UNIVERSITY OF LIVERPOOL

Investigation of Neonatal Mouse Kidney and Endometrial Cell Populations in Explant Culture Combined With an *in-silico* Approach to the Endometrium

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By

David Mathew

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Abstract

Background

The endometrium is a highly dynamic tissue undergoing up to 400 cycles of proliferation, differentiation and breakdown in an average woman's reproductive life. The mechanisms of this proliferative potential are the subject of active study and one candidate is Adult Stem Cells (ASCs). Endometriosis is a benign proliferative endometrial disease resulting from ectopic growth of endometrial tissue comprising glands and stroma with unknown pathogenesis. ASCs have also been implicated in the pathogenesis of endometriosis and attempts have been made in recent years to characterise and isolate this cell population within the endometrium. A great deal of investigation into cellular and molecular processes governing this disease has been undertaken with conflicting results. A useful tool to guide future investigation is the emerging discipline of bioinformatics.

Objectives:

One of the difficulties with investigating endometriosis and endometrial ASCs is the lack of suitable in-vitro models to study the benign yet abnormal growth of endometrial epithelial cells in culture. Ex-vivo culture models using a multicellular culture system are highly desirable for future work and the present study aimed to investigate two of the features associated with ASCs: proliferation and differentiation potential. A crudely fractionated epithelial population of endometrial cells in a neonatal kidney explant model was used to assess this.

The kidney is an ideal tissue for use in co-culture with endometrial cells as the endometrium and the kidney both embryologically derive from the intermediate mesoderm. Furthermore, it has been shown previously that human endometrial cells can generate endometrial-like tissue, including glandular structures, when transplanted into the kidneys of immune-compromised adult mice. To investigate whether an in vitro model using neonatal kidney cells could support the growth and differentiation of endometrial cells, endometrial epithelial cells were grown in a chimeric explant model using kidney tissue isolated from CD1 neonatal mice. Chimeras were generated using a recently developed reaggregation protocol. Controls comprised explants of reaggregated kidneys that did not contain any endometrial cells. Explants were cultured at an air-medium

interface for 2 hours (0 Days), 3 Days and 7 Days respectively before being fixed, processed and analysed with immunofluorescence and RT-qPCR to detect the presence of endometrial-derived glandular structures, and endometrial differentiation markers. Markers of interest for analysis of the chimeras included: Laminin, Progesterone Receptor and Mucin1. A human-specific cytoplasmic antibody was used to identify human cells in the chimeras.

This work was further extended to assess nephrogenesis and endothelial cell survival in the neonatal kidney explants. In the study of the developmental processes governing the mammalian kidney progress has been achieved through the use of embryo explant models. These models are useful for studying early developmental processes such as reciprocal induction but are less suitable for studying later maturation events (e.g. foot process formation in podocytes), due to the fact that the endothelial cell population dies. Podocytes depend upon interactions with the glomerular basement membrane (GBM) to achieve maturity and the GBM is known to be formed, at least in part, from factors secreted by endothelial cells. If an explant model could be established with an intact endothelial population there is potential to study these maturation processes. Markers of interest for neonatal kidney explants included: WT1, Pecam, Synaptopodin, Laminin and Calbindin.

Bioinformatics tools were employed to examine existing microarray data sets of normal endometrium during various stages of the menstrual cycle with the aim of identifying molecular pathways of interest and generating suitable hypotheses. The same process was carried out for endometrium from patients with endometriosis with the future goal of comparing these datasets.

Results:

Analysis of chimeric explants indicated that a large proportion of the endometrial cells died in culture with only a small population present by Day 7 that showed no evidence of forming glandular structures. A degree of nephrogenesis appeared to have occurred in the neonatal kidney explant model with evidence of endothelial cells surviving and forming cord like structures in culture.

Initial investigation of the transcriptomic datasets associated with normal endometrium and patients with endometriosis was submitted to Ingenuity Pathways Analysis (IPA) and

Cytoscape. A large amount of output was obtained and specific pathways suitable for further investigation were identified including: Super Oxide Radicals Degradation, Tryptophan Degradation to 2-amino-3 Semi Aldehyde and Wnt/β Catenin Pathway.

Conclusions:

The three main conclusions were as follows. 1 Human endometrial cells were not able to survive in the chimeric model of *ex-vivo* culture, indicating that this is unlikely to be a suitable model system for studying the formation of endometrial cell-derived glandular structures. 2 Nephrogenesis may be occurring in the reaggreagted neonatal explants, and the endothelial population appeared to have survived and formed capillary like structures *in vitro*. This is an important observation because in explants of embryonic kidneys, the endothelial cells do not survive. 3. Investigation of the molecular processes at play in the normal endometrium and endometriosis has generated some interesting hypotheses that will be further investigated using 'wet' laboratory techniques.

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Glossary

AMM. Amniotic Membrane Model

ASC. Adult Stem Cell

BM. Basement Membrane

BrdU. Bromodeoxyuridine

CAM. Chorioallantotic Membrane

cDNA. Complementary DNA

CFDA SE. Carboxy Fluorescein Diacetate, succinimidyl ester

CFU. Colony Forming Unit

Ct. Cycle to Threshold

DAPI. 4',6-diamidino-2-phenylindole

DAVID. Database for Annotation Visualisation and Integrated Discovery

DMEM. Dulbecco's Modified Eagle Medium

DMSO. Dimethylsulfoxide

dNTP. Deoxyribonucleotide Triphosphate

DTT. Dithiotheritol

ECM. Extra Cellular Matrix

EF. Epithelial Fraction

ELISA. Enzyme Linked Immuno-absorbant Assay

ESCs. Embryonic Stem Cells

FACS. Fluorescence Activated Cell Sorting

FBS. Fetal Bovine Serum

FGF. Fibroblast Growth Factor

FISH. Fluorescence In Situ Hybridisation

FSH. Follicle Stimulating Hornone

g. Relative Centrifugal Force

GBM. Glomerular Basement Membrane

GE. Glandular Epithelium

GnRH. Gonadotrophin Releasing Hormone

HCG. Human Chorionic Gonadotrophin

IDO. Indoleamine 2,3-dioxygenase

IHC. Immunohistochemistry

IPA. Ingenuity Pathways Analysis

IVF. In Vitro Fertilization

KCM. Kidney Culture Medium

LE. Luminal Epithelium

LH. Leutinising Hormone

LRC. Label Retaining Cells

LRT. Label Retaining Technique

MEME. Minimum Essential Medium Eagle

MET. Mesenchymal to Epithelial Transition

MIP. Metastasis Inducing Protein

MM. Metanephric Mesenchyme

MMPs. Matrix Metalloproteinases

MUC1. Mucin1

NBF. Neutral Buffered Formalin

NCBI. National Centre for Biotechnology Information

NGS. Normal Goat Serum

NSAIDs. Non Steroidal Anti Inflammatory Drugs

NSG. Nod SCID (Severe Combined Immune Deficiency) Gamma

NTC. No Template Control

PBS. Phosphate Buffered Saline

PC. Progenitor Cell

PCR. Polymerase Chain Reaction

PDGF-β. Platelet derived Growth Factor Beta

Pecam. Platelet Endothelial Cell Adhesion Molecule 1

PGR. Progesterone Receptor

PTEN. Phosphate and Tensin Homologue

RANTES. Regulated on Activation, Normal T Cell Expressed and Secreted

RCF. Regulators of Cell Fate

ROS. Reactive Oxygen Species

RT-qPCR. Real Time quantitative Polymerase Chain Reaction

RQ. Relative Quantity

SCID. Severe Combined Immunodeficiency

SD. Standard Deviation

SF. Stromal Fraction

SOD2. Superoxide Dismutase 2

SP. Side Population

SPC. Stem/Progenitor Cell

SSEA-1. Stage Specific Embryonic Antigen 1

TA. Transit Amplifying

TAE. Tris-Acetate EDTA

Tbp. Tata Box Protein

TGF-β. Transforming Growth Factor Beta

TIMPs. Tissue Inhibitors of Metalloproteinases

UB. Ureteric Bud

VEGF. Vascular Endothelial Growth Factor

Wt1. Wilms Tumour Protein 1

Chapter 1: Introduction

1.1 Gross Structure of the Uterus

The human uterus is a thick walled muscular organ located within the pelvic cavity. It is roughly 7.5 cm in length and is continuous superiorly with the fallopian tubes, which are encased in the broad ligament. Inferiorly the uterus is continuous with the cervix, vagina and vulva (Figure 1) (Drake RL, 2010). The structure of the uterus is made up of three layers with the outermost layer being parietal peritoneum continuous with the broad ligament. The middle layer is myometrium composed of smooth muscle fibers and the innermost layer of the uterus is the endometrium (Drake RL, 2010).

