic GnRH pulses to increase in both magnitude and frequency, via positive feedback system(11). This produces the so called 'LH surge' during mid-cycle, with a rapid outpouring of LH and to a lesser extent FSH(11). The LH surge initiates a gene cascade in granulosa cells that signal the cell to commence meiotic maturation(13). Furthermore, it induces an inflammatory type reaction at the apex of the Graafian follicle adjacent to the outer surface of the ovarian cortex. Angiogenesis associated with a release of prostaglandins (PG) and cytokines leads to rupture of the follicular wall and ovulation approximately 36 hours after initiation of the LH surge. The empty follicle fills with blood, producing a corpus haemorrhagicum, and the theca and granulosa cell layers of the follicle wall luteinise forming the corpus luteum. In response to LH and human chorionic gonadotrophin (hCG), the corpus luteum produces and secretes progesterone, oestrogen and inhibin. If conception follows, the placenta will subsequently be the main source of progesterone production once it has developed sufficiently(14). Otherwise, progesterone and inhibin levels will fall with regression of the corpus luteum and FSH levels will rise again.

1.1.2 The Uterine Cycle

The endometrium is a dynamic remodelling tissue with the main function of embryo-implantation. In order to prepare for implantation, the upper functionalis layer undergoes well defined cycles of proliferation, differentiation and shedding in response to a prevailing steroid hormonal environment of sequential ovarian steroid exposure(15).

Oestrogens, progesterones and androgens all play vital, but as yet not fully defined roles in the complex mechanisms underlying endometrial development necessary for successful embryo implantation(16). The expression of steroid receptors varies temporally and spatially across the menstrual cycle, contributing to the changes in the impact of steroid hormones on target cells(17). Oestrogen receptors (ER) exist in two isoforms, ERα and ERβ, which are both found in glandular and stromal compartments. Like oestrogen, two isoforms of

progesterone receptors (PR) also exist, PR-A and PR-B. It is of upmost importance to identify the mechanisms regulating endometrial events involved in the normal endometrial functions of implantation and menstruation if we are to elucidate the abnormal endometrial pathologies such as endometriosis or endometrial cancer.

Three distinct phases are described during the uterine cycle: the menstrual phase; the proliferative phase (ProlP), corresponding to the follicular phase of the ovary; and the secretory phase, corresponding to the luteal phase of the ovary.

1.1.2.1 Menstruation Phase:

In the absence of pregnancy, the demise of the corpus luteum causes progesterone and oestrogen levels to fall towards the end of the secretory phase in the menstrual cycle. Withdrawal of progesterone results in shedding of the upper two thirds of the functional layer of the endometrium exposing open blood vessels and glands. This rapid but incomplete degeneration of the functional layer is called menstruation and the first day of menstrual bleed is considered as day 1. During this period the endometrium quickly starts to regenerate with epithelium re-growing most likely at the stumps of the glands. Thereafter two thirds of the luminal surface is covered by epithelium as it grows out of the edges of the cone shaped glands, and epithelial growth is complete within 48 h after shedding(18). The molecular mechanism by which sex steroids induces these events involves complex interactions between the endocrine and immune system.

1.1.2.2 Proliferative phase (ProlP):

The average duration of a cycle is 28 days, with alterations in the length of cycle mainly due to the duration of the proliferation phase (varying from 8-21 days)(7). The ProlP coincides with the follicular phase of the ovarian cycle and takes place between the menstrual phase and ovulation, day 4-14. Increasing circulating oestrogen levels induces remarkable endometrial regeneration, with endometrial proliferation and vascularisation. As a

consequence the endometrium regenerates from an initial 0.5-1mm thick post menstruated endometrium to 5-7mm(18). Increasing circulating levels of oestrogen increases the expression of endometrial ERα, which is essential for the upregulation of PR, therefore endometrium to respond to progesterone during the secretory phase(16). Therefore, pre-ovulatory endometrium contains proliferating cells in glands, stroma and endothelium, leading to relative hypertrophy of the uterine mucosa. Narrow straight glands, a thin surface epithelium and compact stroma are features of the ProlP. Stromal cells are often elongated and spindle shaped(19). Multiple mitoses are present in the "naked nucleus" type of stroma. As growth of the glands and stroma continues, angiogenesis also occurs as ovulation approaches(15). Spiral artery elongation is an important part of endometrial angiogenesis and is crucial for endometrial re-growth(15). Unlike other ovarian hormone receptors, ERβ is found in endometrial endothelial cells. Therefore it is reasonable to deduce that any direct effects of oestrogen on angiogenesis is likely to be mediated by ERβ(16). Oestrogen may also influence endometrial angiogenesis indirectly via actions on their mediators such as angiopoietin 1 and vascular endothelial growth factor.

1.1.2.3. Secretory Phase:

Following ovulation, progesterone dominates the rest of the menstrual cycle as it controls cellular differentiation(20). Differentiation is defined as a gene controlled change in cellular phenotype and function(21). Cellular differentiation transforms the oestrogen primed endometrium into secretory tissue. These morphological changes also reflect the pattern of sex steroid receptor expression across the secretory phase.

This phase of the menstrual cycle is constituted by an early, mid and late secretory phase.

Early secretory phase (ESP)

ESP, day 15-18, is regulated by both oestrogen and progesterone, as shown by microarray studies. Sub-nuclear vacuolation of the glandular epithelium is maximal during the ESP. Eventually vacuoles enlarge and pushes the nuclei into an orderly row. By the end of the ESP, glands are tortuous and stromal vascularity peaks making the endometrium both morphologically and functionally well prepared for implantation during the MSP(14).

Mid secretory phase (MSP)

MSP, day 19-23, is a short period of uterine receptivity towards embryonic implantation, designated as the "window of implantation" (WOI). Endometrial receptivity to implantation is between 6 and 10 days after the LH surge and ovulation(14). This period coincides with peak circulating progesterone levels and is regulated by progesterone alone. The key endometrial events associated with MSP include vacuoles moving into a supra-nuclear position and empty their contents into the lumen via exocytosis. Nuclei then approach the base of the cell. During the MSP there is increased expression of chemokines and cytokines, the presence of increased numbers of leukocytes including uterine natural killer cells, and the onset of pre-decidualisation(15).

Unlike oestrogen, progesterone down-regulates both ERα, PRα and PRβ (inhibiting further action of oestrogen) in the glandular and luminal epithelium and in stromal cells around vessels, thus cellular mitoses are completely blocked and cells commence differentiation(16). It is notable that PR persists in the stroma in the functionalis and is highly expressed by stromal cells in close proximity to the uterine vasculature(16). Thus, the role of progesterone on vascular function is thought to be mediated by the PR-positive stromal cells around blood vessels(16). Stromal proliferation reduces to some extent and commences pre-decidualisation

during the mid-late secretory phase. Pre-decidualisation is an event where stromal cells differentiate beneath the surface epithelium and around blood vessels, a requirement for implantation. In particular, enlargement of nuclei and increase in cytoplasm within peri-arteriolar stromal cells constitutes as the earliest visible pre-decidual change(20). Other histological features of the MSP include maximal glandular secretions and stromal oedema(20).

Late secretory phase (LSP) - Decidualisation

Whilst progesterone is the dominant hormone in the secretory phase, during the LSP ERβ expression is maintained in the stroma, mirroring the decline in endometrial ERα, PR and androgen receptor. Since the functions of ERβ and the actions of progesterone counteract most of the classical ERα mediated actions of oestrogen in the endometrium, the rising ERβ may potentiate progesterone action in the endometrium despite the decline in PR expression. Pre-decidualisation continues into the LSP, irrespective of whether or not a blastocyst is present. The stromal compartment shows the most profound changes with the cells becoming plumper, developing a myofibroblast-like phenotype and increasing production of proteins such as prolactin, insulin-like growth factor binding protein-1 and tissue factor. Additionally, polymorphonuclear leukocyte invasion becomes characteristic in the LSP. Upon implantation, endometrial stromal cells complete decidualisation and terminally differentiate. An impairment of receptivity is often seen in women with endometriosis.

In the absence of pregnancy, a drop in both progesterone and oestrogen levels terminates the secretory phase and the endometrium undergoes dynamic remodelling, resulting in menses and the beginning of the next menstrual cycle.

1.1.2.4 Basalis layer of the endometrium

The lower basalis layer contains dense stroma, glands and large thick walled arteries and serves as a germinal compartment for generating the new functionalis layer each month(8). The stroma is more cellular than in the functionalis layer with a higher nucleo-cytoplasmic ratio(7). Hormonal regulation is different in the basalis and the glands express PR and ER throughout the menstrual cycle, unlike the functionalis(16). This reflects the diverse morphological events that occur in the functionalis in comparison with the basalis layer.

1.1.3 Post menopausal (PM) endometrium

The depletion of the oogonial stem cell population before birth establishes a limited supply of primordial follicles in the ovary(22). With the loss of a cohort of follicles each month during reproductive years, the total number of primordial follicles decreases with increasing age. In the last 12 years or so of reproductive life, there is a much steeper decline in the remaining number of follicles and anovulatory cycles increasingly occur(23). Eventually, only a few follicles remain in the involuting ovaries, causing a permanent cessation of menstruation and this defines the transition into menopause. The loss of oestrogen and progesterone supply causes many physiological effects on a woman's body. Particularly in the endometrium, this loss subsequently ends the cyclical cellular proliferation, differentiation and endometrial shedding. The lack of hormonal stimulation causes the endometrial epithelium to flatten, the necks of tubular glands to close, and the stroma also changes and resembles the basalis stroma of cycling women(20). Additionally, proteinaceous material accumulates in the glands producing a classic histologic picture known as "cystic atrophy"(24). Eventually, these changes contribute to endometrial atrophy and consequently the post-menopausal endometrium is very thin(20). As a consequence, the functionalis layer is very difficult to separate from the basalis.

1.2 – Basement Membrane

BM are specialised sheet like structures which separate cell monolayers (epithelium and endothelium) and connects them to the interstitial matrix, together creating the ECM(25). They possess binding sites for cell adhesion molecules that frequently serve as ligands for cell surface receptors(25). Binding of these receptors to BM proteins initiates intracellular signalling pathways influencing cellular activities(25). Therefore, as well as providing structural support they also mediate many essential functions such as development, proliferation, differentiation and cellular growth(5). They also regulate cell polarity, cell adhesion, spreading and migration via their effects on the cytoskeleton(5, 26).

1.2.1 BM Composition

As seen under the electron microscope after conventional fixation and staining, most BM consist of three layers(27). The innermost layer lying adjacent to the basal plasma membrane of the resting cells, is the lamina lucida. The lamina densa is the middle layer and is electron dense. The outermost third layer, the lamina reticularis, connects the basal lamina to the underlying connective tissue(27).

Depending on the tissue in which they are localised in, BM have heterogeneous molecular compositions and biochemical complexity, which is nearly as diverse as their unique biological functions(25). Their heterogeneity results from variation in the amounts of BM components and the kinds of subtypes used(25). The four major proteins that make up the BM include type four collagen (COLIV), laminin, nidogen/entactin and perlecan. Laminin and COLIV self assemble into suprastructures, and both networks are crucial for BM stability and assembly. Nidogen/entactin and perlecan bridge the laminin and COLIV networks, increase their stability and influence the structural integrity of BM(25). Since COLIV and laminin are the two main proteins needed for BM structure they will both be evaluated in endometrial tissue in this study.

1.2.1.1. Collagen Type IV

Collagens are the major structural component of the ECM, but are also important for tissue architecture organisation and cellular processes(28). COLIV is one of 28 different types of collagen and is the principal component by weight in BM(28). Even though COLIV is a major component of BM, it is not required for BM synthesis and is rather needed for BM maintenance(28). In additional, COLIV mediates cell adhesion to BM through binding with integrin and non-integrin receptors (refer to section 1.2.2-Integrins).

The basic monomers found in COLIV protein are α-chain of which there are six types (α1 to α6 chains). Each α-chain is 400nm long and is composed of three domains: an amino-terminal 7S domain (26 kDa), a middle triple helical collagenous domain (120 kDa) and a non-collagenous (NC1) terminal domain(28), see figure 1.2.

Figure 1.2: α-chain monomer of COLIV.

COLIV is a type of non-fibrillar collagen due to the presence of a NC1 terminal domain and its composition makes each of the six α-chains unique. The collagenous domain consists of repetitive Gly-X-Y amino acid sequence in which X and Y are often proline and hydroxyproline, or lysine and hydroxylysine, providing structural integrity. Numerous short interruptions ensure good flexibility which is characteristic of collagens(29). The 7S domain also consists of interrupted Gly-X-Y repeats.

A protomer is a single unit of COLIV suprastructure and is composed of three α-chains which are linked in their NC1 domains, to create a heterotrimer helix(30). Protomers are synthesised in the golgi apparatus and assemble in the endoplasmic reticulum. Only three different

protomers, α1α1α2, α3α4α5 and α5α5α6, exist despite the 56 possible combinations(5). Following bonding in the NC1 domain, the three α-chains then fully bind in a zipper like fashion and protomer secretion occurs, see figure 1.3(28). During development, α1α1α2 is the most widely expressed protomer and is distributed throughout the BM. During maturation this network gets partially replaced by other protomers that defines the BM particular function(5).

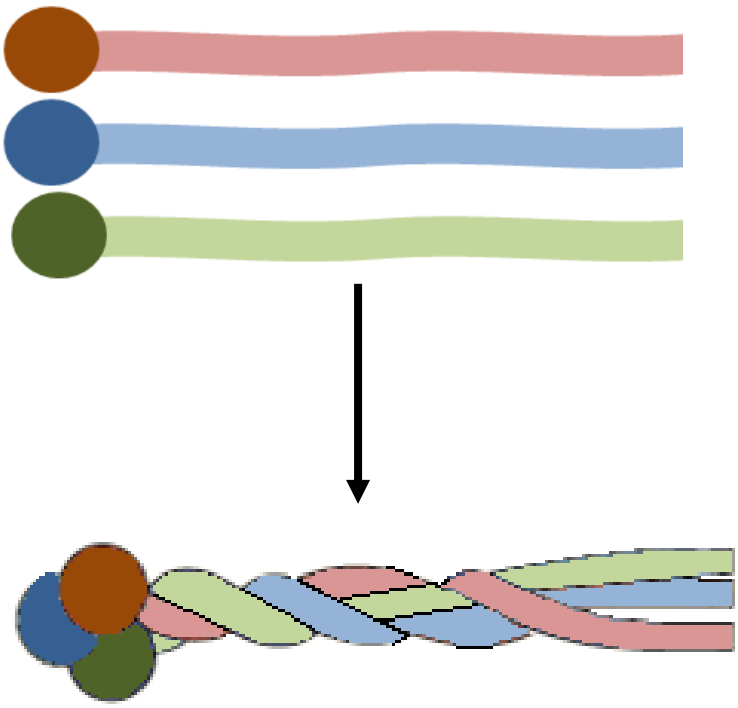


Figure 1.3: NC1 interactions to initiate COLIV protomer formation.

Once secreted, the NC1 and 7S domains of protomers play a critical role for producing the lattice network of collagen which forms the nucleus for a COLIV scaffold to ensure stability. The scaffold evolves into a COLIV suprastructure, with the help of end to end associations and also lateral associations between COLIV protomers(30).

1.2.1.2. Laminin

Laminin is needed for initial BM formation and early development and is the most abundant non-collagenous component in BM(28). It links the BM to the cell surface through interactions with cell surface receptors modulating cell behaviour and also provides a structural role.

Laminins are hetero-trimeric glycoproteins consisting of an α, β and γ chains(5). When these chains assemble together they form a cruciform shape with one long and two or three short arms (figure 1.4). Five α, four β and thee γ chains exist which can assemble into 16 different isoforms of laminin. These isoforms are named in the new nomenclature according to their composition, such that lam-111 consists of α1, β1 and γ1 chains(31). These isoforms are differentially distributed during development and in adult tissues, fulfilling specific functions as key regulators of tissue structure and cell behaviour. Laminin 1 is the first laminin expressed during the development at embryonic stage day 4.5 in mice and is the most abundant type of laminin found in BM(25).

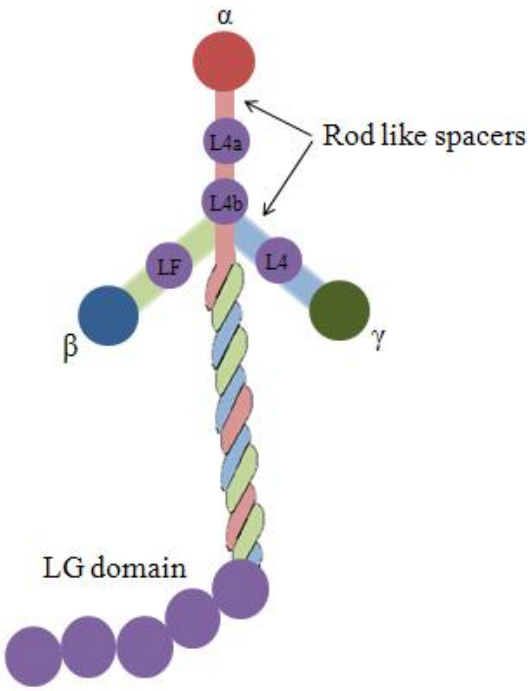


Figure 1.4: Structure of laminin unit

Each chain contains a laminin N-Terminal (LN) domain on the short arm emerging from the long arm which makes each chain unique. The α-chain contains the L4a and L4b globular domains, and the β and γ chains contain LF and L4 gobular domain respectively. Rod like spacers formed by laminin epidermal-growth-factor-repeats separate the globular domains. Laminin initially self assembles by non-covalent bonding between LN domains, and is required for calcium dependent interaction between the three chains(32). The long arm is formed by parts of all three chains, forming the alpha helical coiled coil (LCC) domain(32). The LCC is subsequently flanked by a C-terminal containing five homologous globular motifs collectively known as the LG domain(25). LG domains mediate cell adhesion through binding receptors including integrins, dystroglycan, syndecan and heparan.

1.2.2. Integrins

Many components of the ECM crosstalk with cells through specific transmembrane receptors named integrins, triggering a cascade of intracellular events. Apart from cell adhesion, and linkage of the cytoskeleton to the ECM, integrins act by signalling receptors, mediating growth, differentiation and survival signals from the ECM. Integrins are heterodimers formed by α and β chains linked by disulphide bonds, distributed at the cellular surface in a polarised manner(33). The pairings determine the ligand specificity, which may also be determined by cell type and BM composition. A rearrangement of integrins has been widely reported in the endometrium along the menstrual cycle(34). The following combinations are known to be involved in receptors for COLIV: α1β1, α1β1, α2β1, α3β1, α6β1, α10β1, α11β1, αvβ3 and αvβ5(5). Additionally for COLIV, non-integrin receptors also show COLIV chain specific binding including CD44 and discoidin domain receptor-1. A number of integrins have also been identified as laminin receptors, of which α3β1, α6β1, α7β1 and α6β4 are the major types. These interactions are modulated by the physiological proteolytic degradation of the C-terminal, usually through the loss of LG domains 3-5(32).

1.2.3. Altered Basal Lamina integrity

COLIV and laminin heterogeneity is the major molecular basis for tissue specific BM compositions that create functional diversity across the BM. 15 possible combinations for laminin and 6 different types of collagen IV protomers are differentially expressed throughout the whole body(25). However, if there are aberrations in any of these subtypes after gene mutations, then it may potentially prohibit BM formation(28). In turn, this could result in pathological conditions as seen in the kidney, placenta and eye to name a few. On the other hand, damage of the BM from injury or pathophysiology is followed by BM remodelling. In this case, altered BM integrity triggers cellular activities that aid tissue repair such as recruitment of immune cells and activation of fibroblasts. This then initiates BM remodelling through protein self assembly, deposition and network formation(29).

1.2.4. Basal Lamina in Normal Endometrium

As described in the "Uterine Cycle" section, human endometrial tissues are remodelled every month regulated by cellular proliferation, differentiation, and cell death of epithelial cells. These cellular events not only depend on cell-cell interactions but also cell-matrix interactions. Thus, cyclic remodelling of the endometrium is also thought to be regulated by changes of the BM composition(35). Endometrial BM provides an interface between epithelial and mesenchymal environments, giving structural support and also playing a crucial role in the expression of epithelial phenotype(27). COLIV and laminin have been reported to localise in BM of human endometrial epithelium and shown to undergo a menstrual cycle dependent production in epithelial and stromal cells(36-38). Ovarian hormones are believed to regulate the production of BM, since defective and discontinued BM around glandular epithelium in cases of endometrial hyperplasia and endometrial carcinoma, is observed to be restored after exogenous progesterone treatment(39).

1.2.4.1. Menstrual Phase

Matrix metalloproteinases (MMPs) are a large family of proteases. In the endometrium progesterone has shown to inhibit the action of MMPs, as seen in many microarray studies(40). When LSP ceases and in the absence of pregnancy, the accompanying drop in both oestrogen and progesterone removes this inhibitory influence on MMPs. Expression of MMPs then increases in the endometrium during menstruation and early ProlP, and plays a proteolytic role in tissue breakdown of the endometrium during menstruation, together with plasminogen activator/inhibitor system(41, 42). In particular, MMPs 2 and 9 preferentially degrade BM components, therefore together these proteolytic enzymes degrade the BM supporting the epithelium of the decidualised endometrium and the endometrial blood vessels, among other ECM components of the endometrium(43).

1.2.4.2. Proliferative phase

In the ProlP, important cellular activities take place to recover the thickness of the functionalis layer of the endometrium after menstruation(36). During the ProlP, there is a high cellular mitotic index and a large number of ECM components being produced in the endometrium(2, 42). It has been well established that the proliferative capacity of many cells types is strongly influenced by the ECMs(1).

Surface luminal & glandular epithelium, and vascular endothelium

Both COLIV and laminin have been shown to be localised in BM structures during early ProlP. Using immunohistochemistry (IHC), they are visualised as narrow, continuous bands supporting blood vessels and glandular epithelium only(36, 44). Tanaka et al demonstrated, however, that COLIV immuno-reactivity intensity was significantly decreased in the BM at the luminal and glandular epithelium and vascular endothelium during the late ProlP when compared to other phases of the cycle(35). This may demonstrate that the circulating ovarian oestrogen at this time may partially regulate COLIV production or specific COLIV

proteolytic activities. In contrast laminin expression was seen to be highest throughout the proliferative phase out of the whole menstrual cycle(35).

Tanaka et al used a human endometrial epithelial cell line, that retains many of the intracellular signalling pathways found in normal endometrial epithelial cells, to identify the effects of COLIV and laminin on cellular proliferation and general morphological changes on endometrial cells(35). In particular, laminin was found to enhance endometrial epithelial cell growth and inhibit epithelial apoptosis, in contrast to COLIV(35). This suggests that laminin may play a pivotal role in endometrial epithelial regeneration after menstruation.

Stroma

Unlike epithelial cells, human endometrial stromal cells (ESCs) are found to be immuno-positive for laminin and COLIV(38). This may reveal that in human endometrium COLIV and laminin are produced by stromal cells, but not epithelial cells, and are then transported to intercellular spaces and subsequently to the sub-epithelial BM structures. Towards the end of the PP, expression of both COLIV and laminin increased in the stroma, although the levels increase further in the LSP and deciduas(38).

1.2.4.3. Secretory Phase

In the secretory phase functional changes are characterised by intense ECM rearrangement, aimed at embryo implantation. Implantation consists of a complex series of interactions between embryonic and maternal tissue. Successful implantation in humans requires that the trophoblast penetrates several tissue components in its effort to reach maternal blood supply, including the epithelial lining, BM and underlying stroma(27).

Surface luminal & glandular epithelium, and vascular endothelium

Positive laminin and COLIV immune-reactivity of BM structures around glands and vascular endothelium has shown to persist in the secretory phase(44). Specifically, strong COLIV mRNA signals are observed in BM supporting endothelial cells(45). However, laminin expression has been shown to be significantly reduced in BM supporting the luminal surface epithelium only(35). However, the authors did not conclude what laminin type was reduced, as the antibody to laminin used in this study was polyclonal. This is important as one study investigated the expression of COLVI, laminin-111 and laminin-332 in secretory normal endometrium. They found that COLIV and laminin-111 expression were regular along the BM, whereas laminin-332 appeared uneven and interrupted(42). Therefore this may suggest that the reduced expression of laminin-332 could be so great that it can affect the overall laminin protein integrity in the BM during the secretory phase. As laminin expression decreases along the luminal epithelium along the secretory phase, COLIV expression becomes dominant in the BM supporting the luminal epithelium. As COLIV is seen to inhibit cellular growth and stimulate apoptosis, its strong expression under the LE may contribute to the process of implantation, increasing the embryo-implantation receptivity(46).

Stroma

Many reports show that ovarian steroid hormones mainly regulate the production of COLIV and laminin in ESCs. During the MSP, studies have shown that laminin and COLIV are found in streaks and spots in the stroma(36). By the LSP, COLIV and laminin immuno-reactivity significantly increases(35). Moreover, decidualised ESCs show the strongest expression for both components, which develop into a fully formed aura in a mature decidual cell(38). mRNA studies supports these findings as stronger laminin and COLIV mRNA signals are detected in secretory ESCs and decidual cells, compared to proliferative ESCs(45). In some cases, laminin mRNA signals appear stronger in cells adjacent to

glandular structures than in more peripherally located ESCs(45). This suggests that COLIV and laminin play important biological roles in human decidualised stromal cells. This was confirmed by cell viability assay studies having demonstrated that differentiation and viability of ESCs in the LSP endometrium maybe be auto-regulated by both BM components(35). In particular, laminin has shown to inhibit ESC differentiation(47). The development of an aura of BM is likely to restrict the motility of decidual cells. Thus this static framework may provides an essential environment within which the events of implantation, placentation, immune-modulation and fetal growth occur(36).

1.2.5 Basement Membrane in PM Endometrium

Furness and Lam studied the immuno-localisation of BM component laminin in endometrial samples taken across all phases of the menstrual cycle (n=56) and in PM endometrium (n=11)(48). Results showed that laminin was present in BM supporting glandular epithelium and remained to be intact in all phases of the cycle and was similar in PM endometrium. Iwahashi et al used immunofluorescence to investigate the effects of oestrogen on the ECM in the PM endometrium(24). They found in samples taken from the untreated group, there was immuno-localisation for laminin but none for COLIV(24). In contrast, both BM components were found in the endometrium of oestrogen treated women, and resembled endometrium of cycling women in the proliferative phase. This supporting evidence confirms that circulating levels of ovarian hormones are important for the production of BM components and this is lost after menopause. Additionally, laminin has also been reported to be immuno-localised in BM supporting vascular endothelium in PM endometrium as illustrated by Tanaka et al, but the expression was significantly lower than that seen in cycling endometrium(35).

1.3 Uterine Stem Cells

Stems cells are the fundamental units of biological organisation, playing a critical role in the replenishment and regeneration of dying cells and damaged tissues(49). They are defined as undifferentiated cells capable of reproducing themselves and differentiating into many different cell types(50). During the course of stem cell development, they are first totipotent, being able to differentiate into any cell type in embryonic germ layers (ectoderm, mesoderm and endoderm) and extra-embryonic tissue (trophoblast, placenta and extra-embryonic membranes)(49). Thereafter, they can differentiate into pluripotent stem cells giving rise to cells that form all the three germ layers only. As embryonic development ensues, the differentiation potential becomes increasingly restricted, producing cells that give rise to multiple types of cells within the same germ layer, called multipotent somatic stem cells(51). These cells then usually differentiate along only one lineage producing component cells of the tissue in which they reside, and are known as unipotent somatic cells. Therefore, depending on the stage of development, stem cells can be either classified as either embryonic; derived from blastocysts, or adult stem cells (ASCs); derived from post-embryonic cell lineages(49).

1.3.1 Embryonic stem (ES) cells

ES cells, first isolated from the mouse in 1981, are a type of pluripotent stem cell derived from the inner cell mass of the blastocyst(52). They have the ability to differentiate to form the trophoblast and derivative of all three germ layers(52). Due to their characteristics, ES cells show great promise in understanding organ physiology, for example in the heart and brain(53, 54). However, ES cell studies face hurdles of problems, including risks of teratoma formation and the ethical arguments over the creation, usage and the destruction of human embryos. Therefore the application of ASCs may provide an alternative, and research to determine their full potential is currently being undertaken.

1.3.2 ASCs

ASCs are small populations of quiescent cells found in an undifferentiated state throughout the body after embryonic development(55). They maintain tissue homeostasis through replacement of cells in cellular turnover, thereby contributing to the structural and functional preservation of the tissues during the lifetime of the individual(55). ASCs are defined by their functional properties as seen in vitro:

- High proliferative potential
- Substantial self renewal capacity
- Ability to differentiate into at least one or more lineages
- Clonogenicity

Proliferative potential is determined by analysing the total number of cell population doublings of one cell by serial passage until senescence(4). Maintenance of the stem cell population requires cellular self renewal through mitotic cell division to produce identical daughter stem cells(51). Stem cells are classically identified by their clonogenic activity, defined as the ability of a single cell to self renew and produce a colony when seeded at extremely low densities(49). This well characterised functional activity of ASCs in vitro has been used to demonstrate their existence in various tissues.

There are two types of cell division that maintains this stem cell pool: (a) asymmetric and (b) symmetric cell division.

a) Asymmetric cell division produces an identical daughter cell, which maintains its own population of undifferentiated cells (self renewal), and a more differentiated progenitor cell, see figure 1.5(56). These progenitor daughter cells undergo rapid proliferation, and their daughter cells proliferate producing a more mature progeny known as transit amplifying (TA) cells. TA cells which have properties intermediate between stem cells and end stage differentiated cells. Although they have limited

proliferative potential and inability to self renew, TA cells undergo rapid proliferation and increase in cell number(55). As they undergo rounds of cell division they acquire differentiation markers as part of the cellular amplification process producing numerous terminally differentiated functional cells(51, 55).

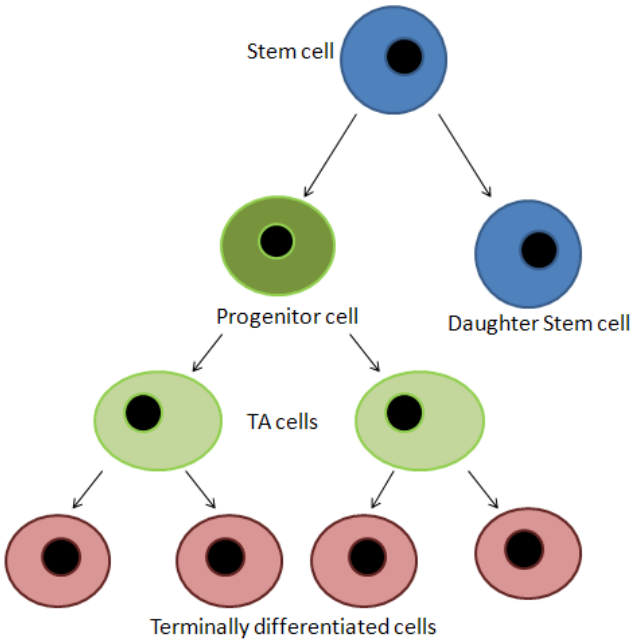


Figure 1.5: Diagram to show asymmetric cell division

b) In contrast to asymmetrical division, symmetrical cell division (see figure 1.6) produces two identical daughter stem cells or two TA progenitor cells.

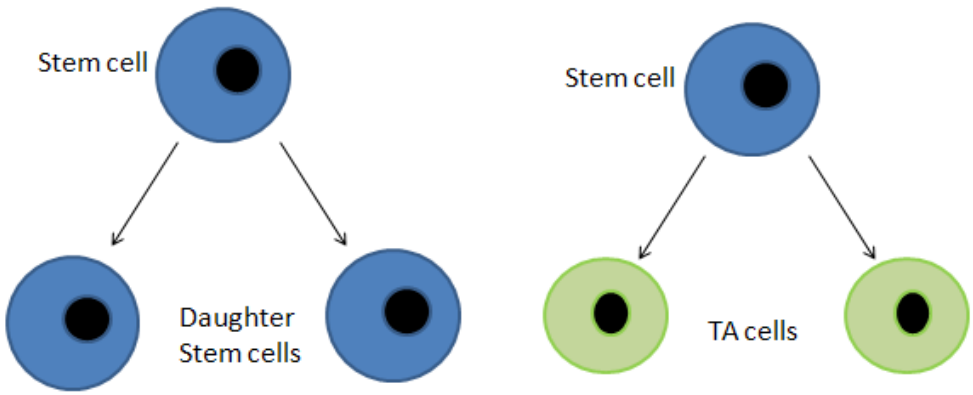


Figure 1.6: Diagram to show symmetrical cell division

Over the past several years there has been a substantial body of literature questioning the fate of ASCs. ASCs have previously been thought to have restricted differentiation potential in order to produce derivatives limited to the tissue which they reside in or within their embryonic germ layer boundaries(51). Nevertheless, this assumption has been greatly challenged as in some cases stem cells have shown to transdifferentiate from one tissue lineage to a different lineage, a process known as plasticity(57). This involves an acquisition of new cellular markers and function of the new cell type without an intervening cell division(51). This plasticity has been detected in mesenchymal stem cells in bone marrow, as these cells are known to traffic via the blood stream and incorporate into damaged tissue and change into many cell types(58). Thus it is now believed that in the setting of tissue damage and a change in the extracellular environment, some ASCs such as haematopoietic stem cells may not be lineage restricted and may be able to differentiate into other cell types in new locations, in addition to their usual progeny of organ of residence(59).

1.3.3. Endometrial Stem/Progenitor Cells

As previously explored, the endometrium is a highly dynamic tissue which undergoes repeated cycles of growth and regression with each menstrual cycle, and undergoes uterine decidualisation in pregnancy(60). Additionally, endometrial growth also follows parturition and in PM women taking oestrogen replacement therapy. This level of cellular turnover is similar to that seen in highly regenerative tissues such as haemopoetic tissue, intestinal epithelium and the epidermis, where ASCs are responsible for generating replacement cells(60). Based on these features, ASCs have also been hypothesised to be involved in the regeneration and development of glandular and stromal components in the endometrium(61). Recent experimental approaches have demonstrated the classical functional properties of ASCs in endometrial tissue.

Chan et al provided the first evidence for the existence of endometrial stem/progenitor cells using cell cloning studies(56). Thereafter, the same group demonstrated endometrial cell clonogenicity, as they found 0.22% of epithelial cells and 1.25% of stromal cells were able to form individual colonies when seeded at clonal density and they also demonstrated the differentiation potential of these cells(62). They reported that the stromal clonogenic cells were able to differentiate into a number of cell types including chondrocytes, adipocytes, osteoblasts and smooth muscle cells which are characteristics of bone marrow and adipose tissue mesenchymal stem cells(63). They further demonstrated a high proliferative potential of endometrial stem cells with epithelial and stromal cell fractions capable of 30-32 population doublings before senescence or transformation, whereas non-stem-like endometrial cells were capable of only 12 population doublings(51).

Colongenicity in endometrial tissue has been tested across all phases of the menstrual cycle, and also in inactive PM endometrium(62). It has been demonstrated that clonogenic potential in epithelial and stromal cells do not differ between the phases of the menstrual cycle and between endometrial samples from reproductive and PM women(61, 64). As PM endometrium only contains the basalis layer, these findings suggests that putative stem/progenitor cells reside in the basalis layer. The lack of difference in clonogenicity between the patient groups also suggests that ovarian hormones may not have a regulatory control over the stem cell activity, since they fall in PM women. In agreement with this, Gargett's group identified mesenchymal stem cells in the endometrium that did not express either PRs or ERα but expressed ERβ(61, 64).

Stem cells can also be identified as a 'side population' (SP) as shown in various adult tissues. This method involves incubation with a DNA-binding dye Hoechst 33342 and then analysing cells using a dual emission wavelength fluorescence activated cell sorting (FACS). Since stem cells are able to pump out the dye via the ATP-binding cassette transporter G2 (ABCG2) in the plasma membrane, they should appear as a low Hoechst population and are

characterised as the side population(60). Given that ABCG2 is a characteristic marker of SP cells, it was used to study the spatial distribution of SP in the endometrium using immunofluorescence(55). Since endometrial functionalis is thought to regenerate from the remaining basalis after menstruation; this suggests that the putative endometrial stem/progenitor cells reside in the basalis. Interesting, ABCG2 cells were distributed evenly throughout the functional and basalis layers of the endometrium(55, 60).

The main problem with the proposed stem/progenitor cells from the endometrium is that the potential stem cells have not been able to conclusively produce an endometrial like tissue in a 3D culture or animal model. Furthermore, there are no markers of endometrial epithelial stem/progenitor cells. For those reasons, although many studies have tried to investigate the source and location of endometrial stem/progenitor cells, their precise origin remains elusive. It has been thought that fetal epithelial and mesenchymal cells remain in adult endometrium and contribute to the regenerative ability of the endometrium(65). Additionally, several lines of evidence suggest that bone marrow-derived cells congregate at sites of tissue damage and incorporate into various organs, including the endometrium(60). Recently, a particular study showed that female mice receiving bone marrow transplantation from male donors, demonstrate genotypically male-derived cells incorporated in their endometrium(66). Therefore, it has been postulated that once bone marrow-derived cells populate the endometrium, they are able to transdifferentiate into endometrial stem/progenitor cells and contribute to the pool of already resident ASCs.

1.3.4 The Role of BM in the Stem Cell Niche

In 1978 Schofield hypothesised that stem cells are embedded within fixed compartments called niches. Recently, mammalian stem cell niches have been described in germinal, haematopoetic, neural, epidermal and intestinal systems(67-70). These physiological microenvironments are highly tissue specific structures, comprising of ECM molecules with additional surrounding cells, which anchor the ASCs. Niches are thought to play a crucial

role in maintaining tissue homeostasis in various ways. Firstly, during periods of inactivity they maintain ASCs in a dormant state (G_0) by signalling inhibitory pathways, protecting them from differentiation, proliferation and apoptotic cues(71). Secondly, they determine the fate of stem cell daughters by balancing stem cell replacement (self renewal) and provision of functional differentiated cells required for organ function(72). Additionally, they provide a protective environment to maintain the genetic fidelity over the stem cells' life span(60).

A well organised ECM is crucial for stem cell niche function and mainly comprises of cadherins to mediate the adhesive interactions between niche cells and stem cells, and integrins to control interactions between ECM and cells(60). It has been proven that breast epithelial stem cells live in a niche where ECM proteins provide an optimal microenvironment(73). As part of this matrix, BM is an important component to which stem cells attach on to(5). The BM is believed to have important function for the structural integrity of stem cells, facilitating the polarisation of cells, which is important for cell division(5). Such an environment may regulate the stem cells and their proliferating and differentiating progenies via the epithelial-mesenchymal cross talk. Therefore, a highly selective specialised ECM is needed to maintain stem cell properties, particularly during development.

Growth factors, including epidermal growth factor (EGF), platelet derived growth factor BB and transforming growth factor alpha have been shown to support endometrial clonogenic activity, and have been suggested to have a role in stem cell niche(61). Interestingly, interactions between ECM and growth factors are integral in tissue repair processes(5). ECM components can directly bind to and release certain growth factors enhancing their activity or protecting them from degradation. This is seen when cleavage of laminin by MMPs releases a protein molecule that can bind to EGF receptor, subsequently enhancing cell motility and migration, hence enabling tissue repair(5).

1.3.5. Stem Cell Markers

Although functional analyses help identify stem cell activity in the endometrium, definite markers for endometrial stem/progenitor cells are unknown and research into indentifying specific markers is ongoing(74). A panel of known stem cell markers, including cytokeratin 5/6 (CK5/6), podocalyxin-like protein 1 (PODXL) and stage specific embryonic antigen (SSEA1) will be used in this study to potentially identify putative stem/progenitor cells in the endometrium sampled from all patient groups studied.

1.3.5.1 CK 5/6

Cytokeratins are the largest subgroup of intermediary filament (IF) proteins, which are preferentially expressed in epithelial tissues(75). IF is part of the cellular cytoskeleton which are very long, chemically stable, unbranched filaments of ~10nm in diameter(76). These filaments braid the nucleus, span the cytoplasm and attach onto cytoplasmic plaques of the typical epithelial cell-cell junctions, known as desmosomes(76). Additionally they attach onto hemidesmosomes that connect a cell to the ECM. Thus this provides a continuum of stability not only between epithelial cells itself, but also to BM and connective tissue compartment of the given epithelium.

Two types of cytokeratins exist: the acidic type 1 cytokeratin (CK 9-20) and the basic type II cytokeratin (CK 1-8)(76). Based on their molecular weight and immuno-reactivity, specific cytokeratin fingerprinting allows the classification of all epithelia. The type of CK expression depends on the type of epithelium and how well differentiated and developed the epithelium is(77). Thus for this reason CKs are used to try morphologically classify stem cells, intermediate TA cells and their progenies, particularly in mammary and prostate tissue(78, 79).

CK5/6 is a marker of differentiated epithelial cells illustrated by its distinctive role in breast cancer histology. The mammary gland consists of a network of ducts that form before birth

which branch out into the mammary fat pad. These ducts are lined with a double-layered epithelium consisting of a basal layer of contractile myoepithelial cells and a luminal layer of specialised glandular cells(80). Further differentiation takes place during puberty when there is a rapid outgrowth of the ducts. Nonetheless, thereafter, breast epithelium remains relatively immature as it is constantly renewed during the menstrual cycle. The final stage of epithelial differentiation occurs during pregnancy and lactation. In order for repeated pregnancies to be sustained, a subgroup of breast cells needs to retain the potential for rapid proliferation and differentiation and this is the function of stem cells, which has also been hypothesised to be the driving force behind mammary carcinogenesis(80). This recognition of the regenerative capacity has led to the recent isolation of stem/progenitor cell-enriched populations from the mouse mammary epithelium(81).

In breast tissue, it has been shown that the luminal epithelial cells express CK 8/18, CK 7 and CK 19, while basal/myoepithelial cells express CK 5/6, CK 14 and CK 17(82). It has been suggested that CK5 positive cells display the phenotypic and behavioural characteristics of stem/progenitor cells for both the glandular and myopepithelial cell lineages(83). Using immunohistochemistry, Bőcker et al stained paraffin sections of breast tissue with CK5/6 and suggested that a breast stem cell is CK5/6 positive in the basal positioned cells that differentiate into all other breast cell types(84) and these results were verified by other studies(85, 86). However, shortly after, Clarke et al studied the expression of CK5/6 in matched pairs of paraffin wax embedded and frozen breast specimens. The staining patterns reported previous CK5/6 positive staining in antigen-retrieved paraffin sections but no CK5/6 only cells were found in frozen sections of the normal breast(87).

Recently, cytokeratin expression was analysed in cultured mouse mammary epithelial cells (MEC) during early and post natal development phases(88). The study found CK6+ putative mammary progenitor cells arise during embryogenesis with distinct temporal and spatial distribution(88). Whereas CK5 expression was considerably lower than CK14, which are two

CKs that are often co-expressed, during the first postnatal weeks of mammary development(88).

Since both breast and endometrial tissue undergo significant changes during the menstrual cycle and are both responsible to ovarian hormones, CK5/6 will be investigated as a marker of endometrial stem/progenitor cells in this study.

1.3.5.2 PODXL

Podocalyxin is a transmembrane glycoprotein, encoded by the PODXL gene, belonging to the sialomucin protein family(89). This protein was originally identified as an important component of glomerular podocytes. Podocytes are highly differentiated epithelial cells with inter-digiting foot processes covering the outer aspect of the glomerular BM. The foot processes of podocytes are covered on their apical surface with an anionic glycocalyx, which PODXL is a major component of(90). It contributes to podocyte characteristics as it is an intensely negatively charged molecule present in large amounts on podocyte foot processes and lines the surface of endothelial cells(91, 92). This negative charge is thought to maintain the distance between foot processes of neighbouring cells and between circulating cells and the endothelium(89). The basal surface of podocyte foot processes is connected to the glomerular BM at focal contacts(93). It has been suggested that glomerular BM components play morphogenetic roles in foot process formation, correlating with the expression of podocalyxin. Nonetheless, there is evidence that podocalyxin inhibits cell binding and spreading to BM components(93). This anti-adhesive effect is thought to contribute to the specialised conformation of podocytes(93). After its discovery, this protein was first cloned in 1995 from rabbit glomerulus, and was termed podocalyxin-like protein 1 (abbreviated as PODXL) sharing many of the characteristics of the original podocalyxin protein(89).

Subsequently, PODXL was found to be expressed in the haematopoietic system with close relation with hematopoietic stem cell marker CD34, and the recently discovered sialomucin, endoglycan(94, 95). Thereafter, PODXL was identified as a marker of malignant testicular tumour expressed on human embryonic carcinoma (EC) cell lines(96). Human EC cell lines are established malignant pluripotent stem cell lines derived from human germ cell tumours, therefore based on these results, PODXL is known as a marker of undifferentiated embryonic stem cells(97). In addition, it is abnormally expressed in subsets of breast, liver, pancreatic and kidney cancer as well as leukaemia(98). Strikingly, it is often associated with the most aggressive cases, and it is likely involved in metastasis(98).

1.3.5.3. Stage specific embryonic antigens (SSEA)

Extracellular glycolipids, cell surface carbohydrates, are the first cellular component encountered by approaching cells, antibodies and other molecules(99). It is well known that the expression patterns and levels of glycolipids drastically change during development, meaning that they can be useful stage-specific marker molecules of developing cells, including stem cells(100). Carbohydrate antigens were first described by Solter and Knowles to show a stage-specific expression in undifferentiated mouse ES cells and undergo marked alterations in their localisation in defined regions of the embryo(101). Therefore, they are of practical value in analysing cell differentiation(101). Significant carriers of stage specific carbohydrates on ES cells are the lactoseries (SSEA-1) and globuloseries (SSEA-3 & SSEA-4)(101).

The epitope of SSEA-1 antibody has shown to correspond to that of Lewis X antigen (LeX), but strictly, is not equal to as LeX.(100). According to the cluster of differentiation (CD) nomenclature, SSEA-1 is also referred to as CD15(100). SSEA1, detected by a monoclonal antibody against mouse EC cells, is also shown to be an established marker of mouse ES cells. It appears first in the late eight-cell mouse embryos (in approximately 50% of ES cells),

later becoming restricted to specific cell types (appearing is less than 10% of ES)(102). Overall, this illustrates the importance of SSEA-1 as a marker for mouse undifferentiated cells(102). The functional role of SSEA-1 is not well understood, however, it is generally considered to be involved in cell adhesion and compaction of the mouse embryo at the morula stage and also in EC cells(102).

In contrast with mouse, human ES and EC cells do not express SSEA-1 in the same manner. During the course of differentiation of human EC cells, there is a transition from SSEA-3^{+}, SSEA-4^{+}/SSEA^{-} to SSEA-1^{+} only phenotype(103). The diminished expression of SSEA-3 and SSEA-4 in the plasma membrane upon differentiation makes them useful markers for pluripotency, and additionally, SSEA1 has been prospectively utilised to isolate multi-potential embryonic stem cells(99). However, it has been postulated that they do not have a functional role in human ES cells as their depletion using inhibitors had no significant effect on the cell's ability to remain undifferentiated(99). Primordial germ cells have shown resemblance to ES cells by their ability to induce teratocarcinomas. Human primordial germ cells with similar properties to ES cells are called embryonal germ (EG) cells, and have been shown to express SSEA-1, SSEA-3 and SSEA-4(102, 104).

Neural stem cells are defined as undifferentiated neural cells, derived from adult brain or from embryos, which yield both neurons and glial cells and are expected to be valuable in therapy in neurodegenerative diseases. Flow cytometric analysis reveal both mouse and human neural stem cell express SSEA-1, at postnatal day 1 and at gestational week 17 respectively(105). Thus, as SSEA1 are expressed in more mature progenitor cells of nervous tissue, this indicates its role as a marker for more differentiated TA cells(100).

Although carbohydrates are complex structures, their cellular expression is highly regulated and appears to be dependent on the pattern of glycosyltransferases, found in the golgi apparatus, modulating post-translational glycosylation(106). The fucosyltransferases (FUT)

are a family of glycosyltranferases that play a crucial role in the developmental control of cell surface glycans(106). In mammals, fucosylated glycans linked to proteins are involved in a wide range of mechanisms such as cell adhesion during development, inflammatory response and leukocyte trafficking(106). Different FUT enzymes transfer different carbohydrates components on glycans on various linkages(106). Interestingly, FUT4, a key enzyme that transfers fucose at α1,3 linkage, is lost during in vitro differentiation of mouse EC cells in parallel to the disappearance of SSEA1(101).

1.4 - Endometriosis

Endometriosis is one of the commonest gynaecological conditions characterised by the presence of endometrial tissue found outside the endometrial cavity. These ectopic lesions are morphologically and biologically similar to normal endometrium, containing functional endometrial glands and stroma. Endometriosis is predominantly found in women of reproductive age, from all ethnic and social groups with an estimated prevalence of 2-22%(107). It should be suspected in women with subfertility, severe dysmenorrhoea, deep dyspareunia and chronic pelvic pain(108). However, the clinical presentation varies depending on the location and severity of the disease process. As well as physical well being, these associated symptoms can also impact on mental and social well being too, creating a huge burden on the economy.

1.4.1. Pathophysiology

There have been significant developments in recent years regarding our understanding of endometriosis, however the pathogenesis of endometriosis still remains unclear and the origin of endometriotic implants has long been an area of active investigation.

Currently there are three theories that describe the aetiology of endometriosis:

THEORY ONE: Menstrual regurgitation

'Menstrual regurgitation' is the most widely accepted theory that was proposed by Sampson in 1927(109). This theory states that fragments of endometrium are transferred through the fallopian tubes in a retrograde manner at the time of menstruation. This is followed by implantation of viable endometrial cells into the peritoneum and pelvic organs to establish ectopic growth of endometrial tissue. Thus, endometriotic cells are thought to have an invasive phenotype similar to metastatic tumour cells. However, the fundermental mechanisms by which menstrual endometrium adhere, proliferates, and established a

functional vasculature in an ectopic site remain to be elucidated. Retrograde menstruation is a universal phenomenon, occurring in at least 76-90% of women undergoing peritoneal dialysis and laparoscopy(110, 111). Nevertheless, given that retrograde menstruation occurs in most women, it is still unclear why some women develop endometriosis whilst others do not. This suggests a primary role of abnormal eutopic endometrial cells (lining the uterine cavity) or an altered peritoneal environment contributing to the establishment of endometriosis.

A Baboon Model for Endometriosis

In order to understand the early and progressive events associated with the establishment of endometriosis, a Baboon model has been developed in which the disease can be induced(112). In this model, menstrual endometrium is harvested from baboons, specifically Papio Anubis, using a pipelle and during laparoscopy the pipelle contents are deposited at three sites: the pouch of Douglas, the broad ligament adjacent to the oviducts and on the uterus. At the subsequent menses, the animals undergo a second laparoscopy and endometrial re-seeding at the same ectopic sites(112). The advantage of the induced model is that the progressive changes in both eutopic and ectopic endometrium can be studied in a non-human primate model at specific times during the menstrual cycle and as the disease process continues(112). Hapangama et al used the baboon model to demonstrate the relationship between eutopic and ectopic endometrial cells(113). They found that during the disease process eutopic endometrial cells acquired a more pro-proliferative potential that contributes to the pathogenesis of endometriosis via repeated retrograde menstruation(113).

THEORY 2: Coelomic Metaplasia

The second theory, coelomic metaplasia, states that endometriosis develops from metaplasia of the cells lining the visceral and abdominal peritoneum induced by a stimulus(114). This hypothesis can account for cases where endometriosis is found in men, pre-pubertal and adolescent girls, distant ectopic sites and endometriosis found in women with congenital

absence of mullerian structures(4). Despite these intriguing findings, evidence to support this particular theory remains elusive.

THEORY 3: Embryonic rest theory

Lastly, the embryonic rest theory proposes that at puberty there is activation of cells of Mullerian duct origin at various sites in pelvic cavity producing endometrial tissue(115). This theory supports the findings of rare cases in which endometriosis lesions are found in males, as the male embryo initially develops female-specific embryological structures(116). However, again, this theory also remains to be unproven and entirely hypothetical.

Link between endometriosis and cancer

For many decades, endometriosis has been suspected to play a role in the aetiology of ovarian cancer. Epidemiological data suggests a link between endometriosis and ovarian cancer with a twofold increased risk of developing ovarian cancer in patients with endometriosis in general and a further fourfold increased risk for high risk endometriosis patients with infertility(117). In ovarian cancer, the metastatic spread of malignant cells first involves the disruption of the mesothelium exposing the underlying ECM which provides an environment of cell adhesion, invasion and growth(118). Similarly, in endometriosis endometrial cells adhere to the exposed mesothelium where the initiate ectopic growth of endometrium. Therefore, endometriosis is thought to exhibit some of the characteristics of tumours since the two diseases involve the metastatic spread of cells.

1.4.2 Diagnosis and Staging

A history and examination can yield a number of significant findings, however none is diagnostic. Similarly, ultrasound scan can only give a presumptive diagnosis of ovarian involvement of endometriosis(119). Surgical exploration of the peritoneal cavity through

laparoscopy followed by histological confirmation is the gold standard for the diagnosis of endometriosis(119). Three clinically distinct forms of endometriosis include endometriotic implants on the surface of the pelvic peritoneum and ovaries (peritoneal endometriosis), ovarian cysts lined by endometrial mucosa (endometriomas) and a complex solid mass comprised of endometriotic tissue blended with adipose and fibromuscular tissue, residing between the rectum and the vagina (rectovaginal endometriotic nodule)(120). During laparoscopy, the peritoneum, fallopian tubes, ovaries and the pouch of douglas are examined for the presence of endometriosis, adhesions, fibrosis and cyst formation. The extent of endometriosis is then staged I-IV according to the American Society for Reproductive Medicine (ASRM) system which correlates well with chances of infertility, see table(121).

Table 1.1 – American Society for Reproduction Medicine Classification of Endometriosis

Stage	**Description**
I	Superficial lesion present with or without a few adhesions.
II	As above, in the presence of deep infiltrating lesions found in the cul de sac
III	As above, in the presence of endometriomas on the ovary in addition to more adhesions
IV	As above with large endometriomas and extensive adhesions.

However, this classification lacks a description of women with pain, and needs to be included since it can be a recurrent and long-standing feature of endometriosis. Additionally, the classification does not take into account of the functionality of different endometriotic implants, nor does it include any biological markers(119). It may be impossible to include all these factors and it is unlikely that an accurate staging system will be introduced until we have a better understand of the pathophysiology behind endometriosis.

Histological features of ectopic lesions include the presence of stromal and endometrial epithelial cells, chronic bleeding and signs of inflammation. Since endometriosis is already established in many women when they first present with symptoms, the initiation of the

disease is difficult to establish. Endometriotic glandular tissue is morphologically heterogenous and, in comparison with eutopic endometrium, the glands appear larger and more disorganised(122).

1.4.3. Management

Treatment of endometriosis needs to be tailored to the individual woman depending on her symptoms, priorities and stage of endometriosis. Treatment of endometriosis entails surgical and pharmacological intervention, either individually or combined. Medical treatment relies on manipulating ovarian hormones and inducing amenorrhoea, and includes combined oral contraceptive pill, analogues of GnRH, or progesterone analogues and aromatase inhibitors(114). These medications are suitable in reducing pain, but their side effect profile, e.g. increased cardiovascular risk, limits their long term use. Additionally recurrence is common on discontinuation and does not improve fertility(114). If fertility is priority, conservative surgery is effective in reducing pelvic pain as well as improving pregnancy. Surgical interventions include excising or ablating endometriotic lesions and the removing adhesions in the peritoneal cavity(114). Overall, currently, there is no universally accepted protocol for the treatment of endometriosis thus treatment is very much individualised.

1.4.4 Basement Membrane in Endometriosis

As previously explored, the endometrium is a highly dynamic tissue and therefore dysregulation of important structural macromolecules of BM may have a role in endometrial pathologies. It has already been established that expression of tenascin, an ECM glycoprotein found in endometrial stroma, is significantly higher in ectopic lesions than in eutopic endometrium, confirmed by western blotting analyses (123). ESCs from women with endometriosis have also shown to have a significantly increased adhesive capacity when placed in tenascin-C, suggesting this ECM protein may have a role in the pathogenesis of endometriosis. Whatever the mechanism by which endometriosis is established, its

development is likely to involve cell adhesion, proliferation, invasion into the underlying tissue, and differentiation of the ectopic endometrial cells. As these cellular events are modulated by ECM components, it is possible that endometriosis may be associated with aberrantly regulated cell-BM interactions.