Figure 1: Anatomy of the Female Reproductive System

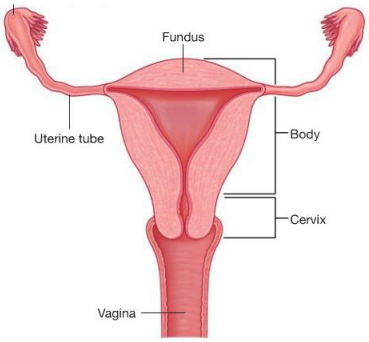


Fig1. Anatomy of the human female reproductive system (Drake RL, 2010)

1.2 Microscopic Structure of the Endometrium

The endometrium is the reproductive tissue lining the uterine cavity. It possesses immense proliferative potential evidenced by the fact that it is able to undergo up to 400 cycles of growth differentiation and shedding within an average woman's reproductive years (Gargett and Masuda, 2010). The endometrium is functionally divided into a superficial 'functionalis' layer which is shed during menstruation and a deeper 'basalis' layer which is retained and from which the new functionalis is regenerated each month.

Histologically the endometrium can be separated into two distinct cell populations, the Epithelium and the Stroma (Figure 2). The epithelium is further subdivided into the Luminal Epithelium (LE, Figure 2A), which lines the uterine cavity serving as the point of contact for the implanting blastocyst and the Glandular Epithelium (GE, Figure 2B), which lines the glands penetrating deep into the basalis (Jabbour et al., 2006). The second population is the stroma (Figure 2C), which constitutes the supporting parenchyma of the endometrium. The stroma and the basalis glands form a rough boarder with the myometrial cells, which make up the body of the uterus (Uduwela et al., 2000).

Figure 2: Microscopic Structure of the Endometrium

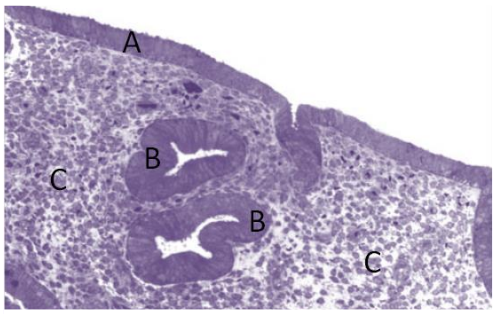


Fig2. Microstructure of the human endometrium. A: Luminal Epithelium, B: Glandular Epithelium, C: Stroma. The functionalis and basalis layers are not histologically distinct. (Gargett, 2008)

1.3 Embryology of the Uterus

The uterus, cervix, fallopian tubes and cranial portion of the vagina are formed from the paired Paramesonephric or 'Mullerian' ducts which were first described in 1825 and derive from the intermediate mesoderm (Gargett). Both the reproductive and urinary systems are derived from this structure which lies between the paraxial and the lateral plate mesoderm along the posterior wall of the abdominal cavity (Sadler, 2004). The luminal epithelium emerges first with the glands being a late fetal development shallow in nature until further development occurs postnatally (Ma, 1973). The stromal cells and myometrium are derived from the urogenital ridge mesenchyme and there is evidence that their differentiation is regulated by the surrounding epithelium (Brody and Cunha, 1989). This highlights the idea of crosstalk between these structures and indicates the importance of studying both cell populations when investigating endometrial physiology (Brody and Cunha, 1989).

1.4 Menstrual Cycle

The human menstrual cycle refers to a set of dynamic changes involving proliferation and differentiation that occur within the premenopausal endometrium according to the corresponding cyclical ovarian hormonal changes, for which the endometrium is the target organ (Figure 3). The cycle can be split into events occurring in the ovary (Figure 3A), and events occurring in the endometrium (Figure 3B). The standardised menstrual cycle is 28 days in duration with day 1 defined as the start of the menstrual phase. Ovulation occurs at approximately day 14 which signifies the transition from proliferative to secretory phase (Gargett, 2008).

Figure 3: Ovarian and Menstrual Cycles

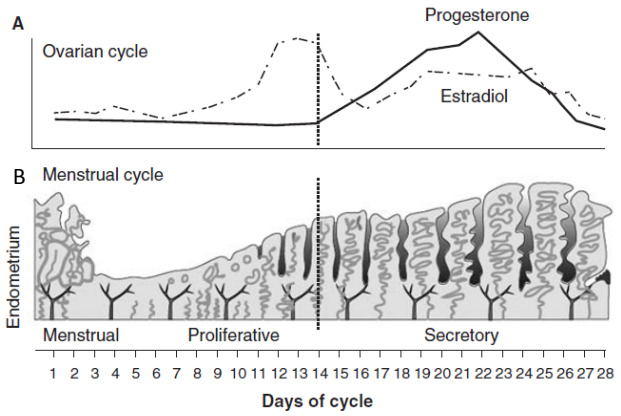


Fig3. The histological and hormonal changes associated with the human ovarian and menstrual cycles.

1.5 The Ovarian Cycle

The ovaries are the female gonads and are responsible for producing the ova which will form the maternal portion of the zygote, they undergo a series of cyclical changes in tandem with the endometrium releasing Oestrogen and Progesterone under the influence of Follicle Stimulation Hormone (FSH) and Luteinising Hormone (LH) (Impey, 2012, Gargett, 2008). The ovarian cycle is divided into follicular and luteal phases (Figure 4).

1.5.1 Follicular phase

The follicular phase is characterised by the pulsatile release of FSH and LH from the anterior pituitary under the influence of Gonadotrophin releasing Hormone (GnRH) (Figure 4A). FSH acts to stimulate the development of several primordial follicles which release oestrogen and inhibin to inhibit FSH in a negative feedback response. As oestrogen levels increase however they begin to exert a positive feedback response stimulating a 'surge' of LH at day 14 (Figure 4A) with follicle rupture and ovulation occurring usually within 36 hours of this event (Gargett, 2008).

Figure 4: Ovarian Cycle

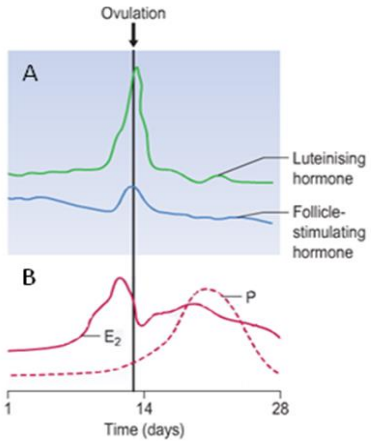


Fig4. Illustration of cyclical hormonal changes during the ovarian cycle. A: Hormones acting on the ovaries, FSH, LH. B: Hormones released by the ovaries, Oestrogen (E_2) and Progesterone (P)

1.5.2 Luteal Phase

Ovulation signifies the beginning of the luteal phase as the ruptured follicle degenerates to form the corpus luteum. This structure secretes both oestrogen and progesterone, though progesterone is dominant, along with inhibin A, the levels of which peak around day 21 (Figure 4B). Progesterone acts to prevent degeneration of the oestrogen primed endometrium and stimulates decidualization (Impey, 2012). Unless the corpus luteum is maintained by Human Chorionic Gonadotrophin (HCG) released as the result of implantation it will degenerate with the subsequent fall in progesterone triggering menstruation (Figure 4B).

1.6 Endometrial Cycle

The endometrial cycle can be split into three broad phases: Proliferative, Secretory and Menstrual each of which are characterised by structural and functional changes highlighting the varied and dynamic nature of this tissue.

1.6.1 Proliferative Phase

The endometrial changes seen in the proliferative phase are thought to be regulated by the dominant hormone in the tissue at this time, oestrogen. The early proliferative phase is characterised by initial repair of the denuded endometrial surface, a process commenced by epithelial cells suggesting that this population is required for stromal responsiveness later in the cycle (Bigsby, 2002). As the proliferative phase continues all cell types multiply under the influence of oestrogen which is thought to assert its effects via Insulin-like Growth Factors along with Epidermal Growth Factors and Transforming Growth Factor Beta (TGF-β) (Gargett et al., 2008). A vast array of additional molecules have also been implicated in the proliferation of the endometrium at this time including: growth factors, cytokines, fibronectin, integrins and matrix metalloproteinases (MMPs) (Thie and Denker, 2002). An interesting aspect of these repair and proliferation processes is that they enable the endometrium to heal without scarring, a phenomenon only previously documented in fetal skin wounds (Samuels and Tan, 1999).

1.6.2 Secretory Phase

The Secretory Phase commences at around day 14 of the cycle and is characterised by functional differentiation of the endometrial tissue to promote an environment suitable for embryo implantation (Gargett, 2008). The key hormone driving these processes is progesteronewhich acts on oestrogen primed cells. The secretory phase can be histologically subdivided into: Early, Mid and Late phases based on the criteria of Noyes et al (Noyes and Haman, 1953).

- Early Secretory Phase: sub-nuclear vacuoles are observed in epithelium
- Mid Secretory Phase: increased gland tortuosity with increased secretory activity. This phase also represents the implantation window (Psychoyos, 1973).
- Late Secretory Phase: the differentiation of stromal cells known as decidualization occurs. This spontaneous non reversible change involves fibroblast type cells close to spiral arterioles becoming enlarged and rounded. Progesterone receptor A is dominant at this time (Alexander et al., 1996).

There are a number of factors secreted into the uterine lumen during the secretory phase, including: proteins, ions, glucose, cytokines, enzymes, growth factors and proteases, collectively known as the histotroph (Kane et al., 1997).

1.6.3 Menstruation

Menstruation is the term used for the sequence of tissue breakdown and endometrial bleeding occurring at the end of each menstrual cycle during which the functionalis layer of the endometrium is shed leaving the basalis intact (Gargett et al., 2008). These changes are initiated by a fall in oestrogen and progesterone preceded by the breakdown of the corpus luteum. Various mechanisms governing menstruation have been proposed, one of which is hypoxia (Markee, 1978) however is it also likely that menstruation is an inflammatory process characterised by tissue oedema, inflammatory cell recruitment and pro inflammatory cytokine release (Finn, 1986). As the endometrial vasculature may play a key role in the tissue breakdown associated with menstruation, studies have considered various pro inflammatory and vasoactive agents associated with this structure e.g. Prostaglandin F2 alpha, Cyclo-oxygenase 2 and Endothelin 1 (Salamonsen et al., 1999a, Salamonsen et al., 1999b).

1.7 Endometriosis

A wide variety of factors contributing to the regenerative capacity of the endometrium have been proposed and research is ongoing. In addition, widening the understanding of normal physiology may provide insights into pathological processes and there are several endometrial diseases which share this characteristic of a large proliferative potential. One such disease is endometriosis which is defined as the presence and growth of tissue similar to endometrium comprising glands and stroma outside of the uterus and represents a major detriment on the quality of life of affected patients (Impey, 2012).

1.7.1 Epidemiology

There is difficulty in accurately documenting the incidence and prevalence of endometriosis as symptoms often do not correlate with severity of disease, and invasive surgical procedures such as laparoscopy are required for definitive diagnosis. In the UK 1-2% of women are diagnosed, though up to 20% of women may have endometriotic lesions which may not be symptomatic (Guideline, 2008). Endometriosis is estimated to cost the UK £2.8 billion per year in days taken off work (EndometriosisUK, 2013).

1.7.2 Clinical Features

The clinical features of endometriosis are broad and highly varied but common signs and symptoms include: pelvic pain, dysmenorrhoea, deep dyspareunia, dyschezia, subfertility and infertility. Other rare symptoms that may suggest extensive disease include: cyclical haematuria, rectal bleeding and bleeding from the umbilicus (Impey, 2012).

1.7.3 Investigations and Diagnosis

Serum CA 125 levels may be raised in patients with endometriosis however this is of limited diagnostic value and reliable biomarkers of the disease are currently lacking. Direct visualisation of endometriotic lesions at laparoscopy is required for definitive diagnosis with or without tissue biopsy and histological analysis (Guideline, 2008). If there is clinical evidence of deeply infiltrating endometriosis, bladder and bowel involvement are assessed with MRI, plus or minus intravenous pyelogram and Barium studies (Guideline, 2008). On inspection of the abdominal cavity lesions may be diffusely spread and thorough investigation is necessary for accurate staging. Common sites include: utero-sacral ligaments, utero-vesical fold, utero-rectal pouch, sigmoid colon and the ovary (endometrioma) (Impey, 2012) (Figure 5A).

There are four different characteristic appearances of endometriotic lesions commonly visualised at laparoscopy (Figure 5B) which may represent different stages of disease activity and different cellular processes. More active lesions appear as red vesicles with white scars or brown spots (powder burn) followed by fibrotic lesions representing more longstanding disease (Impey, 2012). Another laparoscopic finding more commonly found in endometriosis patients is the Allen Masters Peritoneal Window which may be the result of a prolonged inflammatory state (Burney and Giudice, 2012).

Figure 5: Common Sites and Morphology of Endometriotic Lesions

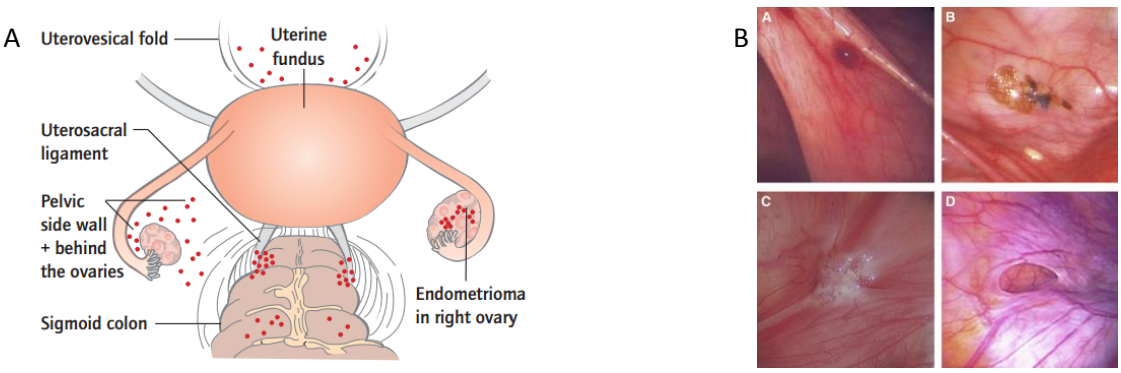


Fig5. Common sites and phenotypes associated with endometriosis lesions. **A.** Common sites of endometriotic lesions within the pelvic cavity. (Impey, 2012) **B.** Phenotypic subtypes of peritoneal endometriosis. (A) Red vesicular lesion. (B) Powder-burn lesion. (C) Fibrotic lesion. (D) Allen-Masters peritoneal defect (Burney., 2012).

1.7.4 Staging of Endometriosis

The revised American fertility society criteria for endometriosis scores the disease extent based on the presence and location of lesions. There are four stages (Impey, 2012):

- Grade 1 (Minimal)
- Grade 2 (Mild)
- Grade 3 (Moderate)
- Grade 4 (Severe)

It should be noted that the severity of disease assessed by the grading system does not correlate well with patient symptoms.

1.7.5 Management of Endometriosis

The treatment of endometriosis can be divided into conservative, medical and surgical treatment. However this is governed primarily by patient symptoms as no treatment is required for asymptomatic lesions found incidentally at laparoscopy. The exception to this are endometriomas which are occasionally removed in this situation as there is a small risk of misdiagnosing ovarian cancer (Guideline, 2008).

- **Medical Management**

First line medical management is analgesia to provide symptom control e.g. Non Steroidal Anti Inflammatory Drugs (NSAIDs) such as mefenamic acid and naproxen (Impey, 2012). However efficacy of this class of drugs in endometriosis has not been proven by randomized control trial and significant adverse effects e.g. gastric ulceration should be considered (Guideline, 2008). Hormonal therapy is also used in endometriosis and is based on the principle that endometriosis is an oestrogen dependent disease noted to regress at times of reduced oestrogen exposure e.g. pregnancy and the menopause. Treatments offered either mimic pregnancy (Combined oral Contraceptive e.g. Microgynon) or the menopause (GnRH Agonist e.g. Zoladex) however these therapies are also associated with side effects e.g. vaginal dryness, hot flushes osteopenia and osteoporosis (Guideline, 2008).

- **Surgical Management**

Surgical management aims to remove the endometriotic lesions from the abdominal cavity and is only considered if medical therapy has been ineffective. At laparoscopy laser or bipolar diathermy can be used to ablate lesions. Other options include radical dissection of lesions or hysterectomy with bilateral salpingo-opherectomy however these options should be considered as a last resort as they entail major surgery and are associated with adhesion formation. Laparoscopy itself has also been associated with endometriosis lesion induction (Harirchian et al., 2012).

1.7.6 Endometriosis and Fertility

Endometriosis is a disease associated with sub-fertility, for example lesions are found in 25% of laparoscopies carried out for subfertility (Impey, 2012); the mechanisms of this sub fertility are not yet clear, though implantation failure is thought to be one mechanism, and this affects management. A Cochrane review has found that medical treatment will not increase fertility if the fallopian tubes are unaffected but that laparoscopic ablation may, particularly when adhesions are present (Jacobson et al., 2002). Many patients with advanced endometriosis will be of reproductive age and the best chance for achieving pregnancy will be In Vitro Fertilisation (IVF). With this in mind it is essential to attempt to understand the processes behind this disease as it impacts upon so many aspects of a patient's life and several different approaches need to be considered.

1.8 Pathogenesis of Endometriosis

Whilst the clinical features of endometriosis are well described the cause of this proliferative disease is still unknown. Several theories have been proposed and these include:

- Retrograde Menstruation
- Coelomic Metaplasia
- Induction Theory
- Mullerian Rest Theory
- Genetic Inheritance
- Stem cell theory

These proposed mechanisms of pathogenesis can be broadly divided into those where ectopic tissue implants onto the abdominal wall e.g. retrograde menstruation, and those where the endometrial tissue arises *de novo* e.g. Coelomic Metaplasia (Gargett, 2008).

1.8.1 Retrograde Menstruation

Retrograde menstruation is the most widely accepted theory for endometriotic lesion induction and was first proposed by Sampson in 1927 (Sampson, 1927). It is suggested that at the point of menstruation some of the shed endometrium, instead of passing through the cervix and vagina, enters the abdominal cavity via the fallopian tubes and implants there, the result being a lesion (Sampson, 1927). There is some clinical evidence to support this theory in the increased prevalence of endometriosis noted in women with uterine outflow obstruction e.g. cervical stenosis (Jenkins et al., 1986).