There are similarities and differences seen in the eutopic endometrium taken from women with no endometrial pathologies (controls) and women with endometriosis. Using IHC, Harrington et al showed that the basalis region of the eutopic endometrium collected from endometriosis patients showed strong staining for COLIV and laminin, irrespective of what phase of the menstrual cycle the sample was collected in(123). This immuno-expression was consistent to what was seen in the endometrium collected from controls. This suggests that basalis layer in the endometrium of patients with endometriosis remains in its preserved functional state across the menstrual cycle. Another study analysed BM components in the LSP endometrium of women with unexplained infertility, a condition closely associated with endometriosis, and also in endometrium from normal fertile controls(37). In contrast to the previous study, all endometrial biopsies were immuno-negative for COLIV and laminin in ESCs and BM supporting blood vessels and glands in the infertility group(37). The lack of endometrial ECM components may have a negative effect on cell recruitment and migration and could result in incomplete embryo-maternal recognition, a possible consequence of which may be implantation failure.

Additionally, remarkable similarities have been observed between eutopic and ectopic endometrial samples in the expression and localisation of BM components. These include similar strong expression of laminin epithelial BM and COLIV in glandular epithelium BM in matched eutopic and ectopic endometrial samples(123, 124). In addition, when studying endometrial stroma, laminin expression appears to be similar between ectopic lesions and of control eutopic endometrium when all sampled during the LSP. Notably, laminin 5 has been

reported to be discontinuous and interrupted in normal secretory endometrium, and this same pattern has also been noted in endometriosis lesions(42).

Proteolytic activity has seen to be increased in ectopic lesions since higher levels of MMP-9 and a higher ratio of MMP-9 to tissue inhibitor of MMP (TIMP)-1 are found, when compared to eutopic endometrium from women with and without endometriosis(42). This could result in enhanced proteolytic potential of endometrial fragments undergoing retrograde menstruation, increasing invasiveness and facilitating the development of endometriotic foci(1). Using collagen invasion assays, Gaetje et al were able to show that cells from peritoneal endometriotic lesions express this invasive phenotype(125). Interestingly, a retrospective analysis showed that in 12 out of 15 samples of fine needle aspirations from endometriotic cysts showed oval structures very strongly stained for COLIV. The authors concluded that repeated bleeding and inflammation, involving proteolytic activity, in ectopic lesions can greatly fragment BM, therefore, assuming this oval shape(126).

Petra et al investigated the role of BM components COLIV and laminin in endometrial cell adhesion and proliferation, both of which are known to take place in the pathogenesis of endometriosis. The authors claimed that as cultured ESCs retain integrin expression in vitro, ESCs can be accepted as a model for investigating the function of integrins and their ligands, and effectively analysing cellular adhesion(1). Using this model, ESCs derived from peritoneal surface and ovarian lesions and from endometrium of women with endometriosis exhibit an increased attachment to BM components. In particular ESCs from ovarian endometriotic lesions exhibited 2.5-3 fold increase in attachment to COLIV and laminin(1). Additionally, ESCs from peritoneal surface lesions exhibited a 1.5-2 fold increase in attachment to COLIV(1). Experiments have shown that endometrial cells do not adhere to intact epithelium but preferentially adheres to sub-epithelial structures(127). In the event of mesothelial injury or rupture of primary ovarian follicle, this may allow peritoneal and ovarian BM components to be exposed thus providing potential sites for attachment. This

interaction between endometrial cells and the sub-epithelial layer is likely to be communicated by integrins(128). Peritoneal fluid also contains released proteolytic fragments of COLIV and laminin during ovulation, increasing the interaction between endometrial cells and ECM components for attachment in the peritoneum.

Endometrial cellular proliferation has shown to be increased in endometriosis(129). It has been thought that the development of ectopic endometrial implants involves proliferation of ESCs subsequent to their attachment to the peritoneal tissue(1). To measure the proliferative capacity of ESCs in different ECM components, DNA synthesis was analysed in both eutopic and ectopic stromal cells(1). These experiments revealed that ESCs derived from eutopic endometrium from women with endometriosis demonstrated a 1.5-3 fold increase in DNA synthesis when separately placed on COLIV, laminin and other ECM components. Additionally, ESCs derived from ovarian lesions exhibited a 2-4 fold increase in DNA synthesis when separately placed on laminin and other ECM components(1). Therefore, these molecules could modify the adhesive and proliferative behaviour of ESCs in menstrual effluent that are transported into the peritoneal cavity through retrograde menstruation.

1.4.5. Stem/progenitor cell function in Endometriosis

Recent characterisation of possible endometrial stem/progenitor cells has provided new insights into endometrial physiology and the pathophysiology of various gynaecological disorders including endometriosis and endometrial cancer. To date there is no direct evidence for the role of stem/progenitor cells in the pathogenesis of endometriosis. However, there are numerous studies that give plenty of indirect evidence for the function of endometrial stem/progenitor cells in the development of endometriotic implants. The array of evidence given can support all theories of the cellular origin of ectopic endometriotic deposits:

Retrograde Menstruation

Recent evidence suggests women with endometriosis have a greater amount of basalis endometrium in their menstrual debris than normal fertile women, as described by Leyendecker et al(130). Fundamental differences have been seen between the cyclical pattern of ovarian hormone receptors of the basalis and functionalis layers in the endometrium. Leyendecker et al used this concept and also explored for any potential similarities or comparisons in PR and ER expression between eutopic and ectopic endometrium. They found that ectopic endometrium mimicked the cyclical pattern of ER and PR of the basalis and was 'out of phase' with the functional eutopic endometrium(130). This study also showed that significantly more basalis layer of the eutopic endometrium was shed in the menstrual flow of women with endometriosis compared with that of healthy controls(130). Thus, in combination with evidence suggesting endometrial stem/progenitor cells mainly reside in the basalis and that women with endometriosis have larger volumes of retrograde menstrual flow, this supports the theory that endometrial stem/progenitor cells are transported in a retrograde fashion and reach peritoneal cavity where they adhere and establish ectopic lesions. It is thought that long term ectopic lesions develop from endometrial stem/progenitor cells and ectopic lesions that easily resolve are established by more mature TA cells(51). Additionally, it is possible that the endometrial stem cell activity may also be abnormally high in endometriosis patients, increasing their capacity to implant and establish themselves as ectopic tissue(51).

Coelomic Metaplasia

Metaplasia of the peritoneal lining has been suggested as a possible cause of endometriosis. However, another source of metaplastic cells is the bone marrow-derived cells, with trans-differentiation capacity, that gain access to the peritoneal cavity via the circulation. Bone marrow-derived cells as an extra-uterine source of stem cells may be responsible for the

observations that support the theory of coelomic metaplasia. Du and Taylor tested this theory by using an experimental model for endometriosis. In this study, samples of wild type endometrium were transplanted into the peritoneal cavity of hysterectomised mice. These mice were then recipients for bone marrow transplantation from LacZ transgenic mice. Ectopic endometrial lesions were later sampled and LacZ expressing stem cells were found to be incorporated into the implants, and were capable of differentiating along epithelial and stromal cell lineages at a frequency of 0.04% and 0.1% respectively(131). Altogether, this suggests that bone marrow-derived cells can not only transdifferentiate into functional endometrial cells after seeding the endometrium, but can also behave similarly outside the endometrial environment. In light of this evidence, bone marrow-derived cells may contribute to the normal endometrial physiology and the development of endometriosis.

Embryonic rest theory

Remnant Mullerian cells have some characteristic stem cell properties of high proliferative potential, multipotency and self renewal. Thus, it is possible that potential stem/progenitor cells can persist in the remnants of the mullerian system, which can form endometriotic implants(4).

Even though there is a vast amount of indirect evidence indicating the role of putative endometrial stem/progenitor cells there is still a lot of unanswered questions. For example it is not known whether there is a difference in the endometrial stem-like cells between women with and without endometriosis. Additionally, even though “retrograde menstruation” is the most widely accepted theory behind the pathogenesis of endometriosis, there is evidence lacking behind the idea that endometrial stem/progenitor cells actually are shed in a retrograde fashion. Extensive research into the role of stem cells in endometriosis is still needed, nonetheless, it is an area difficult to investigate.

1.5. Endometrial Carcinoma

Endometrial carcinoma, a common gynaecological cancer affecting 142,000 women, with an estimated 42,000 deaths per year worldwide (132). In the UK, 7536 women were diagnosed with endometrial cancer in 2007, making it the fourth most common cancer to affect women after breast cancer(133). Endometrial cancer can be divided into two major groups depending on histopathology, epidemiology, and clinical behaviour(133). Type 1 tumours are the most common tumours and constitute oestrogen dependent endometrioid adenocarcinoma and type 2 are more aggressive tumours that carry a poor prognosis. Endometrial cancers are also graded, from I to III, using the International Federation of Gynaecology and Obstetrics (FIGO) grading system and this classification is based on the architecture of the tumour, the appearance of the nucleus in cancer cells and the mitotic index(134).

1.5.1. Basement Membrane in Endometrial Cancer

With the carcinogenesis process, the organised epithelial stromal cellular architecture is lost in the endometrium(2). Altered BM composition and assembly may influence endometrial carcinoma cell growth and invasion and metastasis(135). When analysing BM integrity in accordance with the tissue grade, it has been shown that well differentiated endometrial carcinomas appear to have a preserved intact BM in most areas of the tumour, whereas a progressive lack of these structures seems to parallel an increasing degree of tumour dedifferentiation(135). This disruption or absence may reflect increased degradation or decreased production and deposition of BM material by the tumour cells(135). Since this alteration of the BM is a hallmark of carcinogenesis, for comparison with the BM of the normal and regulated endometrial regeneration and in the benign metastatic disease of endometriosis we examined the BM integrity in endometrial cancer.

1.5.2. Stem Cell Activity in Endometrial Cancer

A small number of cells within the tumours have been shown to have stem cell properties that contribute to its growth and metastases and they are referred to as cancer stem cells (CSCs)(51). CSCs have been identified in acute myeloid leukaemia, breast cancer, glioblastoma and prostate cancer(136). CSCs differ from ASCs in that they are no longer controlled by their stem cell niche(51). Thus, there is no regulation on proliferative activity in turn producing cells comprising the bulk of the tumour. Recently, Hubbard et al demonstrated a role for CSCs in endometrial cancer(3). This comprehensive study showed that in all 34 samples endometrial cancerous tissue, a small population of clonogenic tumour cells were identified and were able to be serially cloned. This indicates that endometrial tumour cells have a self renewal capacity and this property was shown to increase with increasing tumour grade. Additionally, a side population have also been identified in endometrial cancer, also indicating a small population of cells similar to ASCs(137).

1.6 – Summary and Project Hypotheses

The pathophysiology of endometriosis is an area of ongoing debate. It has been suggested that eutopic endometrium from women with endometriosis have altered cellular activities, contributing to the pathogenesis. Many theories have been proposed to establish the cause of endometriosis, two of which include altered endometrial ECM proteins and altered endometrial stem cell activity in the eutopic endometrium.

Therefore, this project will test the following hypotheses:

1. **Is endometrial basement membrane (BM) integrity altered in women with endometriosis?**

   In normal fertile women, endometrial basement membrane (BM) has been shown to have a menstrual cycle dependent production in order to help prepare endometrial receptivity to implantation. BM has shown to be disrupted in the metastatic process of endometrial cancer and has been postulated to be altered in the benign metastatic disease of endometriosis. Thus, we aim to explore in detail whether BM is disrupted in eutopic endometrium collected from women with endometriosis, by assessing the expression of endometrial collagen IV and lamina across the menstrual cycle. In order to gain comparative results, we will also to assess the expression of both BM components endometrium collected from fertile control women.

2. **Is BM disrupted in the pathogenesis of endometrial cancer?**

   Additionally we will explore the expression of BM components in endometrial cancer tissue samples to confirm that they are aberrantly expressed in this malignant disorder. In order to gain comparative results we will also test BM components in endometrium collected from PM women.

3. **What are the expression levels and patterns of stem cell markers CK5/6, PODXL and SSEA-1 in the endometrium of normal fertile controls?**

   It has been postulated that endometrial stem/progenitor cells contributes to the highly regenerative ability seen in the endometrium. However markers for endometrial stem/progenitor cells are lacking. By using known stem cell markers for extra-uterine tissues; PODXL, CK5/6 and SSEA1, we will assess whether these are expressed in the normal cycling endometrium and how expression changes along the course of the menstrual cycle.

4. **Do these stem cell markers (PODXL, CK5/6 and SSEA1) display a different pattern and level of expression in endometrium collected from women with endometriosis?**

   Recent characterisation of possible endometrial stem/progenitor cells has provided new insights into endometrial pathologies. Altered endometrial stem/progenitor cells are thought to play a role in pathogenesis of endometriosis. By using the above stem cell markers, we will also test whether they are aberrantly expressed across the menstrual cycle, in eutopic endometrium collected from women with endometriosis.

5. **Do endometrial cancer cells display stem cell markers PODXL, CK5/6 and SSEA1 and how?**

   Endometrial cancerous cells have shown to possess stem cell properties. By again using the above panel of known stem cell markers, we will investigate if they are expressed by endometrial cancer cells.

6. **Is BM integrity correlated with the stem cell markers investigated?**

   Basal lamina components are also important for the structure and function of stem cell niches. We will investigate for any potential relationships between basal lamina integrity with the expression of endometrial stem/progenitor cell markers in the fertile control, PM and endometriosis groups.

Chapter Two:

METHODS & MATERIALS

2. 1 - Ethical approval

Ethical approval for the project, including collecting human endometrial samples was obtained from Liverpool (Adult) Research Ethics Committee. All samples included were collected from patients attending Liverpool Women's Hospital and they all gave informed written consent. The benign endometrial samples of women in the reproductive age (both fertile control and endometriosis patients) were collected under the ethical approval 09/H1005/55 and 04/Q1505/112. The post menopausal samples and endometrial cancer samples were collected under the ethical approval 11/H1005/4. (For letters of approval from LREC, see appendix 2). A comprehensive literature review (using pubmed, ovid, etc) was performed initially to confirm the originality of the work included in this study.

2.2 - Study Groups

Endometrial biopsies were taken from four different groups of patients. All participants were only included in the study if they met the inclusion criteria. The exclusion criteria for the study were women taking any form of hormonal treatment in the preceding three months, if they were breast feeding or pregnant.

2.2.1 Fertile Control Group

30 endometrial biopsies were obtained from normal fertile women in 3 different time points in the menstrual cycle; the ProlP (n=10), MSP (n=10) and LSP (n=10).

Inclusion criteria:

- Normal ovulating women
- Women undergoing surgical procedures for benign conditions e.g. laparoscopic sterilisation

Exclusion Criteria:

- Any history of abnormal vaginal bleeding
- Women currently breast feeding or pregnant
- History of infertility or recurrent pregnancy loss
- History of endometriosis

2.2.2 Endometriosis Group

30 eutopic endometrial biopsies were obtained from patients with active, peritoneal endometriosis diagnosed concurrently at the time of sampling the endometrium, during the ProlP (n=11), MSP (n=11) and LSP (n=8).

Inclusion criteria:

- Women with regular menstrual cycles
- Surgically diagnosed active peritoneal endometriosis at the time of biopsy taking
- Dysmenorrhoea and/or chronic pelvic pain.

2.2.3 Endometrial Cancer Group

Endometrial samples were also collected from patients with endometrial cancer. Biopsies were obtained from patients with FIGO grade I (n=5), grade II (n=5) and grade III (n=3) endometrioid adenocarcinoma.

Inclusion criteria:

- Patients undergoing surgical treatment with a histological diagnosis of endometrioid adenocarcinoma

2.2.4 Post-Menopausal Group

All patients with endometrial cancer who participated in this study were post-menopausal. In order to have a comparative group for these post menopausal women with endometrial cancer, endometrial biopsies were also taken from post-menopausal women (n=5) who were undergoing hysterectomy for non-endometrial pathology related conditions such as uterine prolapse.

Inclusion criteria:

- Patients undergoing a hysterectomy for benign non-endometrial pathology related causes, e.g. utero-vaginal prolapse

Exclusion criteria:

- History of endometrial pathology
- History of post menopausal vaginal bleeding
- On hormone replacement therapy

2.3 - Participant Identification and Enrolment

Suitable patients for the study were highlighted by checking through theatre lists and reviewing patients' notes. Gynaecology outpatient clinic, pre-operative clinic, and gynaecology ward staff were given appropriate patient information leaflets to pass onto the selected patients as soon as they attended the hospital after being listed for their planned surgery. This allowed patients to read about the background of the study and what it entailed,

before verbal information was given from researchers. Only Good Clinical Practice (GCP) trained personnel who were fully aware of the ethical principals involved in informed consent, were allowed to consent and recruit patients for the study. After verbal consent was given, written consent was also obtained from all patients considered for the study. Patient demographical information including age, weight, height, body mass index (BMI), last menstrual period, cycle regularity, parity and past medical history was obtained from each participant. In the endometriosis group, the disease stage was notified at the time of diagnostic laparoscopy for each patient and was also recorded. See appendix 3 for sample of patient information leaflets, consent forms and patient demographic data form.

2.4 - Collecting the biopsy

Trained professionals obtained endometrial biopsies in a safe and sterile manner from all patients who were consented to take part in the study. Samples were collected after the planned surgical procedure; this ensured that no added discomfort or inconvenience was brought upon the patients. Two types of endometrial samples were collected for the study; full thickness and pipelle samples.

2.4.1. Full thickness samples

For women who underwent a hysterectomy, a full thickness sample was taken immediately after the uterus was removed illustrated in figure 2.1. In order to obtain this biopsy, the removed uterus was first placed on its anterior side on a surgical trolley. Using a carbon steel surgical blade (size 22, Swann-Morton, Sheffield, UK), a median incision was made on the posterior side of the uterus(a), from the uterine fundus down into the cervical canal exposing the endometrial cavity. A 25mm lateral incision was then made within the endometrial cavity, dissecting 10mm into the wall of the uterus(b). The piece of uterine tissue made more accessible was held with a pair of bonney tissue forceps (Phoenix Surgical Instruments Ltd, UK), whilst another median incision was made into the myometrium to free the tissue(c).

This ensured that endometrium, endo-myometrial junction and additionally myometrial layers were all obtained in the biopsy.

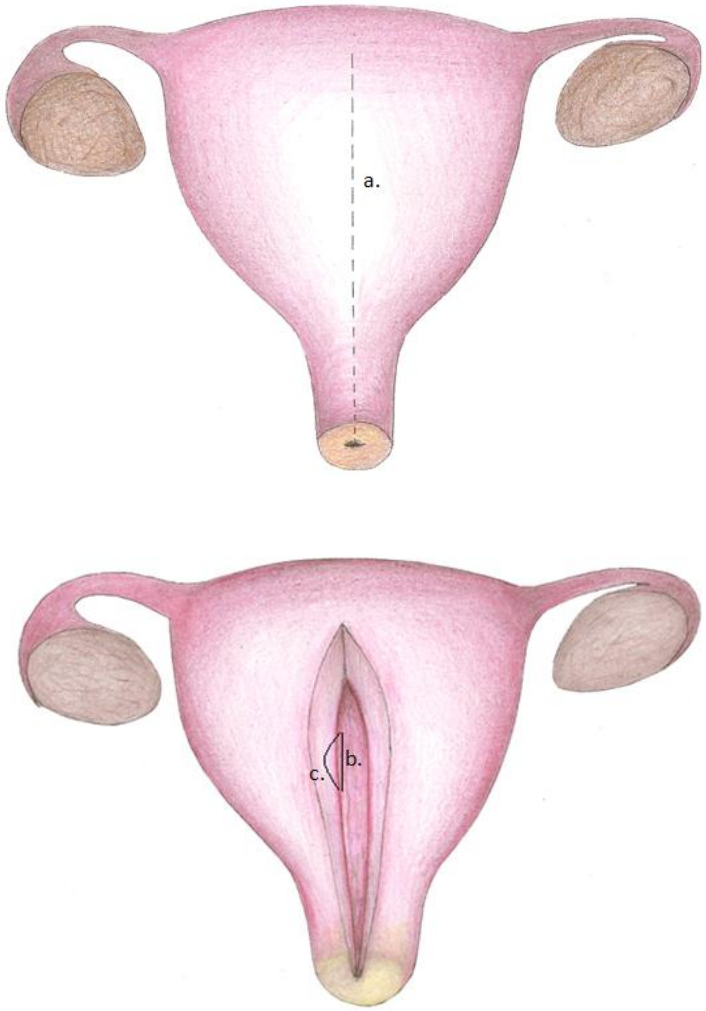


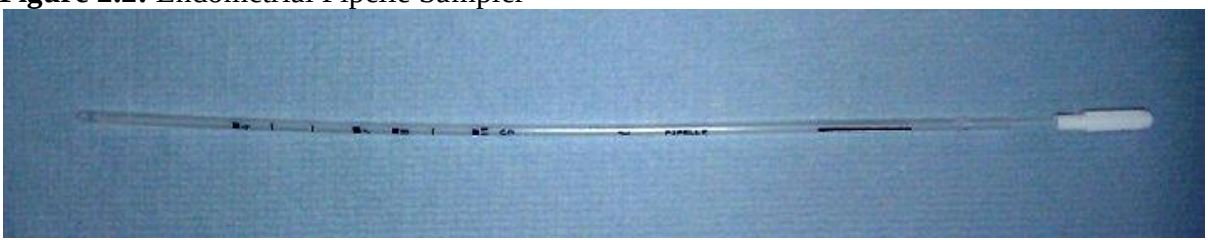
Figure 2.1. Procedure for taking a full thickness endometrial sample.

2.4.3. Endometrial Pipelle Samples

For women who did not undergo a hysterectomy and for all participants in the endometrial cancer subject group, endometrial samples were obtained using a pipelle endometrial sampler at the start of the operation. In theatre, the patient was first placed in the lithotomy position and the external genitalia, vagina and anus were washed thoroughly using sterile aqueous solution containing chlorhexidine gluconate 0.05% (Sterets Unisept, Medlock Medical Ltd, UK). Insertion of a Sims speculum into the vagina exposed the cervix, and a pair of Teales Vulsellum uterine forceps (Phoenix Surgical Instruments Ltd, UK) was used to grip onto the anterior lip of the cervix and bring it down into the vagina. A pipelle, figure 2.2, was then

passed through the cervical canal, into the uterine cavity until the fundus was reached. The length of the uterus and cervical canal was measured using the ruler on the pipelle. The plunger was then pulled back to create a suction effect and draw endometrial tissue into the pipelle. By slowly withdrawing the pipelle out of the uterine cavity, whilst being rotated from side to side, enabled as much of the endometrial tissue to be sampled.

Figure 2.2: Endometrial Pipelle Sampler



2.5 - Processing the Biopsy

To prevent tissues from drying out and necrotic degradation, all endometrial biopsies collected were immediately immersed into a universal tube containing 10% neutral buffered formalin (NBF). This is a special fixative fluid that prevents autolysis, stabilises the microanatomy of the tissue and inhibits the growth of bacteria and moulds that give rise to putrefactive changes. Fixation is the first step of tissue processing, in order to preserve the tissue cells as they naturally occur. The size dimensions of the full thickness and pipelle samples obtained in theatre is adequate to allow enough penetration of NBF through the tissue. All endometrial samples were left in NBF between 24 hours and 5 days to give optimal fixation results.

In order to make the cutting of the sections easier, tissue fixation medium was replaced with wax. This was done by placing samples in a processing machine (Shandon Citadel 1000, Cheshire, UK). This machine took tissue biopsies through a series of incubations in increasing alcohol concentrations to 100% and then xylene and hot paraffin wax, replacing the water content within the tissue. See appendix 4A “Tissue Processing” for more details. Samples were then embedded in paraffin wax blocks, see appendix 4B “Embedding Tissue Biopsies”.

2.6 - Preparation of the Slides

Processed tissues were then embedded in paraffin blocks, with an orientation that was able to give the best representation of its morphology when sliced into sections. Paraffin blocks were cut with 3-5μm thickness using Microm rotary microtome (Microm Ltds, Thame, UK) and were carefully floated onto a pre-warmed water bath, see appendix 4C "Cutting Paraffin Sections". The best sections were selected and were picked up onto aminopropyl triethoxy saline (APES) coated microscope slides, see appendix 4D "APES Coating Procedure". Slides were left positioned in an upright position overnight to help drain off the water.

2.7 - Dating the Endometrial Biopsy

Dating endometrial biopsies was achieved by ensuring consistency across a set of parameters, including date of last menstrual period (LMP) in context with regular cycles, histological dating.

To histologically date samples, haematoxylin and eosin (H&E) staining procedure was used for nuclear and cytoplasmic staining, respectively. For H&E staining of paraffin sections, slides were deparaffinised, rehydrated and then counterstained with haematoxylin. When reacted with tissue, haematoxylin is oxidised to haematein, staining cellular nuclei blue. Staining was differentiated by rapidly immersing slides in acid alcohol. This process of differentiation was arrested by returning slides to water, whereupon the haematein had taken on a blue hue. During tissue dehydration in alcohol, sections were stained with eosin yellowish (tetrabromofluorescein, disodium salt) to obtain full cellular detail, followed by washes in water to remove any excess eosin. See appendix 4E "H&E staining of biopsy tissue samples" for more details.

All H&E stained sections were assessed for tissue integrity and endometrial dating was carried out by a trained consultant gynaecologist. A gynaecological pathologist was able to provide a second independent dating assessment for all samples obtained following a

hysterectomy. For all samples dated by both consultant gynaecologist and gynaecologic pathologist, there were no discrepancies between the two independent dating assessments. Endometrial samples were dated as per the classic paper by Noyes et al in the very first issue of Fertility and Sterility in 1950 (previously discussed in chapter one).

2.8 - Immunohistochemistry (IHC)

IHC involves the application of antibodies to tissue sections to bind to specific antigens on tissue proteins, thus making it possible to visualise the distribution and localisation of specific cellular components within the tissue. The IHC reagent kit used for this study was supplied by Vector ImmPRESS. See appendix 4F "Immunohistochemistry" for full method protocol used. Before the IHC method is discussed, it is important to have a good understanding of the pivotal reagent in IHC: the antibody.

Antibodies belong to a group of proteins called immunoglobulins (Ig) comprising of five major classes: immunoglobulin G (IgG), IgA, IgM, IgD and IgE. These are present in the blood of immunised animals that remains in the serum, also known as the antiserum, after the removal of cells and fibrin. In this study, both polyclonal and monoclonal antibodies were utilised. Polyclonal antibodies are a heterogenous mixture of antibodies, with slightly different specificities and affinities, directed against various epitopes of the same region as shown in figure 2.3.

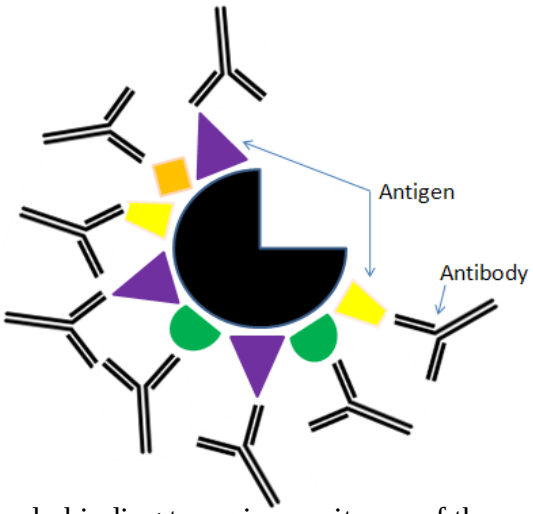


Figure 2.3. Polyclonal antibody binding to various epitopes of the protein.

Polyclonal antibodies are mainly raised in rabbits due to the ease in maintenance of the animal and relative rarity of human antibodies to rabbit proteins. In contrast monoclonal antibodies are a homogenous population of Ig directed against a single epitope that are most commonly raised in mice as shown in figure 2.4.

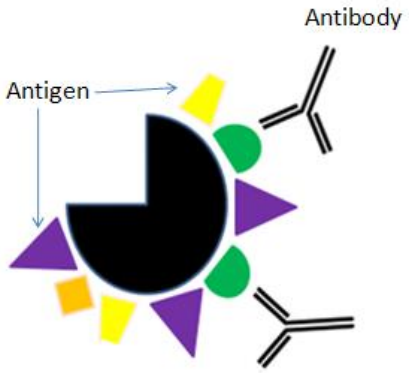


Figure 2.4: Monoclonal antibody binding to a single type of epitope.

When comparing both types of antibodies together, there are advantages and disadvantages to both types. As polyclonal antibodies can recognise multiple epitopes on a single molecule, they are not as subject to the damaging effects of sample processing as are monoclonal antibodies. However, the presence of antibodies to multiple epitopes can increase the chance of cross reactivity with other proteins.

2.9 - Vector ImmPRESS IHC Methodology

2.9.1 Dewaxing

In order for aqueous antibody solution to properly adhere to and penetrate tissue, paraffin wax must be completely removed off the tissue. Slides were first heated for 1 hour at 60˚C or overnight at 37˚C to soften the wax and remove any moisture between the section and the slide which would contribute to the section lifting off the slide. Next, the wax was then taken off the slide by immersing slides in xylene, 100% alcohol and then diminishing concentrations of alcohol until the final buffer was aqueous. See appendix 3G “Preparation of paraffin sections for staining” for more details.

2.9.2 Antigen retrieval

NBF fixation preserves tissue morphology through the formation of cross-linking bonds between tissue proteins. However, this stabilisation can also modify the antigen's epitopes and its electrostatic charges, inhibiting the epitope to react with the paratope of the antibody. Subsequently, this can give weak or false negative staining for IHC detection of certain proteins. This can be corrected by restoring the epitope using various methods of antigen retrieval, see appendix 3H "Antigen Retrieval". A series of preliminary experiments were conducted to validate the antibodies used in this study (refer to chapter 3) and to explore which antigen retrieval method would best suit each individual antibody.

Heat induced epitope retrieval (HIER) and proteolytic induced epitope retrieval (PIER) are two main methods aimed to restore the affinity and avidity of the immune reaction.

The use of heat in HIER methods causes cross-linked protein epitopes to 'unfold' while buffer solutions aid in maintaining the conformation of the unfolded protein. Citrate based buffer (pH6) was required for the use of PODXL antibody. On the other hand, Tris-EDTA buffer (pH9) is very useful for low affinity antibodies or when tissue antigens are not intense, and was used for COLIV, CK5/6 and SSEA1. For HIER methods, slides were immersed in an aluminium pressure cooker and were heated at 120˚C at full pressure for approximately one minute.

Proteinase K (Qiagen, Qiagen house, Fleming Way, Crawly, West Sussex, RH10 9NQ) was used for PIER to prepare sections for laminin staining. This enzyme, isolated from saprophytic fungus *Tritirachium album*, possesses a high specific activity that particularly unmasks antigens of proteins found in the basal lamina. All tissue sections used for laminin staining were incubated in proteinase K at a concentration of 1:25 at room temperature, for approximately 5 minutes. Following all antigen retrieval methods, all slides were incubated in TBS for 5 minutes at room temperature.

2.9.3 Blocking endogenous peroxidise activity

Sometimes tissues contain endogenous enzymes that are similar in specificity to the enzymes used in IHC detection system producing false positive signals. In particular, horseradish peroxidase (HPR) and alkaline phosphatase are the most common enzymes, found in a variety of tissues, known to generate such signals. Alkaline phosphatase is used as part of the avidin-biotin staining methods to localise antigens in tissue sections. However as the Vector ImmPRESS reagent kit is based on an HRP labelled polymer, any non specific staining resulting from endogenous avidin-biotin activity was eliminated. Like with any enzyme, enzymic activity is inhibited when there is excess substrate. Thus, slides were incubated in hydrogen peroxide solution for 10 minutes, as this excess substrate quenched the activity of endogenous peroxidase found in tissues.

2.9.4 Two step staining technique

Vector ImmPRESS IHC protocol particularly follows a two step staining technique. The first involves the application of the primary antibody to the tissue sections to bind with its corresponding antigen (figure 2.5).

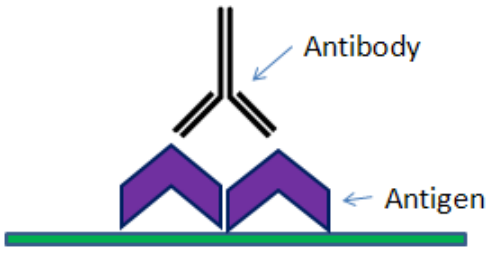


Figure 2.5. Step one of staining procedure: application of primary antibody.

The primary antibodies used and their incubation requirements are shown in the table below. Refer to appendix 5 for manufacturer antibody data sheets.

Table 2.1 - Details of antibodies used in this study with incubation requirements.

Antibody	Clone	Supplier	Concentration	Duration	Temperature
Polyclonal rabbit anti-human laminin	n/a	Dako	1:4000	Overnight	4˚C
Monoclonal mouse anti-human collagen IV	CIV 22	Dako	1:100	Overnight	4˚C
Monoclonal mouse anti-human cytokeratin 5/6	D5/16B4	Millipore	1:350	Overnight	4˚C
Monoclonal mouse anti-human PCLP1	222328	R&D	1:100	1 hour	Room temp.
Monoclonal mouse anti-human SSEA1	MC-480	Biolegend	1:800	Overnight	4˚C

When using long overnight incubation periods, antibodies were incubated at low temperature of 4˚C to prevent over-staining, since antigen antibody reactions reach equilibrium more quickly at higher temperatures. Prior to application of laminin antibody, sections were incubated in hoarse serum to prevent non specific staining.

After incubations with the primary antibody, the second step of staining technique is the application of the appropriate HRP-labelled polymer onto each section (figure 2.6).

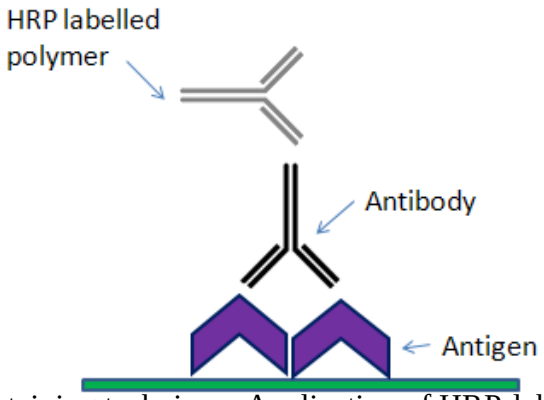


Figure 2.6. Step two of staining technique: Application of HRP-labelled polymer.

HRP contains hematin which forms a complex with hydrogen peroxide (H_2O_2) causing it to decompose. The formation of this enzyme-substrate complex is dependent on the presence of an 'electron donor' to provide the driving force in the continuing catalysis of H_2O_2. 3,3' diaminobenzidinetrahydrochloride (DAB) is a strong electron donor, and was applied to tissue sections to develop the enzyme-substrate reaction. The addition of DAB produced a brown precipitation which is highly insoluble in alcohol and other solvents. As DAB is classified as a potential carcinogen, it was handled and disposed of with appropriate care. Reaction was immediately arrested by immersing slides in water. Between incubations of primary antibody and HRP-labelled polymer and between HRP-labelled polymer and DAB development, all slides were incubated in two washes of TBS for 5 minutes at room temperature. Slides were counterstained with Gills 2 haematoxylin, and were then dehydrated in increasing concentrations of alcohol and then xylene.

2.10 - Controls

In order to test the IHC protocol used in the study, and the specificity of the primary antibodies, special controls were used. In this study, a negative control was used for each IHC staining procedure to assess the specificity of the primary antibodies used. The best form of negative control, for monoclonal antibodies, is to use the same isotope of the primary antibody in the same immunoglobulin concentration which exhibits no specific reactivity with the given tissue tested. For polyclonal antibodies, negative controls should be a dilution of IgG fractions or whole serum from the same animal source. Although ideal, these types of negative controls were not available for each antibody used in the study. Therefore a less optimal negative control was used that contained a mixture of antibodies representing most relevant IgG subtypes depending on what animal the antibody was raised in. This was applied onto sequential sections of a few samples tested in each IHC staining batch.

Whilst validating each antibody, in order to assess whether the most appropriate antigen retrieval method and incubation requirements were used correctly, positive tissue controls were used. Positive control tissues are specific samples that the antibody is known to stain positively. After antibody validation tests, an endometrial sample that was positively stained in the initial staining run was used in each successive IHC runs. This internal positive control was used to assess for any variations in staining between batches of stained sections, which could be due to factors that introduce variations in IHC method.

2.11 - Image Analysis

2.11.1 Collagen IV and Laminin

Laminin and COLIV both stained BM supporting glandular and luminal epithelium and also around vascular endothelium, see figure 2.7.

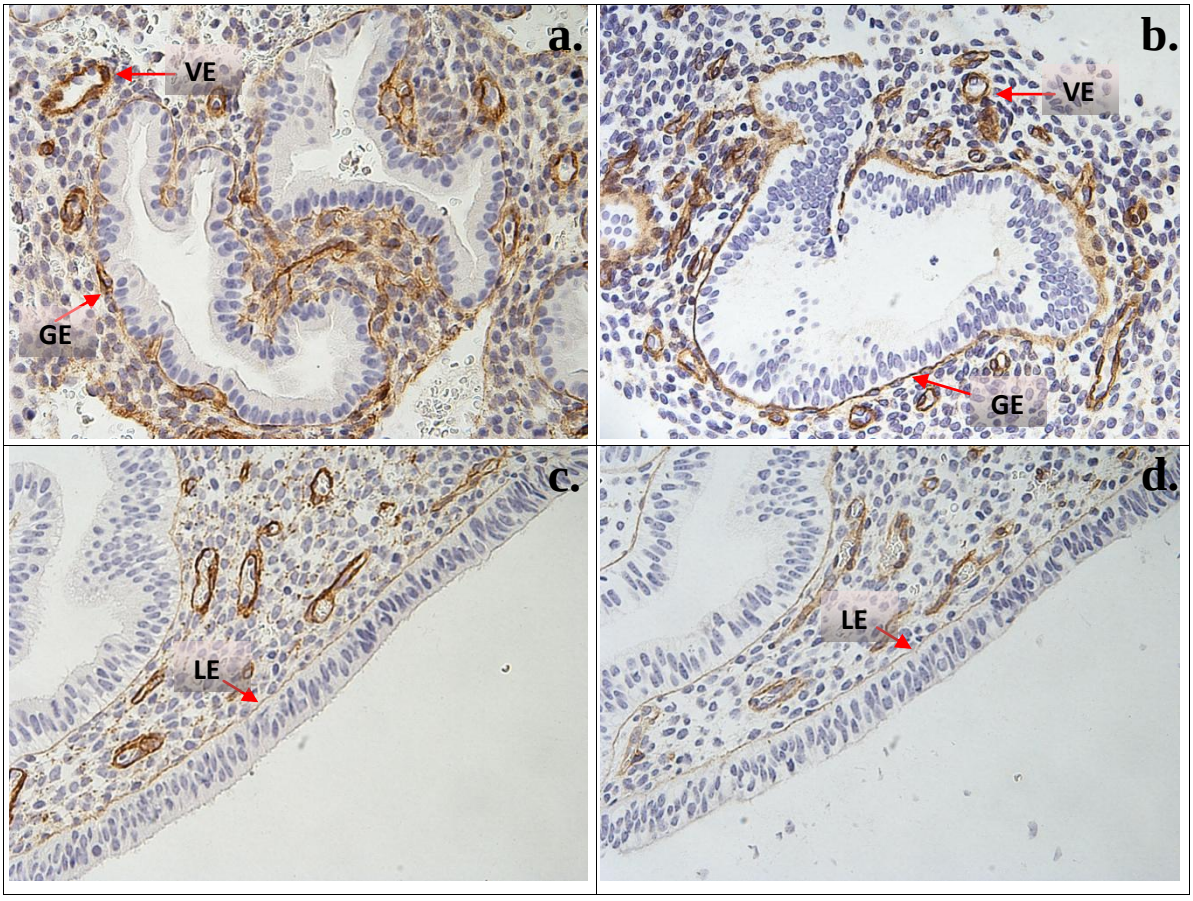


Figure 2.7. Endometrial micrographs showing COLIV (a,c) and laminin (b,d) immune-localisation in BM supporting glandular epithelium (GE) and vascular endothelium (VE) (a,b) and luminal epithelium (LE)(c,d).

To assess BM staining for both proteins, the luminal epithelium and 10 functional layer glands were photographed at X40 magnification. Functional layer glands were identified as the glands being closest to the luminal epithelium (see figure 2.11).

Using a computer assisted image analysis, BM thickness in each photograph was first measured in two places around each gland, and in two places supporting the luminal epithelium, see appendix 6 “Image Analysis” for more details. Each thickness was measured in line with the direction of the cellular polarity as shown in figure 2.8.

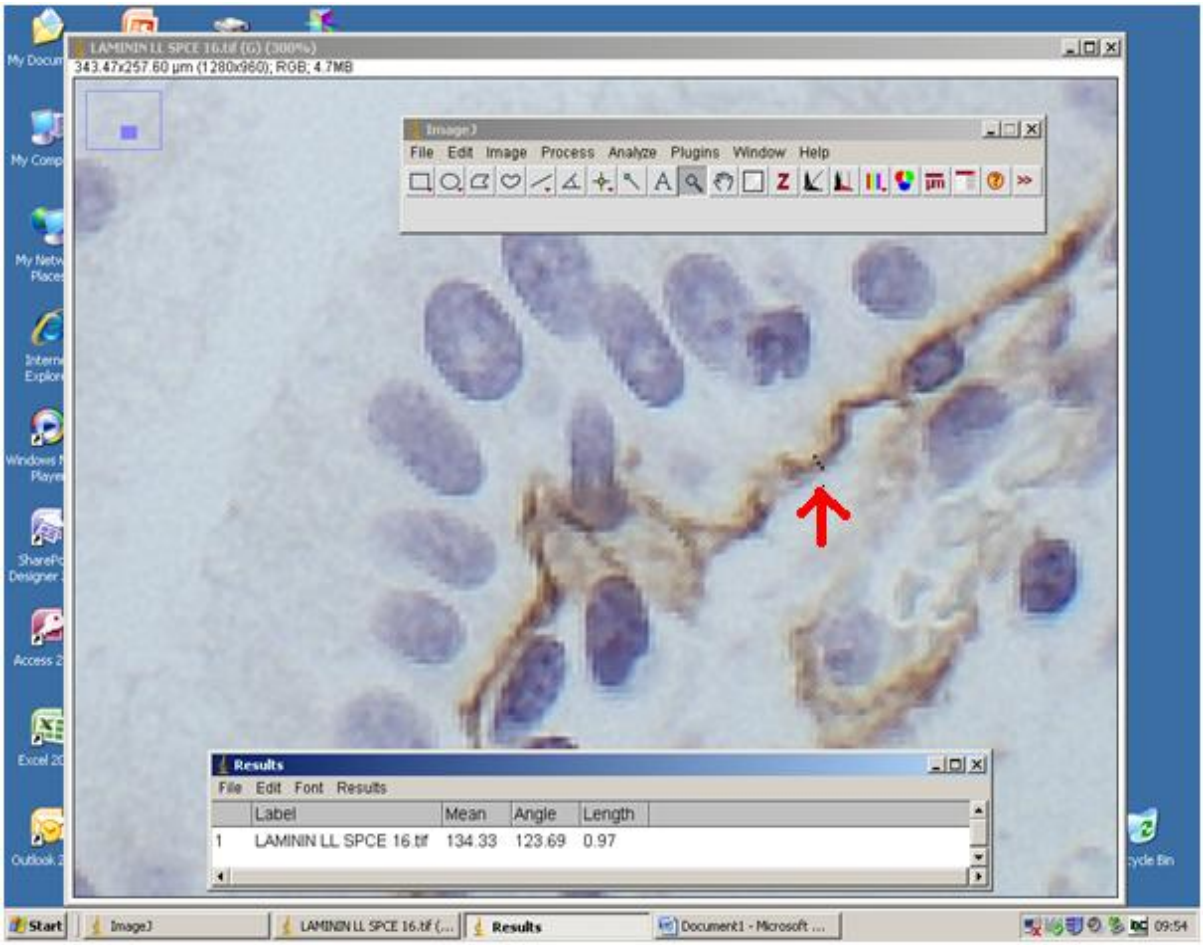


Figure 2.8. Measuring BM thickness using a computer assisted image analysis (Image J).

The clarity of stained BM is mostly not uniform around the whole gland as in some areas the basal lamina appeared ‘hazy’. There are many factors that affect the solidarity and clarity of the BM seen including the angle at which sections are cut, the tortuous spiralling of glands, especially in the late secretory phase and the integrity of the tissue. Therefore, as only two points of the BM around each gland and luminal epithelium were needed, points were chosen

where BM appeared more clearly defined. Only glands in the functionalis were assessed as BM supporting glands in the basalis was more difficult due to the surrounding densely immune-positive stroma for both COLIV and laminin. Staining intensity of BM supporting glands, luminal epithelium and vessels were scored in each photograph using a semi-quantitatively scoring system, scoring between 0-3, see appendix 7A & 7B. Due to areas of dense stromal staining in the basalis layer, BM around glands are obscure therefore only glands in the functionalis layer of the endometrium were assessed for BM thickness and staining intensity for both COLIV and laminin.

2.11.2 PODXL

PODXL mainly stained glandular secretions and the luminal surface of vessels as shown in figure 2.9.

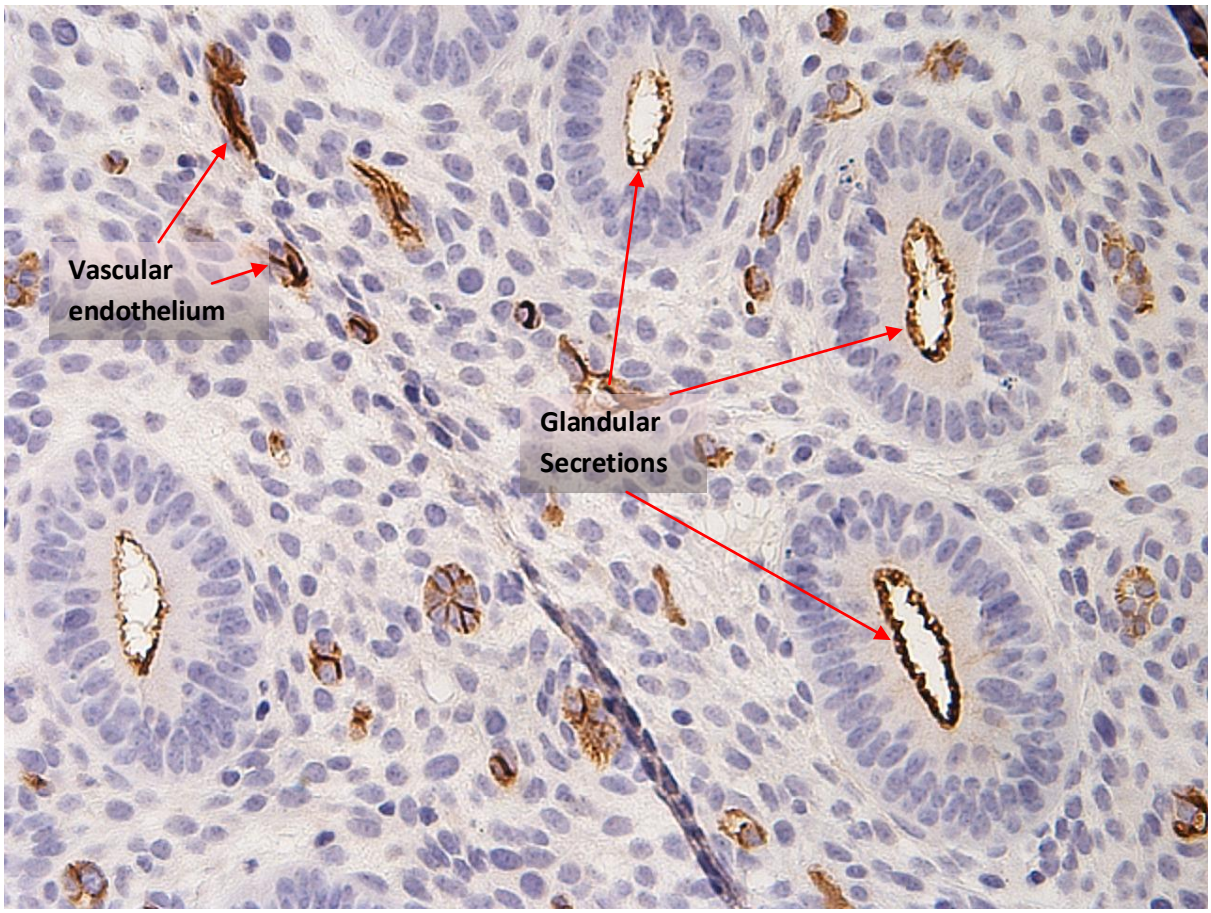


Figure 2.9. Endometrial micrograph showing positive staining for PODXL.

In each tissue section, 10 randomly located glands, from both basalis and functionalis layers, were photographed using x40 magnification. A semi-quantitative modified 'quick score'

method, originally used by Scheissl et al, was utilised to analyse the expression of PODXL in glandular secretions(138). This method takes into account the staining intensity for each immune-positive gland, scoring between 0-3 (0: negative, 1: weak, 2: moderate, and 3: strong, see appendix 7C) and the percentage of the luminal surface of the gland lined with PODXL staining (0≤ 25%: 1, 25≤ 50: 2, 50≤75%: 3, 75≤ 100%: 4). The intensity and percentage scores were then multiplied to give a range of possible scores of 0-12. Additionally, staining intensity of immune-positive PODXL in vessels was also analysed using a semi-quantitative scoring system again scoring between 0-3 (0: negative, 0.5: very weak, 1: weak, 1.5: weak-moderate, 2: moderate, 2.5: strong, and 3: very strong).

2.11.3 CK 5/6

Overall Ck5/6 is a cytoplasmic marker staining the luminal epithelium consistently and occasional glands in the tissue sections as illustrated in figure 2.10.

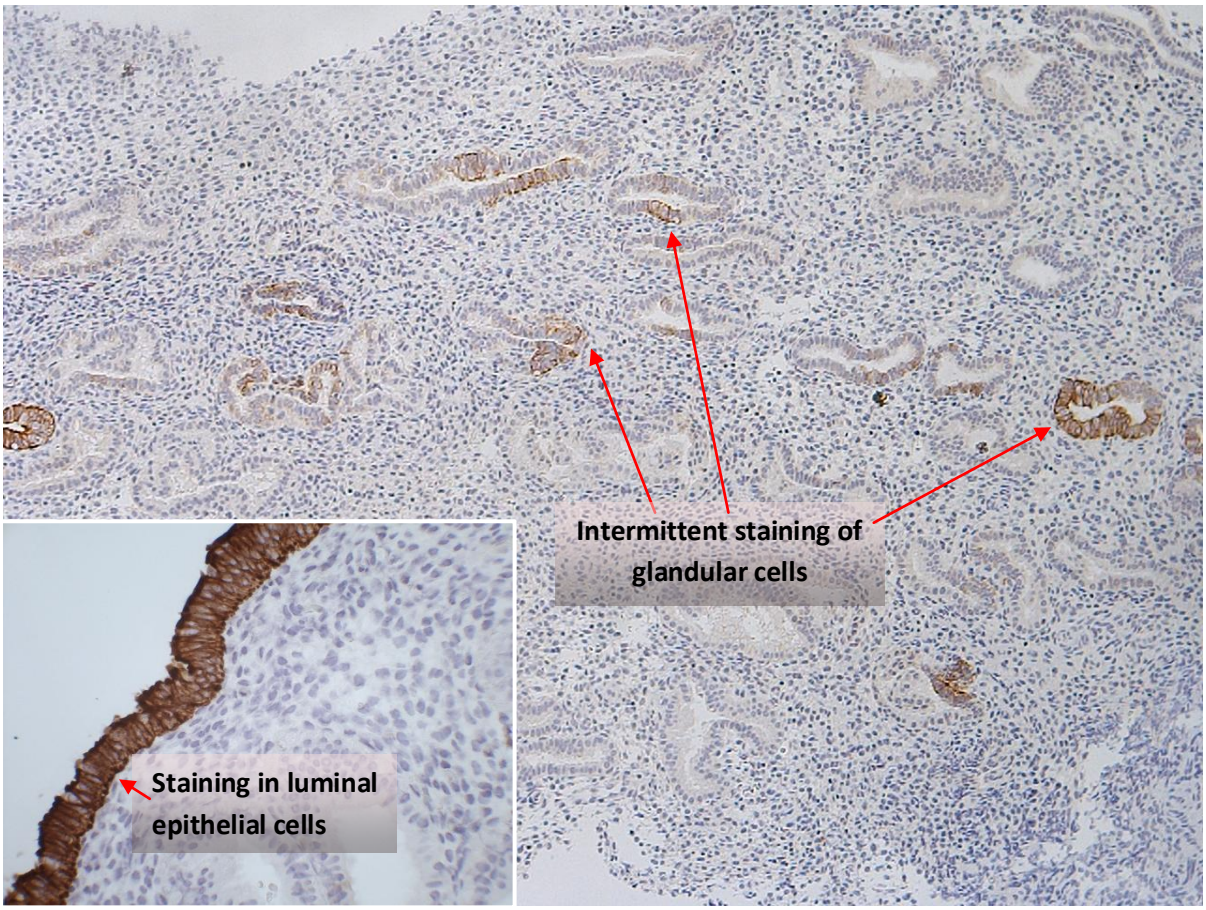


Figure 2.10. Endometrial micrograph showing staining pattern of cytokeratin 5/6 in glands and an insert micrograph showing staining in the luminal epithelium.

To score the frequency of glandular staining, a low power photograph was randomly taken at x10 magnification. The number of glands seen in the micrograph was counted along with the number of positive glands seen. The proportion of positive glands within the section was then calculated. A high power photograph, X40 magnification, of the luminal epithelium was also taken to semi-quantitatively score the staining intensity, scoring between 0-3 (see appendix 7D).

2.11.4 SSEA1

SSEA1 stained the surface of glandular and luminal epithelial cells. After reviewing all control tissue sections stained with SSEA1 it was revealed that there was a big variation in the frequency, proportion of cellular staining and staining intensity between glands in the functionalis and basalis layers of the endometrium. To explore this further 10 glands were assessed in both functionalis and basalis layers of the endometrium. Functionalis glands were located as glands being closest to the luminal epithelium. Basalis layer glands were located as glands closest to the endo-myometrial junction as shown in figure 2.11.

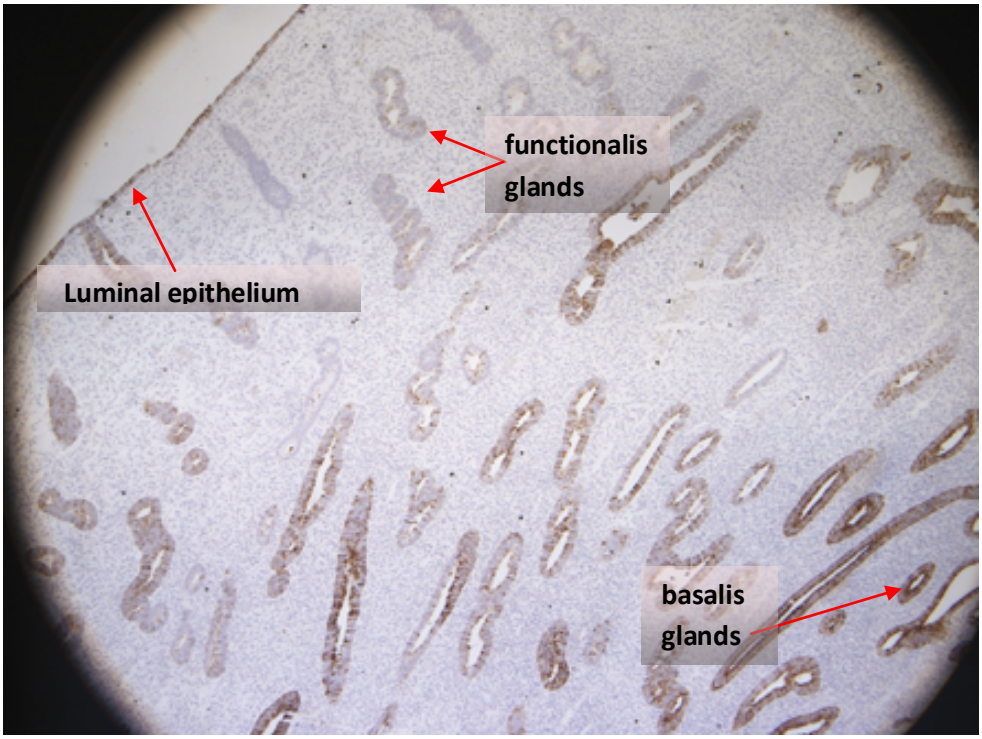


Figure 2.11. Micrograph demonstrating the SSEA1 localisation in glandular and luminal epithelium and also how functional and basalis glands were located.