1.8.2 Coelomic Metaplasia and Induction Theory

Coelomic metaplasia and induction theory are two interrelated ideas implicating metaplasia of extra-uterine cells in the pathogenesis of endometriosis. Coelomic metaplasia states that normal peritoneal tissue is transformed by an as yet undefined stimulus to ectopic endometrial tissue. Similarly induction theory proposes that extra-uterine metaplasia may be induced by either a hormonal or immunological factor, while study is ongoing these 'factors' have not yet been defined (Levander and Normann, 1955). Some indirect evidence for these theories can be found in the form of case reports of ovarian endometriosis in patients with Mayer-Rokitansky-Kuster-Hauser syndrome, a condition characterised by uterine agenesis including absence of the endometrium (Yan et al., 2011).

1.8.3 Mullerian Rest Theory

This theory relates to the development of the female reproductive tract through the Mullerian duct. It is suggested that cells derived from the Mullerian duct maintain a differentiation potential, giving them the capacity to transform into endometrial cells at the onset of puberty under hormonal stimulation (Longo, 1979).

1.8.4 Genetics of Endometriosis

Investigation has been undertaken into the prospect of endometriosis as a familial disease with a genetic basis however as previously mentioned epidemiological study of the disease

is limited due to the requirement for laparoscopy to make a diagnosis (Zondervan et al., 2001, Kennedy et al., 2001). Some correlation has been found but the trait is likely to be complex (Simpson and Bischoff, 2002).

1.9 Proposed Mechanism of Lesion Establishment

Sampson's theory of retrograde menstruation is the most widely excepted model of endometriosis; however it poses several questions that need to be examined in more detail to fully understand the pathogenesis of this disease.

One such issue is the observation that 'retrograde menstruation' occurs in up to 90% of pre-menopausal females (Halme et al., 1984) however only 5-10% experience the disease (Cramer and Missmer, 2002, Missmer and Cramer, 2003), which would suggest other factors are important in determining lesion establishment. In a recent review, Burney and Guidice summarised these as: escape from immune clearance, attachment to peritoneal epithelium, invasion of the epithelium, establishment of local neurovascularity, and continued growth and survival (Burney and Giudice, 2012). Looking at these processes and how they may vary between women with and without the disease has yielded a group of potential biomarkers. However, investigation in this area is still early with many publications offering conflicting evidence. The variation of these putative biomarkers throughout the menstrual cycle is also not always considered in their analysis, a factor which must be taken into account to ensure findings are relevant (May et al., 2011). Bioinformatics is a useful tool which could help gain a 'whole view' of the endometrium as it can consider vast amounts of data, however there is a difference between identifying biomarkers and confirming their role in pathogenesis. The work presented below indicates that further investigation is required in order to more fully understand the pathogenesis of this disease.

1.9.1 Continued Growth and Survival of Endometrial Cells

As retrograde menstruation occurs in such a high percentage of women with only 10-20% developing endometriosis it has been suggested that endometrial cells of patients with the disease are in some way different so as to increase their chances of surviving in the abdominal cavity allowing a lesion to be established. A great deal of work has been carried out in this area however the evidence is conflicting (May et al., 2011). Some studies have looked at regulators of apoptosis and cell cycle with one group proposing evidence for an

anti-apoptotic environment in patients with endometriosis characterised by increased MCL-1 and reduction in Bak expression in secretory phase glandular epithelium (Burlev et al., 2006). Another group has demonstrated up-regulation of the anti-apoptotic gene BCL-2 in the endometrium of affected patients (Wingfield et al., 1995). Telomerase activity is another factor increasing the survival potential of endometrial cells and has been detected increased in secretory phase endometrium of patients with the disease, it was also noted that mean telomere length may also be increased (Hapangama et al., 2008, Hapangama et al., 2009). A further consideration is the presence of endometrial stem cells in endometriosis lesions.

1.9.2 Attachment to the Peritoneal Epithelium and Tissue Remodelling

It is thought that damaged peritoneum could be a risk factor for implantation of ectopic tissue, one *in vitro* study has shown that fragments of endometrial tissue were only adherent in a model of ectopic implantation when extracellular matrix (ECM) was exposed due to induced damage (Gerhard et al., 1991). This is also consistent with work in baboons demonstrating that laparoscopy may be a risk factor for lesion establishment (Harirchian et al., 2012).

1.9.3 Matrix Metalloproteinases

Another potential mechanism of peritoneal damage is the action of MMPs, a group of enzymes associated with degradation of the extracellular matrix and tissue remodelling previously thought to play a role in normal endometrial physiology (Eyster et al., 2002). It is suggested that inappropriate action of these enzymes in patients with endometriosis may result in degradation of the ECM associated with the pelvic peritoneum allowing refluxed endometrial cells to implant and establish lesions (Osteen et al., 2003). Both MMP2 and MMP3 mRNA have been detected upregulated in endometriosis patients, (Di Carlo et al., 2009, Sotnikova et al., 2010, Kyama et al., 2006). These findings have been corroborated using immunohistochemistry, RT-PCR and Enzyme-Linked Immuno-absorbent Assay (ELISA) (Gilabert-Estellés et al., 2007). In a separate study MMP7 was found upregulated in epithelial cells (Matsuzaki et al., 2010b). There is however much conflict within this area of research and some studies have found no difference in expression e.g. of MMP3 (Meola et al., 2010). Tissue Inhibitors of Metalloproteinases (TIMPS) are responsible for inhibiting the action of MMPs and maintaining a homeostatic balance of tissue maintenance and

remodelling. This group of enzymes has also been investigated in endometriosis with similarly conflicting results (Gilabert-Estellés et al., 2003, Ramón et al., 2005, Gilabert-Estellés et al., 2007).

1.9.4 Angiogenesis in Endometriosis

It has been observed in cancer that any tissue growth above a critical mass must establish a blood supply for itself. For this reason angiogenesis has been investigated in endometriosis and interest in this area is enhanced by the finding of a chimeric circulation in the work performed by the Masuda group (Masuda et al., 2007). Vascular Endothelial Growth Factor (VEGF) is a molecule that has been extensively investigated with increased expression detected in the late secretory epithelium of patients with the disease (Donnez et al., 1998). A subtype of the molecule VEGF-A is has also been found upregulated in women with endometriosis as well as down regulation of VEGF receptor 1 and 2 possibly indicating decreased sensitivity (Bourlev et al., 2006). These studies indicated that VEGF might be elevated at a certain point in the cycle in endometriosis, which would make sense as it is a hormone responsive disease. More recent investigation however has suggested increased VEGF expression might persist throughout the cycle (Gilabert-Estellés et al., 2007).

1.9.5 Cytokines, Steroid Hormones and Cell Adhesion Molecules

Many other intra and extracellular molecules have been investigated with regard to their involvement in the pathogenesis of endometriosis. Interleukins are a group of cytokines thought to be differentially expressed in endometriosis. For example IL-1R Type II has demonstrated downregulation in epithelium throughout several studies (Akoum et al., 2001, Kharfi and Akoum, 2001, Kharfi et al., 2002, Lawson et al., 2008) whereas IL-8 has been found up-regulated in epithelial cells (Ulukus et al., 2005). The chemokine 'Regulated on Activation, Normal T Cell Expressed and Secreted' (RANTES) has been found differentially expressed in endometriosis, but further work is needed to fully elucidate its association with the disease as its expression appears to also be cycle phase dependent (Kyama et al., 2008). Other molecules thought to be differentially regulated are vast in number and diverse in function. These molecules have been summarised (May et al., 2011) but some notable examples include: IGF protein 3 (Akoum et al., 1999), Osteopontin (Burney et al., 2007) and Beta Catenin (Matsuzaki et al., 2010a). Cell adhesion molecules such as: Cadherins, Integrins, Selectins, Immunoglobulins are also thought to be important to the pathogenesis of this disease and research is ongoing (May et al., 2011).

1.9.6 Stem Cells in Endometriosis

The cellular and molecular processes investigated in relation of endometriosis often relate to increased cell survival within the peritoneal cavity and proliferation of a cell population in response to a stimulus (in this case, oestrogen). These are properties shared by Adult Stem Cells (ASCs) or Progenitor Cells (PCs) and it is for this reason that ASCs have been hypothesised to play a role in the pathogenesis of endometriosis. Some experimental findings have backed up this hypothesis including investigation of epithelial glands in endometriosis which were monoclonal in some lesions suggesting they descend from a single PC (Gargett and Masuda, 2010). Experimental models of lesion induction in baboons have also demonstrated that introducing endometrial cells into the abdominal cavity was associated with lesion development (Harirchian et al., 2012). As progenitor cells are thought to reside in the basalis it has been suggested that in women with endometriosis these may become inappropriately dislodged and refluxed (Leyendecker et al., 2002).

1.10 Future Directions of Endometriosis Research

It is clear that potential molecules involved in the pathogenesis of endometriosis have been extensively studied using laboratory techniques with potential applications including peripheral blood tests to aid diagnosis and the development of disease modifying pharmacological interventions. While this work is undoubtedly useful there is a great variability in results and to a degree it may be beneficial to introduce a standardised method within the field e.g. in accounting for cycle phase. Another tool that would be useful in guiding this research, and could provide useful insights into cellular and molecular processes occurring in both normal endometrium and endometriosis, is bioinformatics.

1.11 A Bioinformatics Approach to the Endometrium and Endometriosis

The structural and functional complexities of the endometrium pose a great challenge for investigators, particularly when trying to study a proliferative disease such as endometriosis, as our understanding of the normal physiology is incomplete. As previously described a number of potential biomarkers and target molecules have been identified both in normal physiology and disease (May et al., 2011). However, considering that the human genome contains in excess of 22000 genes (Birney et al., 2006), the variable expression of which influences these processes it would be useful to gain a wider perspective on intra and extra cellular events.

Studies using mouse models have provided useful insights into potential disease processes in endometriosis (Masuda et al., 2007). Humans however are more biologically complex organisms and although this is not demonstrated by an increased number of genes within the genome, it can be seen in Protein Superfamily expansion, particularly in the extracellular compartment (Vogel and Chothia, 2006). This is logical as the extracellular compartment is a far less stable environment than the intracellular compartment and as a result adaptations must be more rapid. Given that many of the markers postulated to play a role in the pathogenesis of endometriosis are associated with the extracellular compartment, it is important to observe their expression specifically in humans as the molecular processes may differ between species (Vogel and Chothia, 2006). A potential tool to help overcome these challenges is bioinformatics.

1.11.1 Bioinformatics

Bioinformatics can be most simply described as the 'combination of biology and information technology' and can incorporate any tools or methods used to analyse and manipulate large sets of data (Westhead DR, 2002). This rapidly growing area of investigational biology uses a multidisciplinary approach to increase understanding of complex organisms in a quantitative and discrete way not previously possible. Bioinformatic study consists of three main components:

- The creation of databases allowing storage and manipulation of large biological datasets
- The development of algorithms and statistics to determine relationships between members of these large datasets.
- Use of these tools for analysis and interpretation of various types of biological data, including DNA, RNA and protein sequences, protein structures gene expression profiles and biochemical pathways (Westhead DR, 2002).

Investigation using these components involves the generation of vast quantities of data which represents a great challenge within the discipline as it can seem difficult to know where to begin with analysis. This factor can also be a great strength though as correct interpretation allows structural and functional links to be made between relevant molecules (Arthur, 2008). It is for this reason that bioinformatics must use a multidisciplinary approach as the appropriate scientific knowledge is required to guide research.

With this in mind the three components described are utilised in order to achieve three main objectives: (Arthur, 2008).

- To understand integrated aspects of the biology of organisms, viewed as coherent complex organisations at microscopic and macroscopic levels
- To interrelate sequence, three dimensional structure, expression pattern, interaction and function of individual proteins, nucleic acids and protein-nucleic acid complexes
- To support applications in medicine, agriculture and technology (Arthur, 2008).

The potential of bioinformatics study to aid scientific discovery and influence clinical practice is becoming increasingly realised with several scientific papers in the field of Obstetrics and Gynaecology utilising this tool in recent years (Nguyen et al., 2012). Within this context the main focus is on drug target and biomarker discovery using tools such as: Microarrays, Mass Spectrometry and Proteomic Data Analysis (Arthur, 2008). As it is the case with bioinformatics study that a large amount of information is generated and recorded analysis is not always exhaustive. It was with this in mind that a component of this study was proposed in order to investigate differential gene expression in 'normal' endometrium and endometrium of patients with endometriosis. With this view it is thought that potential drug targets and biomarkers could be discovered and avenues for further 'wet' lab investigation identified.

1.12 Stem Cells

While hypotheses can be generated from *in silico* work it is also important to investigate current models of physiology and disease within the laboratory setting. One such model relating to both normal endometrial function and endometriosis is the idea of stem cells in the endometrium. Stem Cells are defined as a population of undifferentiated cells, the key characteristics of which are self renewal and differentiation into one or more descendant of highly differentiated cell lineage (Till and McCulloch, 1961). They can be subdivided according to their ability to reconstitute tissues associated with an organism: (Wagers and Weissman, 2004)

- Totipotent: (All embryonic and extra embryonic cell types)
- Pluripotent (All cell types of the embryo proper)
- Multipotent (Subset of Cell Lineages)

- Unipotent (Only one mature cell type)

Stem Cells can also be categorised as either Embryonic Stem Cells (ESCs) or Adult Stem Cells (ASCs) (Evans and Kaufman, 1981).

1.12.1 Embryonic Stem Cells

ESCs are defined as pluripotent cells isolated from the blastocyst inner cell mass before implantation and are capable of differentiating into structures constituting all 3 germ layers in vitro (Evans and Kaufman, 1981). ESCs are not widely used for investigation within the endometrium and given the associated ethical concerns this population is not utilised in this study.

1.12.2 Adult Stem Cells

Adult Stem Cells (ASCs) reside within mature tissues and demonstrate the stem cell properties of self renewal and limited differentiation potential, being either multipotent or unipotent (Wagers and Weissman, 2004). This means that whilst maintaining a steady population within their tissue of origin e.g. the endometrium, they are able to form cell colonies specific to their lineage, a concept arising from studies involving Haematopoietic Stem Cells (Harrison, 1980).

ASCs may be responsible for processes such as growth and repair after injury in mature tissue and are believed to be surrounded by a physiological microenvironment referred to as the 'Stem Cell Niche' (Schofield, 1978). This environment is thought to be comprised of a group of supporting 'niche cells' which communicate with their resident ASC stimulating either proliferation or differentiation (Gargett, 2007). Interactions between the ASC and its niche are vital for normal function of adult tissues, a process characterised in gut epithelial tissue (Gargett, 2007).

ASCs have been studied in many tissues, however due to their primitive nature and rarity they have been difficult to isolate. This has been achieved in some tissues e.g. rat neural crest cells (Morrison et al., 1999) and it is hoped that they can be identified and isolated in human endometrium. If this can be achieved, insights into the processes underlying the normal menstrual cycle and proliferative endometrial disease such as endometriosis may be elucidated. Progress within this field would serve to both improve scientific

understanding and aid clinical management of a variety of endometrial pathologies including menstrual disorders and endometriosis.

One tissue in which ASCs have been extensively studied relevant to the endometrium is the intestine. In this tissue the epithelial cells are arranged in a glandular structure similar to that of the endometrium; the gland base, or crypt of Lieberkuhn, has been identified as the area where proliferation occurs. This is evidenced by studies demonstrating activation of the canonical Wnt/Beta Catenin signalling pathway in basal cells, a key regulator of an undifferentiated state associated with ASC activity (Fevr et al., 2007, Valentijn et al., 2013). Being located at the base of the crypt allows these ASC/progenitor cells to be surrounded by a niche, as described by Schofield, composed of extracellular matrix and other cells (Spradling et al., 2001). This would not be possible higher up the crypt as the more differentiated cells migrate upwards through the 'transit amplifying zone' before being shed (Spradling et al., 2001). Specific markers have also been identified in the intestine, notably Musashi1, Hes1 and CD133 (Iovino F, 2009). Any tissue containing ASCs may be associated with a rapid turnover of cells. One danger recognized with this rapid proliferation is the increased risk of mutations which may lead to tumour formation (Wagers and Weissman, 2004). It has been noted in Colon Cancer that tumours expressing the marker CD133 have been demonstrated residing at the base of normal glands leading to the hypothesis that the aberrant proliferation of ASCs may play an early role in the formation of these tumors (Salven et al., 2003, Yin et al., 1997). The progenitor cells isolated in the intestine also show a higher telomerase activity with longer telomere lengths, another ASC property currently being investigated in the endometrium (Schepers et al., 2011, Valentijn et al., 2013)

Given the similarities between endometrial glands and the small intestine epithelial glands this tissue has been used as a model for endometrial research. An example of this is the investigation of Sox9, a molecule associated with proliferation known to be present in the small intestine which a group at the University of Liverpool has recently investigated in the endometrium using IHC (Valentijn et al., 2013).

Much of the work discussed so far focuses on the detection and isolation of Adult Stem Cells from mature tissue. However a recent alternative approach has been aimed at the generation of ASCs from terminally differentiated cells. These cells, with pluripotent properties, were first generated in 2006 by Yamanaka et al generating ASCs from mouse

fibroblasts (Takahashi and Yamanaka, 2006). This was achieved via the retroviral induction of 4 transcription factors: Oct4, Klf4, c-myc and Sox2, the actions of which are thought to reprogram the cell to a pluripotent phenotype (Jaenisch and Young, 2008).

This is an emergent field and several modifications to the procedure including the induction of different transcription factors e.g. Nanog and different methods of induction have been used to reduce potential teratogenicity. Since 2006 iPS cells have also been generated from human cells (Yu et al., 2007).

There are a great number of potential therapeutic applications using this technology e.g. the generation of organs for transplant which, as they would be derived from a patients own cells, would potentially be free from the ethical concerns of using embryonic stem cells and the risks of immune rejection currently associated with transplant surgery. Indeed one recent development was the generation of 'Liver buds' using iPS cells to form hepatocytes (Takebe et al., 2013). However a number of concerns have been raised over the use of iPS cells, primarily the potential for tumour formation given the relatively undifferentiated state of the cells combined with a high proliferation capacity, and recent reports demonstrating immunogenicity of iPS cells (Zhao et al., 2011). There is a great amount of progress still to be made in the field of iPS cells however early results are promising and the potential therapeutic applications are many.