These glands were assessed for (a) positivity of immune-localisation of SSEA1 in glandular cells, (b) staining intensity using a semi-quantitative scoring system, scoring between 0-3 (see appendix 7E) and (c) proportion of glandular cellular staining, scoring between 0-3 (0: no cellular staining, 1: <50% of glandular staining, 2: >50% of glandular staining, 3: 100% of glandular staining). A combined score was then given by multiplying factors a, b and c together. Additionally staining intensity of immuno-positive cells in the luminal epithelium was also assessed using the same semi-quantitative scoring.

2.11.5 – Analysis of PM endometrial samples

As discussed before in chapter 1.1.3, PM endometrium is thin with flat luminal and glandular epithelium with stroma resembling the basalis stroma of cycling women. Whilst analysing the PM samples stained with COLIV and laminin it was interesting to see that there was very little stromal staining across the width of the endometrium. This was in stark contrast with cycling endometrium since the basalis layer contained strong stromal staining, see figure 2.12, rendering analysis of BM supporting glandular epithelium in the basalis. Therefore, taking this into consideration, glands across the width of the PM endometrium could be analysed. However, for the purpose of being consistent, whilst analysing COLIV and laminin staining, only glands closest to the luminal epithelium were assessed.

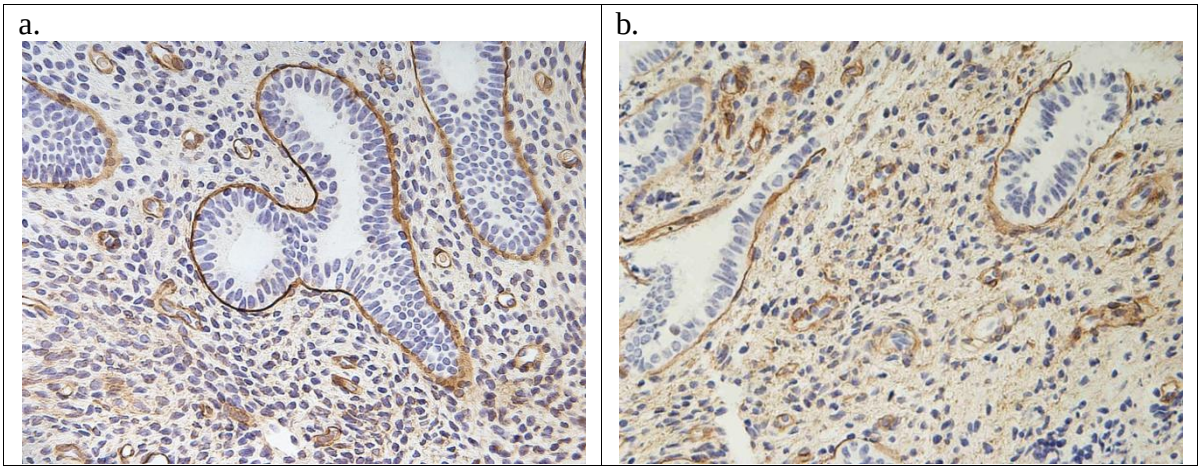


Figure 2.12. Laminin stromal staining in cycling and PM endometrium. Figure shows a comparison of stromal staining for anti-laminin between (a) PM endometrium and (b) Basalis layer of cycling endometrium.

As previously discussed SSEA1 staining analysis was divided into the basalis and functionalis layers of the endometrium. However as PM endometrium is very thin it is very difficult to define whether there are two layers of the endometrium, let alone discuss where the dividing line would be. Due to functional inactivity, PM endometrium is expected to resemble the basalis layer of endometrium of fertile controls, where the cells with regenerative capacity are thought to reside. We therefore considered that all glands assessed in PM endometrium were 'basal' glands.

For PODXL and CK5/6 analysis, random glands will be analysed in PM endometrium, in the same manner as cycling endometrium.

2.12 - Statistical Analysis

The results obtained for this study was analysed using SPSS for window version 16. As the results were unlikely to be normally distributed non-parametric tests were used to analyze the results, such as Kruskal-Wallis (KW) and Mann-Whitney (MW) tests. After a positive ($p<0.05$) KW test, a MW post hoc test was performed to see where the differences lie. Two way analysis of variance (ANOVA), including interactions were used to explore for differences seen between the subject groups. Values of $P<0.05$ were considered significant. Additionally, Pearson's correlation test was also used to assess for any significant relationships between the expression of BM components and stem cell markers.

Chapter Three:

OPTIMISATION OF EXPERIMENT CONDITIONS

In IHC, methods of antigen retrieval and antibody dilutions as well as incubation time and temperature are tightly interwoven in their effect on staining quality. To ensure all antibodies were used at optimal conditions, a number of preliminary experiments were conducted to test each of these factors.

3.1 Optimisation of Laminin Antibody

The type of anti-laminin used, supplied by Dako, is a pan-laminin antibody reacting with all subtypes of laminin. Initially anti-laminin was trialled at a concentration of 1:50, on tissues pre-treated with HIER using citric acid buffer solution, for overnight incubation at 4˚C. Tonsil tissue was used as a known positive control. The staining appeared very strong in all samples tested and the basal lamina could not be accurately recognised illustrated in figure 3.1.

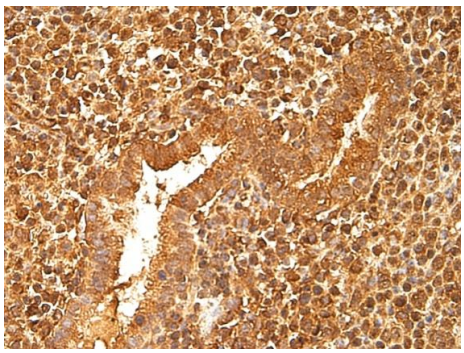


Figure 3.1: Micrograph showing expression of laminin, with antibody used at 1:50, O/N

Consequently, further dilutions of laminin antibody were tested, using concentrations of 1:100, 1:200 and 1:400 (see figure 3.2). Dilutions of the primary antibody, with incubations over long durations allowed for greater economy. Nonetheless, even at the most dilute

concentration, laminin staining still appeared strong and non specific with ill defined BM in all specimens tested. It was noted that the negative control also stained positively indicating either a lack of specificity of the antibody or non-specific background staining.

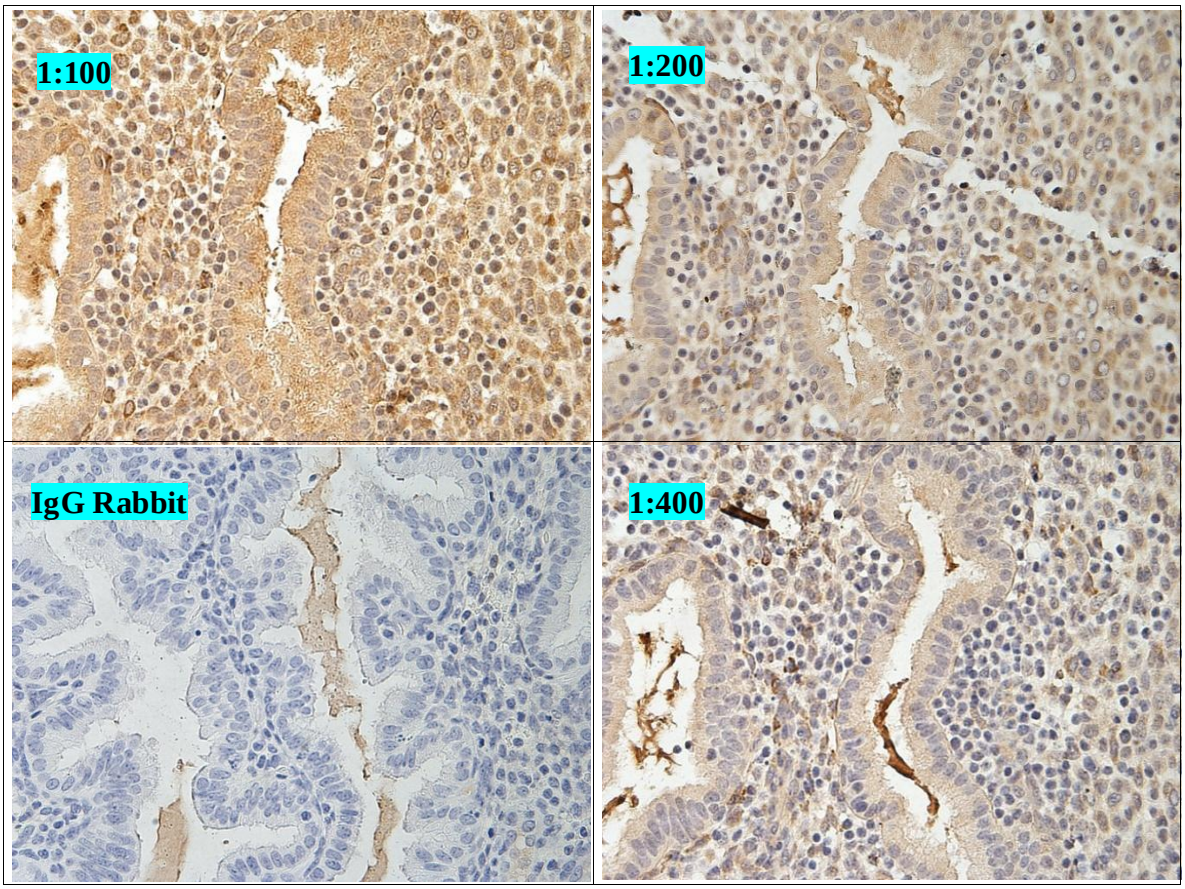
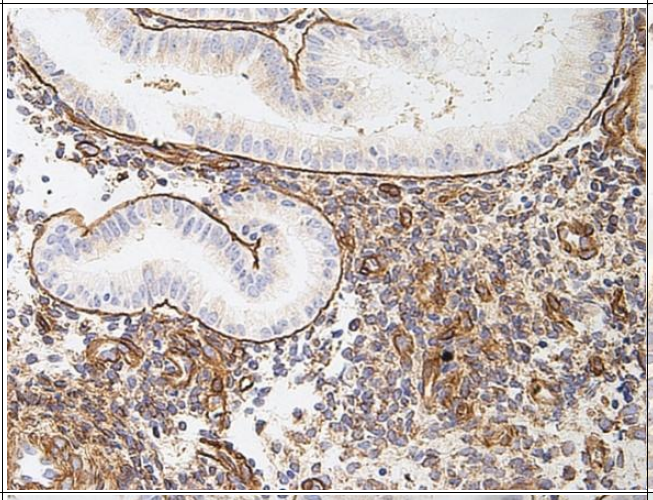
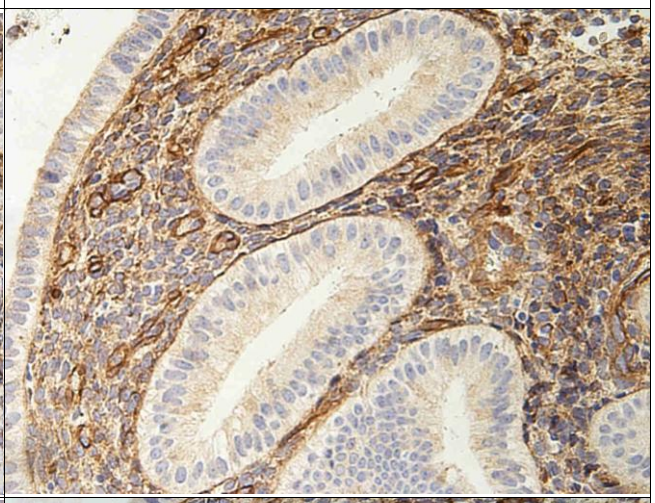
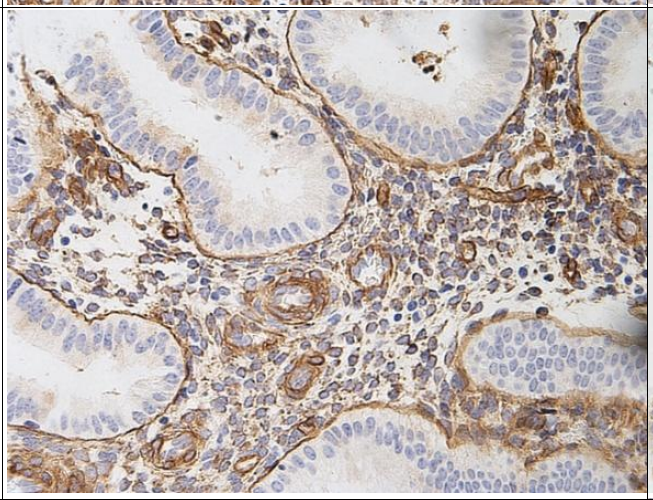
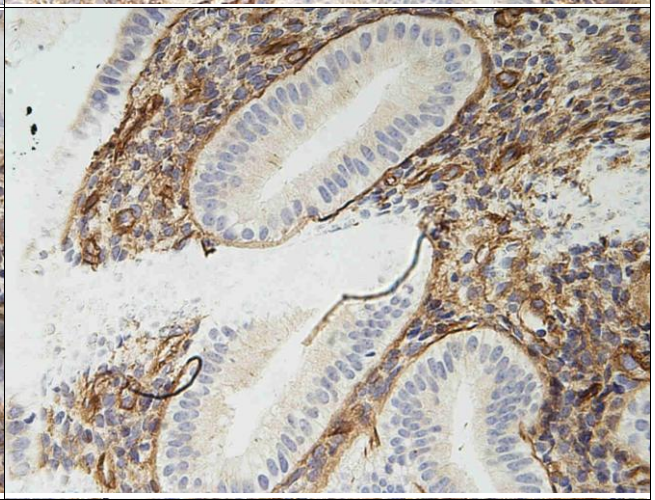
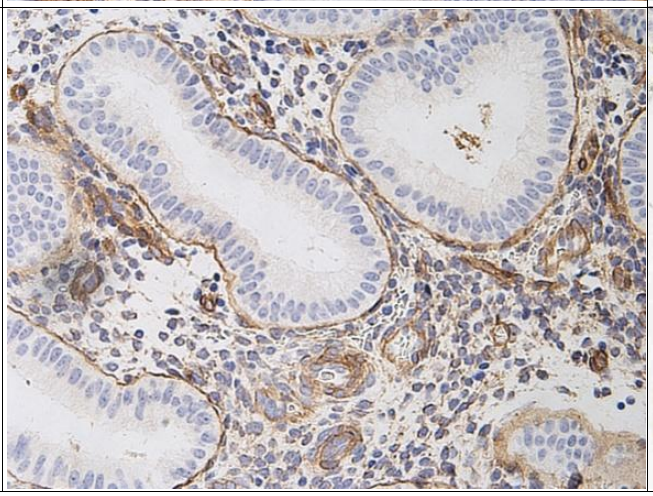
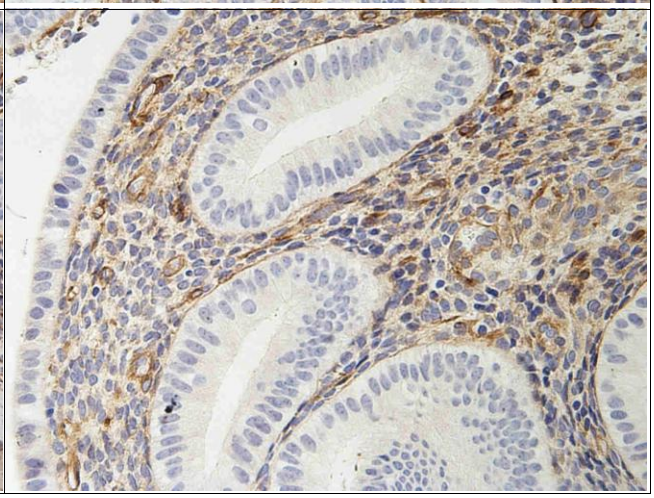


Figure 3.2: Micrographs showing expression of laminin in samples stained with antibody diluted at 1:100, 1:200 and 1:400 and IgG rabbit negative control.

After reviewing the available literature on the use of laminin antibody, we chose the most popular method for antigen retrieval, proteolytic digestion. Therefore, tissue sections were incubated with proteinase K at a concentration of 1:10 for 5 minutes at room temperature. Additionally, horse serum was applied to all sections before the antibody was applied. Horse serum contains antibodies that have been raised against rabbit proteins, thus acts as a protein blocker to minimise non-specific staining. This protein blocker achieves this by competing for the non-specific protein binding sites on the specimen.

Laminin antibody was then tested at 1:500, 1:1000, 1:2000 and 1:4000 for overnight incubation at 4˚C (figure 3.3). All sections were analysed and these preliminary experiments

revealed laminin at a concentration of 1:4000, together with pre-proteolytic digestion and horse serum blocking provided very specific and distinct staining.

	Specimen 1	**Specimen 2**
1:500 laminin dilution		
1:1000 laminin dilution		
1:2000 laminin dilution		

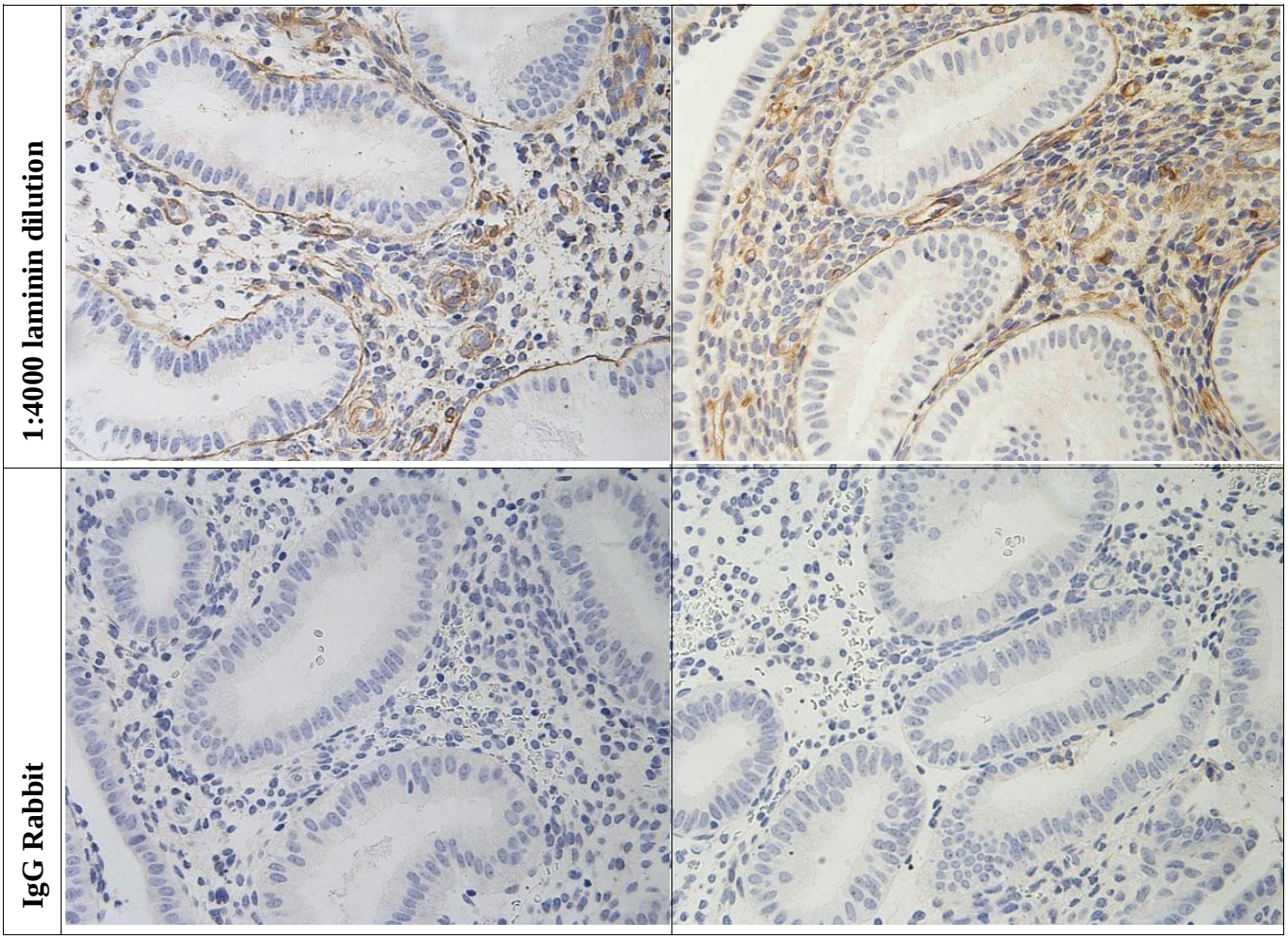


Figure 3.3: Micrographs showing laminin expression in two specimens with antibody concentration used at 1:500, 1:1000, 1:2000 and 1:4000 with their negative controls.

Taking into consideration the fragility brought upon the tissues due to the vigorous nature of the enzyme pre-treatment, proteinase K trialled at more dilute concentrations. As staining around BM was less strong at 1:50, it was decided that 1:25 was an acceptable concentration (see figure 3.4).

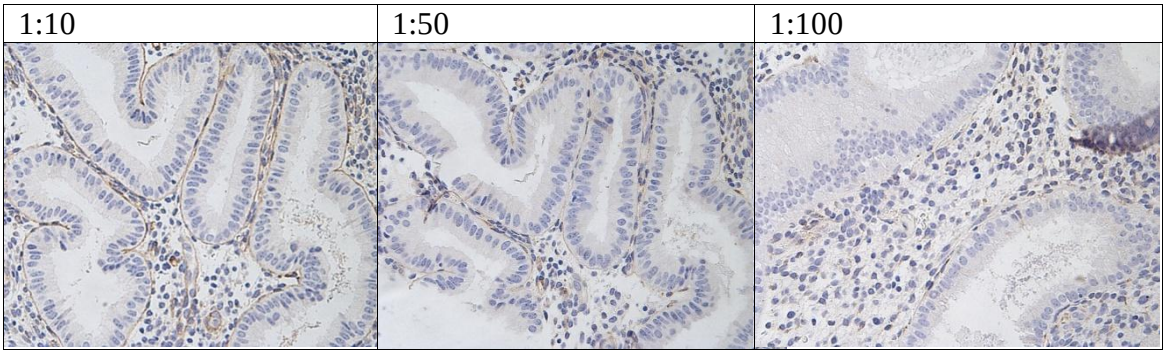


Figure 3.4: Micrographs demonstrating the effects of proteinase K at different concentrations

3. 2 Optimisation of Collagen IV Antibody

COLIV antibody was initially trailed at a concentration of 1:100 on tissues pre-treated with HIER using citric acid buffer solution, for overnight incubation at 4˚C. Placental tissues were used as known positive controls for these preliminary experiments. In all specimens tested, BM supporting glandular epithelium appeared very faint, whereas strong immune-reactivity was occasionally seen in BM supporting vascular endothelium. Thereafter, antibody concentrations at 1:50, 1:100 and 1:200 were considered for overnight incubation at 4˚C, and also for 1 hour at room temperature. As staining appeared very faint in samples stained during both incubation periods, new antigen retrieval methods were explored. A thorough literature review was conducted to investigate how this antibody was used in previous IHC studies. Nonetheless, there was no general consensus on how the antibody was prepared and what treatment conditions were required. Therefore, two additional methods of antigen retrieval were investigated in the same experiment; HIER Tris EDTA and PIER proteinase K. COLIV was tested at the three concentrations previously tested, for overnight incubation at 4˚C. After a comprehensive analysis of all stained sections it was agreed that COLIV at 1:100 using TRIS EDTA for antigen retrieval produced the best staining results (see figure 3.5).

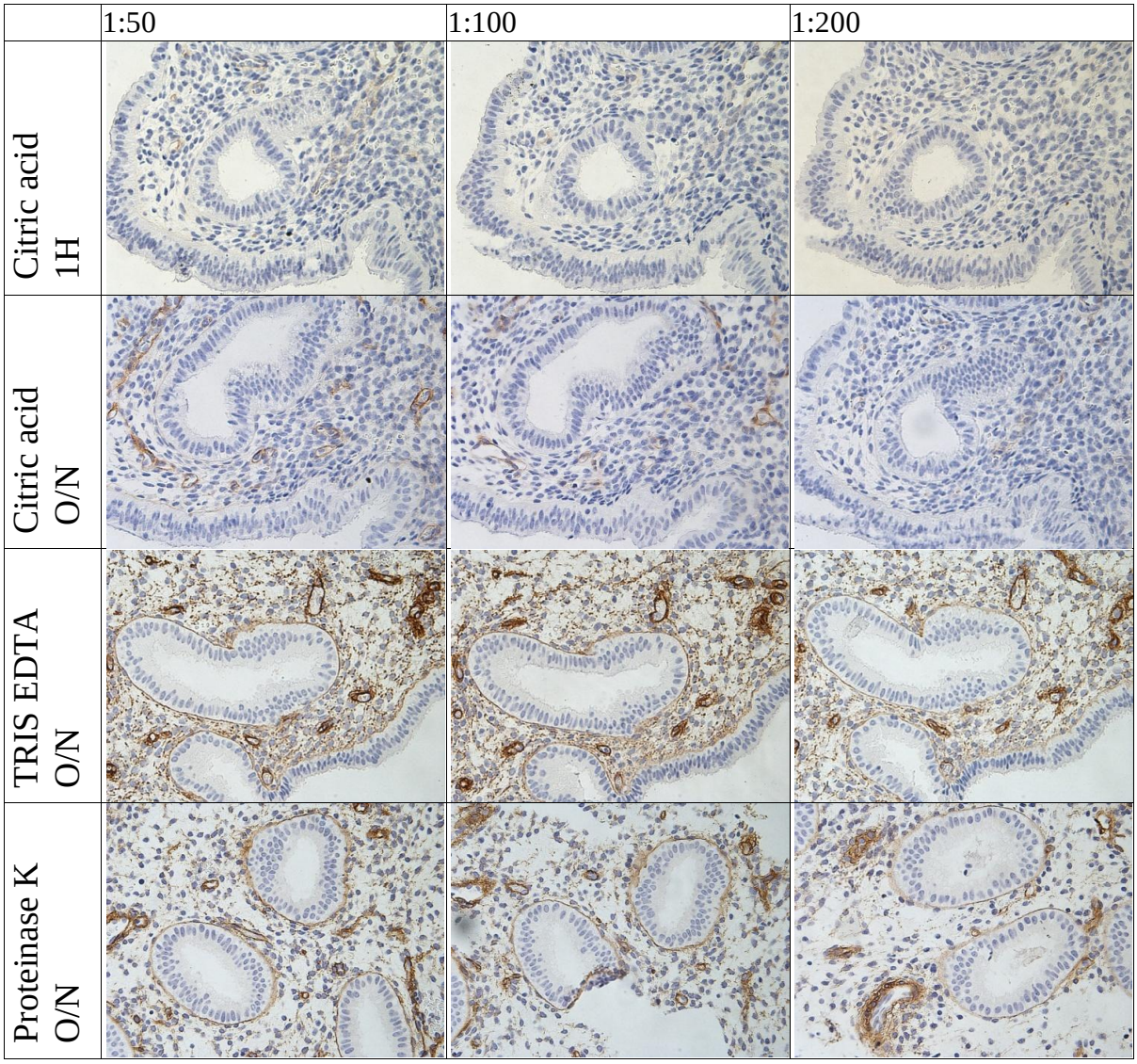


Figure 3.5: Endometrial micrographs demonstrating differential COLIV expression when using different antigen retrieval methods and different concentrations of COLIV

3.3 Optimisation of PODXL Antibody

The supplier of PODXL antibody used in this study recommended a dilution between 1:8 and 1:25. Initially, 1:25 concentration of PODXL was tested, with an incubation period of 1 hour at room temperature, on endometrial sections pre-treated with HIET using standard citric acid. In stained sections very strong staining was seen in glandular secretions, vessels and stroma with some evidence of background staining. Therefore further dilutions were

trialled, including 1:25, 1:50, 1:100 and 1:200 concentrations with an incubation period of 1 hour at room temperature, see figure 3.6. Endometrial tissue sections used in this titration were pre-treated with citric acid using a HIER method.

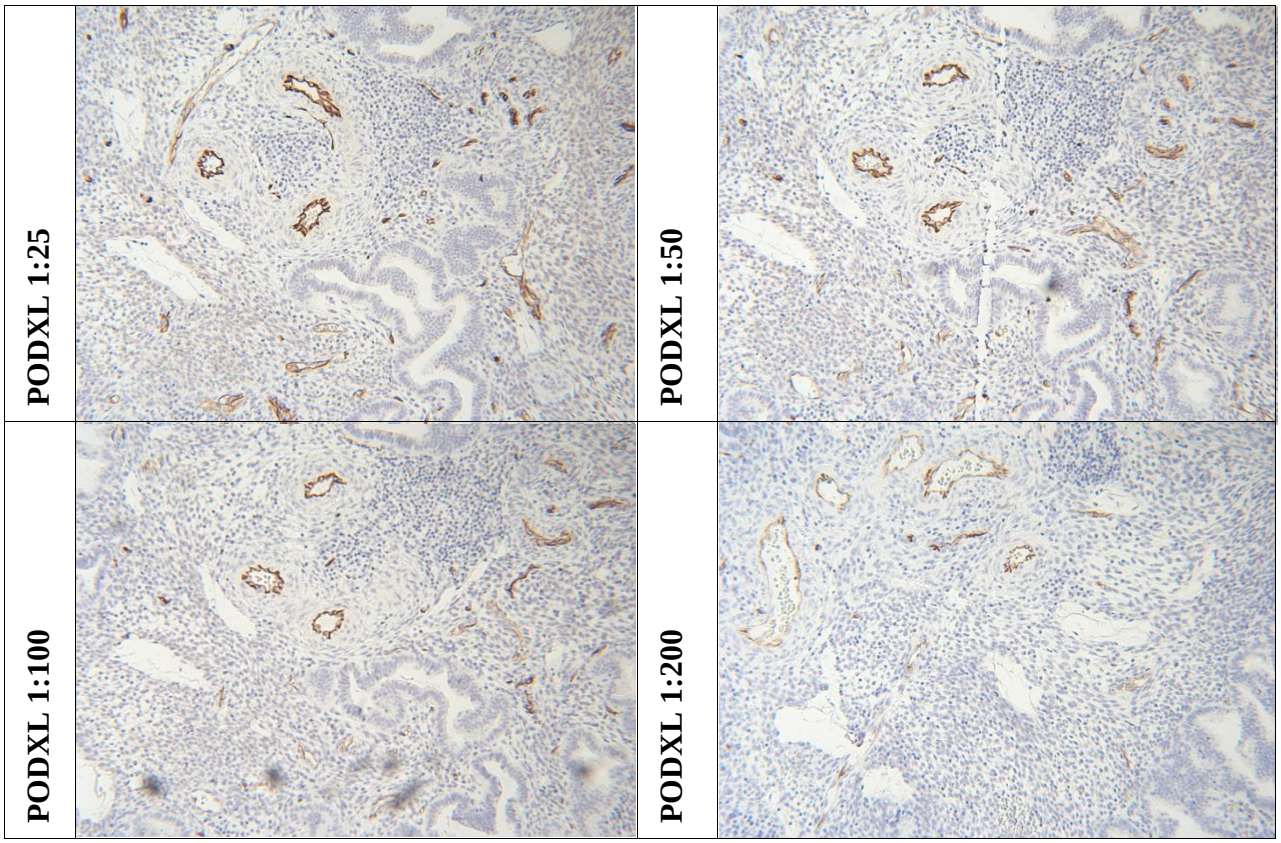


Figure 3.6: Endometrial micrographs to demonstrate variation in staining intensity of PODXL at 1:25, 1:50, 1:100 and 1:200 concentrations.

PODXL antibody dilutions used at 1:25, 1:50 and 1:100 produced very similar staining intensities on tissue sections. However, a marked reduction in the staining intensity was seen at 1:200. Resultantly, it was concluded that 1:100 was the best economical concentration as it produced the same results with the recommended concentration from the manufacturer and is more dilute.

3.4 Optimisation of SSEA1 Antibody

SSEA1 antibody obtained from R&D systems was also tested using a number of antigen retrieval methods and antibody dilutions. Initially a 1:25 dilution was used on a number of tissue sections that were pre- treated with either HIER methods (TRIS EDTA and citric acid) or proteolytic digestion using proteinase K. SSEA1 antibody was incubated on all tissue sections overnight at 4℃. As SSEA1 antibody has previously been reported to be expressed in the liver, sections of liver tissue samples were used a positive controls.

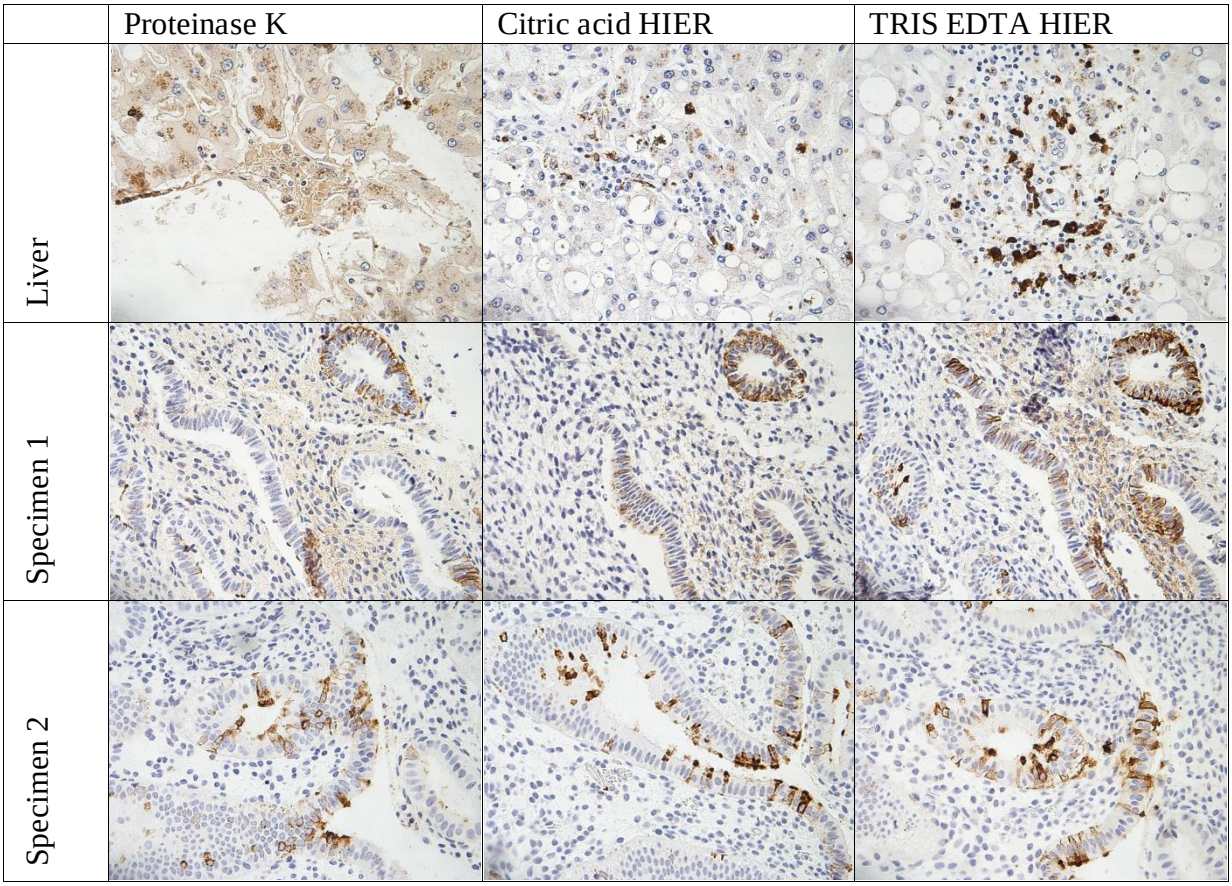
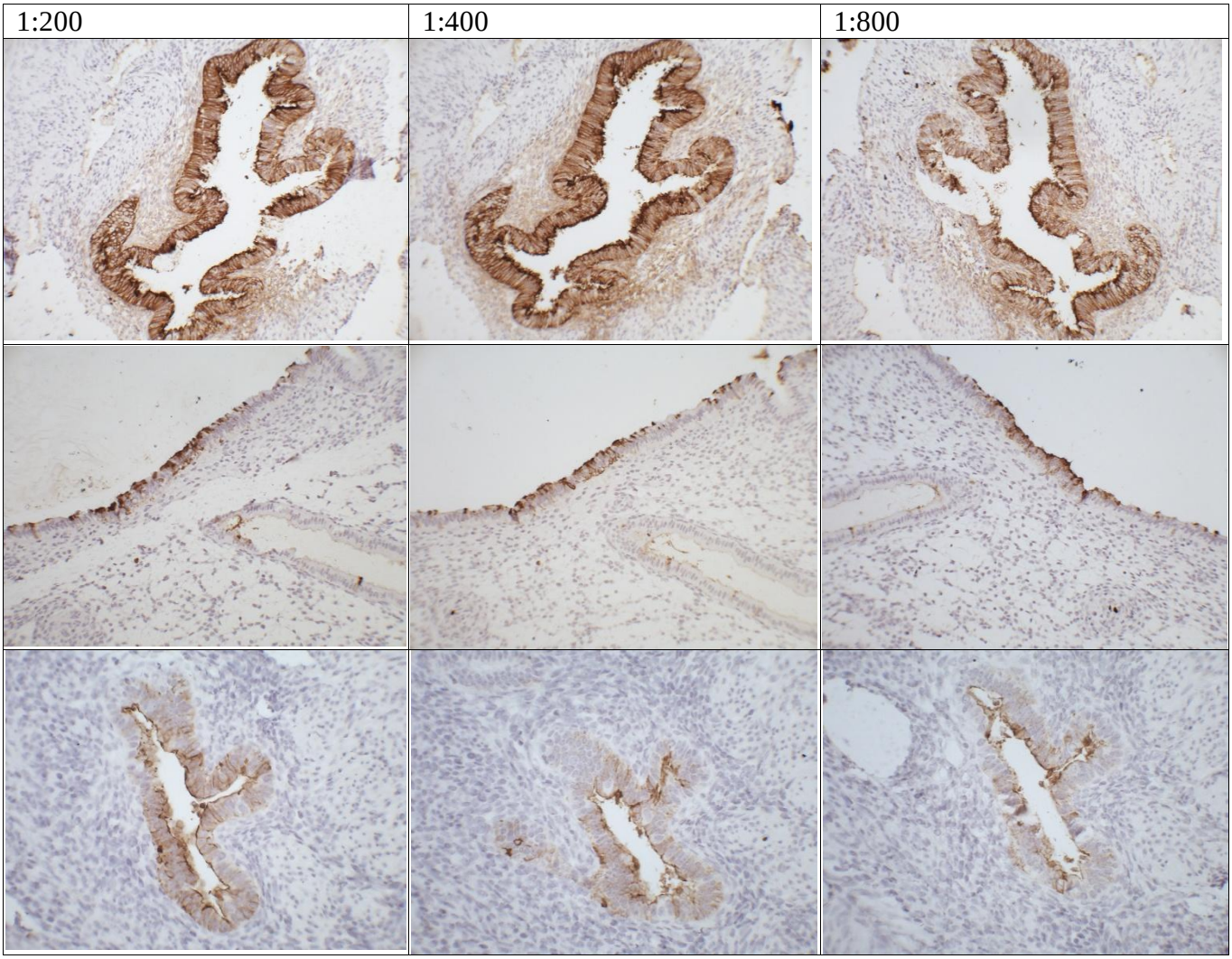


Figure 3.7: Endometrial micrographs to demonstrate differential SSEA1 staining when different antigen retrieval methods are used.

From analysing the staining results shown in figure 3.7 it is clear that TRIS EDTA produced the most clear and strongest SSEA1 staining in all three tissue specimens tested. Titration of the antibody concentration was later performed using SSEA1 antibody purchased from Biolegend. Equivalent concentration of Biolegend SSEA1 antibody to R&D systems SSEA1

antibody was 1:200. Thus further dilutions of Biolegend SSEA1 antibody was tested including 1:200, 1:400 and 1:800, figure 3.8.

Figure 3.8: Endometrial micrographs to show staining in luminal epithelium and strong and weak staining in glandular epithelium at 1:200, 1:400 and 1:800 SSEA1 concentrations



After testing the new SSEA1 antibody at more dilute concentrations, results showed that 1:800 produced very similar results when compared to the most concentrated dilution tested. In conclusion it was decided that 1:800 was used for the rest of the study.

3.5 Optimisation of Cytokeratin 5/6

CK5/6 was tested at a recommended concentration of 1:350, under two different types of HIER methods (citric acid and TRIS EDTA) and two different incubation conditions (overnight at 4ºC and for three hours at room temperature). After comprehensive analysis of all stained sections, it was confirmed that CK5/6 antibody optimally worked when tissue sections are pre-treated with HIER TRIS EDTA and when the antibody is applied for overnight incubation at 4ºC.

Chapter Four:
RESULTS - Fertile Control & PM Endometrial Groups

4.1 – Patient Demographics

Table 4.1: Demographic data of fertile and post-menopausal control subjects

	Age(years)	BMI	Weight (kg)	Height (m)	Parity
Proliferative Phase					
Median	40	27.2	73	1.64	2
Range	37-40	23.1-38.1	63.7-100	1.59-1.69	1-7
Mid-Secretory Phase					
Median	34	25.55	69.5	1.61	2-3
Range	27-44	21.7-30.4	57-83	1.52-1.72	1-4
Late-Secretory Phase					
Median	39	24.3	62	1.65	3
Range	27-45	21.1-41.5	53-113	1.49-1.7	1-4
Post-Menopausal Controls					
Median	71	26.6	61	1.57	3-4
Range	55-77	24.1-31.2	56-76	1.45-1.59	2-5

Table 4.2: Demographic data of subjects in the endometriosis group

	Age(years)	BMI	Weight (kg)	Height (m)	Parity	Disease Stage
Proliferative Phase						
Median	33	29.2	75	1.61	1	1-2
Range	21-49	20.3-37.5	52-110	1.53-1.72	0-3	1-3
Mid-Secretory Phase						
Median	38	24.25	70.5	1.7	1	2-3
Range	21-47	20.5-32.9	58-95	1.63-1.83	0-2	1-4
Late-Secretory Phase						
Median	33	25	72	1.69	0.5	1-2
Range	18-43	20-28.7	56-84	1.59-1.76	0-3	1-2

Table 4.3: Demographic data of subjects in the endometrial cancer group

	Age(years)	BMI	Weight (kg)	Height (m)	Parity
Grade I					
Median	69.5	35.1	89.5	1.62	6
Range	63-83	23.7-45.2	60-115	1.59-1.64	n/a
Grade II					
Median	63	29.4	81	1.62	2-3
Range	50-67	27.9-37.9	69-90	1.54-1.67	2-4
Grade III					
Median	69	29.05	123.5	1.68	4
Range	67-71	26.7-31.4	91-156	1.65-1.7	2-6

Comparing the demographic data between the subject groups revealed a few statistically significant differences. Firstly, the PM group contained women who were significantly shorter (MW test; $p=0.05$) and older (MW test; $p<0.0001$) than the fertile control group. Additionally, women in the endometriosis group were significantly taller (MW test; $p=0.19$) and had a significantly lower number of live births than women in the control group (MW test; $p<0.0001$). Lastly women in the endometrial cancer subject group were significantly heavier (MW test; $p<0.013$) and taller (MW test; $p<0.038$) than women in the PM group, although no statistical significant difference was found between the two groups when comparing BMI. No significant differences were found within the fertile and endometriosis groups across the cycle. Demographic data values are missing for 10 recruited patients which are currently being tracked.

4.2 – Expression of Basal Lamina Components in the Normal Cycling and PM Endometrium

For all results collected from the fertile control and PM groups, please refer to appendix 8A to 8J.

Endometrial BM was visualised as narrow continuous bands supporting the luminal and glandular epithelium and vascular endothelium. Immuno-localisation of COLIV and laminin in BM supporting endometrial samples were analysed for thickness and staining intensities and were evaluated according to the phase of the menstrual cycle.

Collagen IV

Table 4.4. COLIV staining results in the fertile control and post-menopausal group.

	GE BM Thickness (µm)	GE BM Staining Int.	LE BM Thickness (µm)	LE BM Staining Int.	VE BM staining int.
ProlP					
Median	0.851	1.050	0.833	1.500	2.050
Range	0.637 - 0.996	0.700 - 2.000	0.705 - 1.125	0.500 - 1.500	1.450 - 2.550
MSP					
Median	0.711	1	0.89	1	2.2
Range	0.437 - 0.861	0.350 - 1.700	0.000 - 1.345	0.000 - 1.500	0.250 - 2.750
LSP					
Median	0.789	1.625	0.808	1.25	2.2
Range	0.684 - 0.901	0.950 - 2.050	0.000 - 0.985	0.000 - 2.000	1.550 - 2.350
PM					
Median	0.69	1.25	0.67	1	0.9
Range	0.132-3.553	0.150-1.650	0.000-1.175	0.000-1.500	0.100-1.150

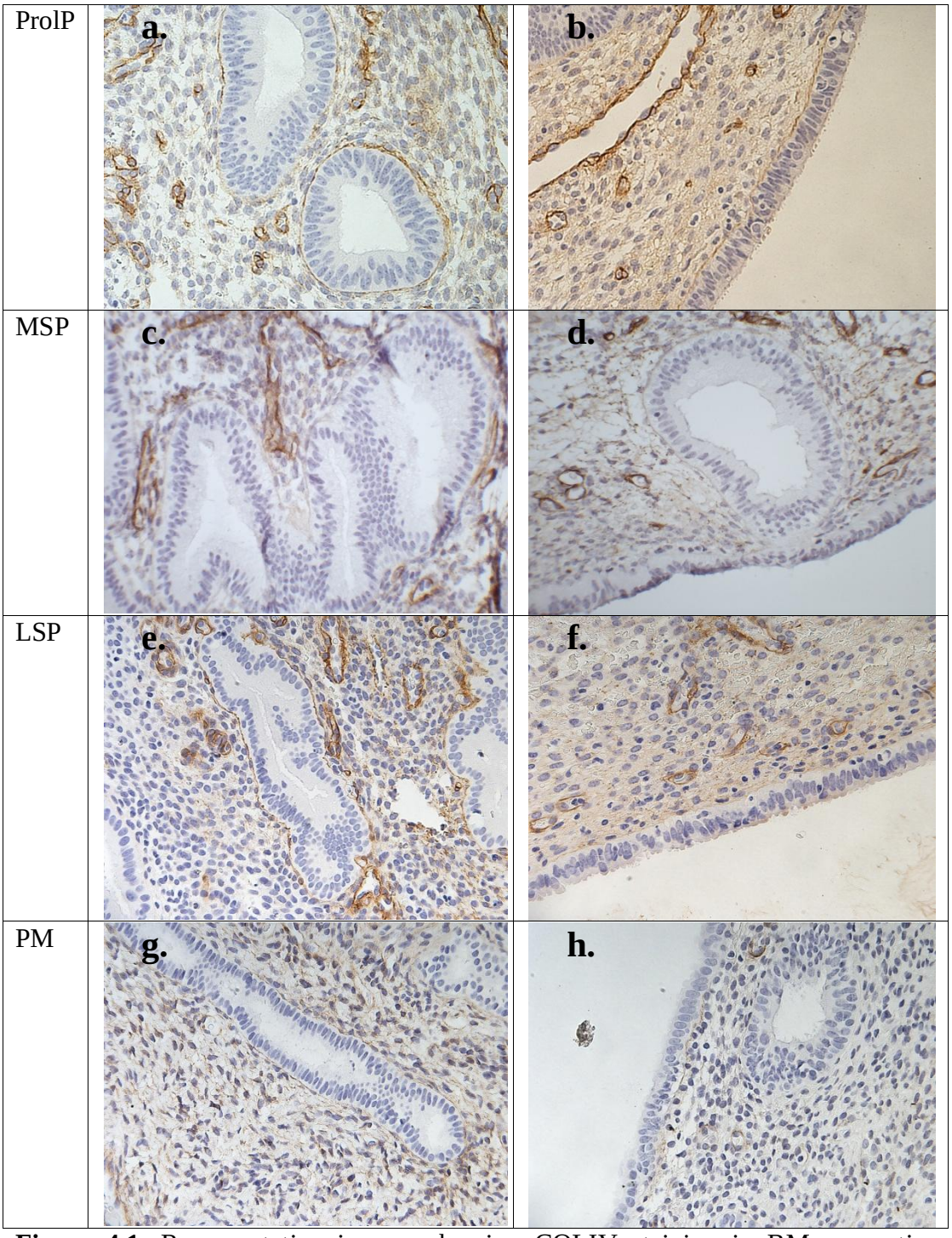


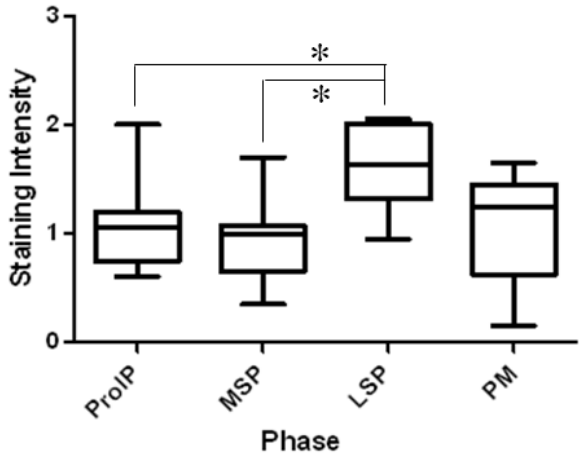
Figure 4.1. Representative images showing COLIV staining in BM supporting the GE (a,c,e,g) and LE (b,d,f,h) in fertile control (ProlP,MSP,LSP) and PM endometrium.

COLIV in BM supporting endometrial glands showed an apparent difference in *thicknesses* across the cycle, with a trend towards average BM thickness being lower in the MSP. However, this difference did not reach statistical significance (KW test; p=0.09). In contrast, the *staining intensities* of COLIV in endometrial BM showed significant differences across the cycle (refer figure 4.1). Firstly, COLIV staining intensities in BM underlying *glandular epithelium* was increased in the LSP than in other phases of the menstrual cycle (KW test;

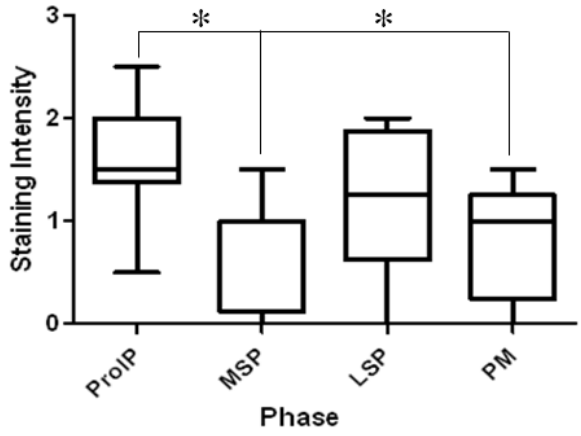
p=0.003, MW test; ProlP p=0.004, MSP p=0.089). Secondly, COLIV staining intensity in BM supporting *luminal epithelium* in the MSP was significantly lower when compared to the ProlP (KW test; p=0.032, MW test; p=0.009).

COLIV staining in PM endometrium showed differences when compared to the fertile control group. Firstly, PM endometrium showed significantly lower COLIV staining intensity in BM supporting the *luminal epithelium* when compared to the ProlP (MW test; p=0.04). Secondly, staining intensity in BM supporting the *vascular endothelium* was significantly lower in the PM group when compared with all phases of menstrual cycle studied (MW test; ProlP p=0.001, MSP p=0.005 and LSP p=0.006), refer fig 4.2.

a. Control: COLIV BM GE Staining Intensity



b. Control: COLIV BM LE Staining Intensity



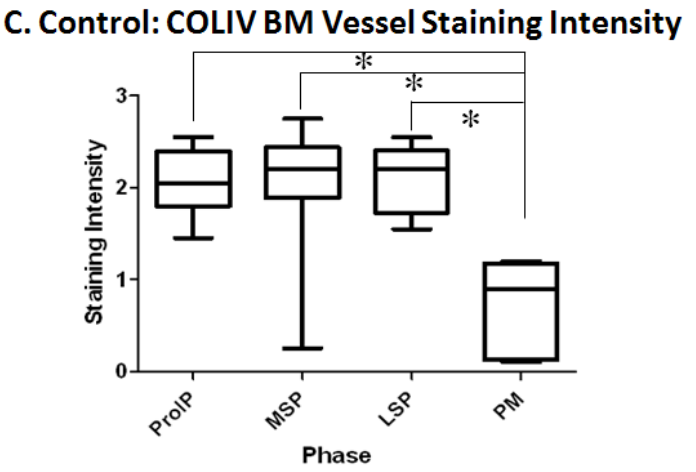


Figure 4.2 Box and whisker plots demonstrating changes seen in COLIV staining intensity in endometrial BM supporting epithelial layers. All asterisks show a statistically significant result. a. COLIV staining intensity shown to be increased in BM supporting GE during the LSP. No significant difference found between cycling endometrium and PM endometrium. b. COLIV staining intensity supporting the LE is significantly decreased in the MSP and PM endometrium when both are compared with the ProlP. C. COLIV staining intensity in BM supporting VE in PM endometrial samples is significantly decreased when compared to cycling endometrium.

Laminin

Table 4.5. Laminin staining results in the fertile control and post-menopausal group.