1.12.3 Adult Stem Cell Plasticity

One of the interesting potential benefits of isolating an ASC population within the endometrium centres on the concept of 'Stem Cell Plasticity'. This property allows an ASC population to 'transdifferentiate', a process by which ASCs could contribute to cell types of different lineages (Wagers and Weissman, 2004). If this is possible then endometrial ASCs, easily obtained in the out-patient clinic and ethically safe, could represent a great source of autologous tissue for transplant. Endometrial ASCs could be extracted from a tissue sample, grown in vitro before differentiation into the population of choice e.g. dopaminergic neurons for to treat Parkinson's disease and transplanted.

This 'plasticity' has been investigated in many tissues including the endometrium (Taylor, 2004) and it is thought that transdifferentiation could depend on the ASC niche (Blau et al., 2001). Focus in this area of research has primarily been on the observation of transdifferentiation and little investigation has been made into the potential mechanisms

underlying this process. A number of alternate mechanisms need to be ruled out in order to accurately demonstrate this phenomenon (Figure 6) (Wagers and Weissman, 2004).

A. ASC Transdifferentiation: Lineage conversion of the cells occurs. A dormant lineage differentiation program is activated to alter the lineage specificity of the cell (Wagers and Weissman, 2004).
B. De-differentiation of an ASC occurs to a more primitive phenotype before re-differentiation This is a phenomenon observed in animal models (Brockes and Kumar, 2002).
C. A heterogeneous population of ASCs is present in the test population.
D. A rare pluripotent stem cell is present in the tissue
E. Cell fusion is taking place. This process is associated with normal cell physiology e.g. in the formation of skeletal myofibroblasts (Anderson, 2000).

Figure 6: Explanations for apparent ASC Transdifferentiation Observed Experimentally

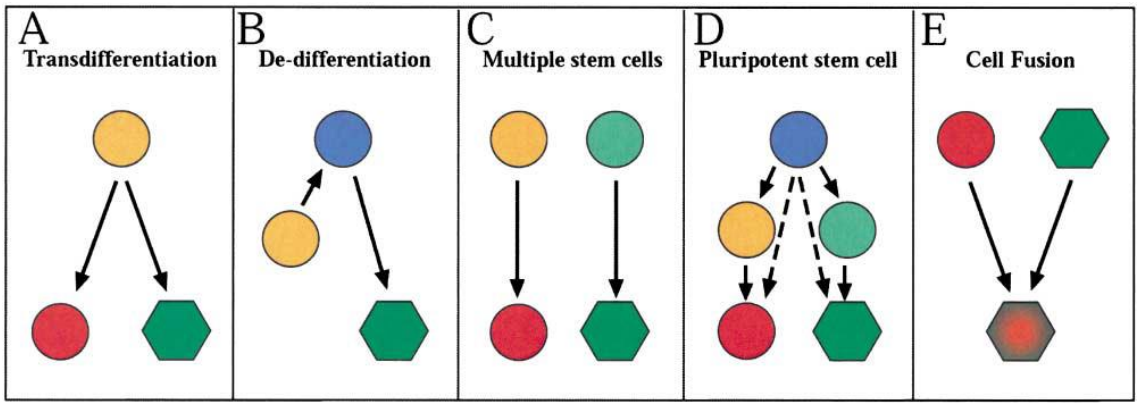


Fig6. Explanations for apparent ACS transdifferentiation observed in culture. When documenting ASC transdifferentiation e.g. in endometrial cells it is essential to account for alternative events. A-E Described above (Wagers and Weissman, 2004).

To date, many of the studies reporting ASC transdifferentiation within the endometrium have not satisfactorily ruled out these events. It is possible either that transdifferentiation within the endometrium is extremely rare, a finding consistent with findings from our laboratory in the University of Liverpool (Da Silva, 2012). Alternatively, progenitor cells within the endometrium could be unipotent; a property associated with the more terminally differentiated Transit Amplifying (TA) cells.

1.12.4 Transit Amplifying Cells

Transit Amplifying cells represent a temporary cell population more differentiated than ASCs but which still have a limited self renewal capacity both *in vitro* and *in vivo* (Seaberg and van der Kooy, 2003). These cells are unipotent and hormone responsive; it is unclear to what extent they are identified in assays of ASC function in the endometrium (Gargett, 2008). In fact one theory is that TA cells and ASCs are in fact the same population of cells exhibiting a differing phenotype depending on the conditions of their niche (Quesenberry et al., 2005).

1.13 Evidence for ASCs in the Endometrium

The high regenerative capacity of the endometrium has long been a source of interest to clinicians and it has been thought for many years that a population of ASCs residing in this environment could account for the vast proliferation potential observed. The first publication recognising this possibility was Prianishnikov in 1978 who outlined a theory of ASCs within the endometrial epithelial population (Prianishnikov, 1978). This paper suggests there are three populations of ACSs in the endometrium divided by their response to oestrogen and progesterone:

- ASCs responsive to oestrogen
- ASCs responsive to oestrogen and progesterone
- ASCs responsive to progesterone only

Prianishnikov thought that the growth of these 'stem cells' was regulated by oestrogen and progesterone acting on the cells directly. It is possible that ASCs themselves are hormone independent but that their nearest descendants (TA cells) become hormone dependent and it was suggested that the ASC and TA cells receptor status would be mediated in an oestrogen dependent manner (Prianishnikov, 1978).

An initial population of TA cells responsive to oestrogen during the proliferative phase would then give rise to a daughter population with both oestrogen and progesterone receptors. These cells would then further differentiate to only become responsive to progesterone. In this way, the progesterone receptor could be seen as a late marker of differentiation of endometrial epithelial cells (Prianishnikov, 1978). This hypothesis was

backed up with the experimental finding that oestrogen will induce the synthesis of progesterone receptors in uterine tissue cells thereby making them susceptible to progesterone (Wiest WG, 1971). Prianishnikov described the characteristics of this putative progenitor population and his theory states that these cells would be found in the base of endometrial glands which lie within the basalis layer not shed during menstruation. This is consistent with studies of ASC distribution in the human gut (Barker et al., 2007) and would seem logical as the ASCs would need to regenerate the endometrium after each menstrual cycle (Prianishnikov, 1978). Functional kinetic studies of human endometrial growth also suggest a progenitor population may be found in basalis tissue (Ferenczy et al., 1979). Further genomic investigation of the endometrium suggested that this basal population may be sparsely distributed, as endometrial glands have been characterised as monoclonal for the deletion of the Phosphate and Tensin Homologue (PTEN) gene. This finding suggests that all cells within this gland and surrounding glands had descended from a common progenitor (Lacey et al., 2008) and is backed up by analysis of endometriosis lesions demonstrating a similar monoclonality, a finding which also implies a stem cell origin of this disease (Van Gorp et al., 2004).

These studies seem to describe a sparsely distributed population of progenitor cells residing in the endometrial basalis responsible for cyclical proliferation; however this would be counter to the theory of induction of endometriosis by retrograde flow as only the functionalis is shed at menstruation. One potential explanation would be lesion induction by hormone responsive TA cells which may be located in the functionalis. Alternatively sections of the endometrial basalis may become dislodged inappropriately and implant in the abdominal cavity (Leyendecker et al., 2002).

In an attempt to both understand endometrial physiology and pathogenesis of endometriosis the characterisation of this progenitor population has been the subject of intense investigation in the 35 years since Prianishnikovs' initial publication. It is currently thought that the endometrium contains two ASC populations, a stromal progenitor and an epithelial progenitor (Gargett, 2008).

1.14 Characterisation of Endometrial Progenitor Cells

Several methods have been utilised with the aim of demonstrating the key characteristics of an ASC in an endometrial cell population, these are generally accepted to be: Self

renewal, Multi/Unipotency, Quiescence and high proliferative capacity (Snippert and Clevers, 2011).

Techniques used include:

- Label Retaining Technique
- Side Population method
- Specific Marker Identification
- *In vivo* xenotransplantation studies.

The final method attempts to demonstrate the 'Gold Standard' for identifying a putative ASC population which is demonstration of *in vivo* re-constitution of the tissue from which the ASC population was isolated (Joseph and Morrison, 2005). The majority of this work has been performed using endometrial stromal cells. This is partly because it is thought that a relatively large population of ASCs will be found here (Holinka and Gurpide, 1987) but also due to the challenges posed by epithelial cell culture, particularly the need for 3D culture to more accurately reflect the *in vivo* environment. As a result work characterising the epithelial progenitor population is sparse.

1.14.1 Label Retaining Technique

The Label Retaining Technique (LRT) aims to identify Label Retaining Cells (LRCs) using the DNA binding dye Bromodeoxyuridine (BrdU) (Chan and Gargett, 2006). The cell nuclei are pulse labelled followed by a 'chase' period in which the tissue is analysed at varying time intervals. Rapidly dividing cells will lose their 'Label' as the BrdU is diluted to undetectable levels. The LRT is based on the feature of 'quiescence' exhibited by ASCs meaning they will retain their 'Label' for longer (Chan and Gargett, 2006). Stained tissues can then be analyzed using Immunohistochemistry (IHC) to identify putative ASC populations and their surrounding niche (Chan and Gargett, 2006).

Using this technique, observations have been made in the endometrium regarding the proximity of stromal LRCs to the surrounding vasculature leading to the hypothesis that this environment may represent the endometrial ASC niche (Kaitu'u-Lino et al., 2012). Another hypothesis generated from the distribution of epithelial LRCs is that the epithelial glands may contain one ASC at the base which is responsible for regenerating the entire gland

(Ferenczy et al, 1979). This is backed up by work demonstrating the monoclonality of endometrial epithelial glands with the HUMARA assay showing clonality of adjacent glands up to 1mm in diameter (Tanaka et al., 2003). The HUMARA assay is a laboratory technique examining monoclonality of cells e.g. in a tumour by looking at X chromosome inactivation. If a group of cells are monoclonal they will all have the same X-chromosome inactivated and are likely to have a common parent cell; this assay specifically examines the Human Androgen Receptor (HUMARA) on the X chromosome (Tanaka et al., 2003).

The LRT is a good method for identifying non-specific quiescent cells, a characteristic associated with ASC populations. Transit Amplifying calls are also of interest in the endometrium however and the LRT would not be suitable for detecting this population due to the rapid proliferation they exhibit (Gargett, Gargett, 2008). For this reason it would be sensible to also use alternative methods to investigate a progenitor population within the endometrium

1.14.2 Side Population Method

Side Population (SP) cells are a group of primitive cells thought to represent an ASC population characterised by the presence of ABCG2 transporter proteins which are not present in mature cells (Zhou et al., 2001). SP cells are identified by staining with Hoechst 33342 dye which is rapidly extruded by the ABCG2 proteins these cells posess but not by the surrounding more differentiated cells. This method has been validated in mouse models to isolate a putative ASC population (Goodell et al., 1996) and the SP phenotype is considered synonymous with an ASC population (Challen and Little, 2006).The labelled cells can then be isolated e.g. using Fluorescence Activated Cell Sorting (FACS).

Cells with the SP phenotype were first identified in human endometrium in 2007 and the population has been identified in both stromal and epithelial cultures *in vitro* (Kato et al., 2007). When isolated in culture these cells initially demonstrated limited proliferative capacity, being mostly in G0 phase of the cell cycle which indicates quiescence (Tsuji et al., 2008). This finding of limited proliferation is contrary to what would be expected of a progenitor population. However, a greatly increased proliferative potential was observed when labelled endometrial SP cells were cultured unfractionated (i.e. all populations together) suggesting that the correct 'niche' may be essential to the proliferation of endometrial progenitor cells (Tsuji et al., 2008).

There are difficulties associated with studies relating to SP cells in the endometrium, some of which aim to demonstrate plasticity of the endometrial progenitor population using isolation of an SP population as a starting point (Miyazaki et al., 2012). The first difficulty comes from the strict requirements necessary to demonstrate the plasticity these studies sometimes claim to exhibit in endometrial SP cells. The second limitation comes from the 2D culturing techniques used in the majority of studies. 2D culture represents a confounding factor as it is not always possible to distinguish between morphological changes known to be associated with culture (Kaur et al., 2004) and those arising as a result of SP plasticity. While these factors limit the ability of SP study to demonstrate ASC plasticity it can be said that an SP population with high proliferative potential can be isolated from the endometrium (Kato et al., 2007). More recent studies attempting to isolate endometrial SP cells suggest that they represent a mixed population of epithelial, endothelial and stromal cells (Masuda et al., 2010).

1.14.3 Specific Marker Identification

Specific markers have been identified for a population of endometrial stromal cells with high proliferative potential; the co-expression of CD146 and Platelet Derived Growth factor Beta (PDGF-B) (Schwab and Gargett, 2007) has identified a group of cells expressing signaling pathways associated with self renewal and multipotency (Spitzer et al., 2012). These cells are mainly found in the basalis and vessels of the functionalis (Schwab and Gargett, 2007). A second candidate marker is W5C5 which was identified by screening endometrial tissue with perivascular markers (Masuda et al., 2012). W5C5^{+} cells have reconstructed stromal tissue in vivo when transplanted under the kidney capsule of severe combined immunodeficient (SCID) NOD/Shi-scid/IL-2Rγnull (NSG) immunodeficient mice (Masuda et al., 2012). It is currently thought that CD146 and PDGF-B represent the same population of stromal cells (Gargett, 2007).

Attempts have also been made to find a specific marker of an epithelial progenitor population. A recent study characterising the endometrial basalis using IHC identified cells expressing markers of proliferation including Sox9 and Nuclear β Catenin located, as with intestinal epithelium, as the base of epithelial glands (Valentijn et al., 2013). Cells with high proliferative potential as evidenced by higher capacity for spheroid formation in 3D matrigel culture were positive for the embryonic stem cell marker Stage Specific Embryonic Antigen 1 (SSEA1) which was proposed as a potential marker of a progenitor population

within the endometrial epithelium, these cells also having longer mean telomere lengths than SSEA1-ve cells (Valentijn et al., 2013). Work into the epithelial progenitor population is much scarcer than the stromal population and attempts to work with this population further may represent and interesting avenue for further research.

1.14.4 *In-vivo* reconstitution Assay

As previously mentioned, the Gold Standard for characterising an ASC population is demonstration of in vivo reconstitution of the tissue from which the cells were isolated (Joseph and Morrison, 2005). A series of studies using this assay on endometrial cells have been carried out by Masuda and co-workers (Masuda et al., 2010). The aim was to build on the previous work identifying the LRC and SP populations to demonstrate regeneration and multipotency of an endometrial progenitor population (Cervelló et al., 2007, Chan and Gargett, 2006, Kaitu'u-Lino et al., 2010, Szotek et al., 2007). As well as being the Gold Standard this model has the advantage that is permits the cells to be studied in a stable long term environment as well as allowing the investigation of differential expression of genes and proteins between the two species (Humans and Mice) through the use of specific antibodies and PCR primers (Masuda et al., 2010).

In an initial study, the parameters of which were also used for subsequent experiments, SP cells (2% of total population) were identified in both stromal and epithelial fractions of human endometrium and isolated using FACS (Masuda et al., 2010). These isolated cells were then transplanted under the kidney capsule of NSG mice which were then treated with a 17-Bestradiol pellet for 8-10 weeks. The kidney capsule was chosen as it provides a rich vascular supply for the transplanted tissue whilst also containing it within a defined region (Xin et al., 2005).

In this study cystic masses were observed in only a few (2/24) mice though staining for human specific markers identified positive cells in many of the animals with some evidence of this population beginning to invade the kidney, however a relatively low level of proliferation was observed and there was no evidence of glandular tissue formation.

The experiment was repeated, this time isolating and labelling the SP cells and mixing them with main population cells before injection under the kidney capsule. This modification was introduced as it was thought that the main population might represent the ‘niche’ the SP

cells would need to proliferate and differentiate (Masuda et al., 2012). The results of this experiment showed a greatly increased proliferation potential of the introduced human cells demonstrated by the formation of large blood filled cystic masses beneath the kidney capsule of a greater number of experimental mice which exhibited hormone dependent changes breaking down in response to progesterone withdrawal in a manner similar to menstruation (Masuda et al., 2012). Characterisation of the cysts also detected the presence of human progesterone receptor which is an indication of an intact oestrogen pathway and mature endometrial cells (Jabbour et al., 2006).

An interesting finding associated with this study is the detection of human derived vessels from the injected endometrial tissue invading the renal parenchyma of the mouse and forming a chimeric circulation as the result of neovascularisation (Masuda et al., 2012). As it is known that angiogenesis is necessary for endometriotic lesion establishment further characterisation was undertaken.

Immunofluorescence demonstrated ABCG2+ve cells distributed in close proximity to endothelial 'vessel like structures' leading to the hypothesis that the epithelial and stromal progenitor population is likely to be perivascular and that in the case of the stromal cells this is backed up by previous work on co-localisation of PDGFR-β and CD146 and previous LRC studies (Schwab and Gargett, 2007, Masuda et al., 2012).

This series of experiments has been useful in investigating the potential ASC population within the endometrium, generating the hypothesis that progenitor epithelial and stromal populations exits, with a perivascular niche, as well as an endothelial population. It is also possible that a progenitor population exists in the functionalis, though the action of TA cells needs to be considered studying this potential population. One limitation however of much of this work is the use of a mouse model.

This is a good animal model to use as it shares several common characteristics with humans, however there are also important limitations to this system. For example mouse endometrium undergoes apoptosis and regeneration instead of menstruation and therefore will not spontaneously develop endometriosis (Lipschutz et al., 1999). Another aspect to consider is the immune compromised nature of these mice which means that several elements of the immune response and inflammatory processes, thought to be crucial to the pathogenesis of endometriosis, cannot be investigated as the ectopic environment does not exhibit an immune response (Awwad et al., 1999). Furthermore, a

recent publication regarding the differences in inflammatory response and sepsis in humans and mice indicates that the two species are also not comparable in this respect (Seok et al., 2013).