	GE BM Thickness (µm)	GE BM Staining Int.	LE BM Thickness (µm)	LE BM Staining Int.	VE BM staining int.
ProlP					
Median	0.962	1.400	0.833	1.000	1.625
Range	0.570-1.332	0.400-2.550	0.000-1.140	0.000-2.000	0.650-2.200
MSP					
Median	0.690	0.825	0.000	0.000	1.225
Range	0.000-1.016	0.000-1.800	0.000-1.005	0.000-1.500	0.200-2.400
LSP					
Median	0.912	1.475	0.810	0.500	1.600
Range	0.491-1.244	0.250-3.500	0.000-1.170	0.000-2.000	0.500-2.250
PM					
Median	0.891	1.400	0.655	1.000	1.500
Range	0.196-1.045	0.200-2.350	0.000-1.060	0.000-2.000	0.050-2.200

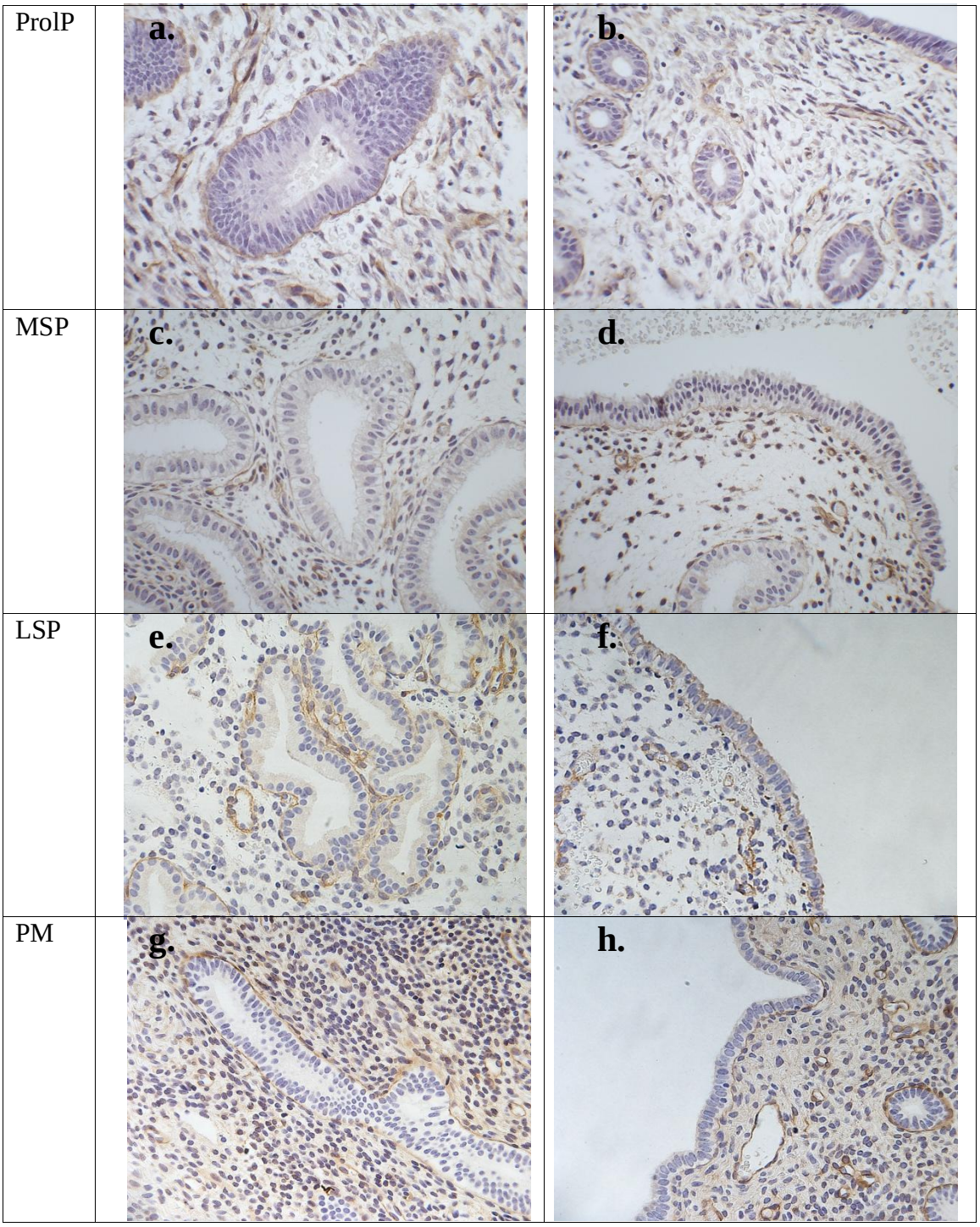


Figure 4.3. Representative images showing laminin staining in BM supporting the GE (a,c,e,g) and LE (b,d,f,h) in fertile control (ProlP,MSP,LSP) and PM endometrium.

As seen for COLIV, immuno-located laminin in BM supporting the glandular epithelium showed a trend towards average BM *thickness* being lower in the MSP (KW test; p=0.046)., and this was statistically significant when compared with the ProlP (MW test; p=0.019) and

suggestive when compared with the LSP (MW test; p=0.075). This trend was also demonstrated for laminin *staining intensity* in BM supporting the glandular epithelium, however this did not reach statistical significance (KW test; p=0.08, MW test; Prolp p=0.052, LSP p=0.052), refer to figure 4.4. When studying the staining intensity of immuno-localised laminin in BM supporting the LE, a significant reduction was also found during the MSP when compared with the ProlP (MW test p=0.043). No differences were found for laminin expression in BM vascular endothelium across the cycle. Additionally, no significant differences in the immuno-expression of laminin were found between fertile control and PM groups.

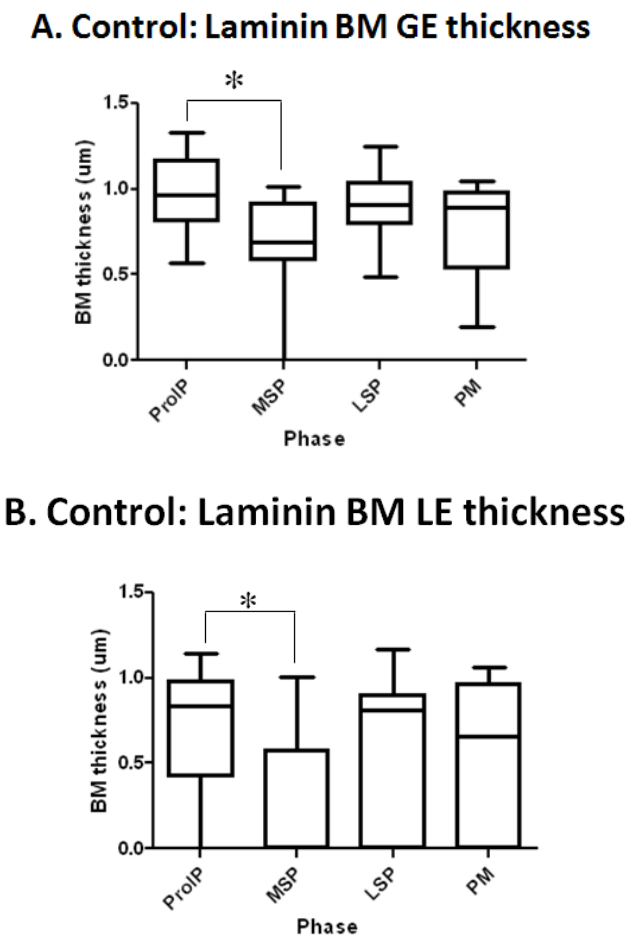


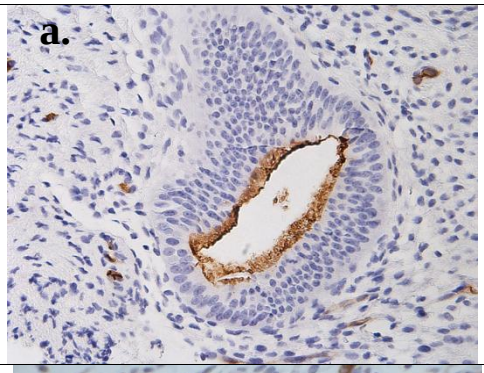
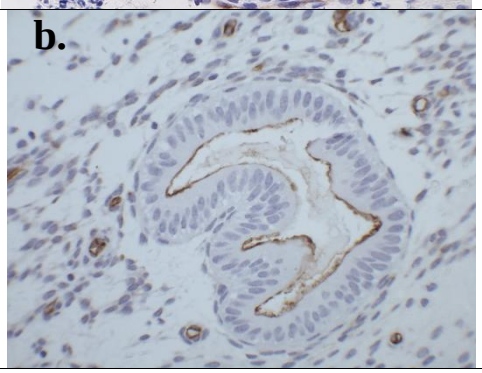
Figure 4.4 Box and Whisker plot showing changes in thickness of immuno-located laminin in endometrial BM. A. BM supporting GE is significantly thinner in MSP than in ProlP. B. BM supporting GE is also significantly thinner in MSP when compared with the ProlP.

4.3 - Expression of Stem Cell Markers in the Normal Cycling and PM Endometrium

PODXL

Table 4.6. PODXL results in the fertile control and post-menopausal group.

	Average combined quick score	Vessel intensity
ProlP		
Median	8.70	1.54
Range	3.05-10.30	1.00-2.70
MSP		
Median	4.95	1.70
Range	0.05-8.00	1.25-2.90
LSP		
Median	0.25	1.92
Range	0.00-4.45	1.50-2.24
PM		
Median	7.00	1.55
Range	3.95-7.50	1.05-1.93

ProlP	
MSP	

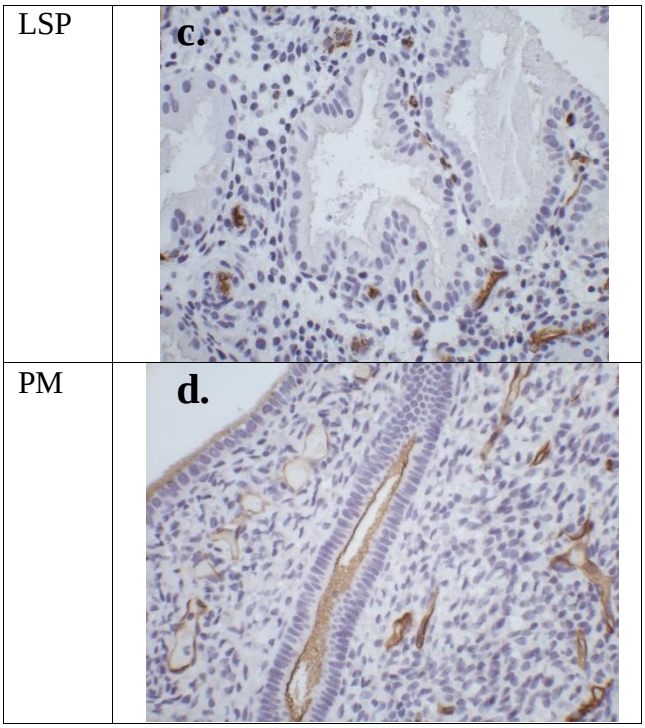


Figure 4.5. Representative images showing PODXL staining in glandular secretions and vascular endothelium in fertile control (ProlP,MSP,LSP) and PM endometrium.

As previously described, PODXL is expressed in glandular secretions and was analysed by combining the staining intensity of immuno-located PODXL with the proportion of the luminal surface of the glandular epithelium covered with PODXL. This gave a 'quick score' for each gland. When evaluating the overall quick-scores across the menstrual cycle, interesting differences were seen. The ProlP showed the highest overall quick scores demonstrating ProlP glands had the highest staining intensities and percentage coverage in glands for immuno-located PODXL. As the menstrual cycle progressed, PODXL quick scores significantly reduced (KW test; $p<0.00001$), as staining intensities in glandular secretions were significantly decreased from ProlP to MSP (MW test; $p=0.03$) and from MSP to LSP (MW test; $p=0.009$). Staining intensity of PODXL expression was also assessed in the vascular endothelium but no significant changes were seen along with the phase of the menstrual cycle. In post-menopausal endometrium, the combined quick-scores resembled to that seen in the ProlP, and were significantly higher than LSP quick-scores (MW test; 0.005).

In contrast, LSP staining intensity for PODXL in the vascular endothelium was significant higher than in PM endometrium(MW test; 0.0028), refer fig 4.6.

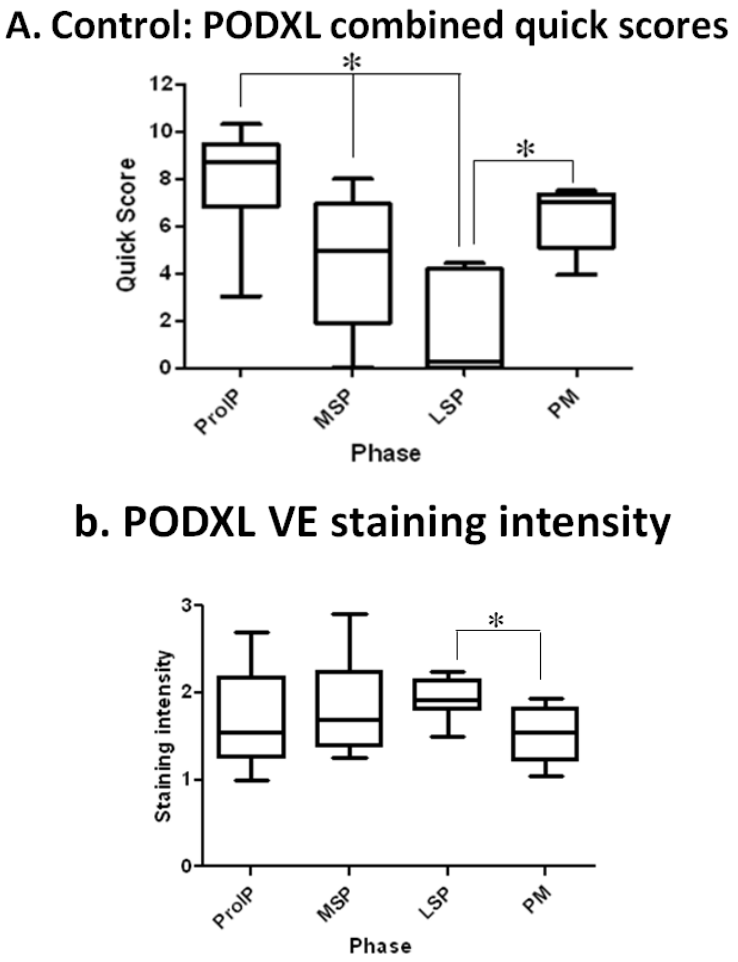
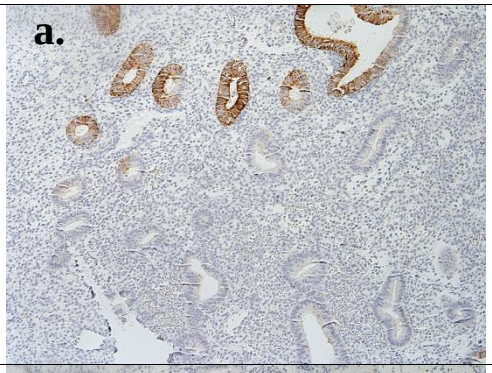
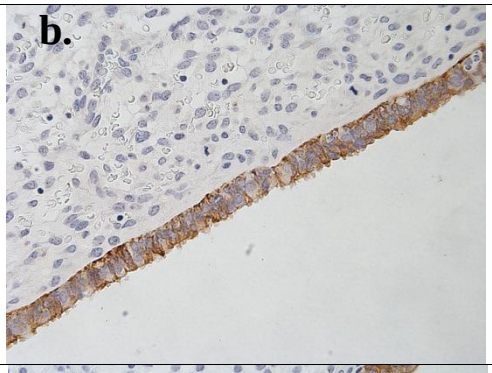
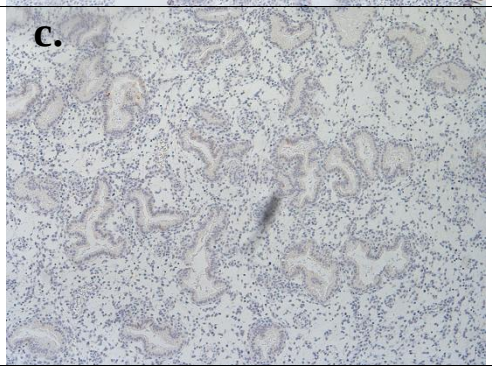
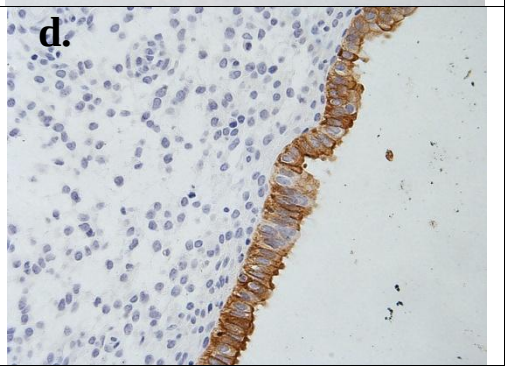


Figure 4.6: Box and Whisker plot to show significant changes in PODXL staining across the menstrual cycle and in PM endometrium. A. PODXL quick-scores significantly reduced along the menstrual cycle. PODXL quickscores in PM endometrium was significantly higher than LSP. B. Staining intensity of PODXL in VE was significantly lower in PM endometrium when compared with the LSP.

Cytokeratin 5/6

Table 4.7. CK5/6 staining results in the fertile control and post-menopausal group.

	% +ve glands	LE staining intensity
ProlP		
Median	40.00	1.25
Range	0.00-100.00	0.00-2.50
MSP		
Median	6.50	2.00
Range	0.00-96.60	0.50-3.00
LSP		
Median	4.15	1.25
Range	0.00-48.00	0.00-2.50
PM		
Median	3.23	0.00
Range	0.00-20.00	0.00-0.25

ProlP		
MSP		

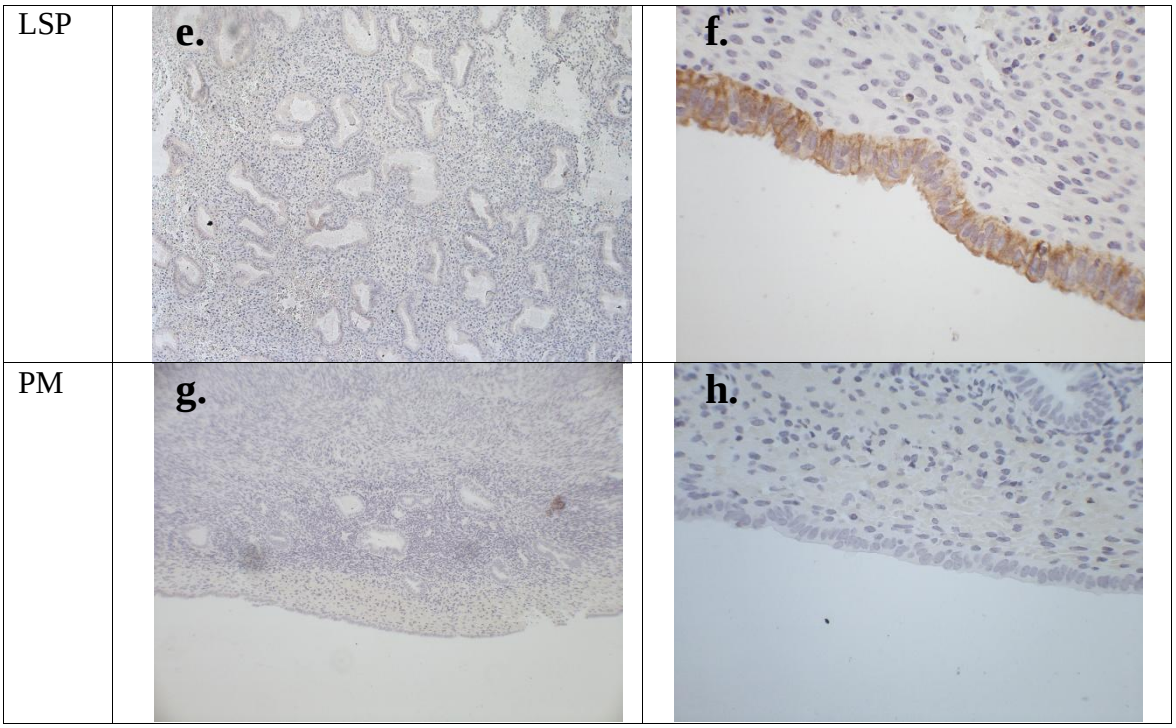
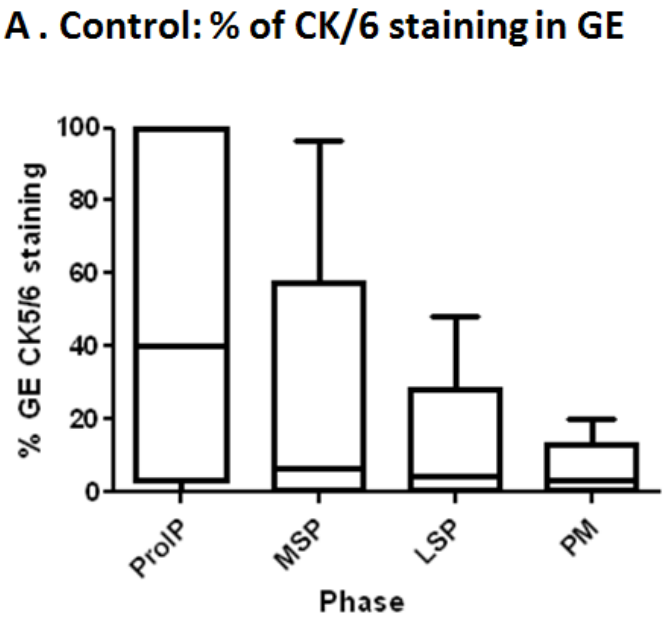


Figure 4.7 Representative images illustrating CK5/6 staining in g**l**andular epithelium (a,c,e,g) and luminal epithelium (b,d,f,h) in fertile control (ProlP,MSP,LSP) and PM endometrium.

There were no significant changes in the percentage of CK5/6+ glands across the phases of the menstrual cycle and between cycling and PM samples. Nonetheless, there was a higher % staining in the ProlP than in the LSP, however this did not reach statistical significance (MW test; p=0.063). Additionally, staining intensity of CK5/6 immuno-localisation in the luminal epithelium was similar in all samples taken from fertile controls. Whereas, in PM samples there was significantly lower staining intensity in the luminal epithelium when compared to ProlP (MW test; 0.0036), MSP (MW test; 0.006) and LSP (MW test; 0.006), see figure 4.8. However, out of the 5 PM samples included for the study, due to differences in the integrity of endometrial biopsies and their orientation in paraffin embedding, only three contained a clearly identifiable luminal epithelium. Therefore, we can not speculate a great deal on luminal CK5/6 staining intensity pattern in PM women with a small sample size.



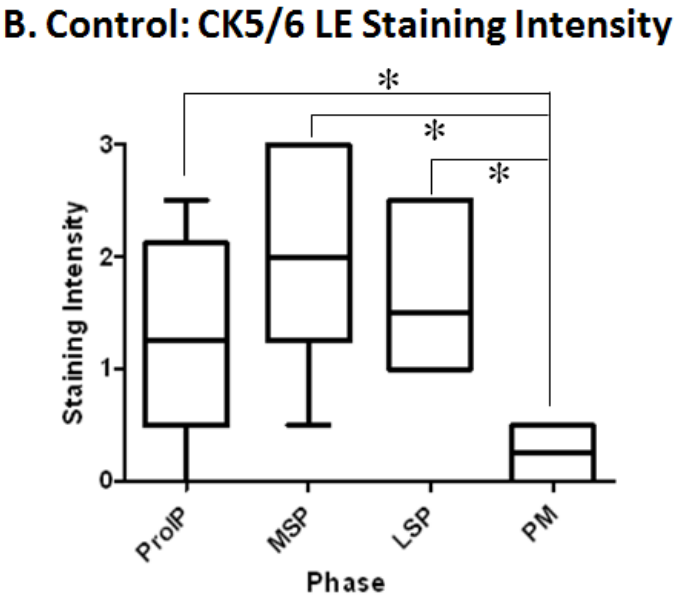


Figure 4.8: Box and whisker plots showing CK5/6 expression throughout the menstrual cycle. A. Proportion of CK5/6 immuno-positivity in GE did not significantly change across the menstrual cycle. B. Graph illustrates the significant reduced of CK5/6 staining intensity in the luminal epithelium in PM endometrium when compared to each tested phase of the menstrual cycle.

SSEA1

Table 4.8. SSEA1 staining results in the fertile control group

	Basalis				Functionalis				
	no. +ve glands	GE staining int.	proportion of GE stained	Quickscore	no. +ve glands	GE staining int.	proportion of GE stained	Quickscore	LE Staining Int.
ProlP									
Median	1.00	1.65	2.80	4.62	1.00	1.13	1.55	1.74	2.00
Range	1.00-1.00	1.35-2.05	2.70-3.00	3.65-6.15	0.70-1.00	0.65-1.85	0.80-3.0	0.52-5.55	1.00-2.50
MSP									
Median	0.45	0.38	0.45	0.17	0.60	0.50	0.60	0.30	0.50
Range	0.20-0.90	0.10-0.65	0.20-1.50	0.02-0.98	0.00-1.00	0.00-1.15	0.00-2.00	0.00-2.30	0.00-0.50
LSP									
Median	0.50	0.50	0.50	0.25	0.60	0.73	0.66	0.47	1.50
Range	0.40-0.90	0.45-1.15	0.40-0.90	0.18-1.04	0.30-1.00	0.20-1.75	0.40-1.80	0.08-3.15	0.00-2.00

Table 4.9. SSEA1 staining results in the PM group

	no. +ve glands	GE staining int.	proportion of GE stained	Quickscore	LE staining int.
PM					
Median	1.00	2.50	2.70	6.25	1.5
Range	0.90-1.00	1.95-3.00	1.90-3.00	1.90-3.00	0.50-3.00

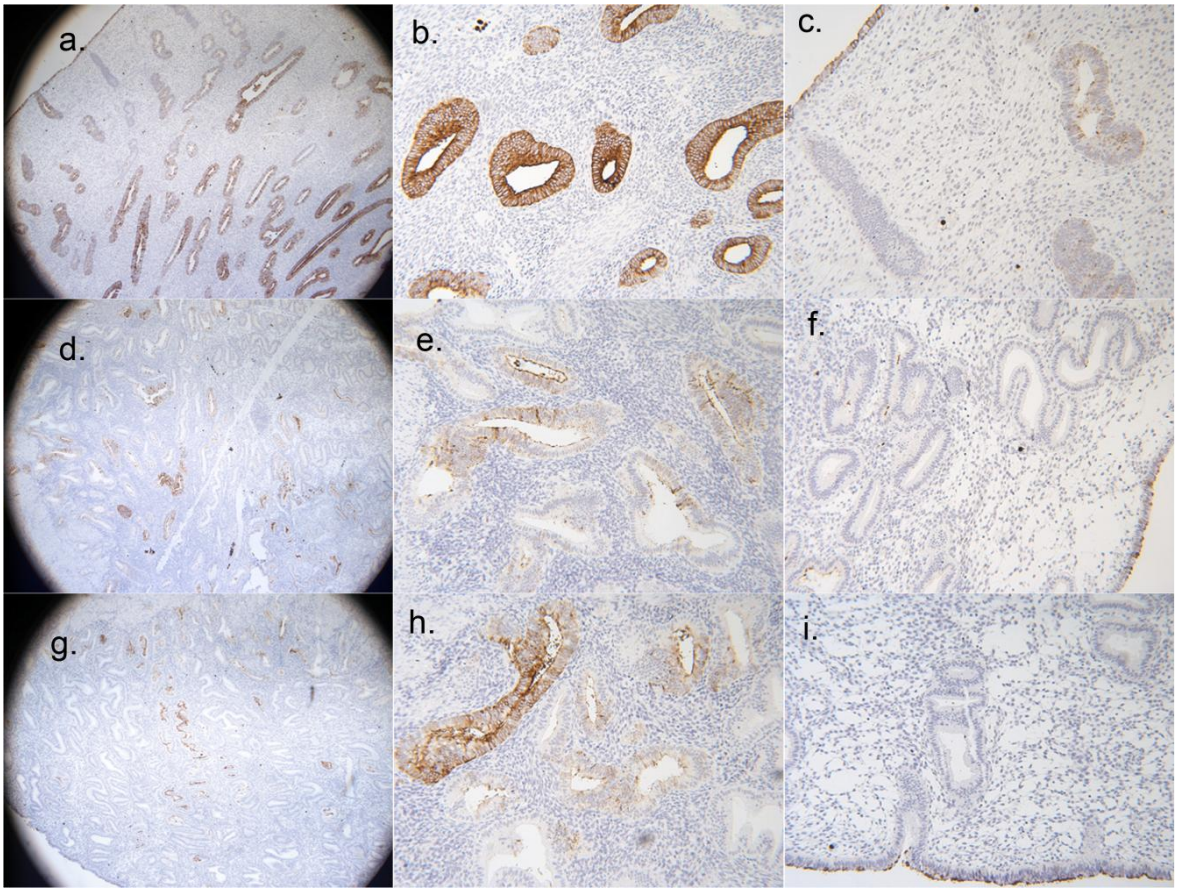


Figure 4.9: Representative micrographs showing SSEA1 staining in fertile control endometrium. Micrographs illustrate staining in ProlP (a-c), MSP (d-f) and LSP (g-i) showing the full thickness of the endometrium (a,d,g), basalis layer of the endometrium (b,e,h) and in the upper functionalis layer and the luminal epithelium (c,f,i).

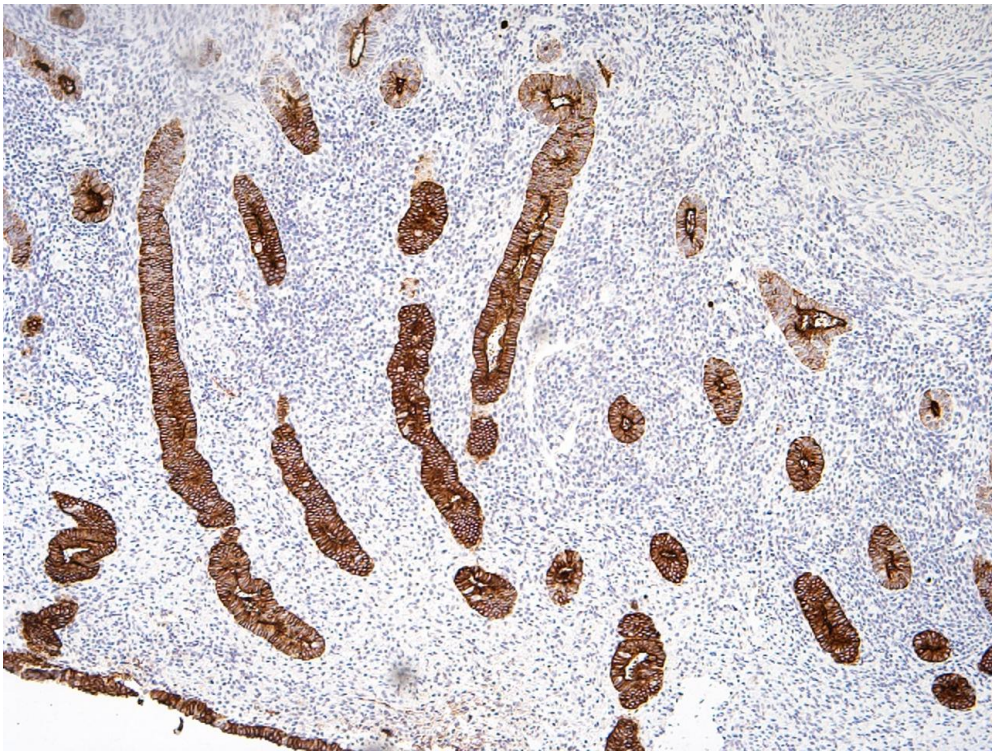


Figure 4.10: SSEA1 localisation in glandular and luminal epithelium in PM endometrium.

As previously discussed, the basal and functional layers were considered as separate entities for analysis of SSEA1. It is generally accepted that when the endometrium is sampled with an endometrial pipelle sampler, only the functional layer of eutopic endometrium is obtained. Therefore, for SSEA1 staining both the full thicknesses and pipelle samples were included for the analysis of the functional layer but only full thickness sections were analysed in the basal layer. Glandular epithelium in both layers of the endometrium were assessed for number of SSEA1 immuno-positive glands, percentage of positive cells and staining intensity of glandular epithelium. A GE staining quick-score was attained by multiplying the three descriptive factors together carried out in both layers in cycling endometrium and also PM endometrium.

i. Basalis Layer

All fertile control full thickness samples taken during the ProlP (n=5), MSP (n=4) and LSP (n=3) had positive glandular epithelial staining in the basalis layer of the endometrium. When studying the ***number of SSEA1 positive glands,*** all 10 basal glands analysed in each ProlP sample were SSEA1 immuno-positive. Additionally, virtually all of the 10 glands analysed in each of the PM sample were strongly positive for SSEA1. Compared with the basal glands of the ProlP, the basal glands of the secretory phase endometrium had significantly lower number of SSEA1 immuno-positive glands (KW test; p=0.012, MW test; MSP p=0.007, LSP p=0.01). This significant decrease is observed when comparing SSEA1 positive basal glands of the PM endometrium with MSP (MW test; p=0.0016) and LSP (MW test; p=0.0036) endometrium.

A similar picture was observed when ***the percentage of SSEA1 positive cells*** were analysed in each positive gland, with basal glands of the ProlP and PM having similar percentages. Additionally, secretory phase endometrium has significantly lower SSEA1 positive basal

glandular cells when compared to the ProlP (KW test; p=0.016, MW test; MSP p=0.014, LSP p=0.024) and PM endometrium (MW test; MSP p=0.016, LSP p=0.036).

SSEA1 staining intensity in basal glandular epithelium showed marked differences according to the phase of the cycle (KW test; p=0.012). Within the fertile control group, basal glandular staining intensity was highest in ProlP samples and decreased significantly across the secretory phase to have the weakest intensity in LSP (MW test; MSP p=0.016, LSP p=0.025). Overall, staining intensity for SSEA1 was seen to be the highest in PM glands being apparently higher when compared with ProlP glands (MW test; 0.0056) and a significantly higher when compared with MSP (MW test; 0.016) and LSP (MW test; 0.0036) glands.

When multiplying the three descriptibe factors to obtained an overall 'Combined SSEA1 quickscore' in both basalis and functionalis layers the same significant differences were mirrored, illustrated in figure 4.11. In the basalis layer, firstly, the overall glandular staining was significantly lower in the secretory phase when compared with the ProlP (MW test; MSP p=0.016, LSP p=0.036) and PM endometrium (MW test; MSP p=0.016, LSP p=0.036). No differences were seen between the MSP and LSP and also between ProlP and PM endometrium.

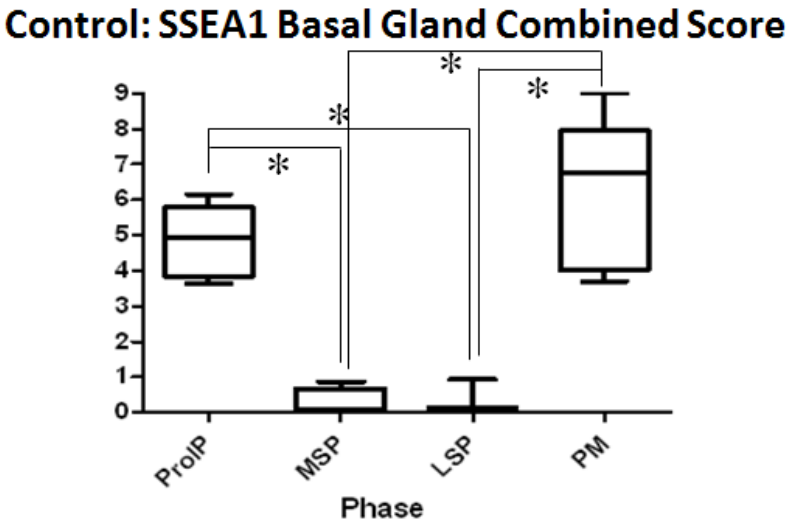


Figure 4.11: Box and whisker plot demonstrating significant decrease in basal SSEA1 glandular staining in the secretory phase when compared with the ProlP and PM endometrium.

ii. SSEA1 in the Functionalis Layer

For all three descriptive factors, functional glands in the ProlP showed significantly higher *number of positive glands* (KW test; p=0.002); *percentage of SSEA1+ cells* (KW test; p=0.013); *and staining intensity* (KW test; p=0.002) in the glandular epithelium when compared to the MSP (MW test; p=0.001, p=0.001, p=0.003 respectively) and LSP (MW test; p=0.023, p=0.005, p=0.089 respectively). However, there was only an apparent difference in the staining intensity of SSEA1 in GE between ProlP and LSP. No significant differences were found between the MSP and LSP. These significant differences were resultantly mirrored when analysing the overall combined scores (KW test; p=0.002). In the functionalis layer, the overall glandular staining was significantly lower in the secretory phase when compared with the ProlP (MW test; MSP p<0.0001, LSP p=0.023). No differences were seen between the MSP and LSP. Figure 4.12 illustrates these overall results in the functionalis.

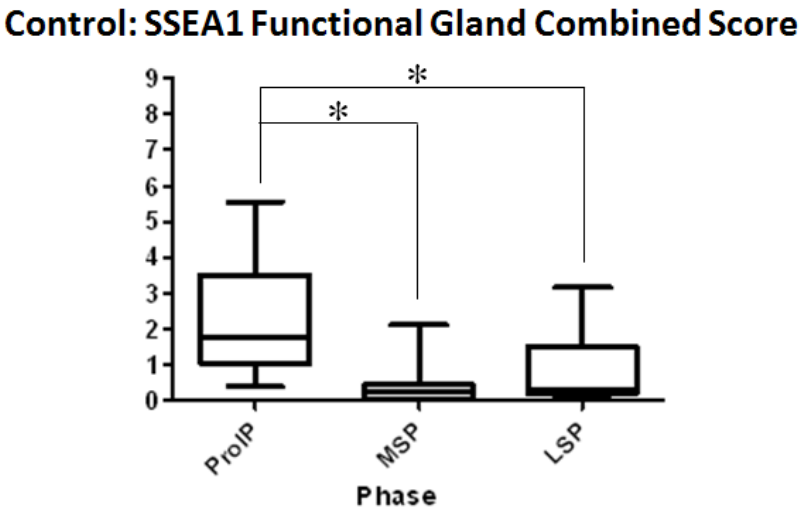


Figure 4.12: Box and whisker plot showing higher SSEA1 glandular staining in proliferative than in secretory phase samples.

Lastly, SSEA1 staining intensity in the luminal epithelium was compared between all phases of the menstrual cycle and in PM endometrium, see fig 4.13. Within the fertile control group SSEA1 staining intensity in the luminal epithelial was lower in the MSP (KW test p=0.001) when compared to the ProlP (MW test; p<0.0001) and LSP (MW test; p=0.065 – apparent difference) and was significantly lower than the staining intensity observed in PM luminal epithelium (MW test; p=0.02).

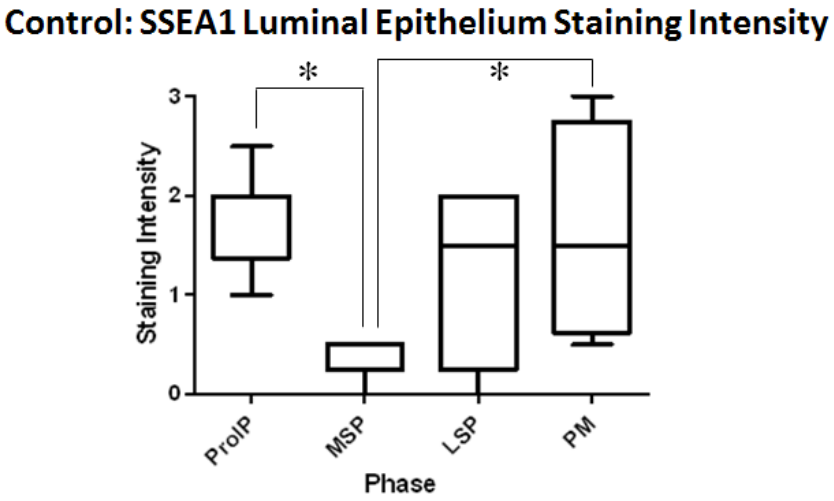


Figure 4.13: Box and whisker plot demonstrating changes in SSEA1 staining intensity in luminal epithelium (LE) across the menstrual cycle. LE staining intensity has shown to be significantly lower in the MSP when compared with the ProlP and PM endometrium.

4.4 Correlation between BM and Stem Cell Markers in Normal Cycling and in PM Endometrium

Expression of stem cell marker PODXL in fertile controls appear to be negatively correlated with the expression of endometrial COLIV. In particular, PODXL combined quick-scores show a negative correlation with the combined scores for COLIV expression BM supporting glands (r=-0.362, P=0.049). Combined score for COLIV was taken by multiplying together the staining intensity and thickness of BM supporting the GE and LE.

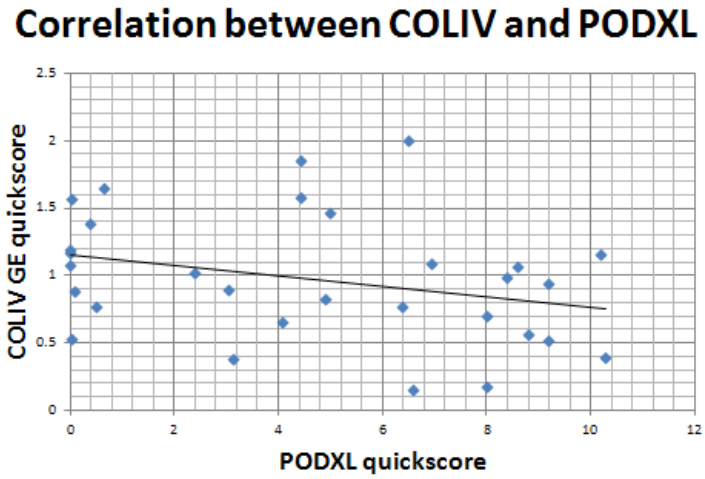


Figure 4.14. Scatter graph to illustrate the negative correlation between combined COLIV expression in BM supporting GE with the PODXL quick-scores, in the fertile control group.

In contrast, the number of positive CK5/6 glands in fertile control endometrium positively correlates with the thickness (r=-0.520, P=0.006) of BM supporting luminal epithelium immuno-located with COLIV.

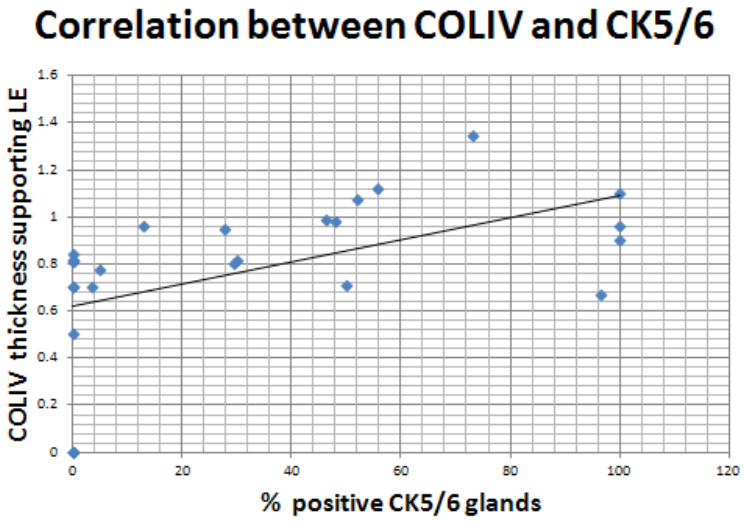
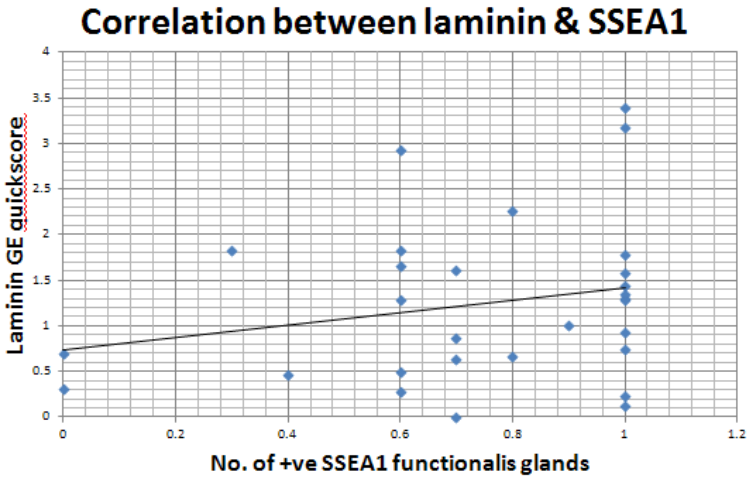


Figure 4.15. Scatter graph to illustrate positive correlation between the thickness of immuno-located COLIV in BM supporting LE with the percentage of CK5/6 glands found in the fertile control group.

Numerous associations were found between BM components and SSEA1 expression in the endometrium. Firstly, a positive correlation was seen between the number of positive functionalis SSEA1 glands of fertile controls, with firstly the combined laminin expression in BM supporting glands (r=0.369, P=0.045) and LE (r=0.418, P=0.027), and secondly the combined COLIV (r=0.392, P=0.047) and in the LE. Additionally, LE immuno-staining intensities of SSEA1 also showed a positive correlation with the combined COLIV staining (r=4.02, P=0.046) and laminin thickness (r=0.47, P=0.013) for BM supporting the luminal epithelium in fertile controls. Additionally, in PM women the overall SSEA1 glandular staining positively correlates with combined laminin scoring in BM around glands (r=0.932, P=0.021) and vascular endothelium (r=0.979, P=0.004). Figure 4.16 graphically illustrates some these findings.



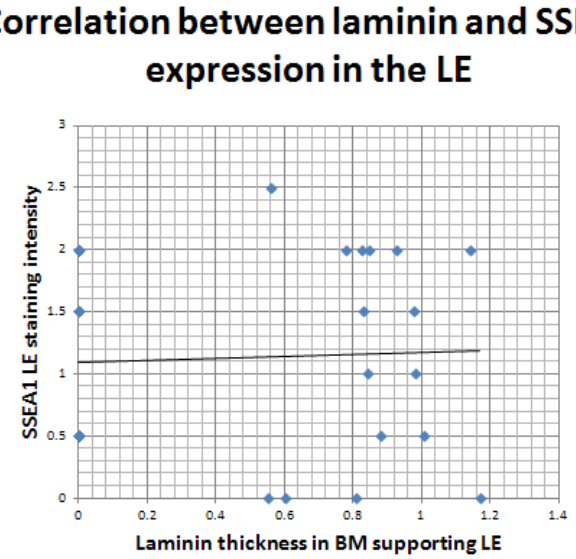


Figure 4.16. Scatter graph to show the positive correlation between the combine laminin quickscore with the number of positive SSEA1 glands in the functionalis layer in the fertile control group. Second scatter graph also shows positive correlation between laminin thickness in the immuno-located BM supporting the LE with the SSEA1 staining intensity in the LE.

Also, associations were found between the different stem cell markers. Interestingly, combined PODXL quick-scores shows a positive correlation with SSEA1 combined gland score for both basalis (r=0.723, P=008) and functionalis (r=0.385, P=0.036) layers of fertile control endometrium, see figure.

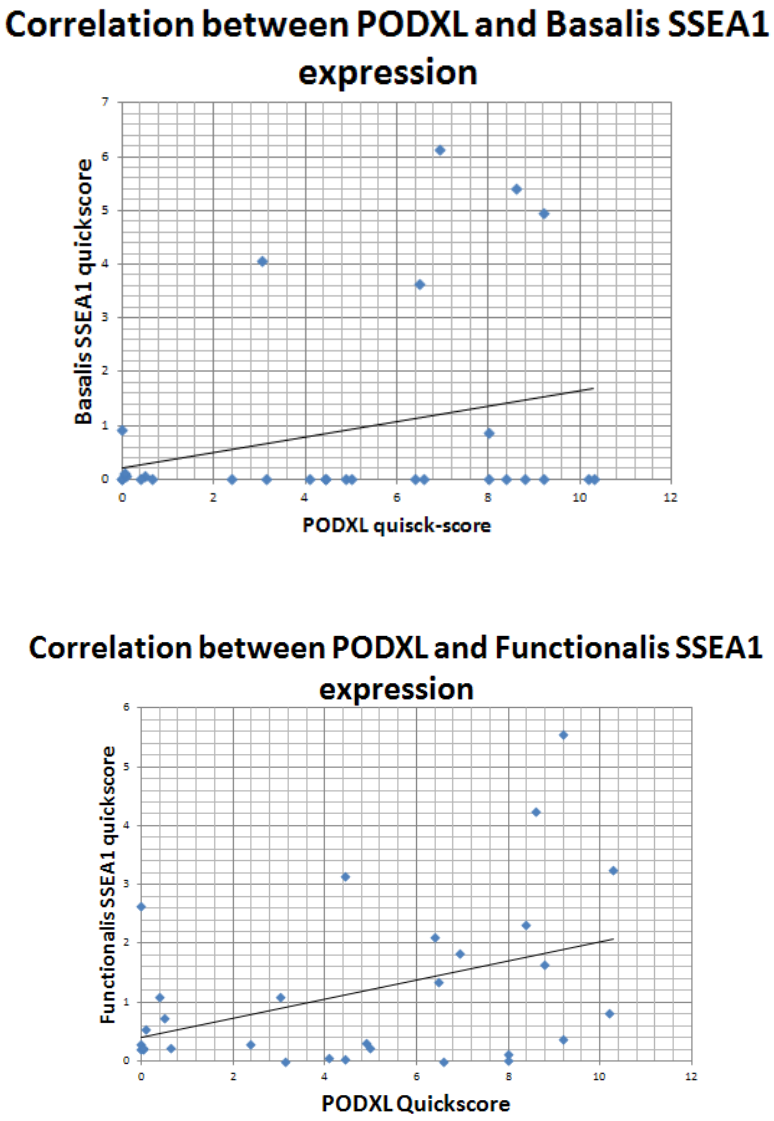


Figure 4.17. Scatter graphs to show positive correlation between PODXL quickscore with the quickscore for SSEA1 GE staining in both basalis and functionalis layers of the endometrium in the fertile control group.

Chapter Five:
RESULTS –Endometriosis group

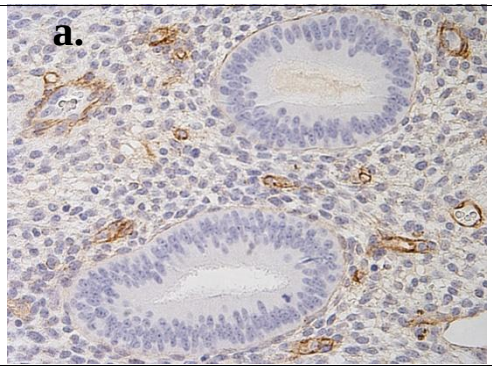
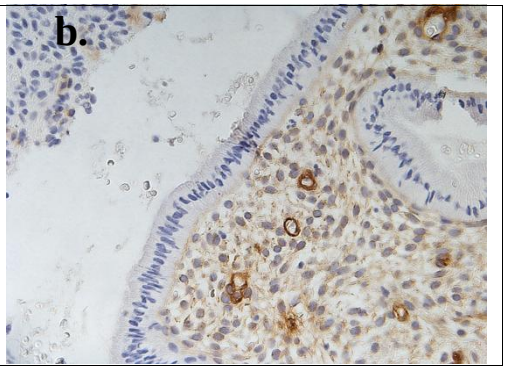
For all results collected from the endometriosis group, please refer to appendix 8K to 8O.

5.1 - Expression of Endometrial Basal Lamina Components in Patients with Endometriosis

Collagen-type four

Table 5.1. COLIV staining results in the endometriosis group

	GE BM Thickness (µm)	GE BM Staining Int.	LE BM Thickness (µm)	LE BM Staining Int.	VE BM staining int.
ProlP					
Median	0.753	1.000	0.880	1.000	1.850
Range	0.00-0.928	0.000-1.85	0.990-1.110	0 – 2.000	0.650-2.650
MSP					
Median	0.663	0.600	0.650	1.000	1.950
Range	0-1.269	0-1.900	0-2.090	0-2.000	0-2.650
LSP					
Median	0.942	1.128	0.720	0.750	2.010
Range	0.128-1.340	0.200-1.500	0-1.455	0-2.000	1.100-2.650

ProlP		

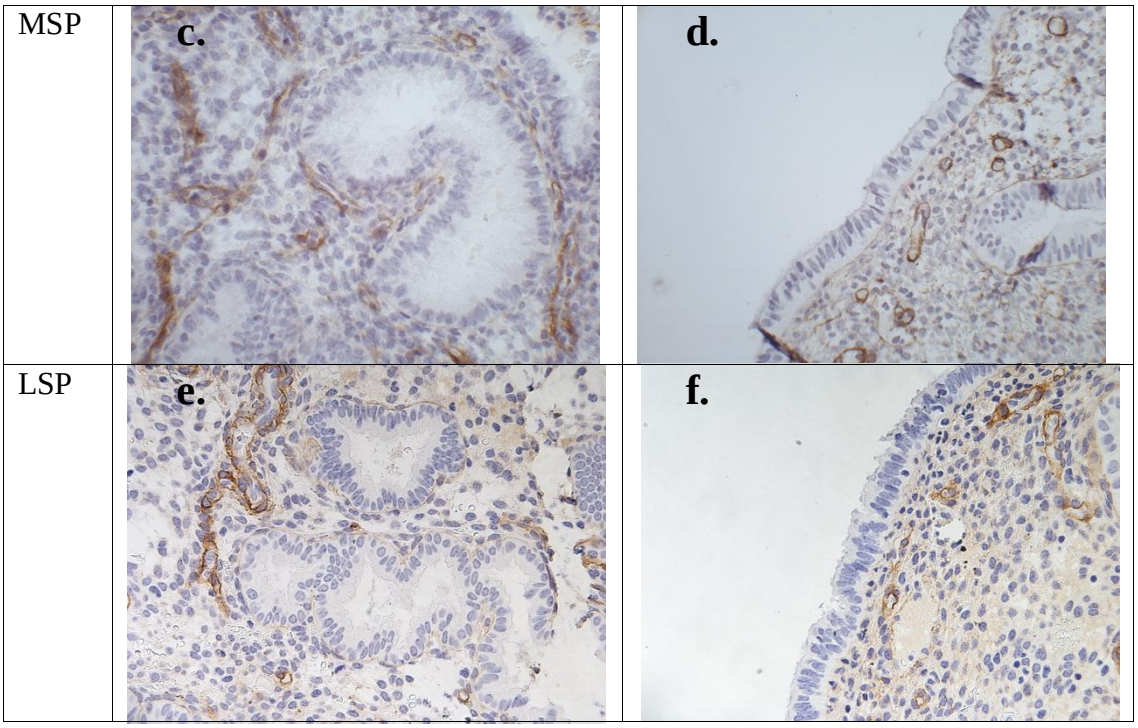


Figure 5.1. Representative images showing COLIV staining in BM supporting the GE (a,c,e) and LE (b,d,f) in cycling (ProlP,MSP,LSP) endometrium samples from women with endometriosis.

Laminin

Table 5.2. Laminin staining in the endometriosis group.

	GE BM Thickness	GE BM Staining Int.	LE BM Thickness	LE BM Staining Int.	VE BM staining int.
ProlP					
Median	0.914	1.000	0.400	0.250	1.500
Range	0.372-9.770	0.250-2.100	0.000-0.950	0.000-2.000	0.600-2.350
MSP					
Median	0.770	1.300	0.513	1.500	1.850
Range	0.630-0.938	0.800-1.700	0.000-1.285	0.000-2.000	1.350-2.400
LSP					
Median	0.856	1.075	1.340	0.500	1.600
Range	0.547-1.043	0.150-2.071	0.000-1.340	0.000-2.000	0.500-2.071

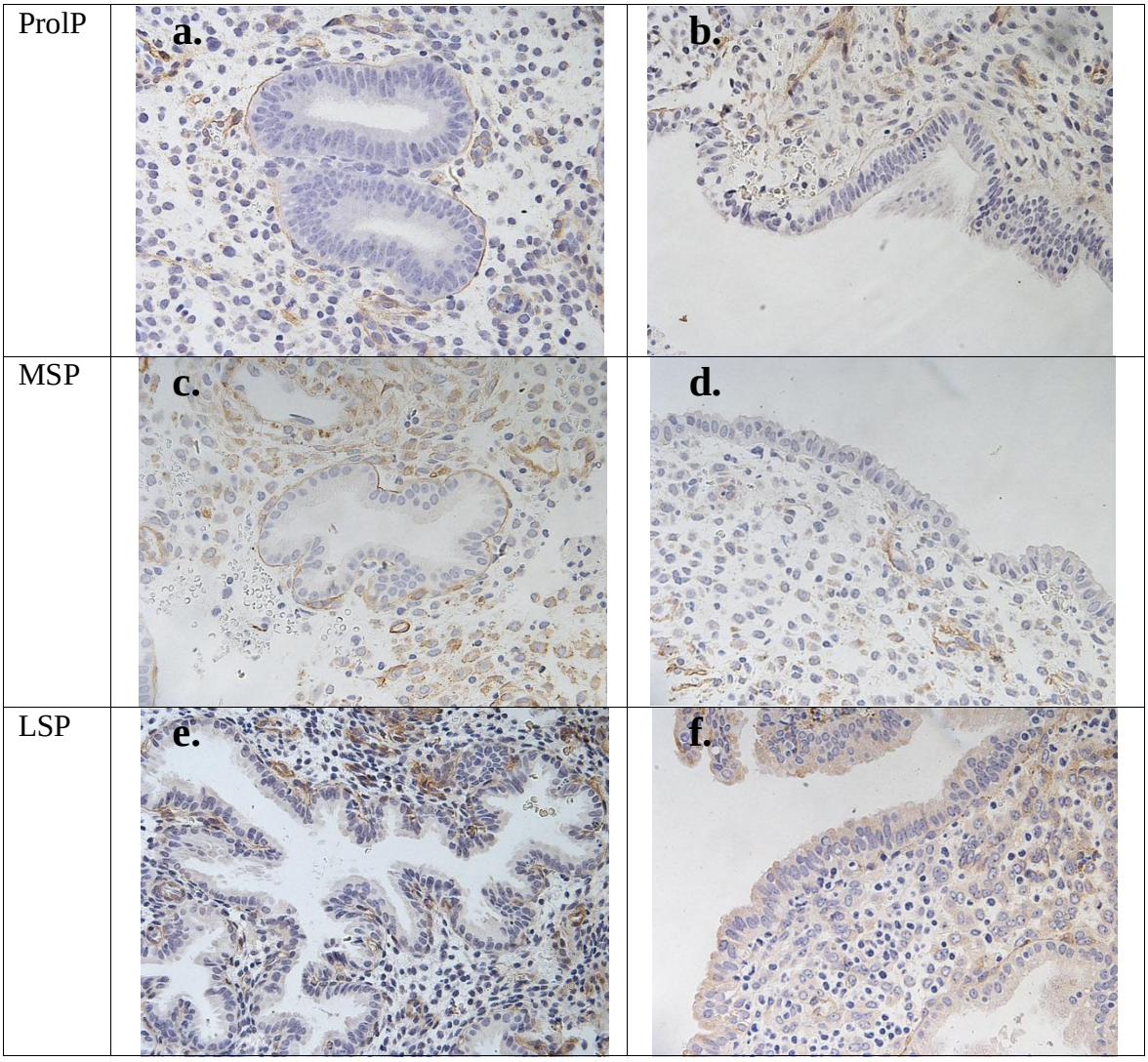


Figure 5.2. Representative images showing laminin staining in BM supporting the GE (a,c,e) and LE (b,d,f) in cycling (ProlP,MSP,LSP) endometrium samples from women with endometriosis.

Although immuno-located COLIV and laminin in endometrial BM were seen to be altered across the menstrual cycle in the fertile control group, no significant changes in BM thicknesses and staining intensities were seen in the endometriosis group.

5.2 - Expression of Endometrial Stem Cell Markers in Patients with Endometriosis

PODXL

Table 5.3. PODXL staining results in the endometriosis group

	Average combined quick score	Vessel intensity
ProlP		
Median	5.00	2.20
Range	0.00-8.65	0.95-2.75
MSP		
Median	2.80	2.30
Range	0.00-8.60	1.30-2.75
LSP		
Median	0.40	1.68
Range	0.00-3.60	1.00-2.25

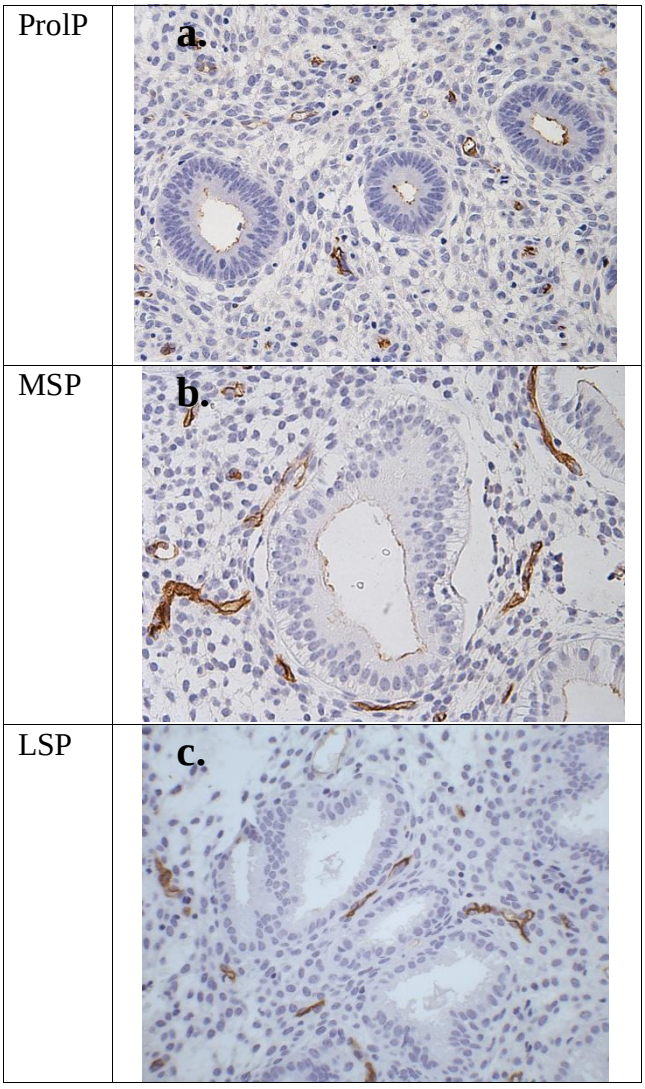


Figure 5.3. Representative images showing PODXL staining in glandular secretions and vascular endothelium in endometrium collected from women with endometriosis.

Similar to the fertile control group, the endometriosis group also showed strong significant changes in PODXL quick-scores within phases of the menstrual cycle (KW test; p=0.014). PODXL quick-scores were highest in the ProlP and significantly decreased as the menstrual cycle progressed, with a significant reduction in scores in the LSP (MW test; p=0.004), see figure 5.4. Additionally, there is a difference in the staining intensity of PODXL expression within the vascular endothelium, with staining intensity in the LSP being lower than in the MSP (MW test; p=0.026).

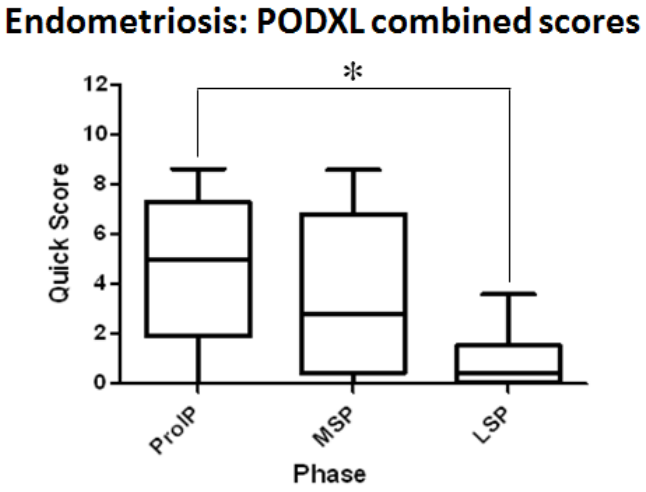


Figure 5.4: Box and whisker plot demonstrating changes in PODXL quick-scores across the menstrual cycle in the endometriosis group.

Cytokeratin 5/6

Table 5.4. Table to show CK5/6 result in eutopic endometrium in the endometriosis group

	% +ve glands	LE staining intensity
ProlP		
Median	19.20	1.00
Range	0.00-100.00	0.00-2.50
MSP		
Median	12.00	1.50
Range	0.00-100.00	1.00-3.00
LSP		
Median	11.15	1.50
Range	0.00-42.90	0.50-2.50

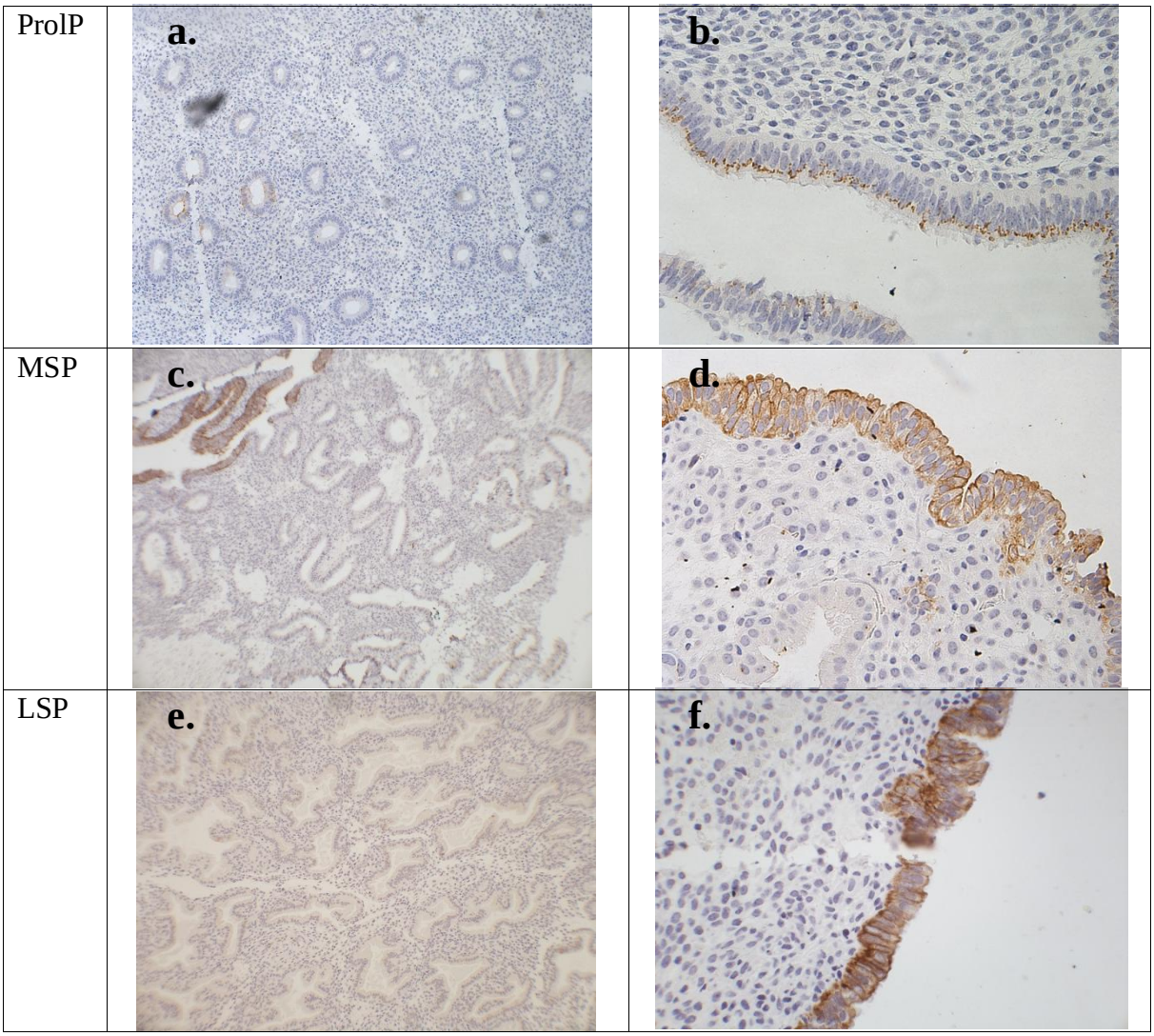


Figure 5.5. Representative images illustrating CK5/6 staining in g**l**andular epithelium (a,c,e,g) and luminal epithelium (b,d,f,h) in eutopic endometrium collected from women with endometriosis.

When analysing the proportion of CK5/6+ glands in a low power field and staining intensity of immuno-located CK5/6 in the luminal epithelium, no changes were seen across the menstrual cycle, and this is consistent with results seen in the fertile control group.

SSEA1

Table 5.5. Table showing results of SSEA1 expression in eutopic endometrium collected from women with endometriosis.

	Basalis				**Functionalis**				
	no. +ve glands	GE staining int.	proportion of GE stained	Quickscore	no. +ve glands	GE staining int.	proportion of GE stained	Quickscore	LE Staining Int.
ProlP									
Median	1.00	1.65	2.80	4.62	1.00	0.85	1.60	1.40	1.50
Range	0.70-1.00	0.95-2.05	1.10-3.00	0-5.37	0.80-1.00	0.50-2.70	1.00-2.80	0.55-6.75	0.50-3.00
MSP									
Median	0.95	1.25	1.50	0.00	0.20	0.15	0.20	0.03	1.25
Range	0.90-1.00	1.20-1.30	1.30-1.70	0.00-2.21	0.00-0.70	0.00-0.70	0.00-0.700	0.00-0.49	0.00-2.50
LSP									
Median	0.60	0.80	0.80	0.79	0.30	0.43	0.30	0.06	1.50
Range	0.20-1.00	0.30-1.30	0.40-1.20	0.024-1.56	0.00-0.80	0.00-0.70	0.00-0.80	0.00-0.28	0.50--2.50

i. Basal Layer

In the endometriosis group, there were no distinct differences in basal gland SSEA1 immuno-staining across phases of the menstrual cycle studied, when analysed for all three descriptive factors previously described, refer figure 5.6. This is in contrast with the fertile control group, where significant difference in basal gland staining was seen across the cycle.

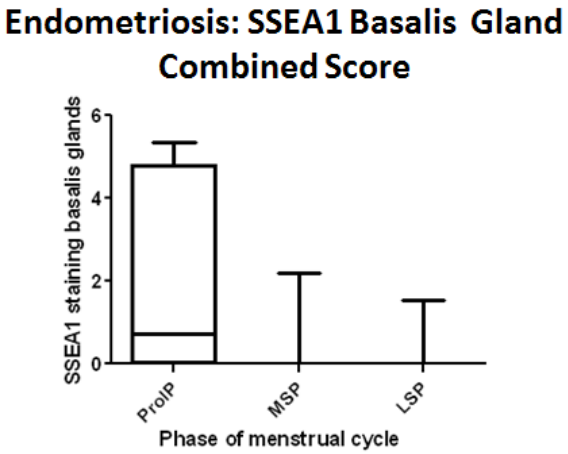


Figure 5.6. Box and whisker plot to demonstrate overall SSEA1 staining in basalis layer glands in the endometriosis group. No significant changes across the menstrual cycle seen.

ii. Functional Layer

In contrast, analysis of SSEA1 immuno-positive glands in the functional layer demonstrated significant changes across the menstrual cycle. For all three descriptive factors, functional glands in the ProlP showed significantly higher *number of positive glands* (KW test; $p<0.0001$); higher *percentages of SSEA1+ glandular cells* (KW test; $p<0.0001$); and higher *staining intensities* (KW test; $p<0.0001$) in the glandular epithelium when compared to the MSP (MW test; $p<0.0001$ all three descriptive factors) and LSP (MW test; $p<0.0001$, $p<0.0001$, $p=0.001$ respectively). These significant differences were resultantly mirrored when analysing the overall combined scores with ProlP functionalis glands with higher SSEA1 glands than secretory phase (KW test; $p<0.0001$, MW test; MSP $p<0.0001$, LSP $p<0.0001$), see figure 5.7. In contrast, staining intensities of immuno-located SSEA1 in the

luminal epithelium remained similar across all three phases of the menstrual cycle tested in the endometriosis group.

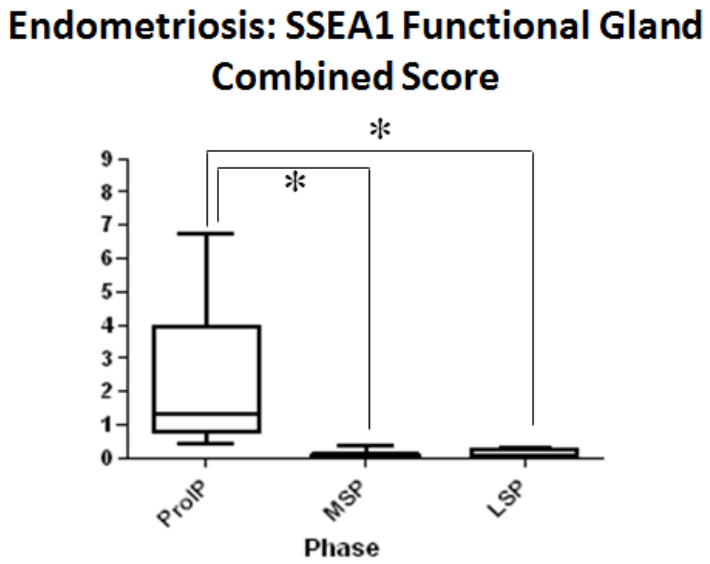


Figure 5.7: Box and whisker plot demonstrating changes in combined SSEA1 functional gland combined scores across the menstrual cycle. Significant reduction in staining in secretory phase with compared with the proliferative phase.

5.3 Correlation between BM and Stem Cell Markers in the Endometriosis Group

When evaluating the relationship between BM components and stem cell marker expression in eutopic endometrium from women with endometriosis, significant trends are observed, some of which have been previously observed in the fertile control group. Firstly, PODXL combined quick-score in the endometriosis group are negatively correlated with COLIV staining intensity in BM supporting glands (r=-276, P=0.026). In contrast, PODXL expression in vessels seem positively correlates with the laminin combined scores in BM supporting luminal epithelium (r=0.313, P=0.02) and vascular endothelium (r=0.386, P=0.002). Additionally, COLIV immuno-staining intensity in BM supporting vessels positively correlates with CK5/6 staining intensity in the luminal epithelium (r=0.328, P=0.013).

Positive correlation is also found between laminin combined scores for staining in BM supporting the glands, with the combined SSEA1 staining score for glands residing in the basalis (r=0.496, P=0.008) and functionalis (r=0.358, P=0.005), and also with SSEA1 staining in the luminal epithelium (r=367, P=0.007).

When looking at the relationship between stem cell markers in the endometriosis group, PODXL combined quick-score positively correlates for all overall SSEA1 staining assessment in the basalis (r=0.596, P=0.001), functionalis (r=0.384, P=0.002) and also in the luminal epithelium (r=0.271, P=0.045).

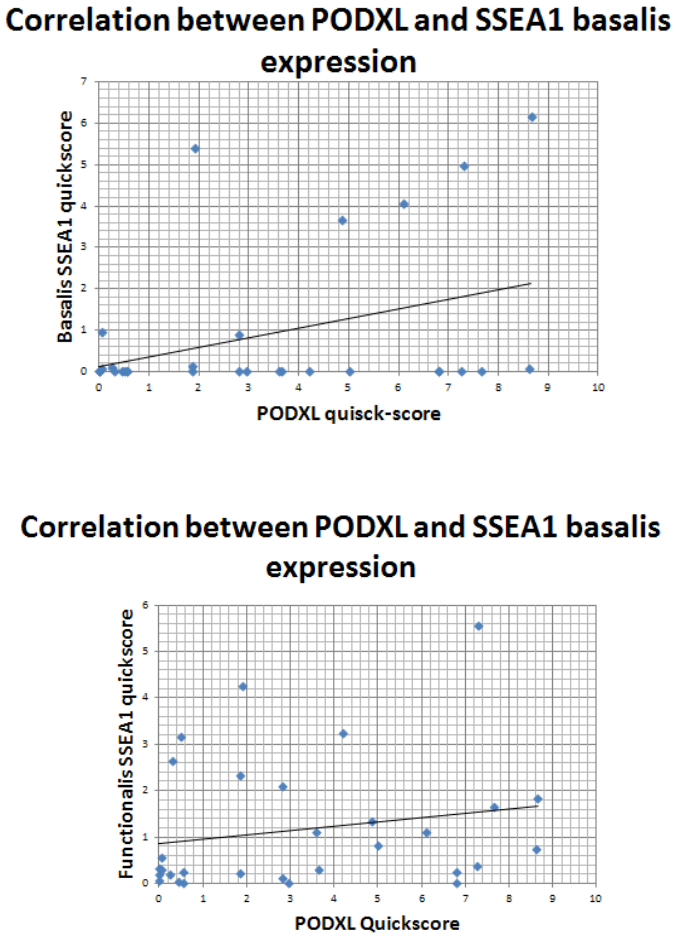


Figure 5.8. Scatter graphs to demonstrate the positive correlation relationships between PODXL expression with basalis and functionalis layer SSEA1 positive glands in the endometriosis group.

5.4 Difference between expression of BM components and stem/progenitor cell markers in eutopic endometrial samples taken from fertile control and women with endometriosis

To analyse the difference in BM components and stem/progenitor cells markers, between the fertile control and endometriosis groups, a two way analysis of variance (ANOVA) statistical test was used. Values of $P<0.05$ were considered significant. When performing ANOVA on all markers, no significant difference was found. However, apparent differences that did not reach statistical significance were seen between the groups. Firstly, there was an apparent difference in the combined laminin score in BM supporting the GE (as it was slightly higher in the MSP in the endometriosis group than in the fertile control group ($F=2.417$, $p=0.099$). Refer to figure 5.9

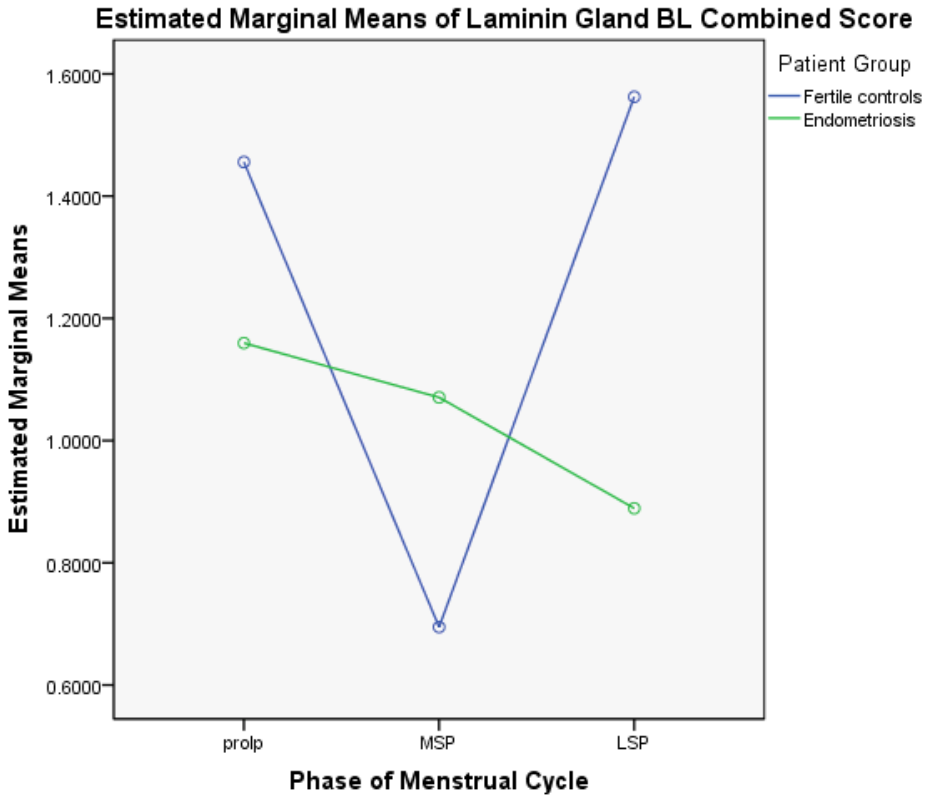


Figure 5.9. Line graph to show estimated marginal means of the combined score of laminin staining immuno-located in the BM supporting the GE. Graph shows in the endometriosis group there is no decrease in overall staining in the MSP when compared with the ProlP and LSP in the endometriosis group, unlike the fertile control group.

Secondly, there was also an apparent difference in the staining intensity in laminin immuno-located in BM supporting vascular endothelium, with staining being higher in the endometriosis group than fertile control group, see figure 5.10 (F=2.491, p=0.092).

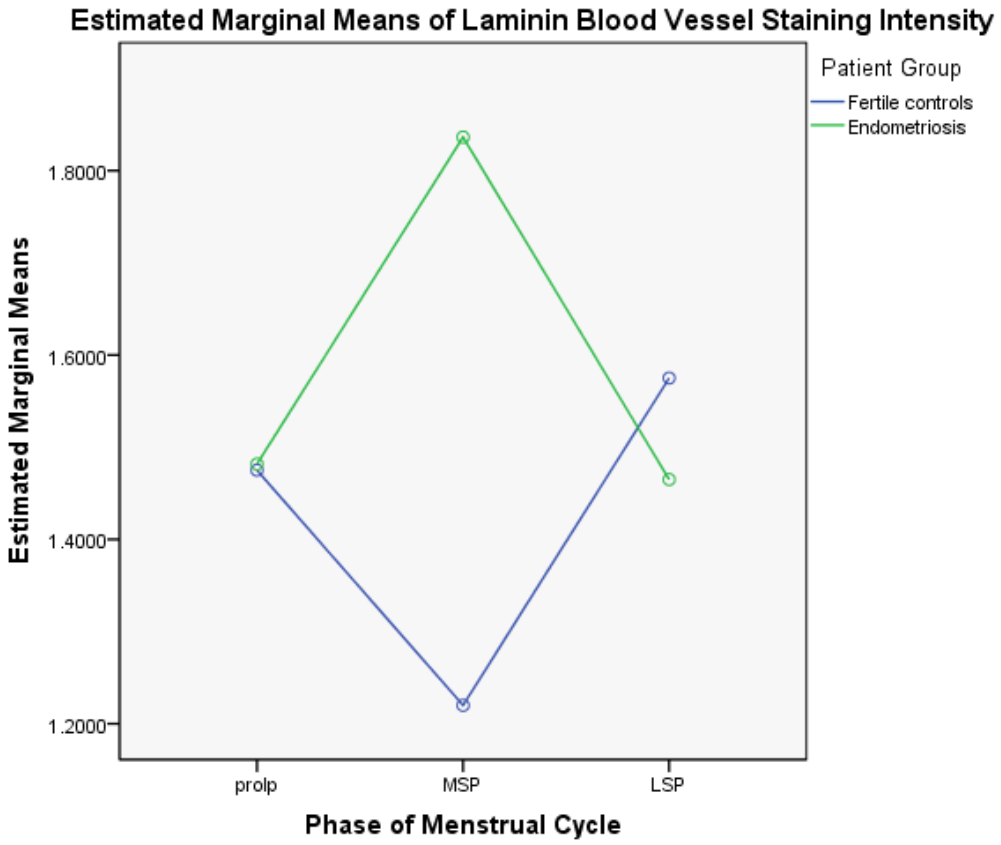


Figure 5.10. Line graph to show estimated marginal means of the laminin staining intensity immuno-located in the BM supporting the vascular endothelium. Graph shows in the endometriosis group there is increase in staining intensity of laminin during the MSP when compared to the fertile control group. However this did not reach statistical significance.

Finally, an apparent difference was also seen between the staining intensity of SSEA1 immuno-localised in the LE between the subject groups, being higher in the endometriosis group than fertile control group, refer to figure 5.11. However, this did not reach statistical significance (F= 2.637, p=0.083).

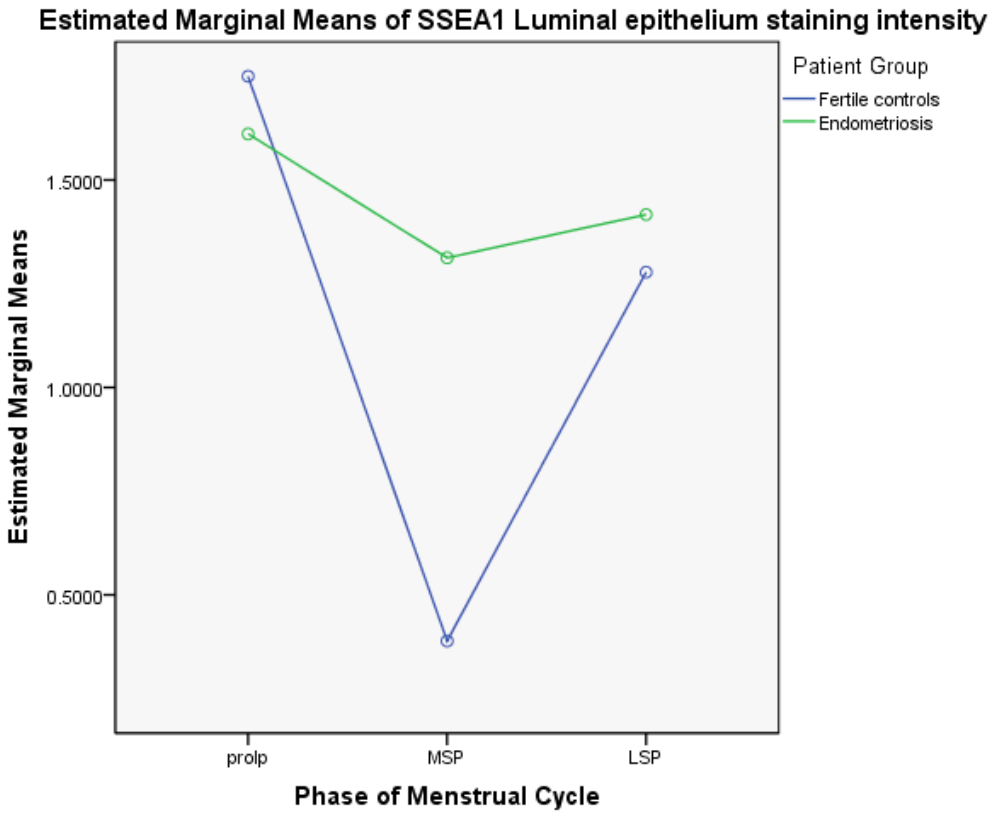


Figure 5.11. Line graph to show estimated marginal means of SSEA1 staining intensities immuno-located in the luminal epithelium. Graph shows in the endometriosis group there is increase in staining intensity of SSEA1 when compared to the fertile control group. However this did not reach statistical significance.

Chapter Six:
RESULTS - Endometrial Cancer Group

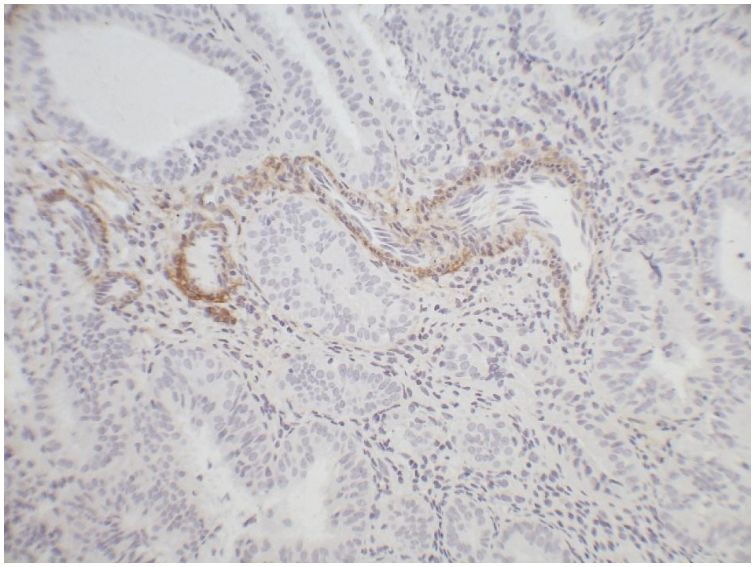
BM components and the panel of stem cell markers were also studied on grade I (n=5), grade II (n=5) and grade III (n=3) endometrioid adenocarcinoma tissue specimens. Due to the disorganisation of uterine tissue seen in this pathological condition, similar quantitative and semi quantitative analysis previously performed on cycling and PM endometrial specimens could not be performed. Therefore only descriptive analysis was made to assess endometrial expression of these markers in this subject group. Varying levels of disorganisation were seen between grade types of endometrial cancer samples, with grade 1 having the least amount of disorganisation and grade 3 having the most, as described in the FIGO classification.

6.1 - Expression of Endometrial Basement Membrane Components in Patients with Endometrial Cancer

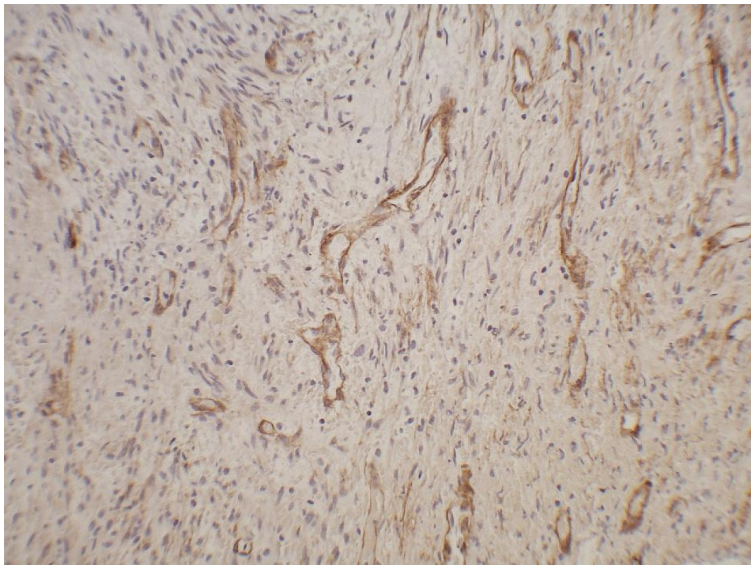
COLIV

In virtually all grade I and II endometrial cancer samples, all glandular structures failed to express COLIV immuno-staining in the supporting BM. As glandular architecture was lost in Grade III endometrial cancer sample, the supporting glandular BM could not be analysed. Vessels in all endometrial cancer samples were identified, even though their normal architecture was altered, and were immuno-positive for COLIV in most samples. Particularly, staining intensity for COLIV in vessels was observed to be increased in ascending levels of endometrial cancer grade type, being strongest in grade III samples. This correlates with cellular staining in cancerous cells, as more cancer cells in grade III endometrial cancer samples were COLIV immuno-positive. Refer to fig 4.12.

(a) Grade I



(b) Grade II



(c) Grade III

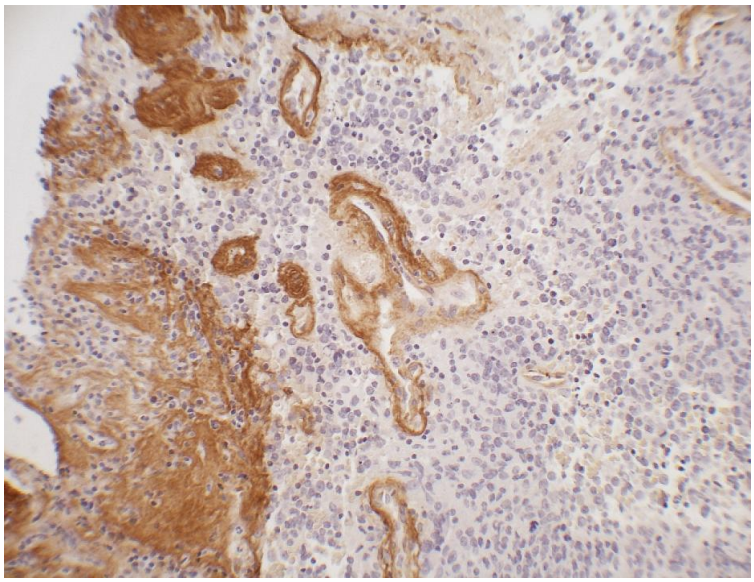
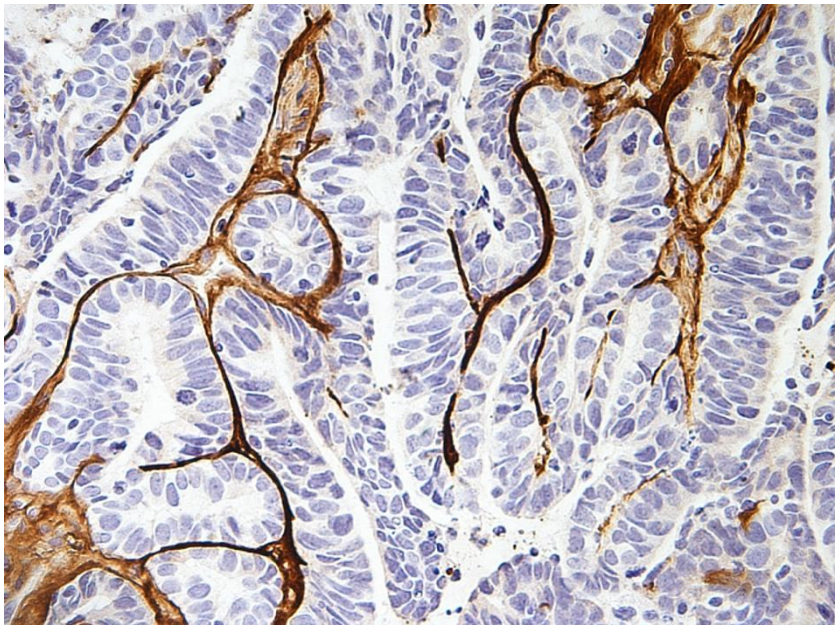


Figure 6.1. Endometrial micrographs showing COLIV immuno-localisation in (a) grade I, (b) grade II and (c) grade III endometrial cancerous tissues

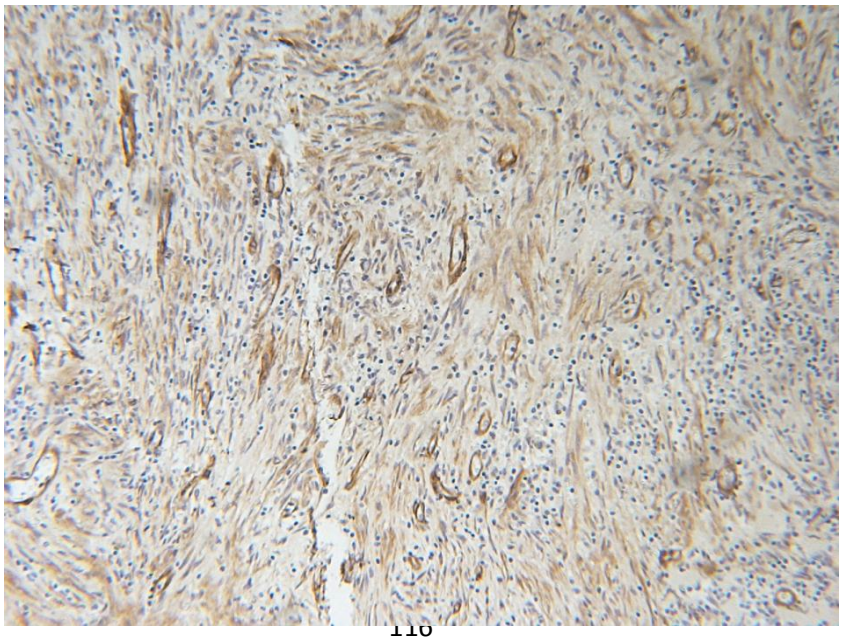
Laminin

Laminin was immuno-positive in all endometrial tissue samples collected from this group with most samples having staining intensities between moderate and strong. In virtually all grade I and II endometrial cancer samples tested, laminin appeared to be positive in elongated linear structures that either resembled BM supporting glandular epithelium or BM supporting endothelium of vessels with lost architecture. Cancer cells seen in all grade types were positive for laminin. In grade II and III samples laminin appeared to be present in BM supporting endothelium of more regular looking vessels and also in the intercellular spaces separating cancerous cells. Refer to fig 4.13

(a) Grade I



(b) Grade II



(c) Grade III

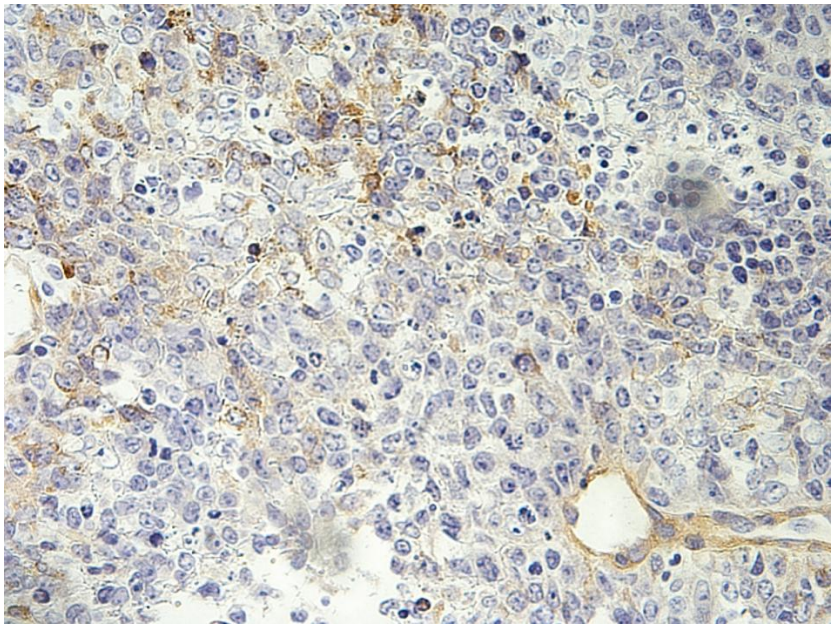


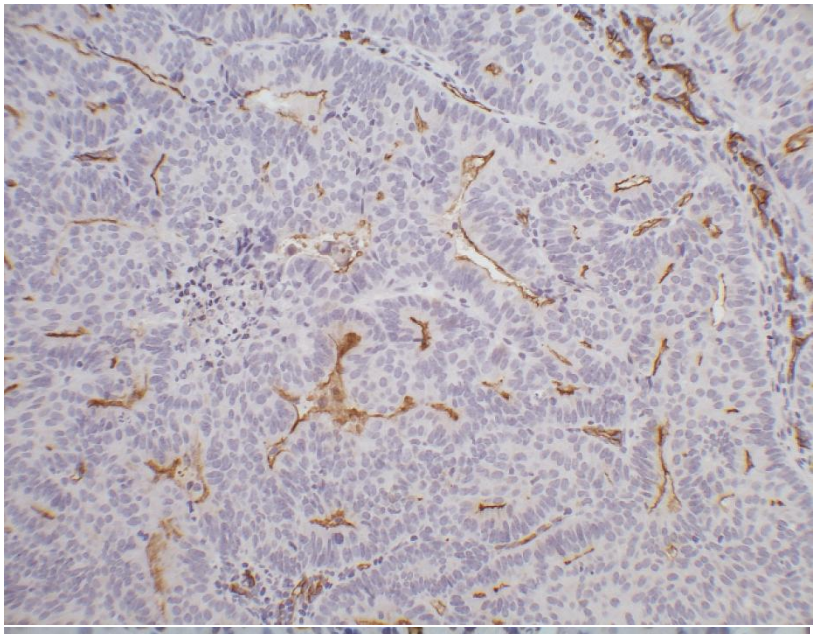
Figure 6.2: Endometrial micrographs showing laminin immuno-localisation in (a) grade I, (b) grade II and (c) grade III endometrial cancerous tissues

6.2 - Expression of Endometrial Stem Cell Markers in Patients with Endometrial Cancer

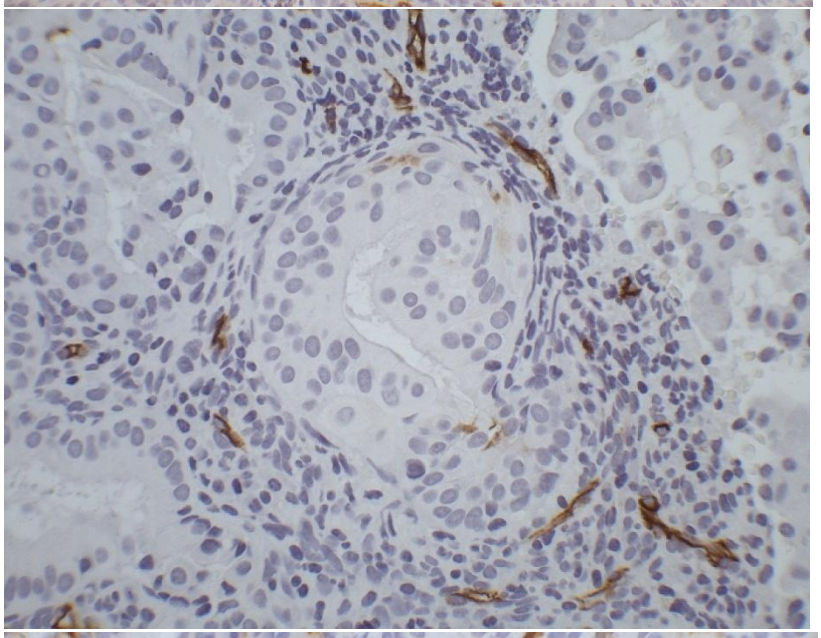
PODXL

All endometrial cancer samples were also tested for PODXL. Cancerous cells in each sample were identified, however, most were observed to be immuno-negative for PODXL. One grade 2 sample appeared to be cellular staining in cancerous cells, see figure 4.14(c). In PM samples, PODXL was densely localised in glandular secretions and in the vascular endothelium, as also seen in the fertile control and endometriosis groups. However, as endometrial tissue samples from endometrial cancer subjects is very disrupted, glands even still present in grade 1 and grade 2 samples were difficult to analyse. Nonetheless, in some samples PODXL staining was still present in glandular secretions. PODXL staining was mainly seen in the vessels in all samples tested. Staining intensities for PODXL staining in vascular endothelium ranged from moderate to strong in this subject group.

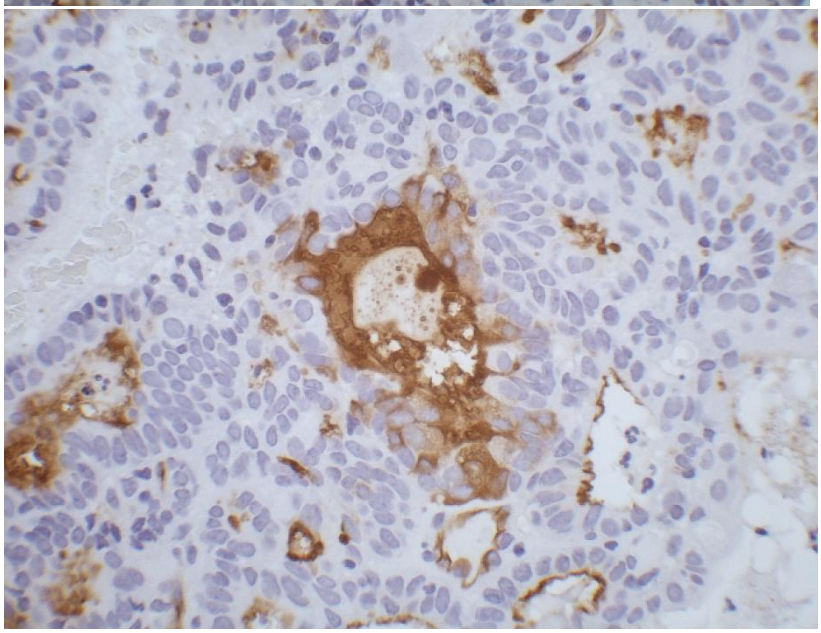
(a) Grade I



(b) Grade II



(c) Grade III



Grade III

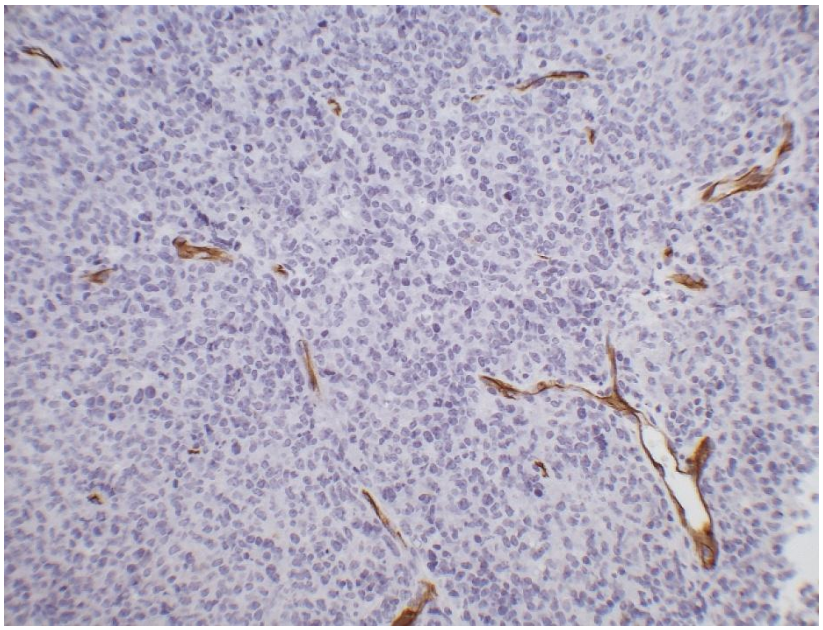
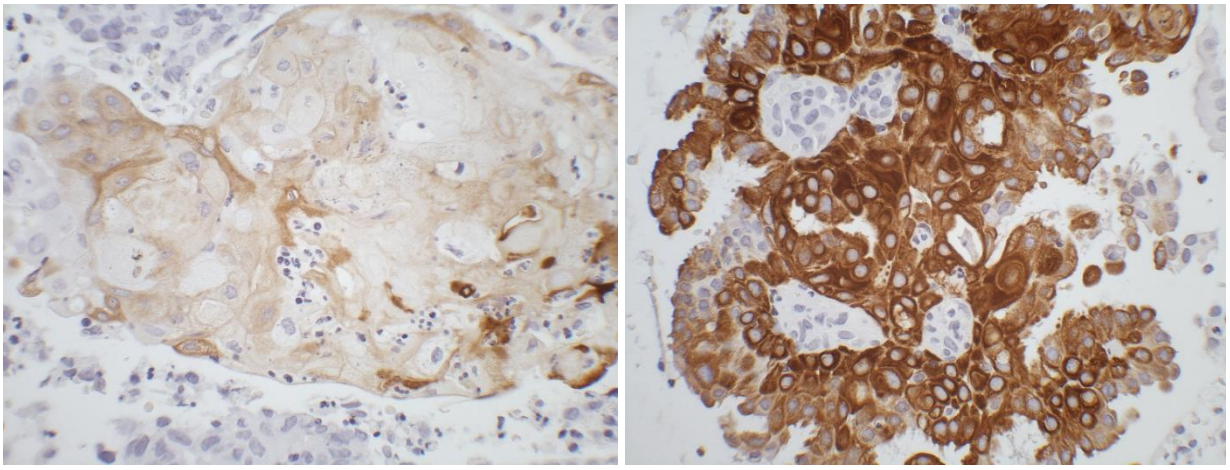


Figure 6.3: Endometrial micrographs showing PODXL immuno-localisation in (a) grade I, (b,c) grade II and (d) grade III endometrial cancerous tissues

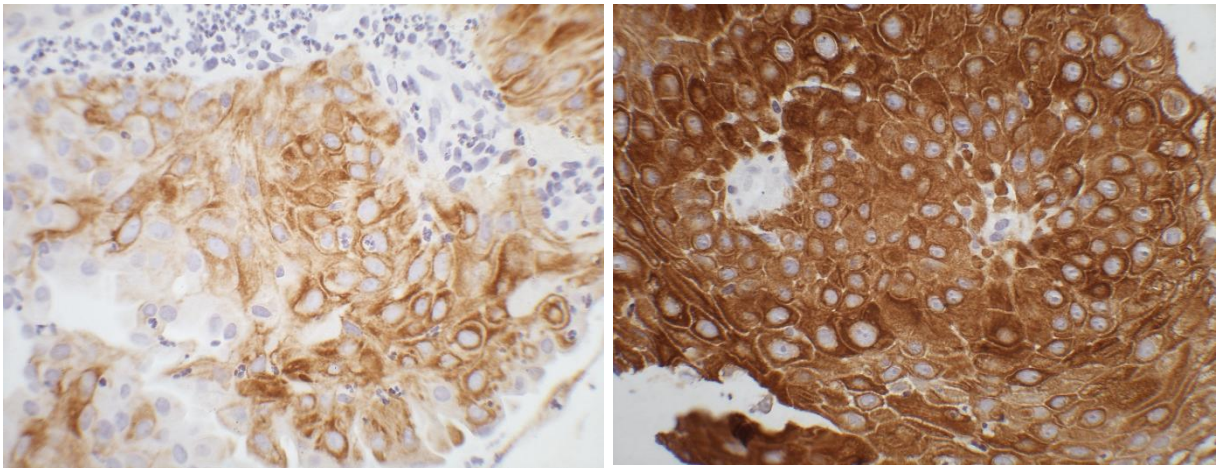
Cytokeratin 5/6

No differences were seen when comparing the CK5/6 immuno-positive staining between grade types of endometrial cancer. Most samples contained weak staining, but at least one sample from each grade type (refer to right hand column of figure 4.15) contained small frequent clusters of moderate-strong CK5/6+ cellular staining in cancerous cells.

(a) Grade I



(b) Grade II



(c) Grade III

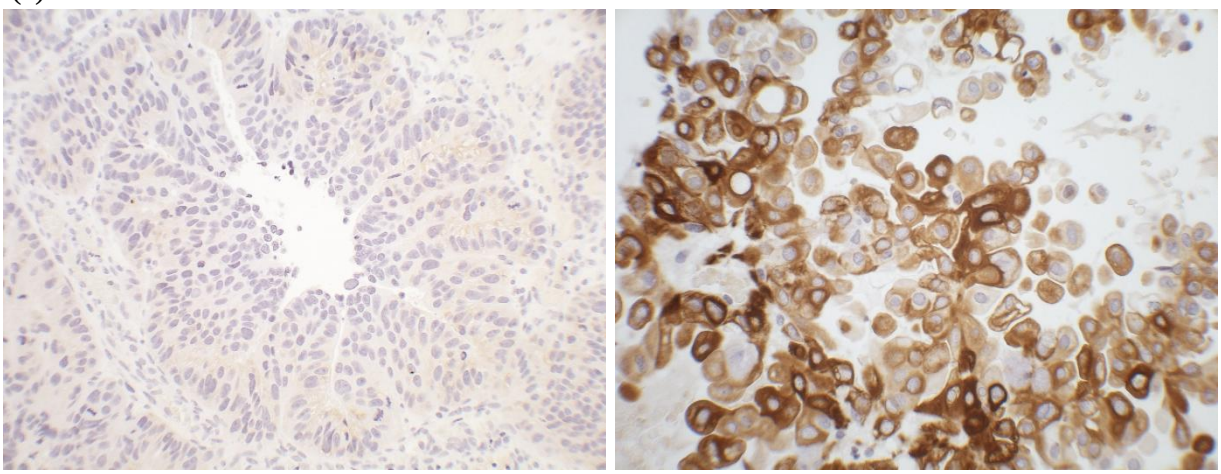
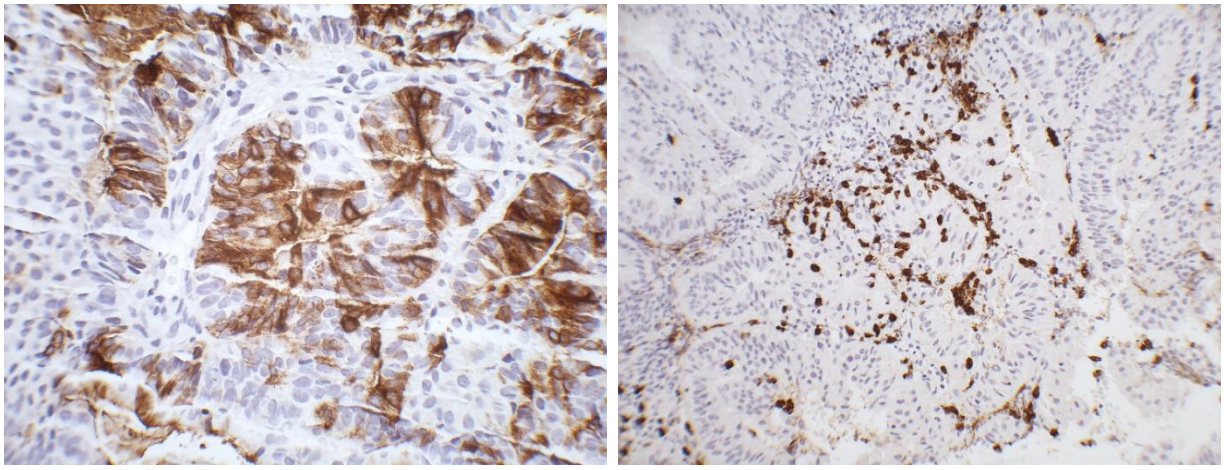


Figure 6.4: Endometrial micrographs showing CK5/6 immuno-localisation in (a) grade I, (b) grade II and (c) grade III endometrial cancerous tissues

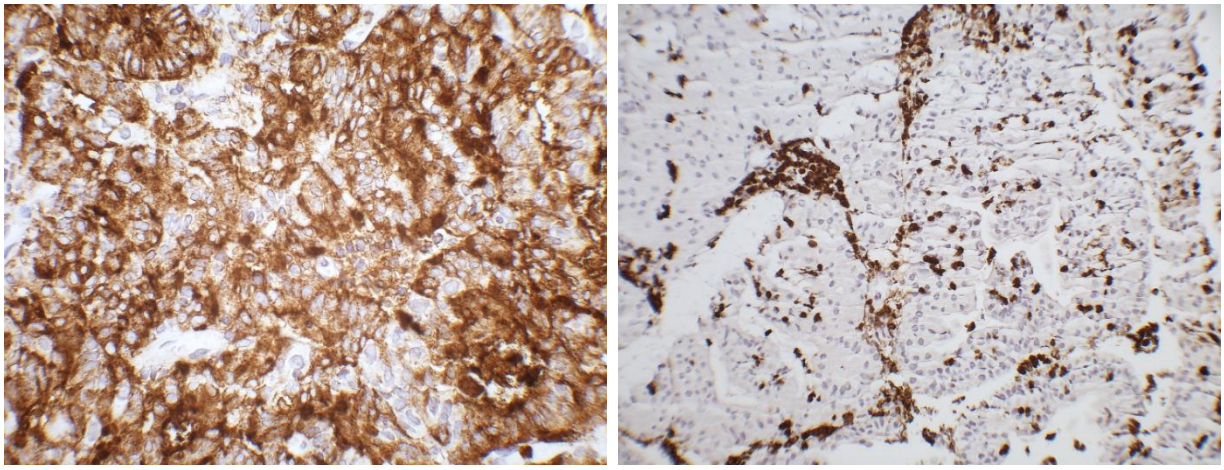
SSEA1

In grade I endometrial cancer samples, architecture of glandular epithelium was altered. However, like in some PM biopsy sections, cells were still intermittently stained within the glandular epithelium. Cellular staining of cancerous cells was mainly seen in grade II and III samples. A couple of endometrial cancer samples from each grade type contained small dense aggregates of cells that were strongly SSEA1+ immuno-stained (refer to right column of figure 4.16).

Grade I



Grade II



Grade III

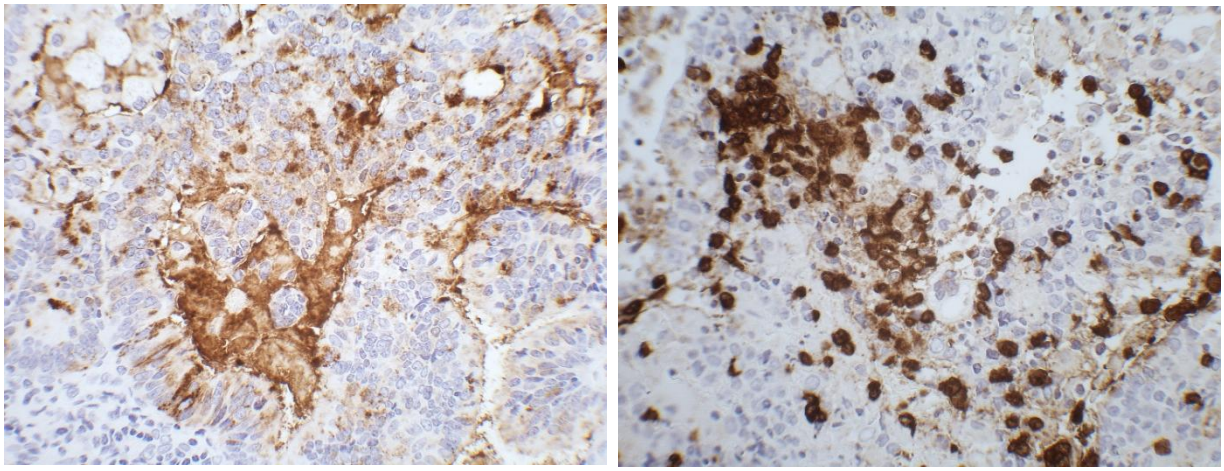


Figure 6.5: Endometrial micrographs showing SSEA1 immuno-localisation in (a) grade I, (b) grade II and (c) grade III endometrial cancerous tissues

Chapter Seven:

DISCUSSION

This was an IHC study exploring the expression of a panel of stem cell markers in conjunction with examining the BM integrity in the endometrium of normal fertile women along the menstrual cycle; of women with the benign metastatic disease endometriosis and of women with the malignant metastatic disease of endometrioid adenocarcinoma.

7.1. Fertile Control Group

Immuno-located COLIV and laminin in endometrial BM was visualised as narrow continuous bands supporting the luminal and glandular epithelium, and vascular endothelium throughout all phases of the menstrual cycle. Within the *fertile control group*, COLIV expression in BM was distinctly altered during the menstrual cycle, with expression peaking in different endometrial structures at different time points of the menstrual cycle. During the ProlP, COLIV expression appeared to be high, although Tanaka et al reported a contradictory decrease of COLIV in the late ProlP in comparison to other cycle phases(35). This contradictory finding could be due to the fact that most of our proliferative endometrial samples were from women in the early- or mid-ProlP. Therefore we could not comment on how the COLIV in BM was altered within the ProlP. Our results show that COLIV expression in the BM supporting the luminal epithelium was significantly lower in the MSP than in other phases of the menstrual cycle. This agrees with previous studies that suggests breakage of BM at the luminal epithelium is necessary for successful implantation of the invasive blastocyst(s)(139). Highest COLIV levels seen in the BM, supporting the glandular and the luminal epithelium during the LSP, also agree with the previous study reported by Tanaka et al(35). As COLIV has shown to inhibit endometrial epithelial cell growth whilst

enhancing epithelial apoptosis, it's dominance during the LSP may suggest a role in preparing the endometrium for the inevitable process of menstruation where endometrium is disrupted and shed at the absence of embryo implantation(35). Expression and activity of endometrial MMPs, proteolytic enzymes that are responsible for breaking down ECM components, are shown to be minimal during the secretory phase(140). Therefore, high expression of COLIV observed in the LSP further validates that MMP activity is suppressed during this period and they will be subsequently up regulated during the actual menstrual shedding.

Tanaka et al, previously showed that laminin is significantly decreased in the BM supporting the luminal epithelium during the secretory phase(35). We also found a similar decrease in laminin expression during the MSP in fertile controls; in our study however, this reduction was found in the BM supporting the glandular epithelium. During the proliferative phase, stromal cells, unlike endometrial epithelial cells, are thought to produce the BM component laminin. Laminin is then transported to the intercellular spaces first and subsequently to the sub-epithelial BM structures(38). However, during the secretory phase laminin mRNA signal appears stronger in stromal cells, particularly in cells adjacent to glandular structures, and has been shown to play a key role in endometrial pre-decidualisation to prepare for embryo implantation(38). Therefore, since the role of laminin in the stroma becomes more important in order to prepare for decidualisation during the MSP, transportation of laminin to the BM supporting structures such as the glandular epithelium, may not occur to the similar extent seen in the ProlP and this is reflected in our results.

In contrast, laminin expression in the BM supporting the luminal epithelium and vascular endothelium remained to be similar throughout proliferative and secretory phases of the menstrual cycle. Laminin is an important protein in regulating intracellular signalling involved in angiogenesis and vasculogenesis(141). Therefore the observed persistent expression of laminin around vessels throughout all the phases of the menstrual cycle

indicates its crucial role in endometrial angiogenesis in the ProlP and in differentiation and remodelling which happens during the secretory phase. BM components supporting the luminal epithelium is needed to modulate cell recruitment, and migration in preparation for embryo-maternal recognition leading to implantation(37). One particular study showed that COLIV and laminin were virtually immuno-absent in endometrium sampled from women with unexplained infertility(37). This strongly suggests that the absence of these crucial ECM components may have a negative effect on the events leading to endometrial receptivity leading to implantation failure. Even though BM is needed to prepare the luminal epithelium for implantation, according to previous authors, a reduction/disruption of the BM components during the MSP allows embryo invasion and implant on the luminal epithelium. However, this study did not see laminin to be reduced during the window of implantation, suggesting either mainly COLIV is down-regulated in the luminal epithelium to coordinate endometrial receptivity to implantation or the reduction of laminin may only occur upon the endometrial-embryo interaction. A thorough examination of the initial events surrounding endometrial-embryo interaction, employing one of the in-vitro culture systems and embryos, will help to answer this question conclusively(142).

CK5/6 has shown to be a marker of undifferentiated cells in the human breast (myoepithelial cells), that is very similar to the endometrium in which they both undergo remarkable morphological changes in response to ovarian steroid hormones(80). CK5/6 was present in glandular and luminal epithelium in the endometrium; however expression did not change across the cycle. As CK5/6 was negatively correlated with COLIV expression in BM supporting the luminal epithelium, this suggests CK5/6 may have a role in the events that prepare for endometrial receptivity to an implanting embryo.

SSEA1 is a cell surface carbohydrate antigen and is the first cellular component encountered by approaching cells and molecules(99). This antigen is known to be present on pluripotent EC, ES and multi-potenital cells illustrating its role as a marker for undifferentiated cells,

however in humans embryos it is present on the more differentiated cells(103). Therefore, SSEA1 has been utilised to isolate multi-potential embryonic stem cells for further characterisation especially in neural stem cells(100). Unlike SSEA1, PODXL has been shown to be a marker of pluripotent stem cells in humans. Complementing its role as a stem cell marker, PODXL is emerging as a marker of human malignancy, including testicular, breast, liver, kidney and blood cell cancers(143).

Expression of both PODXL and SSEA1 in the endometrium varied significantly according to the phase of the menstrual cycle. PODXL immuno-localisation in the functionalis layer was significantly higher in glandular secretions during the ProlP in cycling women. However, PODXL significantly decreased along the menstrual cycle, being weakest in the LSP. This trend was also seen for SSEA1 expression in glandular epithelium in both basalis and functionalis layers of the endometrium. Since dynamic endometrial remodelling occurs along the cycle, the phase dependent changes in the expression of these stem cell markers may indicate a functional relevance. During the ProlP, the functionalis layer undergoes considerable regeneration(18). The high expression of these stem/progenitor cell markers during this time suggests that potential stem/progenitor cells with known high proliferative potential are activated, contributing to the proliferation of the endometrium(51). Later, in the secretory phase, the proliferation in endometrial epithelium is terminated as progesterone exerts its effects on the oestrogen primed endometrium, and induces cellular differentiation(20). Therefore we expect that endometrial stem/progenitor cellular activity ceases from the secretory phase onwards, and this is represented by the significant reduction of endometrial expression of stem call markers PODXL and SSEA1. Even at a comparatively low level, stem cell markers are still expressed during the secretory phase, suggesting that possible endometrial stem/progenitor cells are still present due to their known clonogenic potential in the endometrium(51). In mouse EC cells, FUT4 has been shown to be involved in the regulation of SSEA1(101). Ponnampalam and Rogers have used IHC to test the immuno-

expression of FUT4 in the endometrium. Interestingly they showed that FUT4 localises in the glandular and luminal epithelium which is significantly up-regulated during the ESP and MSP compared with other phases of the menstrual cycle(106). Since we have shown that SSEA1 is significant down-regulated during the secretory phase (MSP and LSP) FUT4 may have a significant functional relevance controlling the expression of endometrial SSEA1. In addition to endometrial regeneration, SSEA1 may also have a role in implantation due to its significant reduction in the BM supporting the luminal epithelium during the MSP. It is likely that may FUT4 regulate the expression of this carbohydrate antigen that facilitate the initial attachment of blastocysts to endometrial epithelium(106)

Overall, these phase dependent changes indicates that circulating levels of ovarian hormones may have regulatory control of the activation of endometrial stem cells, as seen in mammary stem cells, in order to control the endometrial events that lead to implantation(144).

Since there is a positive correlation between PODXL and SSEA1 expression in the endometrial samples tested, this may indicate that these two proteins co-exist in the same endometrial stem/progenitor cell and therefore maybe functionally related. However, functional experiments need to be carried to investigate this further. The expression of these known stem cell markers in both basalis and functionalis layers of the endometrium also encourages the idea that endometrial stem/progenitor cells not only reside in the basalis but are also present within the functionalis layer of the endometrium(55, 60).

When compared to BM components, PODXL expression showed significant negative correlation with the overall COLIV expression in BM supporting glandular epithelium. In the kidney, PODXL is detected in the lining of podocyte foot processes and is connected to glomerular BM at focal contacts, overall contributing to glomerular filtration(90, 93). At these focal contacts PODXL has shown to inhibit spreading of BM components and cell

binding, and is therefore thought to have anti-adhesive effects(93). Therefore this may explain the inverse relationship between endometrial PODXL and COLIV expression.

In contrast, SSEA1 positive functionalis glands positively correlates with both COLIV and laminin expression in BM supporting glandular epithelium. Within stem cell niches, specialised BM is needed to maintain stem cell properties, particularly during stem cell development(71). Thus, the positive relationship seen here suggests a favourable BM integrity is mandatory to modify the cellular behaviour of potential endometrial stem/progenitor cells, in order to function in the highly dynamic functionalis layer of the endometrium.

7.2. Endometriosis Group

In contrast with the fertile control group, no cyclic variation of BM components (COLIV and laminin) was seen in the endometriosis group. Consequently, apparent differences in *laminin* expression were observed between the endometriosis and fertile control groups. These differences included reduced laminin expression in the BM supporting the glandular epithelium, and higher expression in the BM supporting the vascular endothelium in the endometriosis group when compared to the fertile controls.

During the menstrual phase, increased disruption of both functionalis and basalis layers of eutopic endometrium, have been reported in women with endometriosis(130). Laminin is a key protein needed for the initial BM assembly and is a crucial ECM component providing structural support to tissues(32). The decreased laminin expression observed in the BM supporting the glandular epithelium indicates there is altered BM integrity in endometriosis patients, therefore making endometrium more prone to extensive disruption during menstruation. The reduced expression seen may either be due to lack of production of laminin or up-regulation of MMPs and other proteolytic agents in eutopic endometrium from women with endometriosis, events which have also been postulated to occur in endometrial

cancer(48). As experiments have shown that the exposure of sub-epithelial structures is important for cell adhesion events, extensive exfoliation of endometrium is more likely to result in attachment of endometrial debris onto the peritoneal environment when transported in a retrograde fashion(114).

In contrast to the reduced expression in BM supporting glands, an apparent increase in laminin expression has also been observed in BM supporting vessels when compared to fertile controls. Given that laminin is an important protein needed for the intracellular signalling to modulate angiogenesis, the increase in BM supporting vessels may indicate increased angiogenic activity in the endometrium of women with endometriosis(141). Enhanced endometrial angiogenic activity in endometriosis has previously been reported and is thought to contribute to development of ectopic lesions(145, 146).

COLIV expression in the eutopic endometrium in women with endometriosis remained consistent throughout the phases of the menstrual cycle studied, with no particular increase in the LSP, as seen in the fertile controls. This may reflect the attenuated MMP activity seen in eutopic endometrium of women with endometriosis, especially MMP9 that preferentially degrades BM components(147). Increased MMP activity in the endometrium is thought to lead to endometriosis as it increases the invasive properties of the endometrium leading to ectopic implantation after retrograde menstruation(148). Additionally, a previously described study reported absent immuno-localisation of both COLIV and laminin in the endometrium sampled from women with unexplained infertility(37). Due to the absence of demographic information gathered from some of the study participants and study sample sizes we were unable to study the differences of endometrial COLIV and laminin expression between endometriosis patients with and without infertility.

Endometrial expression of PODXL and CK5/6 in the endometriosis group was similar to the fertile control group, with PODXL expression being highest in the ProlP and decreasing along the cycle and no changes in CK5/6 seen across the cycle.

In contrast, SSEA1 expression in the basalis layer of the endometrium was similar across all phases of the menstrual cycle studied. As the endometrial stem/progenitor cells are thought to mainly reside in the basalis layer of the endometrium, the unchanged stem cell marker SSEA1 in the basal layer across the menstrual cycle suggests that stem cell activity also remains unchanged. This is in comparison to the reduced SSEA1 expression in the secretory phase in fertile controls, when it is expected that stem cell activity is thought to be decreased after the ProlP. The persistence of SSEA1 expression into the secretory phase may contribute to the pathophysiology of endometriosis. Leyendecker et al showed that there is increased exfoliation of both basal and functional layers of eutopic endometrium in women with endometriosis during the menstrual phase(130). Our study also supports this finding with evidence of altered endometrial basal lamina seen in the endometriosis group. In addition Hyodo et al conducted a study that showed endometrial injury can also potentially increase SP cells in the endometrium(149). Altogether these previously reported findings, in conjunction with our results, may suggest that there is increased retrograde transportation of basal endometrium that has altered stem cell activity. Consequently, this may contribute to the endometrial growth of ectopic implants contributing to the pathophysiology of endometriosis. To date there is no direct evidence that stem cell activity has a role in the pathogenesis of endometriosis. However, we have shown that SSEA1 is a possible marker of increased endometrial stem/progenitor stem cell activity and this may utilised in the future to implement the characterisation of stem/progenitor cells in ectopic implants. The positive relationship between laminin and PODXL with SSEA1 expression in the endometrium seen in fertile control group, also persists in the endometriosis group indicating the possible regulation between these components still functions in endometriosis.

7.3 Post Menopausal Group

As reported in previous studies, laminin was localised in the BM supporting structures in the oestrogen and progesterone deprived PM endometrium(48). Additionally, there were no significant differences in laminin expression when compared to the fertile control group. This reinforces the assumption that laminin synthesis is constant and regulation is independent of circulating ovarian hormones. However, in contrast with previous reports, COLIV appeared to be present in the PM samples tested, with expression in BM supporting glandular and luminal epithelium similar to mostly all phases of the menstrual cycle tested in fertile controls. Whereas, COLIV expression in BM supporting vascular endothelium was seen to be significantly lower in the PM group when compared to the fertile control group and this was also seen for laminin in a previous report(35). This may be due to the lack of circulating ovarian hormones altogether stimulating less angiogenic activity therefore resulting in less COLIV and/or laminin production in BM supporting endothelial cells.

The transition of PM non-functional endometrium to atrophic endometrium can take up to several years, with proliferative activity persisting for many years(7). The similar change in staining intensities of stem cell markers SSEA1 and PODXL expression in PM endometrium with the basal endometrium taken during the ProlP from cycling women provides confirmatory evidence of this persisting proliferative activity. As previously described, the functional inactivity of PM endometrium is expected to resemble the basalis layer of endometrium of cycling women, where endometrial stem/progenitor cells are thought to reside regenerating the functionalis after menstruation. Since SSEA1 expression in PM endometrium was significantly similar to the ProlP of cycling women, this suggests that stem cells still remain in the endometrium after menopause. This is supported with evidence that the clonogenic activity does not differ between endometrial samples from reproductive and post-menopausal women(61, 64). These stem cells may be regulated by ovarian hormones

since endometrial growth has show to occur when PM women take exogenous oestrogen therapy.

7.4 Endometrial Cancer Group

This study confirms the disruption of both COLIV and laminin BM components in the malignant metastatic disease of endometrial cancer. As we have shown that eutopic endometrium from women with endometriosis have disrupted BM, this further suggests the similar characteristics endometriosis has with malignant disorders.

A small number of cells within the tumours have shown to have stem cell properties that contribute to its growth and metasteses and they are referred to as CSCs, and this has been reported to occur in endometrial cancer. The strong immuno-positivity of CK5/6 and SSEA1 seen in areas of cancerous cells suggests that they may be possible markers of these CSCs that may have evolved from endometrial stem/progenitor cells that were also expressing these markers(51). However, this can only be confirmed by performing functional studies.

7.5 Validity of Stem/progenitor cell markers in the endometrium

Chan et al provided the first evidence for the existence of endometrial stem/progenitor cells using cell cloning studies(56). They demonstrated endometrial cell clonogenicity, as they found 0.22% of epithelial cells and 1.25% of stromal cells were able to form individual colonies when seeded at clonal density and they also demonstrated the differentiation potential of these cells(62). Based on these findings we would expect that less than 1% of endometrial epithelial cells would be stained by a putative endometrial stem cell marker. However, all these possible stem/progenitor cell markers we studied, stained more than 1% of epithelial cells in the paraffin sections. Therefore, these markers are most likely to be markers of progenitor cells that are precursors to differentiated cells. We can say that these markers to

not stain fully differentiated cells due to the inconsistent staining of endometrial epithelial cells, especially for CK5/6 and SSEA1.

7.6 Limitations of the study

No significant differences were observed in the thicknesses of BM supporting glandular epithelium between different phases of the menstrual cycle in cycling endometrium and between cycling and PM endometrium. Additionally, no significant differences in BM thicknesses were found between the fertile control and endometriosis subject groups. Although these findings could represent a true consistency of BM thickness in all fertile control, endometriosis and PM subject groups, it could also be due to factors affecting the measurement of the true BM thicknesses, masking potential differences in BM thicknesses. One major factor affecting the measurement of the true BM thickness was the angle at which the wax embedded tissue biopsies were cut using a microtome. If glands within the paraffin section were cut along the longitudinal axis or perpendicular to the longitudinal axis, staining of BM around gland would appear clean and non-hazy and therefore measurement of the thickness would be accurate. However, if glands were cut at any other angle within the section then this would lead to areas of the BM around the gland to appear hazy making the measurement of thicknesses and scoring staining intensities of the BM difficult and subject to reader bias. This was especially significant in the LSP where glands were very tortuous and the BM of majority of secretory glands was difficult to measure.

It is generally accepted that when the endometrium is sampled with an endometrial pipelle sampler, only the functional layer of eutopic endometrium is obtained. However, this is only an assumption and to date no evidence confirms that this is true, and therefore pipelle endometrial biopsies could also contain a sample of the basalis layer too. This could introduce a potential flaw in our analysis, since glands analysed for SSEA1, PODXL and BM

components in paraffin sections from pipelle samples were all considered as functionalis glands, which potentially can skew the results.

7.7 Future Work

Both laminin and COLIV were also expressed in the stroma of the endometrium. However, due to time deficiencies during the course of the project a comprehensive analysis of stromal staining could not be carried out. This analysis could form as part of future work and would include both scoring the staining intensity of stromal cells with these BM components, and additionally counting the number of immuno-positive stromal cells using assisted computer image analysis software. This analysis can be divided into both basalis and functionalis layers of the endometrium as obvious differences were seen between the two layers. By analysing the stromal staining patterns we can look for any correlation seen with the BM supporting endometrial structures that explain for changes in BM staining across the menstrual cycle.

Although this study assessed the changes in BM and stem cell activity across the menstrual cycle, more patients would need to be recruited to increase the power of the study. More endometrial samples especially taken from women in the ProlP of their menstrual cycle would be needed to observe how the BM changes along the early, mid and late stages of the ProlP. Additionally, since we conclude that it is the alteration of menstrual endometrial debris that is transported in a retrograde fashion leading to endometriosis, we also need to examine the BM integrity and expression of endometrial stem/progenitor cell markers during the menstrual phase. By increasing the sample size in the endometriosis group this will firstly allow us to explore changes of BM integrity and identification of potential endometrial stem/progenitor cells, along with the severity of endometriosis (under the AFS classification). Secondly, it will allow to us compare the expression of these components between endometriosis subjects with and without infertility. Additionally, we could also perform further IHC studies on ectopic endometriosis lesions and compare it with results from eutopic

endometrium from women with endometriosis, especially to see whether potential stem cell markers are also present in ectopic lesions. This is especially important as evidence supporting the presence of stem cells in ectopic implants is lacking. Since there is a well known link between endometriosis and ovarian cancer, we should also look at the ovarian cancer tissue and assess BM integrity along with the expression of the panel of stem cell markers. Additionally, as baboons are commonly used as a model for endometriosis development, we can perform IHC methodologies on both matched eutopic and ectopic endometrial samples collected from these animals, to observe how BM components and stem cell markers are expressed throughout the course of the disease.

Since laminin and COLIV has been previous shown to increase adhesion and proliferation in ectopic endometrial implants, we also need to assess their complement integrins since these receptors modulate the interaction between BM components and endometrial cells(1, 33). Also, we can assess the expression of CD34, a known glycoprotein to mediate the attachment of stem cells to ECM. This will further improve our understanding of how changes within the ECM structure can affect the activity of endometrial stem cells.

Our IHC methodologies have shown the presence of the BM and stem cell markers within the endometrium. In order to test whether these results are not artefact, alternative laboratory methods are needed to confirm these immunohistochemistry results. Other methods include western blotting to give both qualitative and semi-quantitative confirmatory results, and quantitative real-transcriptase polymerase chain reactions (QRT-PCR) to quantify the mRNA levels of the proteins to be tested. Since carbohydrates are formed as a result of post-translational modification of proteins, assessment of mRNA levels cannot be carried out for carbohydrate antigen SSEA1. To analyse the functional properties of the BM components and stem cell markers in endometrial tissue, in-vitro cell culture experiments are needed to assess the effects of these components on cell growth and survival. Conversely, suppressing

the genes for these components in in-vitro experiments, by using silence RNA (siRNA), allows us to analyse what effect this has on endometrial cells.

7.8 Summary

There is growing evidence that the eutopic endometrium of women with endometriosis is different from that of women without endometriosis. BM integrity is known to be altered in the malignant metastatic disease of endometrial cancer and we have shown that it is also altered in the benign metastatic disease of endometriosis, further suggesting similar characteristics between the two pathological conditions. Stem cells are increasingly becoming the focus of many areas of biomedical research. We have tested a panel of known stem cells markers including CK5/6, PODXL and SSEA1 in endometrial samples, all of which show some relationship with the expression of BM components. Interestingly, PODXL and SSEA1 are significantly up-regulated during the ProlP in the endometrium, when we expect stem cells to be most active contributing to endometrial regeneration after menstrual shedding. The aberrant expression of SSEA1 in eutopic endometrium collected from endometriosis patients may indicate an altered activity of possible endometrial stem cells, of which may contribute to its pathophysiology. Extensive research into the role of stem cells in endometriosis is still needed. It is an area difficult to investigate; nonetheless, we provide a stepping stone into providing potential endometrial stem cell markers.

APPENDIX 1
REFERENCES

1. Klemmt PA, Carver JG, Koninckx P, McVeigh EJ, Mardon HJ. Endometrial Cells from Women with Endometriosis have Increased Adhesion and Proliferative Capacity in Response to Extracellular Matrix Components: Towards a Mechanistic Model for Endometriosis Progression. Human Reproduction2007 Dec;22(12):3139-47.

2. Maatta M, Salo S, Tasanen K, Soini Y, Liakka A, Bruckner-Tuderman L, et al. Distribution of Basement Membrane Anchoring Molecules in Normal and Transformed Endometrium: Altered Expression of Laminin Gamma2 Chain and Collagen Type XVII in Endometrial Adenocarcinomas. Journal Molecular Histology2004 Nov;35(8-9):715-22.

3. Hubbard SA, Friel AM, Kumar B, Zhang L, Rueda BR, Gargett CE. Evidence for Cancer Stem Cells in Human Endometrial Carcinoma. Cancer Research 2009 Nov 1;69(21):8241-8.

4. Sasson IE, Taylor HS. Stem Cells and the Pathogenesis of Endometriosis. Annals of the New York Academy Sciences 2008 Apr;1127:106-15.

5. Kruegel J, Miosge N. Basement Membrane Components are Key Players in Specialized Extracellular Matrices. Cellular and Molecular Life Sciences Sep;67(17):2879-95.

6. Dewhurst's Textbook of Obstetrics and Gynaecology. Seventh Edition ed. Edmonds K, editor: Wiley-Blackwell; 2007.

7. Matias Jimenez-Ayala BJ-AP. Cytology of the Normal Endometrium - Cycling and Post-Menopausal. Orell SR, editor2008.

8. Spencer TE, Hayashi K, Hu J, Carpenter KD. Comparative Developmental Biology of the Mammalian Uterus. Current Topics Developmental Biology 2005;68:85-122.

9. Hillier S. The Respective Roles of Gonadotrophins on Follicular Growth and Oocyte Maturation. Journal Gynecologic and Obstetric Biological Reproduction (Paris) 2004 Oct;33(6 Pt 2):3S11-4.

10. Vegetti W, Alagna F. FSH and Folliculogenesis: From Physiology to Ovarian Stimulation. Reproductive BioMedicine Online 2006 Jun;12(6):684-94.

11. Palermo R. Differential Actions of FSH and LH During Folliculogenesis. . Reproductive BioMedicine Online 2007 Sep;15(3):326-37.

12. Messinis IE, Messini CI, Dafopoulos K. The Role of Gonadotropins in the Follicular Phase. Annals of the New York Academy Sciences Sep;1205:5-11.

13. Hillier SG. Gonadotropic Control of Ovarian Follicular Growth and Development. Molecular and Cellular Endocrinology 2001 Jun 20;179(1-2):39-46.

14. Daya S. Luteal Support: Progestogens for Pregnancy Protection. Maturitas 2009 Dec;65 Suppl 1:S29-34.

15. King AE, Critchley HO. Oestrogen and Progesterone Regulation of Inflammatory Processes in the Human Endometrium. The Journal of Steroid Biochemistry and Molecular Biology May 31;120(2-3):116-26.

16. Critchley HO, Saunders PT. Hormone Receptor Dynamics in a Receptive Human Endometrium. Reproductive Sciences 2009 Feb;16(2):191-9.

17. Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine Regulation of Menstruation. Endocrine Reviews 2006 Feb;27(1):17-46.

18. McLennan CE, Rydell AH. Extent of Endometrial Shedding During Normal Menstruation. Obstetrics and Gynecology 1965 Nov;26(5):605-21.

19. Faber M, Wewer UM, Berthelsen JG, Liotta LA, Albrechtsen R. Laminin Production by Human Endometrial Stromal Cells Relates to the Cyclic and Pathologic State of the Endometrium. American Journal of Pathology 1986 Sep;124(3):384-91.

20. William B Robertson. The endometrium. 1981. University of Michigan.

21. Streuli CH, Bailey N, Bissell MJ. Control of Mammary Epithelial Differentiation: Basement Membrane Induces Tissue-Specific Gene Expression in the Absence of Cell-cell Interaction and Morphological Polarity. Journal of Cellular Biology 1991 Dec;115(5):1383-95.

22. Faddy MJ, Gosden RG. A Model Conforming the Decline in Follicle Numbers to the Age of Menopause in Women. Human Reproduction 1996 Jul;11(7):1484-6.

23. Burger HG. The Endocrinology of the menopause. . Journal of Steroid Biochemistry and Molecular Biology 1999 Apr-Jun;69(1-6):31-5.

24. Iwahashi M, Ooshima A, Nakano R. Effects of Oestrogen on the Extracellular Matrix in the Endometrium of Postmenopausal Women. Journal of Clinical Pathology 1997 Sep;50(9):755-9.

25. LeBleu VS, Macdonald B, Kalluri R. Structure and Function of Basement Membranes. Experimental Biology and Medicine (Maywood) 2007 Oct;232(9):1121-9.

26. Beliard A, Donnez J, Nisolle M, Foidart JM. Localization of Laminin, Fibronectin, E-cadherin, and Integrins in Endometrium and Endometriosis. Fertility and Sterility 1997 Feb;67(2):266-72.

27. Dockery P, Khalid J, Sarani SA, Bulut HE, Warren MA, Li TC, et al. Changes in Basement Membrane Thickness in the Human Endometrium during the Luteal Phase of the Menstrual Cycle. Human Reproduction Update 1998 Sep-Oct;4(5):486-95.

28. Van Agtmael T, Bruckner-Tuderman L. Basement Membranes and Human Disease. Cell and Tissue Research Jan;339(1):167-88.

29. Kalluri R. Basement Membranes: Structure, Assembly and Role in Tumour Angiogenesis. Nature Review of Cancer 2003 Jun;3(6):422-33.

30. Wiradjaja F, DiTommaso T, Smyth I. Basement Membranes in Development and Disease. Birth Defects Research Part C: Embryo Today Mar;90(1):8-31.

31. Aumailley M, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, et al. A Simplified Laminin Nomenclature. Matrix Biology 2005 Aug;24(5):326-32.

32. Scheele S, Nystrom A, Durbeej M, Talts JF, Ekblom M, Ekblom P. Laminin Isoforms in Development and Disease. Journal Molecular Medicine 2007 Aug;85(8):825-36.

33. Hynes RO. Integrins: A Family of Cell Surface Receptors. Cell1987 Feb 27;48(4):549-54.

34. Tabibzadeh S. Patterns of Expression of Integrin Molecules in Human Endometrium Throughout the Menstrual Cycle. Human Reproduction 1992 Jul;7(6):876-82.

35. Tanaka T, Wang C, Umesaki N. Remodeling of the Human Endometrial Epithelium is Regulated by Laminin and Type IV Collagen. International Journal Molecular Medicine2009 Feb;23(2):173-80.

36. Aplin JD, Charlton AK, Ayad S. An Immunohistochemical Study of Human Endometrial Extracellular Matrix During the Menstrual Cycle and First Trimester of Pregnancy. Cell and Tissue Research 1988 Jul;253(1):231-40.

37. Bilalis DA, Klentzeris LD, Fleming S. Immunohistochemical Localization of Extracellular Matrix Proteins in Luteal Phase Endometrium of Fertile and Infertile Patients. Human Reproduction1996 Dec;11(12):2713-8.

38. Tanaka T, Wang C, Umesaki N. Autocrine/paracrine Regulation of Human Endometrial Stromal Remodeling by Laminin and Type IV Collagen. International Journal Molecular Medicine2008 Nov;22(5):581-7.

39. Jasonni VM, Bulletti C, Balducci M, Naldi S, Martinelli G, Galassi A, et al. The Effect of Progestin on Factors Influencing Growth and Invasion of Endometrial Carcinoma. Annals of the New York Academy Sciences 1991;622:463-8.

40. Lockwood CJ. Mechanisms of Normal and Abnormal Endometrial Bleeding. Menopause Apr 1;18(4):408-11.

41. Sillem M, Prifti S, Neher M, Runnebaum B. Extracellular Matrix Remodelling in the Endometrium and its Possible Relevance to the Pathogenesis of Endometriosis. Human Reproduction Update1998 Sep-Oct;4(5):730-5.

42. Giannelli G, Sgarra C, Di Naro E, Lavopa C, Angelotti U, Tartagni M, et al. Endometriosis is Characterized by an Impaired Localization of Laminin-5 and alpha3beta1 Integrin Receptor. International Journal Gynecololgical Cancer2007 Jan-Feb;17(1):242-7.

43. Zeng ZS, Cohen AM, Guillem JG. Loss of Basement Membrane Type IV Collagen is Associated with Increased Expression of Metalloproteinases 2 and 9 (MMP-2 and MMP-9) During Human Colorectal Tumorigenesis. Carcinogenesis1999 May;20(5):749-55.

44. Charpin C, Andrac L, Devictor B, Habib MC, Vacheret H, Xerri L, et al. Type IV Collagen Immunostaining and Computerized Image Analysis (SAMBA) in Breast and Endometrial Disorders. Histopathology1989 Jan;14(1):47-60.

45. Soini Y, Alarakkola E, Autio-Harmainen H. Expression of Messenger RNAs for Metalloproteinases 2 and 9, Type IV Collagen, and Laminin in Non-neoplastic and Neoplastic Endometrium. Human Pathology1997 Feb;28(2):220-6.

46. Galan A, O'Connor JE, Valbuena D, Herrer R, Remohi J, Pampfer S, et al. The Human Blastocyst Regulates Endometrial Epithelial Apoptosis in Embryonic Adhesion. Biology of Reproduction2000 Aug;63(2):430-9.

47. Mizuno K, Tanaka T, Umesaki N, Ogita S. Inhibition of cAMP-Mediated Decidualization in Human Endometrial Stromal Cells by IL-1beta and Laminin. Hormone and Metabolic Research1999 May;31(5):307-10.

48. Furness PN, Lam EW. Patterns of Basement Membrane Deposition in Benign, Premalignant, and Malignant Endometrium. Journal of Clinical Pathology 1987 Nov;40(11):1320-3.

49. Weissman IL. Stem cells: Units of Development, Units of Regeneration, and Units in Evolution. Cell2000 Jan 7;100(1):157-68.

50. Watt FM, Hogan BL. Out of Eden: Stem Cells and their Niches. Science2000 Feb 25;287(5457):1427-30.

51. Gargett CE. Uterine Stem cells: What is the Evidence? Human Reproduction Update2007 Jan-Feb;13(1):87-101.

52. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic Stem Cell Lines Derived from Human Blastocysts. Science1998 Nov 6;282(5391):1145-7.

53. Reubinoff BE, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, et al. Neural Progenitors from Human Embryonic Stem Cells. Nature Biotechnology2001 Dec;19(12):1134-40.

54. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, et al. Human Embryonic Stem Cells Can Differentiate into Myocytes with Structural and Functional Properties of Cardiomyocytes. Journal of Clinical Investigations2001 Aug;108(3):407-14.

55. Maruyama T, Masuda H, Ono M, Kajitani T, Yoshimura Y. Human Uterine Stem/progenitor cells: Their Possible Role in Uterine Physiology and Pathology. Reproduction Jul;140(1):11-22.

56. Chan RW, Schwab KE, Gargett CE. Clonogenicity of Human Endometrial Epithelial and Stromal Cells. Biology of Reproduction 2004 Jun;70(6):1738-50.

57. Wagers AJ, Weissman IL. Plasticity of Adult Stem Cells. Cell2004 Mar 5;116(5):639-48.

58. Blau HM, Brazelton TR, Weimann JM. The Evolving Concept of a Stem Cell: Entity or Function? Cell2001 Jun 29;105(7):829-41.

59. Quesenberry PJ, Dooner G, Colvin G, Abedi M. Stem Cell Biology and the Plasticity Polemic. Experimental Hematology2005 Apr;33(4):389-94.

60. Gargett CE, Masuda H. Adult Stem Cells in the Endometrium. Molecular Human Reproduction 2010 Nov;16(11):818-34.

61. Teixeira J, Rueda BR, Pru JK. Uterine Stem Cells. Stembook2008.

62. Gargett CE. Identification and Characterisation of Human Endometrial Stem/progenitor Cells. The Australian and New Zealand Journal of Obstetrics and Gynaecology2006 Jun;46(3):250-3.

63. Schwab KE, Gargett CE. Co-expression of Two Perivascular Cell Markers Isolates Mesenchymal Stem-like Cells From Human Endometrium. Human Reproduction 2007 Nov;22(11):2903-11.

64. Schwab KE, Chan RW, Gargett CE. Putative Stem Cell Activity of Human Endometrial Epithelial and Stromal Cells During the Menstrual Cycle. Fertility and Sterililty2005 Oct;84 Suppl 2:1124-30.

65. Snyder EY, Loring JF. A Role for Stem Cell Biology in the Physiological and Pathological Aspects of Aging. Journal of American Geriatrics Society2005 Sep;53(9 Suppl):S287-91.

66. Taylor HS. Endometrial Cells Derived from Donor Stem Cells in Bone Marrow Transplant Recipients. Journal of the American Medical Association2004 Jul 7;292(1):81-5.

67. Wilson A, Trumpp A. Bone-marrow Hematopoietic-Stem-Cell Niches. Nature Reviews Immunology2006 Feb;6(2):93-106.

68. Ito M, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, et al. Stem Cells in the Hair Follicle Bulge Contribute to Wound Repair but not to Homeostasis of the Epidermis. Nature Medicine2005 Dec;11(12):1351-4.

69. Sancho E, Batlle E, Clevers H. Signaling Pathways in Intestinal Development and Cancer. Annual Review of Cell and Development Biology2004;20:695-723.

70. Doetsch F. A Niche for Adult Neural Stem Cells. Current Opinion in Genetics and Development2003 Oct;13(5):543-50.

71. Du H, Taylor HS. Stem Cells and Female Eeproduction. Reproductive Sciences2009 Feb;16(2):126-39.

72. Li L, Xie T. Stem Cell Niche: Structure and Function. Annual Review of Cell and Development Biology 2005;21:605-31.

73. Brisken C, Duss S. Stem Cells and the Stem Cell Niche in the Breast: an Integrated Hormonal and Developmental Perspective. Stem Cell Review2007 Jun;3(2):147-56.

74. Gargett CE. The Endometrium: A Novel Source of Adult Stem/Progenitor Cells Stem cell Biology and Regenerative Medicine2009;Part IV

391-404.

75. Coulombe PA, Omary MB. 'Hard' and 'soft' Principles Defining the Structure, Function and Regulation of Keratin Intermediate Filaments. Current Opinion Cellular Biology2002 Feb;14(1):110-22.

76. Moll R, Divo M, Langbein L. The Human Keratins: Biology and Pathology. Histochemistry and Cellular Biology2008 Jun;129(6):705-33.

77. Alam H, Sehgal L, Kundu ST, Dalal SN, Vaidya MM. Novel Function of Keratins 5 and 14 in Proliferation and Differentiation of Stratified Epithelial Cells. Molecular Biology of the Cell Nov;22(21):4068-78.

78. Mohammadizadeh F, Naimi A, Rajabi P, Ghasemibasir H, Eftekhari A. Expression of Basal and Luminal Cytokeratins in Breast Cancer and their Correlation with Clinicopathological Prognostic Variables. Indian Journal Medical Sciences2009 Apr;63(4):152-62.

79. Schalken JA, van Leenders G. Cellular and Molecular Biology of the Prostate: Stem Cell Biology. Urology2003 Nov;62(5 Suppl 1):11-20.

80. Tischkowitz MD, Foulkes WD. The Basal Phenotype of BRCA1-related Breast Cancer: Past, Present and Future. Cell Cycle2006 May;5(9):963-7.

81. Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, et al. Generation of a Functional Mammary Gland from a Single Stem Cell. Nature2006 Jan 5;439(7072):84-8.

82. Bhalla A, Manjari M, Kahlon SK, Kumar P, Kalra N. Cytokeratin 5/6 Expression in Benign and Malignant Breast Lesions. Indian Journal of Patholgy and Microbiology2010 Oct-Dec;53(4):676-80.

83. Bocker W, Moll R, Poremba C, Holland R, Van Diest PJ, Dervan P, et al. Common Adult Stem Cells in the Human Breast Give Rise to Glandular and Myoepithelial Cell Lineages: A New Cell Biological Concept. Laboratory Investigation2002 Jun;82(6):737-46.

84. Boecker W, Buerger H. Evidence of Progenitor Cells of Glandular and Myoepithelial Cell Lineages in the Human Adult Female Breast Epithelium: A New Progenitor (Adult Stem) Cell Concept. Cell Proliferation2003 Oct;36 Suppl 1:73-84.

85. Deugnier MA, Faraldo MM, Janji B, Rousselle P, Thiery JP, Glukhova MA. EGF Controls the In Vivo Developmental Potential of a Mammary Epithelial Cell Line Possessing Progenitor Properties. Journal of Cell Biology2002 Nov 11;159(3):453-63.

86. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In Vitro Propagation and Transcriptional Profiling of Human Mammary Stem/progenitor Cells. Genes and Development2003 May 15;17(10):1253-70.

87. Clarke CL, Sandle J, Parry SC, Reis-Filho JS, O'Hare MJ, Lakhani SR. Cytokeratin 5/6 in Normal Human Breast: Lack of Evidence for a Stem Cell Phenotype. Journal of Pathology2004 Oct;204(2):147-52.

88. Sun P, Yuan Y, Li A, Li B, Dai X. Cytokeratin Expression During Mouse Embryonic and Early Postnatal Mammary Gland Development. Histochemistry and Cellular Biology2009 Feb;133(2):213-21.

89. Kershaw DB, Thomas PE, Wharram BL, Goyal M, Wiggins JE, Whiteside CI, et al. Molecular Cloning, Expression, and Characterization of Podocalyxin-like protein 1 from Rabbit as a Transmembrane Protein of Glomerular Podocytes and Vascular Endothelium. The Journal of Biological Chemistry1995 Dec 8;270(49):29439-46.

90. Kershaw DB, Beck SG, Wharram BL, Wiggins JE, Goyal M, Thomas PE, et al. Molecular Cloning and Characterization of Human Podocalyxin-like Protein. Orthologous

Relationship to Rabbit PCLP1 and Rat Podocalyxin. The Journal of Biological Chemistry1997 Jun 20;272(25):15708-14.

91. Seiler MW, Venkatachalam MA, Cotran RS. Glomerular Epithelium: Structural Alterations Induced by Polycations. Science1975 Aug 1;189(4200):390-3.

92. Seiler MW, Rennke HG, Venkatachalam MA, Cotran RS. Pathogenesis of Polycation-induced Alterations ("fusion") of Glomerular Epithelium. Laboratory Investigation1977 Jan;36(1):48-61.

93. Economou CG, Kitsiou PV, Tzinia AK, Panagopoulou E, Marinos E, Kershaw DB, et al. Enhanced Podocalyxin Expression Alters the Structure of Podocyte Basal Surface. Journal of Cellular Science2004 Jul 1;117(Pt 15):3281-94.

94. Doyonnas R, Kershaw DB, Duhme C, Merkens H, Chelliah S, Graf T, et al. Anuria, Omphalocele, and Perinatal Lethality in Mice Lacking the CD34-related Protein Podocalyxin. The Journal of Experimental Medicine2001 Jul 2;194(1):13-27.

95. Sassetti C, Van Zante A, Rosen SD. Identification of Endoglycan, a Member of the CD34/podocalyxin Family of Sialomucins. The Journal of Biological Chemistry 2000 Mar 24;275(12):9001-10.

96. Cheung HH, Davis AJ, Lee TL, Pang AL, Nagrani S, Rennert OM, et al. Methylation of an Intronic Region Regulates miR-199a in Testicular Tumor Malignancy. Oncogene2011 Mar 7.

97. Bhattacharya B, Miura T, Brandenberger R, Mejido J, Luo Y, Yang AX, et al. Gene Expression in Human Embryonic Stem Cell Lines: Unique Molecular Signature. Blood2004 Apr 15;103(8):2956-64.

98. Nielsen JS, McNagny KM. The Role of Podocalyxin in Health and Disease. The Journal of American Society of Nephrology2009 Aug;20(8):1669-76.

99. Lanctot PM, Gage FH, Varki AP. The Glycans of Stem Cells. Current Opinion in Chemical Biology2007 Aug;11(4):373-80.

100. Yanagisawa M, Yu RK. The Expression and Functions of Glycoconjugates in Neural Stem Cells. Glycobiology2007 Jul;17(7):57R-74R.

101. Muramatsu T. Developmentally Regulated Expression of Cell Surface Carbohydrates During Mouse Embryogenesis. Journal of Cellular Biochemistry1988 Jan;36(1):1-14.

102. Muramatsu T, Muramatsu H. Carbohydrate Antigens Expressed on Stem Cells and Early Embryonic Cells. Glycoconjugate Journal2004;21(1-2):41-5.

103. Kannagi R, Cochran NA, Ishigami F, Hakomori S, Andrews PW, Knowles BB, et al. Stage-specific Embryonic Antigens (SSEA-3 and -4) are Epitopes of a Unique Globo-series Ganglioside Isolated from Human Teratocarcinoma Cells. The EMBO Journal1983;2(12):2355-61.

104. Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, et al. Derivation of Pluripotent Stem Cells from Cultured Human Primordial Germ Cells. Proceedings of the National Acaddemy of Sciences of the United States of America 1998 Nov 10;95(23):13726-31.

105. Klassen H, Schwartz MR, Bailey AH, Young MJ. Surface Markers Expressed by Multipotent Human and Mouse Neural Progenitor Cells Include Tetraspanins and Non-protein Epitopes. Neuroscience Letters2001 Oct 26;312(3):180-2.

106. Ponnampalam AP, Rogers PA. Expression and Regulation of Fucosyltransferase 4 in Human Endometrium. Reproduction2008 Jul;136(1):117-23.

107. Endometriosis, Clinical Knowledge Summaries (June 2009).

108. Verkauf BS. Incidence, Symptoms, and Signs of Endometriosis in Fertile and Infertile Women. Journal of Floride Medical Association1987 Sep;74(9):671-5.

109. JA S. Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity

American Journal of Obstetrics and Gynaecology1927;14:422-69.

110. Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde Menstruation in Healthy Women and in Patients with Endometriosis. Obstetrics and Gynecology1984 Aug;64(2):151-4.

111. Blumenkrantz MJ, Gallagher N, Bashore RA, Tenckhoff H. Retrograde Menstruation in Women Undergoing Chronic Peritoneal Dialysis. Obstetrics and Gynecology1981 May;57(5):667-70.

112. Fazleabas AT. A Baboon Model for Inducing Endometriosis. . Methods in Molecular Medicine2006;121:95-9.

113. Hapangama DK, Turner MA, Drury J, Heathcote L, Afshar Y, Mavrogianis PA, et al. Aberrant Expression of Regulators of Cell-fate Found in Eutopic Endometrium is Found in Matched Ectopic Endometrium Among Women and in a Baboon Model of Endometriosis. Human Reproduction2010 Nov;25(11):2840-50.

114. Caroline Overton CD, Lindsay McMillan, Robert W Shaw. The Atlas of Endometriosis. Third Edition ed: Informa Healthcare; 2007.

115. Batt RE, Smith RA, Buck GM, Severino MF, Naples JD. Mullerianosis. Progress in Clinical Biological Research 1990;323:413-26.

116. Witz CA. Current Concepts in the Pathogenesis of Endometriosis. Clinical Obstetrics and Gynecology1999 Sep;42(3):566-85.

117. Nyhoj L, Mathiesen R, Jochumsen KM. Association Between Endometriosis and Ovarian Cancer. Ugeskr Laeger2010 Nov 29;172(48):3336-9.

118. Sawada M, Shii J, Akedo H, Tanizawa O. An Experimental Model for Ovarian Tumor Invasion of Cultured Mesothelial Cell Monolayer. Laboratory Investigation1994 Mar;70(3):333-8.

119. Endometriosis and Infertility. Fertility and Sterility2006 Nov;86(5 Suppl 1):S156-60.

120. Bulun SE. Endometriosis. The New England Journal of Medicine2009 Jan 15;360(3):268-79.

121. Revised American Society for Reproductive Medicine Classification of Endometriosis: 1996. Fertility and Sterility1997 May;67(5):817-21.

122. Bergqvist A, Ljungberg O, Myhre E. Human Endometrium and Endometriotic Tissue Obtained Simultaneously: A Comparative Histological Study. International Journal of Gynecologic Pathology1984;3(2):135-45.

123. Harrington DJ, Lessey BA, Rai V, Bergqvist A, Kennedy S, Manek S, et al. Tenascin is Differentially Expressed in Endometrium and Endometriosis. Journal of Pathology1999 Jan;187(2):242-8.

124. Stovall DW, Anners JA, Halme J. Immunohistochemical Detection of Type I, III, and IV Collagen in Endometriosis Implants. Fertility and Sterility1992 May;57(5):984-9.

125. Gaetje R, Kotzian S, Herrmann G, Baumann R, Starzinski-Powitz A. Invasiveness of Endometriotic Cells in Vitro. Lancet1995 Dec 2;346(8988):1463-4.

126. Hemachandran M, Nijhawan R, Srinivasan R, Rajwanshi A. Collagenous Bodies in Endometriotic Cysts. Diagnostic Cytopathology2004 Nov;31(5):330-2.

127. Koks CA, Groothuis PG, Dunselman GA, de Goeij AF, Evers JL. Adhesion of Menstrual Endometrium to Extracellular Matrix: the Possible Role of Integrin alpha(6)beta(1) and Laminin Interaction. Molecular Human Reproduction 2000 Feb;6(2):170-7.

128. Witz CA. Cell Adhesion Molecules and Endometriosis. Seminars in Reproductive Medicine2003 May;21(2):173-82.

129. Hapangama DK, Turner MA, Drury JA, Quenby S, Saretzki G, Martin-Ruiz C, et al. Endometriosis is Associated with Aberrant Endometrial Expression of Telomerase and Increased Telomere Length. Human Reproduction2008 Jul;23(7):1511-9.

130. Leyendecker G, Herbertz M, Kunz G, Mall G. Endometriosis Results From the Dislocation of Basal Endometrium. Human Reproduction2002 Oct;17(10):2725-36.

131. Du H, Taylor HS. Contribution of Bone Marrow-derived Stem Cells to Endometrium and Endometriosis. Stem Cells2007 Aug;25(8):2082-6.

132. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I. Endometrial cancer. Lancet2005 Aug 6-12;366(9484):491-505.

133. Saso S, Chatterjee J, Georgiou E, Ditri AM, Smith JR, Ghaem-Maghami S. Endometrial cancer. British Medical Journal2011;343:d3954.

134. Alkushi A, Abdul-Rahman ZH, Lim P, Schulzer M, Coldman A, Kalloger SE, et al. Description of a Novel System for Grading of Endometrial Carcinoma and Comparison with Existing Grading Systems. American Journal of Surgical Pathology2005 Mar;29(3):295-304.

135. Stenback F, Risteli J, Risteli L, Wasenius VM. Basement Membrane Laminin and Type IV Collagen in Endometrial Adenocarcinoma: Relation to Differentiation and Treatment. Oncology1985;42(6):370-6.

136. Pardal R, Clarke MF, Morrison SJ. Applying the Principles of Stem-cell Biology to Cancer. Nature Review on Cancer2003 Dec;3(12):895-902.

137. Kato K, Takao T, Kuboyama A, Tanaka Y, Ohgami T, Yamaguchi S, et al. Endometrial Cancer Side-population Cells Show Prominent Migration and Have a Potential to Differentiate into the Mesenchymal Cell Lineage. American Journal of Pathology 2009 Jan;176(1):381-92.

138. Schiessl B, Innes BA, Bulmer JN, Otun HA, Chadwick TJ, Robson SC, et al. Localization of Angiogenic Growth Factors and Their Receptors in the Human Placental Bed Throughout Normal Human Pregnancy. Placenta2009 Jan;30(1):79-87.

139. Blankenship TN, Given RL. Loss of Laminin and Type IV Collagen in Uterine Luminal Epithelial Basement Membranes During Blastocyst Implantation in the Mouse. The Anatomical Record1995 Sep;243(1):27-36.

140. Goldman S, Shalev E. The Role of the Matrix Metalloproteinases in Human Endometrial and Ovarian Cycles. European Journal of Obstetrics and Gynecology and Reproductive Biology2003 Dec 10;111(2):109-21.

141. Simon-Assmann P, Orend G, Mammadova-Bach E, Spenle C, Lefebvre O. Role of Laminins in Physiological and Pathological Angiogenesis. International Journal of Developmental Biology;55(4-5):455-65.

142. Rashid NA, Lalitkumar S, Lalitkumar PG, Gemzell-Danielsson K. Endometrial Receptivity and Human Embryo Implantation. Americam Journal of Reproductive Immunology Jul;66 Suppl 1:23-30.

143. Schopperle WM, DeWolf WC. The TRA-1-60 and TRA-1-81 Human Pluripotent Stem Cell Markers are Expressed on Podocalyxin in Embryonal Carcinoma. Stem Cells2007 Mar;25(3):723-30.

144. Lydon JP. Stem cells: Cues from Steroid Hormones. Nature Jun 10;465(7299):695-6.

145. Kim SH, Choi YM, Chae HD, Kim KR, Kim CH, Kang BM. Increased Expression of Endoglin in the Eutopic Endometrium of Women with Endometriosis. Fertility and Sterility2001 Nov;76(5):918-22.

146. Kumar P, Wang JM, Bernabeu C. CD 105 and Angiogenesis. Journal of Pathology1996 Apr;178(4):363-6.

147. Chung HW, Wen Y, Chun SH, Nezhat C, Woo BH, Lake Polan M. Matrix Metalloproteinase-9 and Tissue Inhibitor of Metalloproteinase-3 mRNA Expression in Ectopic and Eutopic Endometrium in Women with Endometriosis: A Rationale for Endometriotic Invasiveness. Fertility and Sterility2001 Jan;75(1):152-9.

148. Pitsos M, Kanakas N. The Role of Matrix Metalloproteinases in the Pathogenesis of Endometriosis. Reproductive Sciences2009 Aug;16(8):717-26.

149. Hyodo S, Matsubara K, Kameda K, Matsubara Y. Endometrial Injury Increases Side Population Cells in the Uterine Endometrium: A Decisive Role of Estrogen. The Tohoku Journal Experimental Medicine2011;224(1):47-55.

APPENDIX 2
LREC Approval for Research Project

National Research Ethics Service
North West 2 Research Ethics Committee - Liverpool Central
Room 181
Gateway House
Piccadilly South
Manchester
M60 7LP

Telephone: 0161 237 2336
Facsimile: 0161 237 2383

05 October 2009

Dr Dharani K Hapangama
Clinical Senior Lecturer / Honorory Consultant in Obstetrics & Gynaecology
University of Liverpool
University Department, First Floor
Liverpool Women's Hospital,Crown St
Liverpool
L8 7SS

Dear Dr Hapangama

Study Title:	**The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis**
REC reference number:	**09/H1005/55**
Protocol number:	**1.0**

Thank you for your letter of 14 September 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC. A list of the sub-committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research

governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		
REC application	2.2	
Protocol	1.0	02 July 2009
Investigator CV		
GP/Consultant Information Sheets		
Letter from Sponsor		02 July 2009
Referees or other scientific critique report		
Participant Information Sheet	2	09 September 2009
Participant Consent Form	2	09 September 2009
poster healthy + baby		
poster endometriosis		
Response to Request for Further Information		14 September 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of

changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H1005/55	Please quote this number on all correspondence

Yours sincerely

Professor Sobhan Vinjamuri
Chair

Email: carol.ebenezer@northwest.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers"

Copy to: *Mrs Gillian Vernon*

NHS

National Research Ethics Service

North West 2 Research Ethics Committee - Liverpool Central
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Tel: 0161 625 7818
Fax: 0161 237 9427

22 March 2010

Dr D Hapangama
Clinical Lecturer
Liverpool Women's Hospital
Crown St
Liverpool
L8 7SS

Dear Dr Hapangama

Study title: **Telomere Length, Expression of Telomerase and hTert in Normal and Pathological Endometrium**

REC reference: **04/Q1505/112**

This study was given a favourable ethical opinion by the Committee on 15 February 2004.

It is a condition of approval by the Research Ethics Committee that the Chief Investigator should submit a progress report for the study 12 months after the date on which the favourable opinion was given, and then annually thereafter. To date, the Committee has not yet received the annual progress report for the study, which was due on 14 February 2010. It would be appreciated if you could complete and submit the report by no later than 22 April 2010.

Guidance on progress reports and a copy of the standard NRES progress report form is available from the National Research Ethics Service website.

The NRES website also provides guidance on declaring the end of the study.

Failure to submit progress reports may lead to a suspension of the favourable ethical opinion for the study.

04/Q1505/112:	**Please quote this number on all correspondence**

Yours sincerely

C. Ebenezer (Mrs)
Committee Co-ordinator

E-mail: carol.ebenezer@northwest.nhs.uk

Copy to: *Dr Lynne Webster, Liverpool Women's Hospital*

This Research Ethics Committee is an advisory committee to North West Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Liverpool Women's NHS
NHS Foundation Trust

Crown Street
Liverpool
L8 7SS

Tel: 0151 708 9988
www.lwh.nhs.uk

14th April 2011

Dr Dharani Hapangama
Clinical Senior Lecturer / Honorary Consultant in O&G
Liverpool Women's Hospital
University Department, First Floor
Crown Street
Liverpool L8 7SS

Direct dial: 0151 702 4346
Email: Gillian.vernon@lwh.nhs.uk

Dear Dharani

ID: LWH0877 – Study of the Role of Metastasis Inducing Proteins and cell fate regulators in the pathogenesis of Endometrial Cancer

Following submission of project documents, associated paperwork and approvals to the Trust's R&D Department, I am pleased to inform you that your research project has been approved by the R&D Director. This approval relates to the documentation listed below:

- Ethics approval letter [11/H1005/4] dated 11th April 2011
- Protocol [version 1.0] 9th December 2010

The research is registered on the Trust's R&D database under the reference LWH0877, which I would be grateful if you could quote in all future correspondence regarding the project.

The Sponsor(s) of this research project under the Research Governance Framework for Health and Social Care (RGF) are the Trust and the University of Liverpool.

Having gained approval to conduct this research under the auspices of Liverpool Women's NHS Foundation Trust, you will be expected to comply with the principles and guidelines set out in ICH Good Clinical Practice and the Department of Health RGF. Please refer to your delegated duties outlined overleaf.

I would like to take this opportunity to wish you the best of luck with this research and to request a copy of the final report and any subsequent publications.

Yours sincerely

Vernon

Gillian Vernon
Research & Development Manager

Liverpool Women's

APPENDIX 3
Study Patient Information Leaflets 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
NHS Foundation Trust

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

"Endometrial stem cell Study"

Role of endometrial stem cells in endometriosis.

Version 2: Biopsy of Endometrium and endometriosis lesions

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). The inner-lining of the womb (endometrium) has these stem cells that 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 80 women with endometriosis (you must have been off all hormonal contraception for at least 3 months), who have regular periods. If you have endometriosis and is scheduled to have surgery to remove endometriosis lesions 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 be taken from you. Once the operation is over, a small sample of the endometriosis tissue that has already been removed will also be taken for the research.

Therefore, **NO** extra surgery will be performed for the study. A ***blood sample*** will also be taken from your veins.

How is the endometrial pipelle sample done?

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. Since 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?

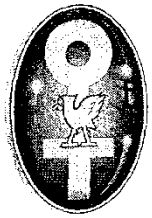
There are no special compensation arrangements in place to compensate you in case if taking part in this research project harms you. However, if you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to 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. Any surplus tissue after being used for this study, will be stored in the Liverpool Women's Hospital and may be used for other ethically approved research.

If you are interested in taking part, please contact Dr. Dharani Hapangama (0151- 702 4114 or 0151 706 9988, bleep 141) 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
Hospital

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

Liverpool Women's
NHS Foundation Trust

Study number: LWH0813
Patient Identification Number for this trial:

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.

Researcher: Dr Dharani Hapangama
Clinical Senior Lecturer / Consultant in Obstetrics & Gynaecology Division of Perinatal and Reproductive Medicine School of Reproductive and Developmental Medicine First Floor Liverpool Women's Hospital Crown Street Liverpool L8 7SS

Age:

Height (cm):
Weight (kg):
Body mass index:

Smoking history:

Parity:
Miscarriage:
TOP:

Days of bleeding:
Cycle length:
LMP:

Endometriosis stage:

APPENDIX 4
STANDARD OPERATING PROCEDURES (SOP)

4A. TISSUE PROCESSING

Background

Formalin fixed tissue is dehydrated, cleared then impregnated with paraffin wax using the automated Shandon Citadel 1000 processing machine. The processing setting for most sample types is ***programme A*** which operates using the schedule provided below. The total processing time is 18 ¾ hours, therefore the processor is generally run overnight. Starting the processor at 2pm will result in completion of the cycle at 8:45 the following morning.

Programme A Processing Schedule:

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

Equipment information:

Citadel processor (serial number CA 1390 EO 608) manufacturer: Thermo electron Corporation. Manufacturer's address: 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England.

Maintenance and contract:

Maintained/serviced by Thermo Fisher Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England. Tel: 01928 562 541, Fax: 01928 562 512.

Solvent Information:

Ethanol and xylene are purchased from Chemistry solvent stores (University of Liverpool). The wax (Histoplast PE REF8330) is purchased from Thermo Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England. The 10% neutral buffered formalin is purchased from Sigma Life science, Fancy Road, Poole, Dorset, BH12 4QH.

Health and Safety Precautions

Wear a laboratory coat, safety spectacles and nitrile gloves. Xylene and formalin are harmful by contact to skin and eyes or if ingested or inhaled. Refer to the risk assessments and COSHH forms before starting procedure.

Overnight processing procedures requires the completion of permits provided in safety circular SCR14/3 (link to document provided below). A 'Yellow permit' (for long term operation) should be prepared by the DSC or laboratory manager and the copy secured in a clear plastic folder on the door outside laboratory 4. The 'Pink' permit (valid for the duration of the experiment only) should be prepared by the processor user and the document should be placed on or adjacent to the apparatus. Information that needs to be recorded on the pink copy includes:

a) experimental details

b) times and dates of experiment

c) emergency procedures

d) name, address and telephone number of the processor user

e) signature of researcher concerned and a countersignature from the laboratory manager/DSC.

PROTOCOL

A Preparation of Solvent Containers on Processing Machine:

Between processing runs, ethanol solutions are stored in labelled bottles to prevent evaporation. These are found in the metal solvent cabinet under the bench in Laboratory 4. Therefore the solvent containers must be re-filled before tissue processing commences. To access the processor solvent container, press the ***raise*** button on the hand held controller**.** Remove the evaporation covers from the top of the processor then press ***rotate*** or ***check/fill*** button. The rotate function will cause the operating head assembly to advance one position

and the check/fill option rotates the head three positions. Remove the container using the metal handles and add 1.5L of the required ethanol solution (the top of the bevelled edge on the solvent container is approximately 1.5L). Repeat process until all ethanol containers are filled. It is also advisable to check the level of the xylene, formalin and the wax containers on a regular basis. If fresh 100% ethanol is used to top up or prepare solutions for the processor ensure the ethanol log is updated with the volume used. The ethanol log is situated in DSC office (room 1128).

B Sample Processing Procedure

1. Label plastic cassettes with the sample ID number using pencil.
2. Carefully remove the tissue from the formalin pot using forceps and place into the small wire inserts. Secure the inserts into the plastic cassettes and re-check the sample ID. Dispose of the used formalin in the red labelled waste container which is stored below the fume hood.
2. Arrange the cassettes so they are loosely spaced in the processing baskets
3. Press ***Go To 1*** on the hand held controller**,** slot the processing baskets onto the holder over the formalin and place weight on top of the basket.
4. Press ***Lower*** button on the hand held controller (to lower the basket into the formalin).
5. Check the time is correct by pressing ***clock*** (alter time by pressing the + button or whilst holding in the clock button if necessary).
6. Select the appropriate processing time function:

Date of Processing	**Processing Time**	**Hand Held Controller Action**
Monday-Thursday	Before 2pm	Press ***delay on*** button
Monday-Thursday	After 2pm	Press ***autostart*** button
Friday	Before 2pm	Press ***days delay***, hold this in, scroll to 2 days using the + button then press ***delay on.***
Friday	After 2pm	press ***days delay,*** hold this in, scroll to 1 day using the + button, then press ***delay on***

7. Complete the citadel 1000 user log (the folder is situated by the embedding staition in laboratory 4) with sample, solvent and programme information..
8. When the processing cycles are completed, switch on the Shandon Histocentre 3 embedding machine.

9. Press ***raise*** button on the hand held controller and remove baskets from holder.
10. Place blocks into heated reservoir in the embedding machine.
11. Wipe processing baskets with absorbent towels to remove surplus wax then place equipment into the oven (heated to ~100°C) for several minutes to remove any residue.
12. Soak biopsy inserts in xylene for several hours/overnight to remove wax residues. Leave the inserts in the fume hood to air dry
13. Remove the alcohols from the processing machine using the method described in section A and pour solutions in the labelled storage bottles using a funnel. The storage bottles are then placed in the flammable solvent metal storage cabinets.
14. Update the citadel 1000 processing user log.

C Disposal of Waste Solvents

The solvents must be changed on a regular basis (depending on time of year and processor usage) to ensure efficient processing of the tissue samples. Waste solvents must be placed in the red solvent waste containers and identified clearly with the appropriate labels provided by Lisa Heathcote or Jo Drury. Contaminated waste wax can be discarded by pouring the molten solution into a container and cooling until the wax is set. The wax container can then be disposed of in the LWH clinical waste. Refer to the waste disposal protocol (SOP 25) for further information.

4B: EMBEDDING TISSUE BIOPSIES

BackgroundACKGROUND

The Shandon Histocentre provides easy convenient embedding of histological specimens. Prior to operating the unit, be sure to read and observe the safety instructions in the operator manual.

EQUIPMENT INFORMATION:

Plastic cassette

Shandon HistoCentre 3 Embedding Station

Hot forceps

Metal Moulds

HEALTH AND SAFETY REQUIREMENTS

Normal laboratory precautions, including the wearing of gloves, laboratory coat, safety goggles and the use of a fume cupboard, should be taken when handling the samples. Refer to the risk assessments and COSHH forms before starting procedure. Place all waste in clinical waste bin.

METHOD:

1. When the processing cycles are completed, switch on the Shandon Histocentre 3 embedding machine.
2. Press ***raise*** button on the hand held controller and remove baskets from holder (use paper towels to ensure that wax doesn't drip on the floor).
3. Place blocks into heated reservoir in the embedding machine.
4. Wipe processing baskets with absorbent towels to remove surplus wax then place equipment into the oven (heated to ~100°C) for several minutes to remove any residue.
5. Take a metal mould from the heated unit, place it underneath the wax tap and half-fill it with wax.
6. Take a cassette and place on the heated area by the wax tap to prevent solidification of the wax.
7. Open the cassette, and transfer the sample to the wax-filled mould using hot forceps.
8. Move the mould to the "cold spot" at the front of the dispensing unit
9. Position tissue to bottom surface of metal mould using hot forceps to stabilise tissue in place.
10. Cover with a plastic cassette (number upwards) and hold in place by applying pressure with hot forceps on top of cassette.
11. Top up wax by hovering above the heated area and place on pre-cooled cooling block.
12. Using forceps tap against top of plastic cassette to release any air bubbles within the liquid wax.
13. Leave on cooling area* for at least 30 minutes
14. Cassette should come cleanly away from the metal mould. If necessary use forceps or a scalpel to loosen the edges.

15. Store the block in the metal storage cabinet (trays 5 and 6) in numerical order (room 1125).

16. Place the metal biopsy inserts into a container of xylene to clean off the wax.

4C: CUTTING PARAFFIN SECTIONS

Background

Preparation of 3-5 micron thick sections from wax – embedded tissue is a commonly used histological technique. This SOP will describe how to prepare these using the Microm HM335 rotary microtome and attaching sections onto aminopropyl triethoxy silane (APES) coated slides.

HEALTH AND SAFETY REQUIREMENTS

Wear a laboratory coat and nitrile gloves. Care needs to be taken when performing this procedure because there is a risk of sharps injury from the microtome blades. Refer to the physical hazards safety circular, risk assessments and COSHH forms before starting procedure. Also ensure waste wax sections are cleaned from the floor and the general working area at the end of the procedure to prevent risk of slip/trip injuries. Place all waste tissue sections in the clinical waste bags and the used microtome blades and slides into the yellow clinical waste sharps bins. DO NOT COMMENCE WORK WITH THE MICROTOME UNTIL SUFFICIENT TRAINING HAS BEEN PROVIDED AND AUTHORISATION HAS BEEN RECEIVED FROM LISA HEATHCOTE OR JO DRURY.

EQUIPMENT INFORMATION:

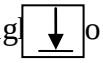
- Microm HM335 rotary microtome is supplied by MICROM Company. Manufacturer's address: Microm UK Ltd., 8 Thame Park Business Centre, Leinman Road, Thame, OX9 3XA. Further information can also be obtained on http://www.microm-online.com.
- Microscope slides (twin frost size 26 x 76mm-Printed "IVD CE" 90º ground edges, catalogue number MAE-1000-03P Pack of 1000) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Coverslips 22 x 22mm, 22 x 40mm and 22 x 50 are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- Microtome blades (MB Dynasharp Catalogue Reference 3050836) are purchased from Thermo Scientific 93 – 96 Chadwick Road, Astmoor, Runcorn, Cheshire, WA7 1PR England.

- Forceps, paint brush, section dryer and water bath are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

METHOD:

1. Carefully fill the water bath with distilled water ensuring the electrical connection points remain dry.
2. Turn on water bath from the plug and set the power switch from O to I
3. The water will need to be heated to 40℃. The best way do to this is to first turn the temperature dial to 10 (maximum setting) for 7-8 minutes and then turn the dial down to 2.5. If the temperature of the bath is above 40℃, pour in distilled water to lower the temperature.
4. Scrape away any surplus wax from the edges of the cassette to ensure a good fit in the holder then place the tissue block in the refrigerator. Cool the block for at least 30 min prior to cutting.
5. Ensure that the handwheel is locked in the upper range of the vertical movement by turning the lever downwards.
6. Press the reverse course feed button  to move the knife carrier away from the specimen.
7. Turn the clamping lever on the blade holder to the front and swing the protective bracket forwards. Insert the feather S35 blade into the slot behind the clamping plate ensuring the blade is level on the rail (use forceps to manipulate the blade if necessary). Use half of the blade for cutting at a time. Preferably use left side first and then the right side.
8. Return the clamping lever to the original position (in line with the black frame of the blade) to lock the blade in place. The protective bracket should also be returned to the original position when the microtome is not in use to minimise the risk of sharps injury.
9. The blade angle is pre-set to 10-12° and should not require adjustment. These settings should remain the same to ensure minimal amounts of tissue are wasted when different laboratory workers prepare sections from the same block.
10. Insert the specimen against the 'fixed jaw' of the universal cassette clamp and secure by pulling the lever to the front. Ensure that each specimen cassette is always inserted in the same orientation (horizontal placement in the universal cassette clamp with the specimen number on the left) and inserted to the far left of the clamp.

11. Unlock the handwheel by turning the lever upwards. Rotate handwheel in a clockwise direction until the centre of the tissue block is level with the blade holder. Then lock the handwheel by turning the lever downwards.
12. Press the forward course feed button  to move the blade carrier near to the specimen. To establish whether the blade is close to the specimen view the distance between the two at a side angle. Also, gently move the handwheel up and down to see if the specimen is not touching the blade. When you are satisfied that both blade and specimen are close to each other move the handwheel so the specimen is away from the blade, this prevents scoring the specimen block in the middle.
13. The required section and trimming thickness are set by means of a circular knob on the left of the instrument. Press the circular control knob to switch between section (FEED) and trimming (TRIM) thickness. The corresponding LED will be displayed on the operating control panel:
    - Green LED lights up when the FEED function is used
    - Yellow LED lights up when the TRIM function is selected.
14. When a new tissue block is used, select TRIM and turn the control knob until the corresponding LED is set at 20µm.
15. Unlock the handwheel and start trimming by rotating the handwheel in a clockwise direction. Once you begin to see specks on tissue in the sections change the TRIM settings from 20µm to 10µm.
16. Continue to trim on the new setting until a representative amount of tissue is exposed in the tissue block.
17. Select FEED and turn the control knob until the corresponding LED is set at the required section thickness (usually 3-5µm). Rotate the handwheel until the first section generated. Gently hold the end of the section with forceps and continue to cut until a ribbon (consisting of approximately 6 sections) is produced. Discard the first few sections when first cutting on the feed section as usually the first few are too thick.
18. Carefully float the sections onto the pre-warmed water bath. Leave or a minute or so until the wrinkles in the section disappear. Separate the sections by applying gentle pressure using forceps. Select the best sections and float onto APES-coated slides. It is considered to be good laboratory practice to attach each section in the same orientation on the surface of the slide where you can read the IVD sign.
19. Label the frosted part of the slide with the specimen ID and thickness (using a pencil). Place slides in a rack to dry at room temperature for several hours (preferably over night).

20. When not cutting for a few minutes the specimen block moves slightly out of place which potentially can create thicker sectioning when you go back to cutting. To avoid creating thicker sections press the reverse course feed button very slig  o move the knife carrier away from the specimen by a small amount.
21. During the cutting procedure a build up of wax on either side of the blade carrier. This can potentially cause scoring of the section. Using a soft brush, brush in an upward direction against the blade carrier to remove the wax, this is to prevent blunting of the blade and cutting down of the brush if a downward direction was used.
22. If the sections start to wrinkle during the cutting procedure, then the tissue block has become too warm and needs to be refrigerated for at least 10 minutes. To remove the tissue block, press the reverse course feed button until the specimen is safely away from the blade holder. Return the safety bracket to the upright position. Remove the tissue block by pressing the lever on the universal cassette clamp.
23. Remove any unused tissue sections from the water bath surface using kitchen roll.
24. When the microtome blade needs to be replaced, return the tissue block to the furthest position using the course feed button and lock the handwheel. Unlock the lever on the blade carrier and place the microtome blade into the yellow clinical waste sharp bin. NEVER try to remove the microtome blade when the tissue block is close to the blade edge because there is a risk of sharps injury and also the risk of damaging the tissue block.
25. When the sections have dried, place the slides into a staining rack and bake for 60 minutes in a slide drier (pre-warmed to 60°C), or 37º C overnight. Place slides in a suitable 'dust free' container until required for the immunohistochemical staining procedure.

4D: APES COATING PROCEDURE

Background

The Aminopropyl triethoxy silane (APES) coating procedure is used to improve tissue adhesion by producing a positive charge on the surface of the slide. The protocol described below was modified from the original method described by Maddox and Jenkins in 1991 (full reference provided in the appendices).

HEALTH AND SAFETY REQUIREMENTS

The APES concentrate is a toxic chemical that is moisture sensitive and requires storage at 0-5°C; it is an irritant and can cause burns. Normal laboratory precautions, including the

wearing of gloves and the use of a fume cupboard, should be taken when handling the concentrate solution. Refer to the risk assessments and COSHH forms before starting procedure. Place all waste solutions into a labelled red solvent waste can and attach a solvent label (available from Lisa Heathcote or Jo Drury by request).

EQUIPMENT INFORMATION:

- Microscope slides (twin frost size 26 x 76mm-Printed "IVD CE" 90º ground edges, catalogue number MAE-1000-03P Pack of 1000) are purchased from Liverpool Women's Hospital (NHS) purchasing department.
- APES solution (catalogue number A3648) is purchased from Sigma-Aldrich Company Ltd. UK distributor address: The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT.
- Staining racks and glass jars are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

METHOD:

26. Place slides into plastic racks. Each rack holds 24 slides
27. Prepare a 2% APES working solution by mixing 5ml APES with 245ml 100% ethanol, or for the bulk method 40ml APES with 1960ml 100% ethanol. The APES solution is stable for several hours; therefore coat as many racks as possible to save reagents and time.
28. Prepare a glass staining trough containing 100% ethanol

Position 1: APES working solution

Positions 2 – 4: distilled water

29. Place slides racks in 100% ethanol. Dip several times to dislodge dust etc., and then drain the staining racks on absorbent paper towels.
30. Incubate the slides for 5 minutes in APES working solution.
31. Rinse slides in three changes of distilled water (2 minutes incubation for each rinse).
32. Drain slides on absorbent towels and air dry overnight, or dry in a section dryer for 1 hour at 37°C.

33. Discard the first change of distilled water. Place the staining jar containing fresh distilled water at the last rinse location (position 5). Therefore the 2nd change of water has becomes the first etc.

34. Repeat steps 5 through to 9 for each rack of slides.

35. Transfer the coated slides to a dust free container labelled 'APES' and the date of coating (APES slides are stable for approximately 3 months).

4E: HAEMATOXYLIN AND EOSIN STAINING OF BIOPSY TISSUE SAMPLES

Background

The Haematoxylin and Eosin stain is probably the most widely used stain in Histopathology/ Cytopathology. It has the ability to demonstrate an enormous amount of tissue structures. The haematoxylin component stains cell nuclei blue / black with good intranuclear detail whilst the eosin stains cell cytoplasm and most connective tissue fibres in varying shades of pink.

Health and Safety Precautions

The main health and safety risks to this procedure arise from chemicals/reagents that are potentially hazardous, gloves must be worn at all times. Eosin Y aqueous contains (formaldehyde: 50 – 00 – 0), causes burns, and may cause sensitisation by skin contact. In addition, it is toxic by inhalation, in contact with skin and if swallowed. Haematoxylin (Gill 2) contains (ethylene glycol: 107 – 21 – 1; aluminium sulphate 10043 – 01 – 3; acetic acid: 64 – 19 – 7). This is harmful if swallowed; very toxic by inhalation; causes severe burns, and is irritating to eyes.

METHOD

Work in a fume hood

For paraffin sections:

1. Dewax prior to staining: 10 min Xylene 1

10 min Xylene 2

5 min 100% Ethanol 1

5 min 100% Ethanol 2

1 min 90% Ethanol

1 min 70% Ethanol

2. 2 min water

3. Gills 2 Haematoxylin

4. Rinse in tap water

5. Acid alcohol dip

6. Blue in tap water (approximately 5 min)

7. 70% alcohol – 45seconds

8. 95% alcohol - 45seconds

9. Eosin Y, aqueous 4 min

10. Water rinse x 2

11. Incubate water 2 min

12. 95% alcohol 20 - 30s

13. 100% alcohol 1: - 45seconds

14. 100% alcohol 2: - 45seconds

15. Xylene 1: 5mins

16. Xylene 2: 10mins

17. DPX and mount

4F: IMMUNOHISTOCHEMISTRY – GET FROM JO

Background

Immunohistochemical staining is a valuable tool for detecting specific antigens in tissues. In order to perform the standard staining procedure, first the tissue section has to be prepared (SOP 04, 05 and 06), deparaffinized and then rehydrated (SOP 08). Antigen retrieval (SOP 09) is then performed to break any protein cross-links, therefore unmasking the antigens and epitopes in the tissue sections. The Vector immPRESS ^{TM} reagent kit, HRP is a two-step Immunohistochemical staining technique. This system is based on an HRP labelled polymer which is conjugated with secondary antibodies. The labelled polymer does not contain avidin or biotin. Consequentially non-specific staining resulting from endogenous avidin-biotin activity in liver, kidney, lymphoid tissues and cryostat sections is eliminated or significantly reduced. The interpretation of any positive staining or its absence should be complemented by morphological and histological studies with proper counts. Any endogenous peroxidase activity is quenched by incubating the specimen with Peroxidase block. The specimen is

incubated with an appropriately characterised and diluted mouse primary antibody, followed by incubation with the labelled polymer. Staining is completed by incubation with 3'3-Diaminobenzindine (DAB)+ substrate - chromogen which results in a brown–coloured precipitate.

Definition

This SOP will explain how to stain tissue using an unlabelled antibody. Please read entire procedure before staining sections. Perform all antibody and staining incubations in a humid chamber and do not allow sections to dry out. Isotype and system controls should also be run. Carefully time all tests. Do not touch test specimens on slides during the staining procedure.

Health and Safety Precautions

The main health and safety risks to this procedure arise from chemicals/reagents that are potentially hazardous, such as hydrogen peroxide (H_2O_2), xylene, ethanol, and DAB (3'3-diaminobenzindine). Gloves must always be worn when working with any of these chemicals.

EQUIPMENT INFORMATION

Staining dishes

Humidified chamber

Cover slips

General points:

Ensure that solutions cover the whole specimen – if necessary use a piece of Parafilm to spread.

Antibody information:

All current antibody datasheets are held in a labelled lever arch file in room 1128. Additional information on appropriate antibody concentration, antigen retrieval conditions and incubation time/temperature is held in an Excel spreadsheet (C:\Documents and Settings\jadrury.livad\My Documents\Dharani\Dharani abs.xlsx).

Reagent information:

TBS: 6 g/l Trizma base (T1503, Sigma-Aldrich) + 8.7 g/l NaCl (S7653, Sigma-Aldrich). Adjust pH to 7.6 with HCl.

BSA: A3803 (Sigma Aldrich)

H_2O_2: H1009 (Sigma-Aldrich)

ImmPRESS anti-mouse IgG kit (50 ml) (MP-7402, Vector Laboratories)

ImmPRESS anti-rabbit IgG kit (50 ml) (MP-7401, Vector Laboratories)

ImmPRESS anti-goat IgG kit (50 ml) (MP-7405, Vector Laboratories)

ImmPACT DAB (120 ml) (SK-4105, Vector Laboratories)

Shandon Gill 2 Haematoxylin (Thermo Scientific)

1% acid alcohol: 1 ml HCl/100 ml 70% ethanol

Shandon Consul-Mount (Thermo Scientific)

Supplier information:

Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT. Tel: 0800 717181. Fax: 0800 378785. Web: www.sigma-aldrich.com.

Thermo Scientific, Bishop Meadow Road, Loughborough, LE11 5RG. Tel: 01509 231166 Fax: 01509 231893.

Vector Laboratories Ltd. 3, Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS. Tel: 01733 237999. Fax: 01733 237119. Web: www.vectorlabs.com.

METHOD (Paraffin sections):

1. Place slides in a staining dish containing TBS and incubate 5 minutes at room temperature.
2. Prepare 0.3% H_2O_2/TBS (2.5 ml 30% H_2O_2 + 247.5 ml TBS)
3. Incubate slides in 0.3% H_2O_2/TBS bath 10 minutes at room temperature.
4. Prepare humidified chamber by placing folded paper towels in the centre gulley's and soaking with distilled water.
5. Decant H_2O_2/TBS and tap off any remaining solution. Carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.

6. Place slides in a staining dish filled with TBS and incubate 5 minutes at room temperature.
7. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
8. Mark area to be stained with DAKO hydrophobic marker pen ensuring that the tissue is surrounded with sufficient space to allow spreading of antibodies.
9. Place slides in a staining dish filled with TBS and incubate 5 minutes at room temperature.
10. Prepare antibody diluent (TBS/0.5 % BSA eg. 250 µl 10% BSA + 4750 µl TBS)
11. Prepare appropriate dilutions of each antibody, allowing 50 µl per section.
12. Tap off any remaining solution, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
13. Place slides in humidified chamber.
14. If necessary, add one drop horse serum block to each section, spread with parafilm to ensure that the whole section is covered, and incubate 20 min at room temperature.
15. Tap off the serum block and proceed to step 17.
16. Steps 14 and 15 can be omitted if staining is clean without blocking.
17. Apply 50µl of the appropriate antibody to each section, spread with parafilm to ensure that the entire section is covered and incubate 30-120 min at room temperature or overnight at 4°C. See C:\Documents and Settings\jadrury.livad\My Documents\Dharani\Dharani abs.xlsx for specific conditions for each antibody.
18. Tap off the antibody solution onto paper towels prior to placing the slides in a staining dish filled with TBS.
19. Incubate 5 minutes at room temperature.
20. Decant TBS and refill.
21. Incubate 5 minutes at room temperature.
22. Tap off any remaining TBS, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
23. Return slides to the humidified chamber and apply 1 drop labelled polymer-HRP. Ensure that the polymer matches the primary antibody.
24. Spread with parafilm to ensure that the entire section is covered and incubate 30 min room temperature.
25. Tap off the polymer solution onto paper towels prior to placing the slides in a staining dish filled with TBS.
26. Incubate 5 minutes at room temperature.

27. Decant TBS and refill.
28. Incubate 5 minutes at room temperature.
29. Prepare substrate/chromogen solution: 30µl/1ml substrate (require 50 µl per section).
30. Tap off any remaining TBS, carefully wipe around the specimen and the back of the slides with a tissue to remove excess liquid.
31. Return slides to the humidified chamber and apply 50 µl substrate/chromagen solution.
32. Spread with parafilm to ensure that the entire section is covered and incubate 10 min room temperature.
33. Place slides in staining rack and immerse immediately in tap water to stop the reaction.
34. Turn on fume hood in lab 4.
35. Counterstain using filtered Gill 2 haematoxylin in lab 4. Immerse for 1 min 30s.
36. Immerse in tap water and rinse until water is clear.
37. Dip briefly in acid alcohol, and immediately back into tap water (5 min).
38. Incubate 1 min in 70% ethanol.
39. Blot off excess and incubate 1 min in 90% ethanol.
40. Blot off excess and incubate 3 min in 100% ethanol.
41. Repeat step 40.
42. Blot off excess and incubate 5 min in xylene.
43. Blot off excess and incubate 10 min in xylene.
44. Remove a few slides at a time and apply sufficient mountant to cover the section.
45. Choose an appropriately sized coverslip and apply to the slide.
46. Remove air bubbles by using a cocktail stick or yellow pipette tip to gently press on the coverslip and "chase" bubbles to the edge.
47. Leave to dry in fume hood.

3G: PREPARATION OF PARAFFIN SECTIONS FOR STAINING

Background

Factors that prevent adhesion of sections to blank slides are usually attributed to moisture under the section, and almost always become apparent after heat antigen retrieval. The method of heating slides prior to immunohistochemical staining ensures tissue sections adhere to the glass slide. It is important the sections are not over-baked, however, as this could increase background and decrease target staining.

In order to prepare slides for staining, tissue must be deparaffinised using xylene and gradually rehydrated with descending grades of alcohol.

Health and Safety Precautions:

Wear a laboratory coat, safety spectacles and nitrile gloves. Xylene is harmful by contact to skin and eyes or if ingested or inhaled. Work in the fume hood for dewaxing procedure. Refer to the risk assessments and COSHH forms before starting procedure.

Materials

- Staining racks and glass jars are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.
- Ethanol and xylene are purchased from Chemistry solvent stores (University of Liverpool).
- Section Dryer. Model E28.5. Supplier information: Raymond A. Lamb, Eastbourne, BN23 6QE, England.

Method

1. Switch on Section Dryer Model E28.5. Adjust temperature using the temperature dial (see below for temperature required).
2. Label slides with the stain/antibody and concentration to be used, and date in pencil.
3. Place slides for staining in metal slide racks.
4. Heat to 60^{o}C for 60 minutes or overnight at 37 ^{o}C.
5. Dewaxing solutions are in staining dishes in the fume hood in room 1125. Place the slide rack in xylene-1 for 10 minutes. Long forceps can be used to facilitate the transfer of the slide rack between dishes.
6. Drain off excess xylene, and transfer to the dish labelled "xylene-2" for 10 minutes.
7. Drain off excess xylene, and briefly blot on paper towel.
8. Place the slide rack in the dish labelled "100% ethanol-1" for 5 minutes.
9. Drain off excess ethanol, and transfer to the dish labelled "100% ethanol-2".
10. Drain off excess ethanol, briefly blot on paper towel and transfer to 90% ethanol for 1 minute.
11. Drain off excess ethanol, briefly blot and transfer to 70% ethanol for 1 minute.

12. Drain off excess ethanol, briefly blot and transfer to a clean staining dish containing tap water.

3H: ANTIGEN RETRIEVAL

BACKGROUND

The mechanisms of formalin-fixation have been thought to be due to the formation of cross-linking bonds between tissue proteins, stabilising them to withstand subsequent processing (Mason and O'Leary 1991). Whilst preserving tissue morphology, the formation of cross linking bonds may, however modify the antigen's epitopes and/or it's electrostatic charges, thus producing weak or false negative staining during immunohistochemical detection of certain proteins. Restoring the epitopes enables the antigen to react with the paratope of the antibody. Methods of proteolytic pre-treatment and heat retrieval of tissue antigenicity aim to restore the avidity of the immune reaction.

Proteinase K is a type of proteolytic agent which is isolated from the saprophytic fungus *Tritirachium album*. It possesses a high specific activity which remains stable over a wide range of temperatures and pH values with substantially increased activity at higher temperature. It particularly unmasks antigens of proteins found in the basement membranes including laminin and collagen IV.

There are 2 commonly used heat retrieval buffers, which vary in their pH. Heat causes cross-linked protein epitopes to 'unfold' (in manner similar to DNA denaturation), while buffer solutions aid in maintaining the conformation of the unfolded protein. The citrate based solution is designed to break the protein cross-links, therefore unmask the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibodies. Tris-EDTA is very useful for low affinity antibodies or when tissue antigens are not intense. This buffer works well for many antibodies, but it often gives high background staining (maybe due to endogenous biotin revealed after this pretreatment), so primary antibody can often be highly diluted.

HEALTH AND SAFETY REQUIREMENTS

Care needs to be taken when performing heat based retrieval procedures because there is a risk of burns from the hotplate and pressure cooker. There is also a risk of chemical burns from sodium hydroxide and there is a mild irritant effect from citric acid. Suitable personal protection equipment (Nitrile gloves, safety glasses and a lab coat etc) should be worn. Refer to the physical hazards safety circular, risk assessments and COSHH forms before starting

procedure. DO NOT COMMENCE WORK WITH THE PRESSURE COOKER UNTIL SUFFICIENT TRAINING HAS BEEN PROVIDED AND RECEIVED AUTHORISATION FROM LISA HEATHCOTE OR JO DRURY. THE SEAL ON THE PRESSURE COOKER SHOULD BE INSPECTED PRIOR TO EVERY USE AND REPLACED ANNUALLY.

EQUIPMENT INFORMATION:

- Proteinase K supplied by QIAGEN. QIAGEN HOUSE, Fleming Way, Crawley West Sussex, RH10 9NQ
- Diluent
- Humidity Chamber
- Sodium Hydroxide pellets and citric acid are supplied by VWR International Ltd, Poole, BH15 1TD England (Future orders will be obtained from Sigma or Thermo Fisher).
- Trizma base (T1503, Sigma- Aldrich Chemical Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT. Tel: 0800 717181. Fax: 0800 378785. Web: www.sigma-aldrich.com)
- EDTA (disodium, dehydrate) is supplied by VWR International Ltd, Pole, BH15 1TD. Future orders will be obtained from Sigma (E4884).
- Tefal Clipso Easy 6L pressure cooker and Russell Hobbs hotplate are obtained commercially from John Lewis department store. Annual Insurance check TBC. The seal/gasket needs to be replaced annually. Gasket (part number SA793145) is obtained directly from: http://www.homeandcook.co.uk refer to section accessories/pressure cookers. 2010 price £8.50 +£1.50 delivery
- Slide racks are supplied by Raymond A Lamb Ltd. Manufacturer's address: Units 4 & 5, Parkview industrial estate, Eastbourne, East Sussex, BN23 6QE England.

- Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA Solution, pH 9.0):
  Tris Base 12.1 g
  EDTA 3.7 g
  Distilled water 2 l to make 5x stock solution
  Mix to dissolve, pH is usually at 9.0. Store this solution at room temperature for 3 months or at 4°C for longer storage.

METHOD A (Proteinase K):

- Prepare a volume of proteinase K with a 1:25 concentration of the working stock that is sufficient for 50µl per section. Allow larger volume for larger sections.
- Once all slides have been de-waxed place into distilled water
- Prepare humidified chamber
- Remove slides from the staining dish and wipe the backs of the slides and place onto the humidified chamber.
- Distribute 50µl of proteinase K onto each section and spread over whole of tissue using a piece of parafilm. Take care whilst spreading to ensure sections do not get dried or damaged in any way.

- All sections should be incubated with proteinase K for 5 minutes at room temperature.
- After 5 minutes tap the solution off the slide and place slides in a glass staining dish filled with TBS.
- See SOP 12 for immunohistochemistry protocol.

METHOD B (Heat based antigen retrieval):

1. Either:
   (a) Prepare a 10mM solution of citrate buffer, pH=6.0 by adding 3.15g citric acid to 1.5 litre of distilled water and adjust pH to 6.0 with 2M NaOH.
   Or:
   (b) Dilute stock 5x Tris-EDTA buffer, pH=9.0 by adding 300 ml to 1.2 l of distilled water.
2. Place buffer in the pressure cooker and place on the hotplate.
3. Turn on the hotplate to the maximum heat setting.
4. Loosely place the lid back on pressure cooker. Bring the buffer to a rolling boil.
5. Immerse the slide rack in the buffer using long forceps.
6. Engage the lid, turn the valve to pressure symbol and press the lid down until it clicks.
7. Set timer for 1 minute, when steam starts to vent, start timer. After 1 minute, turn off the hotplate and release the pressure by turning the vent to the "steam" symbol, and cool as quickly as possible by transferring the pressure cooker to the sink and running under cold water.
8. Transfer the slides back to the glass staining jar containing distilled water.
9. Transfer to TBS ensuring that the tissue does not dry out.
10. See SOP 12 for immunohistochemistry protocol.

APPENDIX 5
ANTIBODY DATA SHEETS

DakoCytomation
Denmark A/S · Produktionsvej 42 · DK-2600 Glostrup · Denmark · Tel. +45 44 85 95 00 · Fax +45 44 85 95 95 · CVR No. 33 21 13 17

Polyclonal Rabbit
Anti-Laminin
Code No./ Code/ Code-Nr. Z 0097

Intended use
For in vitro diagnostic use. Polyclonal Rabbit Anti-Laminin is intended for use in immunocytochemistry. The antibody labels laminin, which is present in the basement membrane. The antibody may be useful in the characterization of basement membrane preservation, e.g. in breast cancer (1, 2) and adenocarcinomas of the lung (3). Differential identification is aided by the results from a panel of antibodies. Interpretation must be made within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

Introduction
Laminins are large heterotrimeric basement membrane glycoproteins composed of an α−, a β− and a γ−chain. At present 5 α−chains, 3 β−chains, and 3 γ−chains are known to form at least 15 different isoforms (4). The laminin isoforms have different tissue- and developmental-specific localizations, and more than 20 cell surface receptors have been identified for laminin, including integrins, proteoglycans, lectins, gangliosides, sulphatides, amyloid precursor protein and galactosyltransferases (5, 6). Laminins have been found to promote cell adhesion, migration, protease activity, proliferation, tumour growth, angiogenesis and metastasis.

Reagent provided Purified immunoglobulin fraction of rabbit antiserum provided in liquid form in 0.1 mol/L NaCl, 15 mmol/L NaN3.
Protein concentration g/L: See label on vial.

Immunogen Laminin isolated from the rat yolk sac tumour cell line L2.

Specificity The antibody labels laminin in basement membranes. Traces of contaminating antibodies have been removed by solidphase absorption with human and rat plasma proteins as well as rat immunoglobulins. In crossed immunoelectrophoresis, using 12.5 µL antibody per cm2 gel area against 2 µL human plasma, 2 µL rat plasma or 20 µg of rat immunoglobulins, only one precipitate corresponding to laminin appears. Staining: Coomassie Brilliant Blue. As demonstrated by immunocytochemistry, the antibody cross-reacts with the laminin-equivalent protein in man, mouse (7) and rat (8).

Precautions
1. For professional users.
2. This product contains sodium azide (NaN3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
3. As with any product derived from biological sources, proper handling procedures should be used.

Storage
Store at 2-8 °C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the user must verify the conditions. There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed, which cannot be explained by variations in laboratory procedures, and a problem with the antibody is suspected, contact our Technical Services.

Specimen preparation Paraffin sections: The antibody can be used for labelling paraffin-embedded tissue sections fixed in formalin. Pretreatment of tissues with DakoCytomation Proteinase K, code No. S 3020 is recommended. Heat-induced epitope retrieval with DakoCytomation Target Retrieval Solution, pH 6, code No. S 2369, DakoCytomation Target Retrieval Solution, code No. S 1700, DakoCytomation Target Retrieval Solution, pH 9, code No. S 2368, or DakoCytomation Target Retrieval Solution, High pH, code No. S 3308, was found inefficient. The tissue sections should not dry out during the treatment or during the following immunocytochemical staining procedure.

Staining procedure
Dilution: Polyclonal Rabbit Anti-Laminin, code No. Z 0097, should be diluted in DakoCytomation Antibody Diluent, code No. S 0809, and may be used at a dilution range of 1:25-1:50 when applied on formalin-fixed, paraffin-embedded sections of tonsil and using 5 minutes pre-treatment at room temperature with DakoCytomation Proteinase K, code No. S 3020, and 30 minutes incubation at room temperature with the primary antibody. Optimal conditions may vary depending on specimen and preparation method, and should be determined by each individual laboratory.
The recommended negative control is DakoCytomation Rabbit Immunoglobulin Fraction (Solid-Phase Absorbed), code No. X 0936, diluted to the same protein concentration as the primary antibody. Unless the stability of the diluted antibody and negative control has been established in the actual staining procedure, it is recommended to dilute these reagents immediately before use, or dilute in DakoCytomation Antibody Diluent, code No. S 0809. Positive and negative controls should be run simultaneously with patient specimen.
Visualization: DAKO LSAB□+/HRP kit, code No. K 0679, and DAKO EnVision□+/HRP kits, code Nos. K 4008 and K 4010 are recommended. Follow the procedure enclosed with the kit.

Performance characteristics
Extracellular matrix labelled by the antibody display staining of the basement membrane. Normal tissues: The antibody was found to label basement membranes in human tonsil and kidney. Abnormal tissues: In a study of 220 cases of ER-positive non-invasive breast lesions, labelling of 20 cases with the antibody revealed no distinct immunoreactivity in the surrounding disrupted myoepithelial cell layers, suggesting disruption of the basement membrane also. In a study of 53 cases of lung carcinomas of the adenocarcinoma mixed bronchioloalveolar subtype, labelling with the antibody was lost in collapsed alveolar regions in 40/53 cases and in scarred regions in 53/53 cases indicating complete destruction of the basement membrane in these areas.

DakoCytomation Denmark A/S · Produktionsvej 42 · DK-2600 Glostrup · Denmark · Tel. +45 44 85 95 00 · Fax +45 44 85 95 95 · CVR No. 33 21 13 17
Monoclonal Mouse
Anti-Human
Collagen IV
Clone CIV 22
Code No./ Code/ Code-Nr. M 0785

Intended use
For in vitro diagnostic use.
Monoclonal Mouse Anti-Human Collagen IV, Clone CIV 22, is intended for use in immunocytochemistry. The antibody labels type IV collagen and is a useful tool for the identification of basement membranes. Differential identification is aided by the results from a panel of antibodies. Interpretation must be made within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

Introduction
Collagen IV is a major constituent in particular of the lamina densa of basement membranes and ultrastructurally it looks amorphous. Basement membranes are thin extracellular matrices separating parenchymal, endothelial, and epithelial cells from underlying connective tissue. In glomerular and tubular basement membranes 40% of the protein is type IV collagen. Detection of intracellular type IV collagen is generally difficult owing to a low concentration. In newly formed capillaries found in the inflammatory sites of rheumatoid arthritis synovium, intracellular type IV collagen may, however, be detected (1). Structurally the protein consists of four domains. One of these, the triple helical collagen IV domain, is formed by the association of two polypeptide chains of type α1 and one of type α2. This domain is 340 nm long and is highly crosslinked by disulphide bridges (1, 2). Diagnostic applications of collagen type IV immunostaining have mostly centered around the demonstration of basal lamina in invasive tumours. In particular, the demonstration of an intact basal lamina has been used to distinguish benign glandular proliferations, such as microglandular adenosis and sclerosing adenosis, from well-differentiated carcinoma, like tubular carcinoma of the breast (3).

Reagent provided
Monoclonal mouse antibody provided in liquid form as cell culture supernatant dialysed against 50 mmol/L Tris/HCl, pH 7.2, and containing 15 mmol/L NaN_3. Clone: CIV 22 (2). Isotype: IgG1, kappa. IgG concentration: see label on vial.

Immunogen
Purified pepsin fragments of type IV collagen isolated from human kidney (2).

Specificity The specificity of the antibody has been assessed in radioimmunoassay (RIA) where it recognizes an epitope present in the human type IV collagen in native conformation, whereas it does not recognize the reduced and alkylated protein in the denatured state. Staining of immunoblots with the antibody was negative, further indicating that the antibody recognizes a conformational epitope on type IV collagen (2). In immunoblotting or RIA, no cross-reactivity of the antibody with isolated human collagen types I, II, III and V could be detected (2). As demonstrated by RIA, the antibody cross-reacts with the collagen IV-equivalent protein in cow (2).

Precautions
1. For professional users.
2. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
3. As with any product derived from biological sources, proper handling procedures should be used.

Storage
Store at 2-8 °C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the user must verify the conditions. There

are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact our Technical Services.

Specimen preparation Paraffin sections: The antibody can be used for labelling paraffin-embedded tissue sections fixed in formalin. Pre-treatment of tissues with heat-induced epitope retrieval is required. Optimal results are obtained with DakoCytomation Target Retrieval Solution, code No. S 1700. Less optimal results are obtained with 10 mmol/L citrate buffer, pH 6.0, or 10 mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0. However, DakoCytomation Target Retrieval Solution, High pH, code No. S 3308 and pre-treatment of tissues with proteinase K were found inefficient. The tissue sections should not dry out during the treatment or during the following immunocytochemical staining procedure. Frozen sections and cell preparations: The antibody can be used for labelling acetone fixed, frozen sections (5, 6).

Staining procedure
Dilution: Monoclonal Mouse Anti-Human Collagen IV, code No. M 0785, may be used at a dilution range of 1:25-1:50 when applied on formalin-fixed, paraffin-embedded sections of human kidney and using 20 minutes heat-induced epitope retrieval in DakoCytomation Target Retrieval Solution, code No. S 1700, and 30 minutes incubation at room temperature with the primary antibody. Optimal conditions may vary depending on specimen and preparation method, and should be determined by each individual laboratory. The recommended negative control is DakoCytomation Mouse IgG1, code No. X 0931, diluted to the same mouse IgG concentration as the primary antibody. Unless the stability of the diluted antibody and negative control has been established in the actual staining procedure, it is recommended to dilute these reagents immediately before use, or dilute in DakoCytomation Antibody Diluent, code No. S 0809. Positive and negative controls should be run simultaneously with patient specimen. Visualization: DAKO LSAB™+/HRP kit, code No. K 0679, and DAKO EnVision™+/HRP kits, code Nos. K 4004 and K 4006, are recommended. For frozen sections and cell preparations, the DakoCytomation APAAP kit, code No. K 0670, is a good alternative if endogenous peroxidase staining is a concern. Follow the procedure enclosed with the selected visualization kit. Automation: The antibody is well-suited for immunocytochemical staining using automated platforms, such as the DakoCytomation Autostainer.

Performance characteristics Normal tissues: The antibody shows the characteristic labelling of basement membranes in a variety of tissues and organs tested, including kidney, skin, striated and smooth muscle, spleen, lymph node, lung, placenta and tendon. In spleen and lymph nodes, the expected fragmented labelling of the discontinuous basement membranes of the sinusoids is observed, whereas other blood vessels exhibit a linear, continuous labelling. In kidneys, basement membranes of capillaries, parts of the mesangial matrix and the Bowman's capsule, and the tubular basement membranes are labelled by the antibody. The only basement membrane showing negative labelling with the antibody is that of the corneal epithelium. All structures others than basement membranes are consistently negative with this antibody (2). Abnormal tissues: In congenital epidermolysis bullosa (EB), the antibody allowed a rapid distinction between the two major variants, EB simplex, and EB dystrophica (4). Around dilated vessels of port-wine stains, the antibody labelled much broader belts than around vessels in normal skin (5), and in angioimmunoblastic lymphadenopathy, vascular proliferation and small amounts of intercellular collagenous fibrils were reliably revealed by the antibody (6).

Human Podocalyxin Antibody

Monoclonal Mouse IgG_{2A} Clone # 222328

Catalog Number: MAB1658

DESCRIPTION

Species Reactivity	Human
Specificity	Detects human Podocalyxin in direct ELISAs and Western blots. In Western blots, no cross-reactivity with recombinant mouse Podocalyxin or recombinant human Endoglycan is observed.
Source	Monoclonal Mouse IgG_{2A} Clone # 222328
Purification	Protein A or G purified from hybridoma culture supernatant
Immunogen	Mouse myeloma cell line NS0-derived recombinant human Podocalyxin Ser23-Arg427 Accession # AAB61574
Formulation	Lyophilized from a 0.2 μm filtered solution in PBS with Trehalose. See Certificate of Analysis for details.

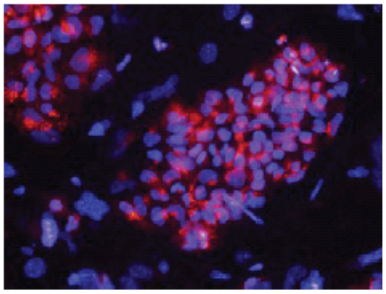
APPLICATIONS

Please Note: *Optimal dilutions should be determined by each laboratory for each application. General Protocols are available in the Technical Information section on our website.*

	Recommended Concentration	Sample
Western Blot	1 µg/mL	Recombinant Human Podocalyxin
Flow Cytometry	2.5 µg/10^6 cells	BG01V human embryonic stem cells
Immunocytochemistry	8-25 µg/mL	See Below

DATA

Immunocytochemistry



Podocalyxin in BG01V Human Stem Cells. Podocalyxin was detected in immersion fixed BG01V human embryonic stem cells using 10 µg/mL Mouse Anti-Human Podocalyxin Monoclonal Antibody (Catalog # MAB1658) for 3 hours at room temperature. Cells were stained with the NorthernLights™ 557-conjugated Anti-Mouse IgG Secondary Antibody (red; Catalog # NL007) and counterstained with DAPI (blue). View our protocol for Fluorescent ICC Staining of Cells on Coverslips.

PREPARATION AND STORAGE

Reconstitution	Reconstitute at 0.5 mg/mL in sterile PBS.
Shipping	The product is shipped at ambient temperature. Upon receipt, store it immediately at the temperature recommended below.
Stability & Storage	**Use a manual defrost freezer and avoid repeated freeze-thaw cycles.** ● 12 months from date of receipt, -20 to -70 °C as supplied. ● 1 month, 2 to 8 °C under sterile conditions after reconstitution. ● 6 months, -20 to -70 °C under sterile conditions after reconstitution.

BACKGROUND

Podocalyxin, also known as Podocalyxin-like protein-1 (PCLP1 or PODXL), is a type I transmembrane glycoprotein. It belongs to the CD34/Podocalyxin family of sialomucins that share structural similarity and sequence homology (1). Podocalyxin is a major sialoprotein in the podocytes of the kidney glomerulus and is also expressed by both endothelium and multipotent hematopoietic progenitors (2). It has been identified as a novel cell surface marker for hemangioblasts, the common precursors of hematopoietic and endothelial cells.

References:

1. Li, J. *et al.* (2001) DNA Seq. **12**:407.
2. Hara, T. *et al.* (1999) Immunity **11**:567.

BioLegend
The path to legendary discovery™

Product Data Sheet

Purified anti-mouse/human CD15 (SSEA-1)

Catalog # / Size: 125601 / 25 µg
125602 / 100 µg

Clone: MC-480

Isotype: Mouse IgM, κ

Immunogen: Mouse F9 Teratocarcinoma Stem Cells (X-irradiated)

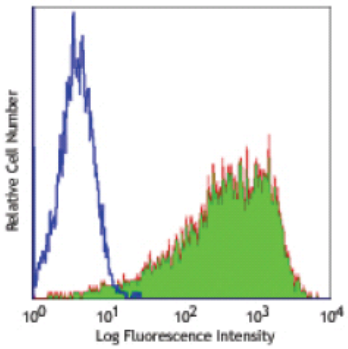
Reactivity: Mouse, **Cross-Reactivity:** Human

Preparation: This antibody is at >85% purity.

Formulation: Phosphate-buffered solution, pH 7.2, containing 0.09% sodium azide.

Concentration: 0.5 mg/ml

Storage: The antibody solution should be stored undiluted at 4°C.



F9 (mouse embryonic carcinoma cell line) stained with purified MC-480, followed by anti-mouse IgM-PE

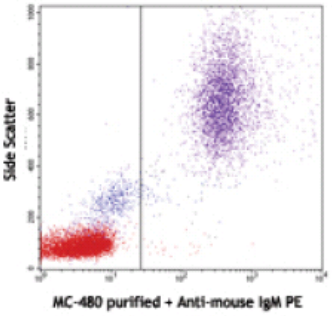
Applications:

Applications: FC - *Quality tested*
IP, WB, IHC - *Reported in the literature*

Recommended Usage: Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. For immunofluorescent staining, the suggested use of this reagent is ≤0.125 µg per million cells in 100 µl volume. It is recommended that the reagent be titrated for optimal performance for each application.

Application Notes: Additional reported applications (for the relevant formats)include: immunoprecipitation[1], Western blotting[1] and immunohistochemistry[1] of acetone-fixed frozen tissue sections and formalin-fixed paraffin-embedded sections.

Application References: 1. Solter, D. and Knowles, B.B., 1978. *Proc. Natl. Acad. Sci. USA.* 75:5565.



Human peripheral blood lymphocytes, monocytes, granulocytes stained with purified MC-480, followed by anti-mouse IgM PE

Description: The MC-480 antibody reacts with mouse and human Stage-Specific Embryonic Antigen-1 (SSEA-1). It is a Lewis blood group related carbohydrate antigen known as X-hapten, Lewis X, 3-FAL, 3-fucosyl-N-acetyllactosamine, or CD15.The expression pattern of SSEA-1 antigen is different from human and mouse. In mouse, SSEA-1 is expressed on embryonic stem cells (ES), embryonal carcinoma cells (EC), 8-cell to blastocyst embryos, and a subset of embryonic inner cell mass. The expression on murine ES cells is decreased upon differentiation. In human, however, SSEA-1 is not found on undifferentiated ES cells, but its expression is upregulated along with differentiation. CD15 is highly expressed on adult human granulocytes. It has been reported that SSEA-1 plays a role in cell adhesion and regulation of cell differentiation.

Antigen References:
1. Solter, D. and Knowles, B.B., 1978. *Proc. Natl. Acad. Sci.* USA. 75:5565.
2. Harris, J.F., *et al.* 1984. *J. Immunol.* 132:2502.
3. Gooi, H.C., *et al.* 1981. *Nature* 292:156.
4. Cui, L., *et al.* 2004. *J. Histochem. Cytochem.* 52:1447.

Related Products:

Product	Clone	Application
Purified Mouse IgM, κ Isotype Ctrl	MM-30	FC, ICC, ICFC, IF, IHC, IP, WB
Cell Staining Buffer		FC, ICC, ICFC
RBC Lysis Buffer (10X)		FC, ICFC

Anti-Cytokeratin 5, 6, clone D5/16B4

Monoclonal Antibody

Cat. # MAB1620

Lot # LV1825820

pack size: 50 µg

Store at 2-8°C

FOR RESEARCH USE ONLY

MILLIPORE

Certificate of Analysis

Applications	Species Cross-Reactivity	Antibody Isotype	Epitope/ Region	Host Species	Molecular Weight	Accession #
WB, IH(P), IF	H, M, R	IgG1	N/A	M	Varies	NP_000415

Background

The cytokeratins are a family of intermediate filaments found in almost all epithelial cells. They are divided into four subclasses depending on their patterns of reactivity and electrophoretic characteristics and are also arranged in a numbered series of 20 or more according to molecular weight and isoelectric point (Quinlan et al. 1985). Types 5 and 6 of cytokeratin (CK5/6) are in high frequency in mesothelial cells, as opposed to cells of adenocarcinoma. CK5/6 has been shown to be a reliable marker for mesothelioma and squamous cell carcinoma of the lung and Bowen's disease. It does not react with pulmonary adenocarcinoma.

Presentation

Purified mouse monoclonal IgG1 in buffer containing 0.02 M Phosphate buffer, 0.25 M NaCl, pH 7.6 with 0.1% sodium azide.

Concentration

1 mg/mL

Specificity

Reacts with human cytokeratin 5 on immunoblots of cytoskeletal preparations of epidermis and non-cytokeratinizing epithelium (Abstract: European Symposium of the Biology of the Cytoskeleton, Helsinki, 18-21, 6, 89, 1989) and on tissue sections. Shows reactivity with cytokeratin 6 and weak reactivity with cytokeratin 4 on immunoblots. No cross-reactivity with cytokeratins 1, 7, 8, 10, 13, 14, 18 or 19. MAB1620 recognizes basal cells and a part of the stratum spinosum in the normal pavement epithelium. The antibody may be useful for distinction of low differentiated pavement epithelium carcinoma and adenocarcinoma. The antibody works on formalin fixed tissues.

Species Cross-reactivity

Human. Expected to cross-react with mouse and rat.

Immunogen

Purified cytokeratin 5 (Exp. Cell Res. (1986) 162:114; J. Invest. Dermatology (1987) 88:191).

Molecular Weight

Cytokeratin 4 (59 kDa), Cytokeratin 5 (58 kDa) and Cytokeratin 6 (56 kDa).

Method of Purification

Protein A Purified

Storage and Handling

Stable for 6 months at 2-8°C in undiluted aliquots from date of receipt.

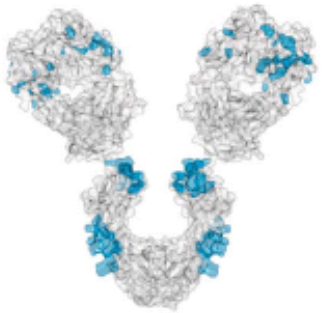
Control

A431 cell lysate, human bladder tumor tissue.

Quality Control Testing

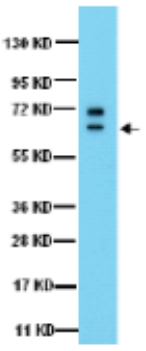
Routinely evaluated by Western Blot on A431 lysates.

Western Blot Analysis: 1:500 dilution of this lot detected **cytokeratin** 5/6 on 10 ug of A431 lysates.



References

1. Mikaelian, I., *et al.* (2004). *Toxicology Pathology.* 32:181-191.
2. EQAS 2002 Evaluation, A. Dodson, ed., UK-NEQAS, (2002). *Immunocytochemistry Journal.* 1(3).
3. Clover, J., *et al.* (1997). *Histopathology.* 31:140-143.
4. Mischke, D. and Wild, G., (1987). *J. Invest. Dermatology.* 88:191.
5. Wild, G. and Mischke, D., (1986). *Exp. Cell Res.* 162:114.
6. Quinlan, R.A., (1985). *Ann N Y Acad Sci.* 1985;455:282-306.



Western Blot Analysis: Representative lot data. A431 lysate was resolved by electrophoresis, transferred to PVDF membrane and probed with anti-**cytokeratin** 5/6 (1:500 dilution of this lot). Proteins were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a chemiluminescence detection system.

Arrow indicates protein **cytokeratin** 5/6 (~60/70 kDa). Expected MW is 62 kDa, observed MW for cytokeratin 5/6 is 56-70 kDa.

Additional Research Applications

Immunohistochemistry (frozen or paraffin sections): A 1:50-1:100 dilution from a previous lot was used for paraffin, high-temperature antigen retrieval required (Pressure cooker, Citrate/EDTA buffer pH 6.0, 4 minutes at full pressure).

Immunofluorescence: A previous lot of this antibody was used in Immunofluorescence.

Optimal working dilutions must be determined by end user.

Important Note: During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. For products with volumes of 200 µL or less, we recommend gently tapping the vial on a hard surface or briefly centrifuging the vial in a tabletop centrifuge to dislodge any liquid in the container's cap.

PROTOCOL

Western Blot

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on cell lysate and transfer the proteins to a PVDF membrane. Wash the PVDF membrane twice with water.
2. Block the blotted PVDF membrane in freshly prepared 5% BSA with 0.05% Tween®-20 for 1 hour at room temperature with constant agitation.
3. Incubate the PVDF with the recommended dilution of anti-Cytokeratin 5/6 diluted in freshly prepared 5% BSA for 1 hour at room temperature or overnight with agitation at 2-8°C.
4. Wash the PVDF 3 times with TBST.
5. Incubate the PVDF in the secondary reagent of choice (a goat anti-mouse HRP conjugated IgG, Catalog # AP124P 1:1000 dilution was used) in 5% milk for 1 hour with agitation at room temperature.
6. Wash the PVDF 3-5 times with TBST.
7. Use Spray and Glow Catalog # 17-373 to visualize results. Use as directed.

APPLICATION NOTES FOR MAB1620

Paraffin Embedded Sections: Formalin-fixed paraffin embedded sections are dewaxed. Before adding the antibody the section should be treated with pronase at 37°C.

Frozen Sections: Ideal frozen sections (4-5 µm) are obtained from shock frozen tissue samples. The frozen sections are air dried and then fixed with acetone for 10 minutes at -20°C. Excess acetone is allowed to evaporate.

1) If necessary, block unspecific binding sites by overlaying the sections with 20 µL fetal calf serum for 30 minutes at 37°C in a humid chamber.
2) Cover the preparation according to the size of the section with a suitable volume of properly diluted MAB1620 and incubate for one hour at room temperature in a humid chamber.
3) Immerse the slides briefly in PBS and wash three times for three minutes each in PBS.
4) Wipe slides dry, except the area of the section. Cover the section with 10-20 µL of a solution of anti-Mouse IgG-FITC or anti-Mouse IgG-enzyme and incubate for one hour at room temperature or at 37°C in a humid chamber.
5) Wash the slide three times for three minutes each in PBS.

The section should not be allowed to dry out during the steps. If using an enzyme conjugate, the preparation is covered with a substrate solution and incubated at room temperature until a clearly visible color develops.

RELATED PRODUCTS (specific)

cat #	description
MAB3224	Anti-Basal Cell Cytokeratin
MAB3412	Anti-Cytokeratin AE1/AE3, recognizes acidic & basic cytokeratins, clone AE1/AE3
IHC2025-6	IHC Select® Anti-Cytokeratin AE1/AE3 (Pan cytokeratins), prediluted, clone AE1/AE3
IHC2029-6	IHC Select® Anti-Cytokeratin High MW, prediluted, clone 34betaE12
IHC2026-6	IHC Select® Anti-Cytokeratin Type I, prediluted, clone AE1
IHC2027-6	IHC Select® Anti-Cytokeratin Type II, prediluted, clone AE3
04-587	Anti-Cytokeratin 5
CBL267	Anti-Cytokeratin 5, 14, clone LH8
MAB3228	Anti-Cytokeratin 5, 8
IHCR2030-6	IHC Select® Anti-Cytokeratin 5, 6, prediluted, clone D5/16B4
IHC2030-6	IHC Select® Anti-Cytokeratin 5, 6, prediluted, clone D5/16B4
AP124P	Goat anti-Mouse IgG, Peroxidase Conjugated, H+L
AP192F	Donkey anti-Mouse IgG, FITC Conjugated

RELATED PRODUCTS (non-specific)

cat #	description
IPVH00010	Immobilon-P 26.5 cm x 3.75 m Roll PVDF 0.45 um
IPFL00010	Immobilon-FL 26.5 cm x 3.75 m Roll PVDF 0.45 um
IPVH07850	Immobilon-P 7 x 8.4 cm PVDF 0.45 mm (sheet) 50/pk
ISEQ00010	Immobilon-P SQ 26.5 cm x 3.75 m 1 roll PVDF 0.2 um
ISEQ07850	Immobilon-P 7 x 8.4 cm PVDF 0.2 mm (sheet) 50/pk
IPFL07810	Immobilon-FL 7 x 8.4 cm PVDF 0.45 mm (sheet) 10/pk
WBKLS0050	IMMOBILON WESTERN CHEMILUM HRP SUBSTRATE 50 mL
17-373SP	Spray & Glow™ ECL Western Blotting 40 mL
2060	Re-Blot Western Blot Recycling Kit
2500	Re-Blot Plus Western Blot Recycling Kit
B2060-175GM	Blot Quick Blocker Membrane Blocking Agent 175G
2170	CHEMIBLOCKER-1LT
20-200	IMMUNOBLOT BLOCKING REAGENT 20G
DAB150-MS	IHC SELECT® DAB KIT SINGLE SPECIES-MOUSE 150 TESTS; 1 kit

APPENDIX 6
IMAGE ANALYSIS SOP

Immunohistochemically stained endometrium was previously analysed manually down the microscope but this was subject to wide inter-observer variability. There was potential for error from observer fatigue, and inter-observer quality control was not possible as different areas of endometrium were likely to be examined. There was also no guarantee that the same area had not been analysed more than once. Digital image analysis offers greater reproducibility, quality control and may be faster than manual counting. This SOP will explain how to capture images of stained endometrial biopsies using NIS Elements-F and Eclipse Net and how to measure BL thickness using Image J.

Health and Safety Precautions

The main health and safety risks to this procedure arise from the use of Display Screen Equipment (DSE). DSE risks are the possibility of upper limb disorders (e.g. repetitive strain injury (RSI)), eye strain and eye fatigue and the effects of mental stress. In order to minimise these risks, users should endeavour to create a comfortable work position and layout of work, ensure that the screen is regularly cleaned and avoids glare and reflections, take short breaks or changes of activity, report and problems straight away and arrange to have eyesight checks. Users should have a copy of the HSE leaflet "Working with VDUs" if they work on DSEs for at least one continuous hour on 10 occasions.

EQUIPMENT INFORMATION

Setup 1

- Nikon Biophot Microscope, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon DS-5M camera head 5M pixel, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon Digital Sight DS-U1 digital control unit, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon C-Mount TV adaptor 0.6x, Nikon Corporation, Tokyo 100-8331, Japan
- Eclipsenet software, developed by Laboratory Imaging s.r.o. for Nikon Instruments Europe B.V., 1170AE Badhoevedorp, The Netherlands

Setup 2 (supplied by Jencons-PLS)

- Nikon Eclipse 50i Microscope, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon DS-Fi1 digital camera Head 5M pixel, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon Digital control unit DS-U2 USB, Nikon Corporation, Tokyo 100-8331, Japan
- Nikon C-Mount TV adaptor, 0.63x, Nikon Corporation, Tokyo 100-8331, Japan
- NIS-Elements-F software, developed for Nikon Instruments

Both systems

- Personal computer (minimum specification 1GB RAM, 2.8GHz processor)
- Adobe Photoshop CS2 software, Education Version. Adobe Systems Incorporated. 345 Park Avenue, San Jose, CA 95110-2704 USA www.adobe.com
- MacBiophotonics Image J http://www.macbiophotonics.ca/downloads.htm

Methods

1. Turn on the PC and for "Setup 2", log on to the University Managed Network.
2. Switch on the Nikon Digital Sight Box and wait until a continuous green light is displayed.
3. Load the Image Capture Software (Eclipse Net for Setup 1, and NIS-Elements F for Setup 2) by double clicking the icon on the desktop with the mouse.
4. Turn on the microscope.
5. Check the condenser

    - Place a slide on the microscope stage, and move to an area containing no tissue.
    - Use the 4x objective and close the diaphragm (dial on the bottom right hand side of the microscope).
    - Adjust the condenser until the diaphragm is sharply in focus (just changing from blue to red "halo").

6. Check settings:

Setup 1	Setup 2
Nikon DS-U1 Camera Driver tool box: AE P (autoexposure program) AE lock on (tick box) Live fast: 640x480 full Live quality: 1280x960 Interlaced	Mode – Normal Resolution – 640 x 480 (fast focus) 1280 x 960 (quality capture) AE lock on (tick box) Exposure – Mode – Auto exposure

Exposure and gain are automatically adjusted by the software. Compensation: +1.0-+1.6 EV Contrast: High Sharpness: High	AE comp +1.0 EV - +1.6 EV (+1.0 will be darker) Colour – Contrast – High Sharpness - high (Scene mode| commands|advanced)

Nb. Higher resolution images can be taken (2560x1920), but these are very large and should not be used for analysis with ImageJ.

7. Ensure that the objective selected in the software matches the objective being used on the microscope.
8. Check exposure – for Setup 1, use "probe" (⬚) to select area of interest; For Setup 2, click "AE area" and a box will appear.

   - If necessary adjust the size and position of the box to an area that is clear and white.
   - Click "Autowhite" (under white balance for Setup 1, and colour section of camera settings for Setup 2).
   - It is only necessary to "autowhite" once for each slide and objective combination.

9. Check calibration - click " ⊢┤ " for Setup 1 or "scale" for Setup 2 - scale bar with µM appears. For Setup 1, calibration settings are unlikely to require adjustment. For Setup 2, calibration must be performed for each new user.
10. Calibrate each objective using the scale slide.

    - Starting with the 4x objective, focus on the scale bar ensuring that the highest possible focus number is obtained in the focus bar at the bottom of the software screen by adjusting first the coarse, then the fine focus knobs on the microscope.
    - Ensure that the image displayed is on "1:1".
    - For Setup 1, Click on Calibrations|Recalibrate, ensure correct objective name is displayed and click "OK". For Setup 2, Click on Camera|calibrate.
    - Click at both ends of the largest length that can fit on the screen, and enter the length in the text box (ensure units are µm). Nb. 1 mm = 1000 µm. Click "OK".
    - Repeat for each objective.
    - Nb. This procedure should only need to be performed once for each new user.
    - Check calibration by applying scale bar and moving over the calibration slide image.
    - The colour, style, line thickness and font for the scale bar can all be changed by Right hand mouse clicking on the scale bar and changing the settings as required.

11. Scan the whole tissue section on slide, firstly on 4X, then 10X then 40X, this will give a good indication of which areas to photograph. Always check the negatives on each slide as no staining should be observed.
12. For each new sample, create a new working folder. For Setup 1, click Camera|Capture & Save Options. In "Save to file" section, select directory and prefix if desired for autocapture, ensuring that file format is "tif". For Setup 2, click File|options|working folder|New folder.

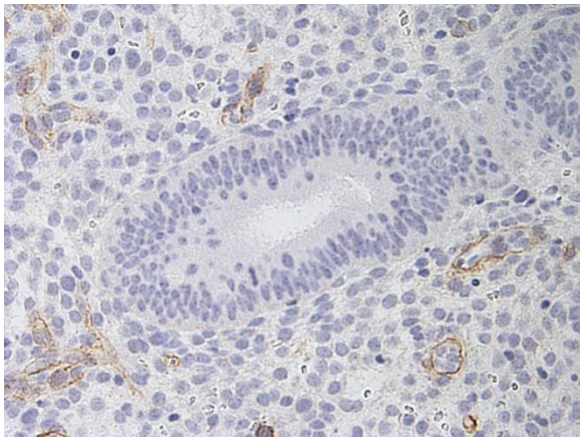
Procedure: Measuring BM Thickness

1. Before measuring BM thicknesses, size dimensions should be calibrated. To do this, first open an image with an already inserted scale bar (50μm). Zoom into the picture making the size of the scale bar as big as possible in the window. Click on the line button and carefully draw a line along the whole length of the scale bar. Click on analyse button on image J and then click on set scale. Change size to 50 and units to um. Click on global calibration setting and then ok.
2. Open the desired endometrial micrographs through image J. Zoom into the micrograph as much as possible without greatly sacrificing the resolution of the image. Using the line button again, measure the BM along the direction of the cell polarity. Points of measurement should be the most well defined stained BM for both COLIV and laminin.
3. Once satisfied with the line measurement click 'm' key to measure the BM thickness in μm. Measurements should automatically appear in an excel spreadsheet.

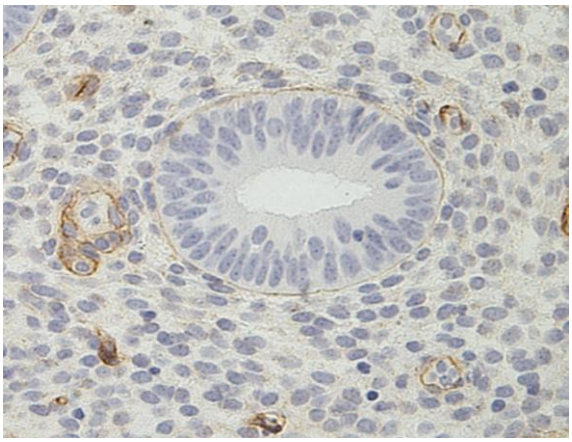
APPENDIX 7
STAINING INTENSITY ASSESSMENT

7A: Collagen IV

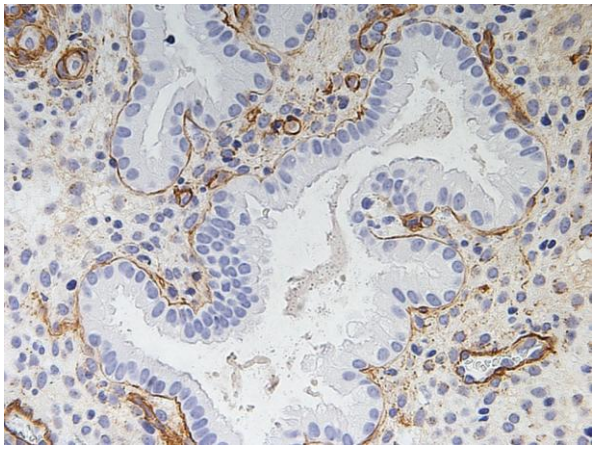
NIL: 0



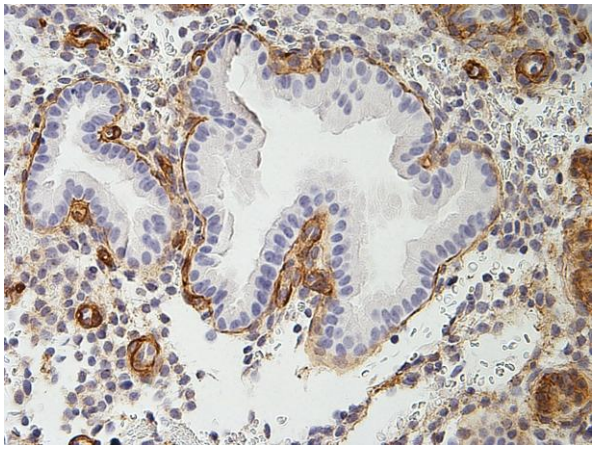
WEAK: 1



MODERATE: 2

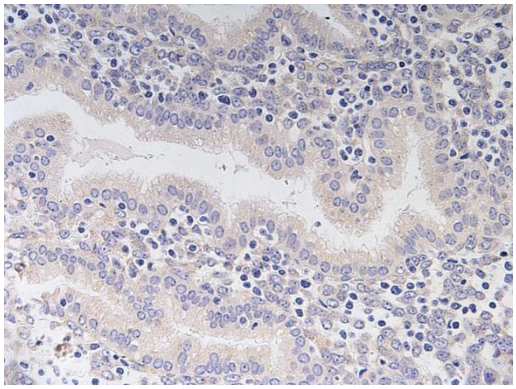


STRONG: 3

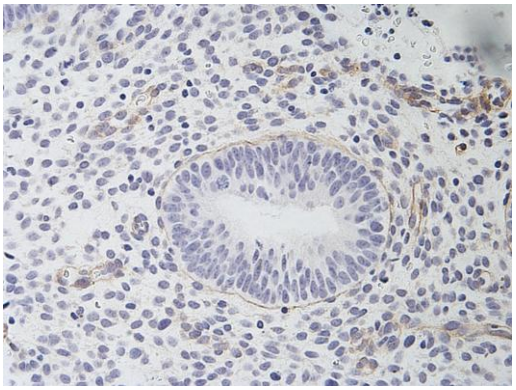


7B: Laminin

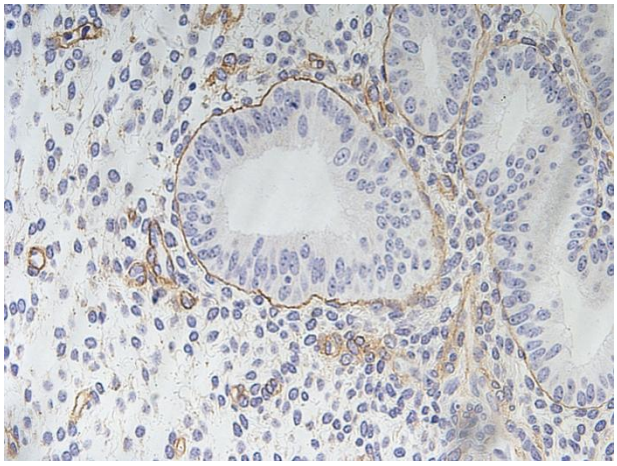
NIL: 0



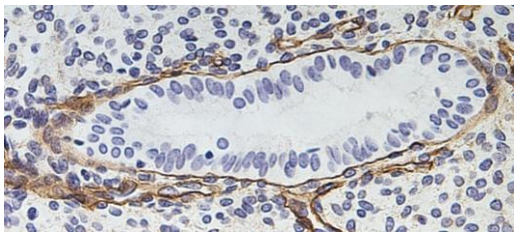
WEAK: 1



MODERATE: 2

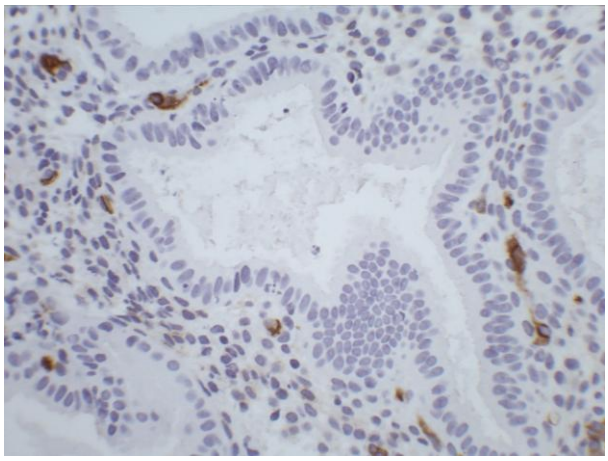


STRONG: 3

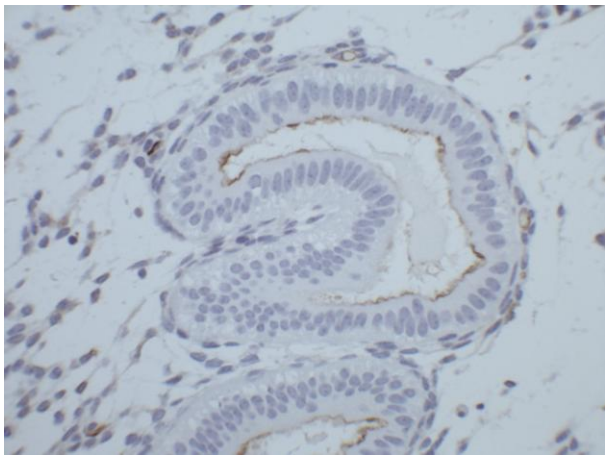


7C: PODXL

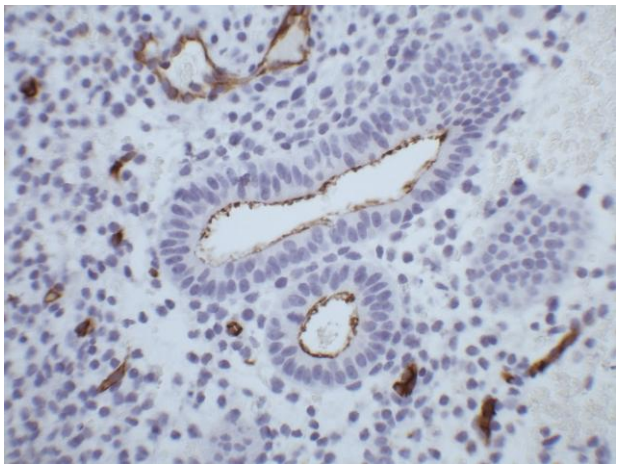
NIL: 0



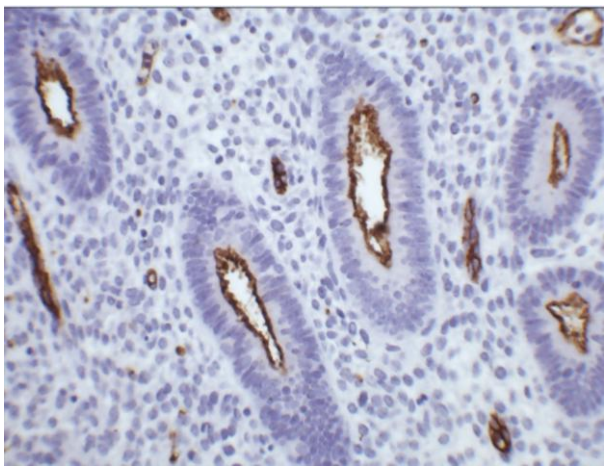
WEAK: 1



MODERATE: 2

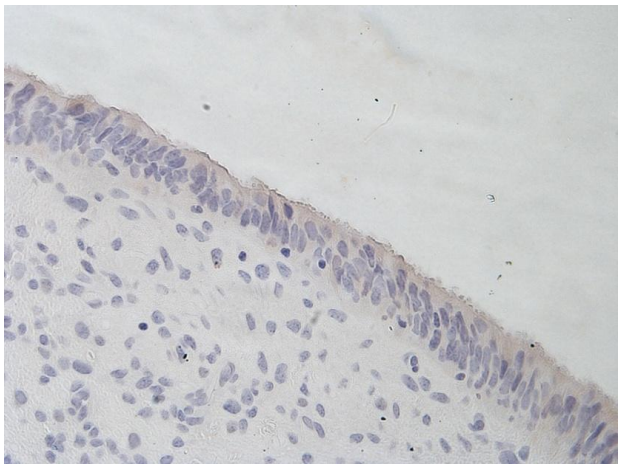


STRONG: 3

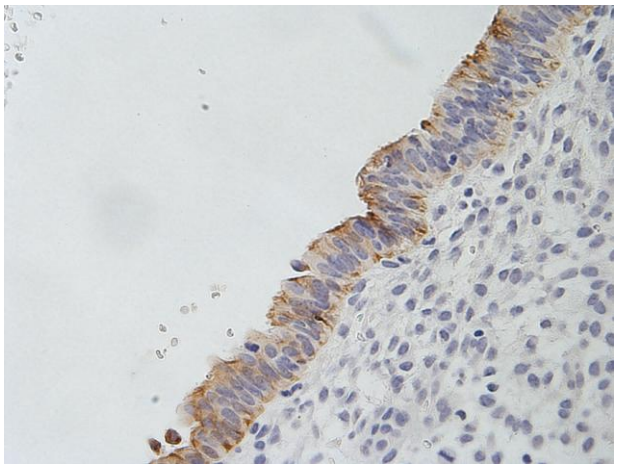


7D: CK 5/6

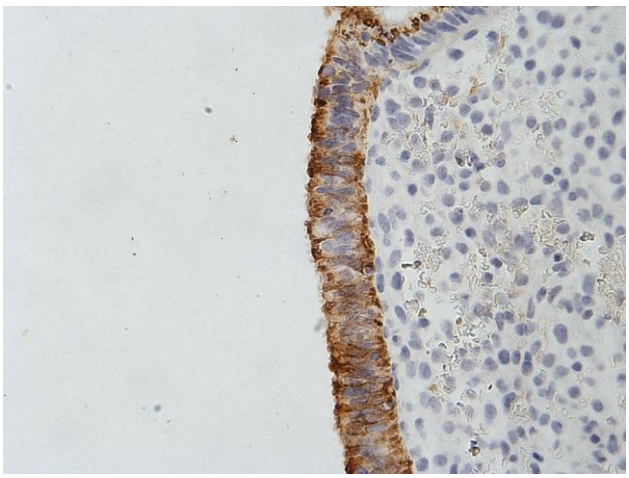
NIL: 0



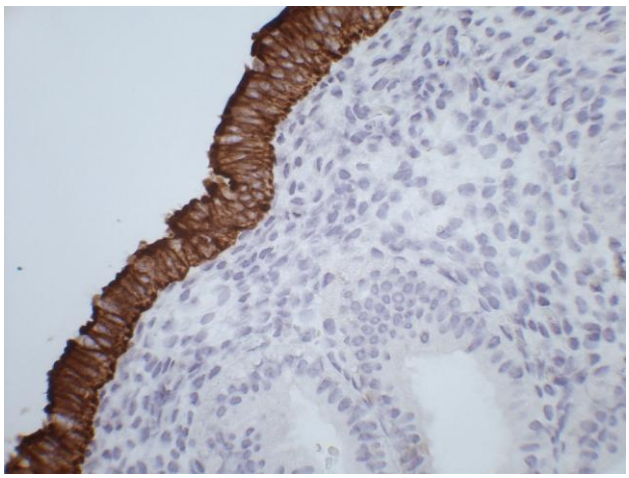
WEAK: 1



MODERATE: 2

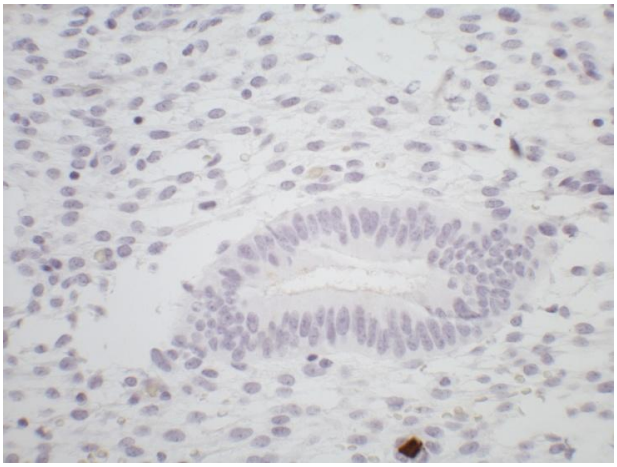


STRONG: 3

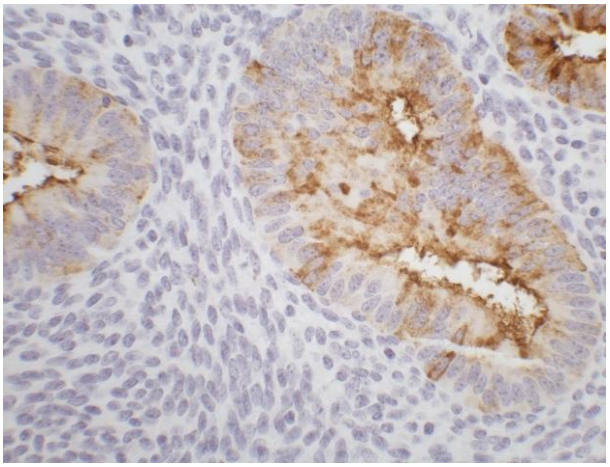


7E: SSEA1

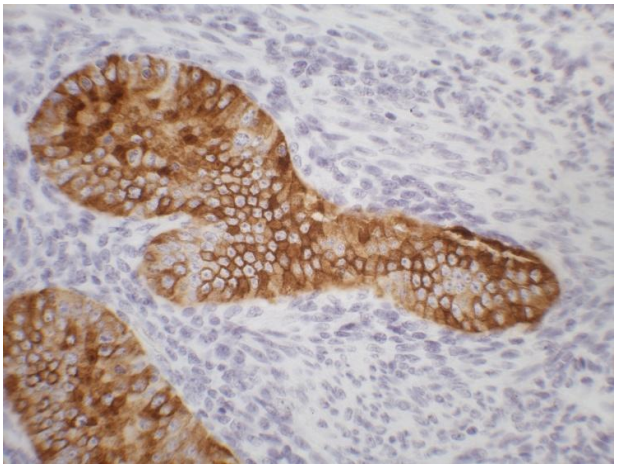
NIL: 0



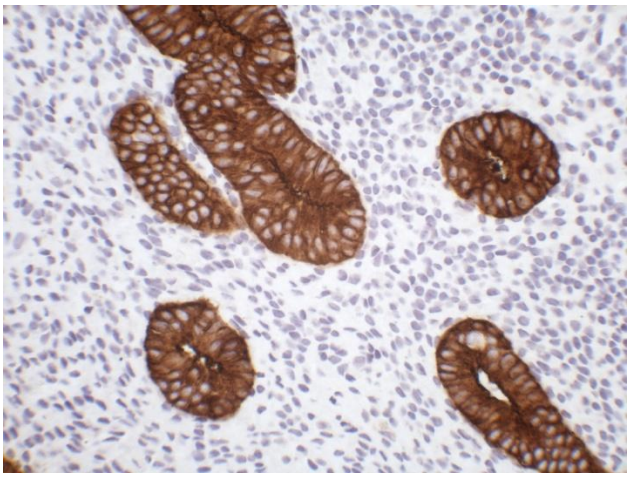
WEAK: 1



MODERATE: 2



STRONG: 3



APPENDIX 8
STUDY RESULTS

8A. Results of COLIV expression in fertile control group

COLLAGEN IV CONTROLS							
PP	**AV gland thickness**	**AV gland intensity**	**Gland QS**	**AV LEE thickness**	**AV LEE intensity**	**LE QS**	**AV vessel intensity**
1	0.712	0.750	0.534	0.705	1.500	1.058	1.450
2	0.852	1.100	0.937	0.905	1.500	1.358	1.900
3	0.743	0.750	0.557	0.965	0.500	0.483	1.650
4	0.729	0.700	0.510	0.705	2.000	1.410	1.850
5	0.888	1.200	1.065	0.710	1.500	1.065	2.300
6	0.996	2.000	1.992	0.845	2.500	2.113	2.550
7	0.851	1.150	0.979	0.775	1.500	1.163	2.350
8	0.899	1.200	1.078	0.820	1.000	0.820	2.550
9	0.637	0.600	0.382	1.125	1.500	1.688	2.100
10	0.894	1.000	0.894	1.100	2.000	2.200	2.000
Median	**0.851**	**1.050**	**0.915**	**0.833**	**1.500**	**1.260**	**2.050**
WOI							
1	0.778	1.050	0.817	0.820	0.500	0.410	2.550
2	0.878	1.150	1.010	0.670	1.000	0.670	2.250
3	0.437	0.350	0.153	N/A	N/A		0.250
4	0.861	1.700	1.463	0.990	1.500	1.485	2.750
5	0.661	0.800	0.528	0.960	1.000	0.960	1.850
6	0.725	1.050	0.761	N/A	N/A		2.200
7	0.698	1.000	0.698	1.075	1.000	1.075	2.400
8	0.505	0.750	0.379	0.000	0.000	0.000	1.900
9	0.765	1.000	0.765	1.345	1.000	1.345	2.200
10	0.484	0.350	0.169	0.000	0.000	0.000	1.950
Median	**0.711**	**1.000**	**0.729**	**0.890**	**1.000**	**0.815**	**2.200**
LSP							
1	0.684	0.950	0.650	N/A	N/A		1.750
2	0.787	2.000	1.573	0.505	1.500	0.758	2.300
3	0.721	1.650	1.189	0.805	1.000	0.805	1.650
4	0.901	2.050	1.847	0.000	0.000	0.000	2.400
5	0.803	2.050	1.646	N/A	N/A		2.100
6	0.718	1.500	1.077	0.950	2.000	1.900	2.400
7	0.864	1.350	1.166	0.810	1.000	0.810	2.050
8	0.791	1.750	1.384	0.985	2.000	1.970	2.350
9	0.735	1.200	0.882	0.705	0.500	0.353	1.550
10	0.978	1.600	1.565	0.820	1.500	1.230	2.550
Median	**0.789**	**1.625**	**1.287**	**0.808**	**1.250**	**0.808**	**2.200**

8B. Results of Laminin expression in fertile control group

LAMININ CONTROLS							
PP	**AV gland thickness**	**AV gland intensity**	**GLAND QS**	**AV LEE thickness**	**AV LEE intensity**	**GLAND QS**	**AV vessel intensity**
1	0.957	1.050	1.005	0.825	1.500	1.238	0.800
2	0.966	1.350	1.304	0.840	1.000	0.840	1.550
3	1.332	2.550	3.395	0.975	2.000	1.950	2.200
4	1.151	1.400	1.611	0.980	1.000	0.980	1.750
5	0.570	0.400	0.228	0.000	0.000	0.000	0.650
6	0.849	1.700	1.442	0.560	0.500	0.280	1.250
7	0.916	1.400	1.282	1.140	0.500	0.570	1.700
8	0.712	1.300	0.925	0.000	0.000	0.000	0.800
9	1.228	1.450	1.781	0.780	1.000	0.780	2.100
10	1.056	1.500	1.584	0.845	1.000	0.845	1.950
Median	**0.962**	**1.400**	**1.373**	**0.833**	**1.000**	**0.810**	**1.625**
WOI			0.000			0.000	
1	0.914	0.950	0.868	0.000	0.000	0.000	1.100
2	0.712	0.700	0.498	0.600	1.500	0.900	1.350
3	0.669	1.050	0.702	0.000	0.000	0.000	1.500
4	1.016	1.800	1.829	1.005	1.500	1.508	1.850
5	0.000	0.000	0.000	0.000	0.000	0.000	0.200
6	0.929	1.450	1.346	N/A	N/A		2.400
7	0.547	0.500	0.274	0.000	0.000	0.000	0.600
8	0.618	0.500	0.309	0.000	0.000	0.000	0.950
9	0.601	1.100	0.661	0.550	0.500	0.275	0.850
10	0.712	0.650	0.463	0.000	0.000	0.000	1.400
Median	**0.690**	**0.825**	**0.580**	**0.000**	**0.000**	**0.000**	**1.225**
LSP			0.000			0.000	
1	0.914	2.000	1.828	N/A	N/A		1.800
2	0.874	0.850	0.742	0.000	0.000	0.000	1.400
3	0.910	3.500	3.185	0.880	1.000	0.880	1.600
4	0.994	1.300	1.292	0.000	0.000	0.000	1.600
5	1.244	2.350	2.923	0.000	0.000	0.000	1.900
6	1.001	1.650	1.652	0.810	0.500	0.405	2.200
7	0.491	0.250	0.123	0.000	0.000	0.000	0.500
8	1.158	1.950	2.257	1.170	2.000	2.340	2.250
9	0.712	0.900	0.640	0.925	1.000	0.925	0.900
10	0.819	1.200	0.982	0.830	0.500	0.415	1.600
Median	**0.912**	**1.475**	**1.472**	**0.810**	**0.500**	**0.405**	**1.600**

8C. Results of PODXL expression in fertile control group

PODXL CONTROLS		
Proliferative	Average combined quick score	Vessel intensity
1	10.2	1.95
2	9.2	1.53
3	8.8	1.3
4	9.2	2.39
5	8.6	1
6	6.5	1.55
7	8.4	1.125
8	6.95	1.4
9	10.3	2.7
10	3.05	2.11
Median	**8.7**	**1.54**
Window of Implantation		
1	4.9	1.89
2	2.4	2.07
3	6.6	1.5
4	5	1.4
5	0.05	1.25
6	6.4	2.78
7	8	2.9
8	3.14	1.389
9	0.5	2
10	8	1.35
Median	**4.95**	**1.695**
Late secretory Phase		
1	4.1	1.8
2	4.45	1.8
3	0	1.95
4	4.45	1.8
5	0.65	2.24
6	0	2.12
7	0	2.2
8	0.4	2.12
9	0.1	1.5
10	0.05	1.89
Median	**0.25**	**1.92**

8D. Results of CK5/6 expression in fertile control group

Cytokeratin 5/6 Controls		
Proliferative	% +ve glands	LE SI
1	3.60	0.00
2	100.00	2.50
3	100.00	2.00
4	0.00	1.00
5	50.00	2.50
6	0.00	0.50
7	5.00	0.50
8	30.00	1.50
9	55.60	2.00
10	100.00	1.00
Window of Implantation		
1	0.00	0.50
2	96.60	3.00
3	0.00	3.00
4	46.20	1.50
5	13.00	2.00
6	0.00	N/A
7	52.00	1.50
8	0.00	2.00
9	73.00	3.00
10	0.00	1.00
Late secretory Phase		
1	8.30	
2	0.00	1.00
3	29.60	N/A
4	0.00	1.00
5	11.10	N/A
6	27.80	2.50
7	0.00	1.50
8	48.00	2.50
9	0.00	1.00
10	0.00	1.50

8E. Results of SSEA1 expression in fertile control group

SSEA1 CONTROLS									
	Basal				Functional				
PP	no. +ve glands	Av Gland SI	Av % of glands	Combined Quickscore	no. +ve glands	Av Gland SI	Av % of glands	Combined Quickscore	LE SI
1					0.9	0.75	1.2	0.81	2
2	1	1.65	3	4.95	1	1.85	3	5.55	1
3					1	1.1	1.5	1.65	1.5
4					0.7	0.65	0.8	0.364	1
5	1	2	2.7	5.4	1	1.7	2.5	4.25	2
6	1	1.35	2.7	3.645	1	0.75	1.8	1.35	2.5
7					1	1.55	1.5	2.325	2
8	1	2.05	3	6.15	1	1.15	1.6	1.84	1.5
9					1	1.35	2.4	3.24	2
10	1	1.45	2.8	4.06	1	1.1	1	1.1	2
WOI									
1	0.2	0.1	0.2	0.004	0.7	0.65	0.7	0.3185	0.5
2					0.6	0.8	0.6	0.288	0
3					0	0	0	0	0.5
4					0.6	0.65	0.6	0.234	0.5
5	0.5	0.35	0.5	0.0875	0.7	0.35	0.8	0.196	0.5
6					1	1.05	2	2.1	N/A
7	0.9	0.65	1.5	0.8775	0.6	0.35	0.6	0.126	0.5
8					0	0	0	0	0.5
9	0.4	0.4	0.4	0.064	0.8	1.15	0.8	0.736	0
10					0.2	0.1	0.2	0.004	0.5
LSP									
1					0.4	0.35	0.4	0.056	2
2					0.3	0.2	0.4	0.024	0.5
3					1	1.55	1.7	2.635	N/A
4					1	1.75	1.8	3.15	2
5					0.6	0.65	0.6	0.234	0
6					0.6	0.55	0.6	0.198	1.5
7	0.9	1.15	0.9	0.9315	0.6	0.8	0.6	0.288	0
8					1	1.1	1	1.1	2
9	0.4	0.45	0.4	0.072	0.8	0.85	0.8	0.544	1.5
10	0.5	0.5	0.5	0.125	0.7	0.43	0.71	0.21371	2

8F. Results of COLIV expression in PM control group

COLLAGEN IV POST MENOPAUSAL							
PM	**AV gland thickness**	**AV gland intensity**	**Gland qs**	**AV LEE thickness**	**AV LEE intensity**	**LEE QS**	**AV vessel intensity**
1	1.057	1.650	1.744	0.925	1.000	0.925	1.150
2	3.553	1.250	4.441	1.175	1.500	1.763	0.900
3	0.132	0.150	0.020	0.000	0.000	0.000	0.150
4	0.561	1.100	0.617	0.670	1.000	0.670	0.100
5	0.690	1.250	0.863	0.545	0.500	0.273	1.200
Median	**0.690**	**1.250**	**0.863**	**0.670**	**1.000**	**0.670**	**0.900**

8G. Results of Laminin expression in PM control group

LAMININ POST MENOPAUSAL							
PM	**AV gland thickness**	**AV gland intensity**	**Gland QS**	**AV LEE thickness**	**AV LEE intensity**	**LEE QS**	**AV vessel intensity**
1	0.8855	1.05	0.929775	0	0	0	0.65
2	1.045	2.35	2.45575	0.655	1	0.655	2.2
3	0.1955	0.2	0.0391	0	0	0	0.05
4	0.891	1.4	1.2474	0.865	2	1.73	1.5
5	0.913	1.55	1.41515	1.06	2	2.12	1.65
Median	**0.891**	**1.4**	**1.2474**	**0.655**	**1**	**0.655**	**1.5**

8H. Results of PODXL expression in PM control group

PODXL PM		
PM	Average combined quick score	Vessel intensity
1	3.95	1.4
2	7	1.55
3	7.5	1.05
4	7.2	1.7
5	6.286	1.929
Median	**7**	**1.55**

8I. Results of CK5/6 expression in PM control group

CK5/6 post menopausals controls		
Post menopausals	% +ve glands	LE SI
1	20.00	n/a
2	6.45	0.50
3	3.23	0.00
4	0.00	0.00
5	0.00	0.50

8J. Results of SSEA1 expression in PM control group

SSEA 1 POST MENOPAUSALS						
PM	no. +ve glands/10 glands	Av Gland SI	No. of G with +ve GS	Av % of glands	LE SI	QS
1	0.9	2	10	2.4	N/A	4.32
2	1	3	10	3	0.5	9
3	1	1.95	10	1.9	1	3.705
4	1	2.55	10	2.7	3	6.885
5	1	2.5	10	2.7	2	6.75

8K. Results of COLIV expression in endometriosis group

COLLAGEN IV ENDOMETRIOSIS							
PP	**AV gland thickness**	**AV gland intensity**	**AV gland QS**	**AV LEE thickness**	**AV LEE intensity**	**AV LE QS**	**AV vessel intensity**
1.000	0.621	0.600	0.373	N/A	N/A		1.000
2.000	0.826	1.400	1.156	1.110	2.000	2.220	2.650
3.000	0.681	0.650	0.442	0.890	0.500	0.445	1.400
4.000	0.753	1.000	0.753	N/A	N/A		2.000
5.000	0.863	1.850	1.597	0.665	0.500	0.333	2.400
6.000	0.812	1.350	1.096	0.880	1.000	0.880	1.850
7.000	0.000	0.000	0.000	0.000	0.000	0.000	0.650
8.000	0.619	0.500	0.310	0.920	1.000	0.920	2.000
9.000	0.132	0.100	0.013	N/A	N/A		0.950
10.000	0.865	1.000	0.865	0.855	1.000	0.855	1.800
11.000	0.928	1.650	1.530	N/A	N/A		2.300
Median	**0.753**	**1.000**	**0.753**	**0.880**	**1.000**	**0.855**	**1.850**
WOI							
1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2.000	0.054	0.050	0.003	0.000	0.000	0.000	0.900
3.000	0.056	0.050	0.003	0.650	0.500	0.325	0.500
4.000	0.526	0.400	0.210	N/A	N/A		1.150
5.000	0.663	0.650	0.431	0.000	0.000	0.000	1.950
6.000	0.694	0.950	0.659	0.890	2.000	1.780	2.200
7.000	0.411	0.600	0.246	0.565	1.000	0.565	2.050
8.000	0.733	0.750	0.550	1.560	1.500	2.340	2.200
9.000	1.094	1.900	2.079	0.785	1.500	1.178	2.250
10.000	1.269	0.450	0.571	2.090	2.000	4.180	0.910
11.000	1.814	1.750	3.175	N/A	N/A		2.650
Median	**0.663**	**0.600**	**0.431**	**0.650**	**1.000**	**0.565**	**1.950**
LSP							
1.000	1.340	0.550	0.737	N/A	N/A		1.100
2.000	1.070	0.450	0.481	0.000	0.000	0.000	1.150
3.000	0.840	1.500	1.260	0.000	0.000	0.000	2.600
4.000	0.794	1.357	1.077	N/A	N/A		2.071
5.000	0.985	0.900	0.886	1.455	0.500	0.728	2.200
6.000	0.128	0.200	0.026	0.620	1.000	0.620	1.550
7.000	0.986	2.000	1.972	1.075	2.000	2.150	2.650
8.000	0.899	1.400	1.259	0.820	1.000	0.820	1.950
Median	**0.942**	**1.129**	**0.982**	**0.720**	**0.750**	**0.674**	**2.011**

8L. Results of laminin expression in endometriosis group

LAMININ ENDOMETRIOSIS							
PP	**AV gland thickness**	**AV gland intensity**	**GLAND QS**	**AV LEE thickness**	**AV LEE intensity**	**LEE QS**	**AV vessel intensity**
1	1.391	2.050	2.852	0.950	2.000	1.900	2.200
2	0.977	1.550	1.514	0.800	0.500	0.400	1.600
3	0.914	0.950	0.868	0.000	0.000	0.000	1.100
4	0.680	0.600	0.408	N/A	N/A	N/A	0.700
5	1.039	1.850	1.922	0.000	0.000	0.000	1.750
6	1.042	2.100	2.188	0.970	1.500	1.455	2.350
7	0.444	0.350	0.155	0.000	0.000	0.000	1.200
8	1.108	1.650	1.828	0.900	1.000	0.900	2.250
9	0.431	0.250	0.108	N/A	N/A	N/A	1.050
10	0.745	1.000	0.745	0.000	0.000	0.000	1.500
11	0.372	0.450	0.167	N/A	N/A	N/A	0.600
Median	**0.914**	**1.000**	**0.868**	**0.400**	**0.250**	**0.200**	**1.500**
WOI							
1	0.630	0.800	0.504	0.000	0.000	0.000	1.700
2	0.684	1.300	0.889	0.490	2.000	0.980	2.150
3	0.728	1.650	1.200	1.285	2.000	2.570	2.150
4	0.770	1.550	1.194	N/A	N/A		2.400
5	0.882	1.150	1.014	0.535	2.000	1.070	2.100
6	0.897	1.700	1.524	0.980	2.000	1.960	1.700
7	0.752	1.100	0.827	0.000	0.000	0.000	1.850
8	0.654	1.000	0.654	0.715	1.000	0.715	1.450
9	0.938	1.200	1.125	0.000	0.000	0.000	1.500
10	0.911	1.400	1.275	N/A	N/A	N/A	1.350
11	0.924	1.700	1.571	N/A	N/A	N/A	1.850
Median	**0.770**	**1.300**	**1.125**	**0.513**	**1.500**	**0.848**	**1.850**
LSP							
1	0.864	1.250	1.079	N/A	N/A	N/A	1.700
2	0.898	0.150	0.135	0.525	0.500	0.263	1.900
3	0.849	1.200	1.018	0.000	0.000	0.000	1.350
4	1.043	2.071	2.160	N/A	N/A	N/A	2.071
5	0.666	0.650	0.433	0.000	0.000	0.000	0.500
6	0.942	1.500	1.413	0.825	2.000	1.650	2.050
7	0.547	0.550	0.301	0.700	0.500	0.350	0.650
8	0.605	0.950	0.575	1.340	1.500	2.010	1.500
Median	**0.856**	**1.075**	**0.796**	**0.613**	**0.500**	**0.306**	**1.600**

8M. Results of PODXL expression in endometriosis group

PODXL ENDOMETRIOSIS		
Proliferative	Average combined quick score	Vessel intensity
1	5.000	2.350
2	7.300	2.550
3	7.650	2.500
4	7.250	2.280
5	1.900	0.950
6	4.850	2.200
7	1.850	1.300
8	8.650	2.750
9	4.200	1.300
10	6.100	2.150
11	0.000	1.750
Median	**5.000**	**2.200**
Window of Implantation		
1	3.650	1.900
2	2.950	2.750
3	6.800	2.750
4	0.250	2.300
5	2.800	2.650
6	2.800	2.500
7	6.800	1.690
8	8.600	2.700
9	0.550	1.800
10	0.000	1.300
11	0.450	1.950
Median	**2.800**	**2.300**
Late secretory Phase		
1	0.300	1.375
2	0.500	1.600
3	0.560	2.250
4	0.000	1.330
5	0.050	1.000
6	3.600	2.083
7	0.050	1.800
8	1.850	1.750
Median	**0.400**	**1.675**

8N. Results of CK5/6 expression in endometriosis group

Cytokeratin 5/6 Endometriosis		
Proliferative	% +ve glands	LE SI
1	15.80	0.00
2	7.10	1.00
3	19.20	1.50
4	84.60	N/A
5	0.00	1.50
6	30.80	0.50
7	0.00	0.00
8	100.00	2.50
9	100.00	1.00
10	0.00	1.00
11	26.70	N/A
Window of Implantation		
1	100.00	1.00
2	0.00	2.00
3	12.00	1.50
4	0.00	1.00
5	100.00	1.00
6	22.20	2.00
7	23.80	3.00
8	0.00	2.50
9	6.70	1.50
10	0.00	1.50
11	47.40	N/A
Late secretory Phase		
1	0.00	0.50
2	0.00	1.00
3	42.90	2.50
4	23.00	N/A
5	0.00	1.50
6	14.30	2.00
7	42.90	1.00
8	8.00	2.00

8O. Results of SSEA1 expression in endometriosis group

SSEA1 ENDOMETRIOSIS									
	Basal				Functional				
PP	no. +ve glands	Av Gland SI	Av % of glands	Combined Quickscore	no. +ve glands	Av Gland SI	Av % of glands	Combined Quickscore	LE SI
1					1	2.7	2.5	6.75	3
2					1	0.75	1.5	1.125	2
3					1	0.5	1.6	0.8	0.5
4	1	1.6	2.9	4.64	0.8	0.55	1	0.44	N/A
5	1	1.6	3	4.8	0.9	0.85	1.6	1.224	0.5
6	1	2.05	2.5	5.125	1	1.9	2.5	4.75	1.5
7	0.7	0.95	1.1	0.7315	0.8	0.55	1.3	0.572	1
8					1	1.35	2.5	3.375	2.5
9	1	1.85	2.9	5.365	1	1.4	2.8	3.92	2
10					1	1.45	1.6	2.32	1.5
11	1	1.7	2.7	4.59	0.9	0.85	1.7	1.3005	N/A
WOI									
1					0.2	0.1	0.2	0.004	2.5
2					0.3	0.25	0.3	0.0225	1.5
3					0.2	0.1	0.2	0.004	2
4					0.2	0.15	0.2	0.006	N/A
5					0.5	0.5	0.5	0.125	0.5
6					0	0	0	0	0
7					0.7	0.7	0.7	0.343	1
8	1	1.3	1.7	2.21	0	0	0	0	2
9					0.4	0.4	0.5	0.08	1
10					0	0	0	0	N/A
11	0.9	1.2	1.3	1.404	0.6	0.5	0.6	0.18	N/A
LSP									
1					0.2	0.2	0.2	0.008	N/A
2					0.6	0.7	0.6	0.252	2.5
3					0.4	0.6	0.4	0.096	0.5
4					0	0	0	0	N/A
5	0.2	0.3	0.4	0.024	0	0	0	0	0.5
6					0.6	0.45	0.7	0.189	2
7	1	1.3	1.2	1.56	0.2	0.4	0.2	0.016	1.5
8					0.8	0.45	0.8	0.288	1.5