1.15 The Future of Stem Cells in the Endometrium

Progress has been made in isolating the putative ASC population in the endometrium, particularly within the stromal compartment. Assays used have demonstrated some of the features of ASCs and some specific markers have been identified though it is possible that these represent more differentiated TA cells with a third possibility being that ASCs and TA cells represent the same population with a different phenotype dependent on the niche (Quesenberry et al., 2005). Further work will be needed in the future to characterise and isolate this elusive population though it is important to consider the niche when conducting these experiments. However it can be said with a degree of certainty that a population of ASC/progenitor cells has been demonstrated within the endometrium and that the proliferative potential exhibited by this organ can be attributed to their action.

1.16 Disease Models of Endometriosis

As an enigmatic, proliferative disease, endometriosis has been the subject of active investigation for several years. This has led to the establishment of 'disease models', two categories of which are *in vitro* and animal models. These models are useful in modeling the disease and also share some overlap with the study of an endometrial progenitor population. Whilst these models have provided some useful insights into the disease there are also associated limitations which constrain the interpretation of the findings.

1.16.1 *In vitro* Models

The *in vitro* study of endometriosis has several advantages as the methods used are cheap and easily reproducible. A variety of strategies have been previously employed including: Chick Chorioallantotic Membrane (CAM), Peritoneal Explants, Amniotic Membrane Model (AMM) and Peritoneal Mesothelial Cell (PMC) monolayer. These methods attempt to model the interactions between endometrial cells and the peritoneum in humans in order to study the processes of attachment and invasion in endometriotic lesion establishment.

The use of CAM in endometriosis research has allowed investigators to monitor processes essential to lesion establishment e.g. Angiogenesis (Maas et al., 2001) and invasion of cells,

however this particular technique is limited in that it does not allow the study of specific marker expression (Griffith et al., 2010). Other methods can be used to compensate for this and the AMM is one example. The AMM allows the modelling of invasion of endometrial cells onto a strip of de-epithelialised amniotic membrane which can then be analysed by immunocytochemistry for marker expression (Liotta et al., 1980). One limitation to this technique is that endometrial cancer cell lines have been used to model the invasion process which do not represent normal physiology (LaRocca and Rheinwald, 1984).

As human endometriosis is the condition under study, explant models using human tissue have been developed. The culture of endometrial cells on a PMC monolayer has produced some useful observations, notably that an intact peritoneum is protective against attachment of endometrial cells and consequently endometriotic lesion establishment (Groothuis et al., 1999). The attachment of both endometrial epithelial and stromal cells to PMCs in monolayer has also been modeled and found to be a rapid process occurring within 1 hour with invasion occurring by 24 hours, providing an insight into lesion establishment (Witz et al., 2001).

The use of 2D culture methods however is problematic due to morphological changes previously identified, an issue particularly pertinent for epithelial cells. This limitation is being overcome however with the use of 3D culture in some developing models (Valentijn et al., 2013). This is achieved by culturing cells in Matrigel, a widely used basement membrane matrix derived from mouse sarcoma (Kleinman et al., 1986) and studies using this method have demonstrated that endometriotic lesion cells are as 'invasive' as endometrial cancer cells (Zeitvogel et al., 2001).

1.16.2 Animal Models

A different experimental approach to models of endometriosis is exemplified by animal models, using techniques which attempt to observe aspects of the disease pathogenesis in the *in vivo* setting. Several different animals have been used with the two most important being baboons and mice.

Baboons represent a good model for studying endometriosis as only humans and non-human primates spontaneously develop the disease (Story and Kennedy, 2004) This is logical if Sampson's theory is considered as only animals that shed their endometrium have the capacity for retrograde menstruation (Lipschutz, 1999). To study the disease in

baboons, lesions have been experimentally induced either by creating a uterine outflow obstruction or by inserting endometrial tissue laparoscopically (D'Hooghe et al., 1999). These studies have demonstrated the importance of immune interactions when studying lesion establishment as well as the role of peritoneal inflammation (D'Hooghe et al., 2001). However they are limited by the physiological differences between humans and non human primates (Legrand et al., 2009) as well as ethical considerations.

A large amount of work has been carried out on mouse models of endometriosis. These have the advantage of being relatively cheap to perform with fewer ethical considerations and are divided into homologous and heterologous models. Homologous murine models involve the transplant of endometrial tissue from syngeneic animals into an immunocompetent host allowing the study of lesion development (Cummings and Metcalf, 1995). The animal's hormonal exposure is controlled by ovariectomy at the beginning of the experiment followed by exogenous oestrogen treatment (Tirado-González et al., 2010).

Heterologous murine models involve the transplant of human endometrial tissue into immunocompromised e.g. *SCID* mice. This allows the study of human tissue in an ectopic environment and several variations of the model have been developed (Story and Kennedy, 2004, Zamah et al., 1984, Nisolle et al., 2000). A notable example is the work described by Masuda et al, studying the proliferative potential of human endometrial cells transplanted under the kidney capsule of NSG mice (Masuda et al., 2007). There are advantages and disadvantages to this model previously described however it has been a key focus in the development of the field of endometrial research due to its relative inexpensiveness and the desire of investigators to use *in vivo* models.

It is clear that to be physiologically accurate *ex vivo* models of endometriosis need to comprise human endometrium derived from patients, rather than cell lines. Furthermore, a model system should permit the analysis of specific marker expression and of immune interactions with the ectopic environment. In addition, models should be relatively cheap, reproducible and free from ethical concerns. The current literature does not provide a completely satisfactory model for the disease and there is scope for further development.

1.17 The Kidney

Following the work done by Masuda et al transplanting endometrial cells into live *SCID* mice, the potential to investigate the epithelial population of progenitor cells was considered using a similar concept for the present study. Investigating the behaviour of these cells in a different niche would be of interest to see if a progenitor population could exhibit the ASC characteristics of proliferation and differentiation. The mouse kidney would be considered a suitable environment as the kidney and endometrium share a common embryological origin and an explant model would be developed using tissue from culled mice in accordance with the principles of the 3 Rs. Developing a kidney explant model would also provide the opportunity to investigate aspects of glomerular maturation.

1.17.1 Embryology of the Mammalian Kidney

Both the kidney and the endometrium are embryologically derived from a cell population termed the intermediate mesoderm. While development of the mammalian kidney represents a complex set of processes it can be broadly divided into 3 stages:

- Pronephros
- Mesonephros
- Metanephros

These structures are similar in all mammals; however, some details specific to mice are included in this report. In mice, the pronephric tubules arise from the pronephric duct at 8 days of gestation. These tubules are transient structures and the rostral component of the structure they comprise soon degenerates to form the 'Wolffian duct' (Saxen, 1987). As this degeneration occurs a new set of tubules are induced in the adjacent mesenchyme forming a structure known as the mesonephros. The Mesonephros does not represent a functioning kidney structure and also regresses via apoptosis. However it does have 2 functions:

- To act as a source of haematopoietic stem cells
- To persist in males to form the epididymis and vas deferens (Wintour et al., 1996)

The final structure in the kidney development of mammals is the metanephros, which is formed through a series of interactions referred to as 'reciprocal induction' between the

mesenchymal and epithelial cell populations of the developing kidney, the metanephric mesenchyme (MM) and the Ureteric Bud (UB) (Gilbert, 2010).

1.17.2 Reciprocal Induction

The MM acts upon the UB structures, causing them to branch, and the UBs induce the MM to undergo MET so that they form condensed aggregates adjacent to the UBs. These aggregates will eventually form glomeruli and progress through a series of well-described developmental structures:

- Comma Shaped Body
- S-Shaped Body
- Capillary Loop
- Mature Glomerulus

It during the capillary loop structure that the epithelial cells of the MM differentiate into the mature cell types found in the nephron (Grobstein, 1955). The first glomeruli with capillary loops can be detected at day 14 of gestation in rodents and will continue to form until one week postnatally (Gilbert, 2010). The nascent nephrons fuse with branching UB structures, which upon maturation, give rise to the mature collecting ducts and calyces, thus forming a continuous path for the renal filtrate. This process of nephron formation has been documented *in vitro* and it has been observed that when the UB and MM are separated and cultured in isolation there is no branching or differentiation of either cell population; each requires signals and interactions with the other to form the mature nephron (Grobstein, 1955).

1.17.3 Vasculogenesis in the Developing Kidney

It is clear that 'reciprocal induction' represents an immensely complicated set of biological processes, the cellular mechanisms of which have been the subject of recent study. One area of particular interest is the origin and development of endothelial cells and capillaries. This population has only become the subject of active research in recent years, this may be due to the discovery of the potential role it plays in the formation of the

Figure 7: Dual Secretion of GBM Components

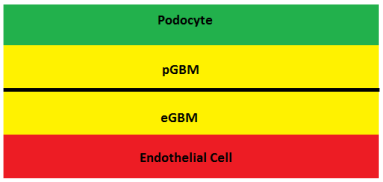


Fig7. Glomerular Basement Membrane components are formed from both Podocyte and Endothelial Cell populations (Abrahamson, 2009).

glomerular basement membrane (GBM) (Abrahamson, 2009).

The 'Comma' and 'S-Shaped' bodies described during nephron formation have their characteristic appearance due to the formation of a space into which primitive endothelial cells (angioblasts) migrate in order to for the glomerular capillaries, known as the 'vascular cleft' (Abrahamson, 2009). These angioblasts differentiate into endothelial cells and assemble a thin layer of basement membrane matrix around themselves (Figure 7) which separates them from the podocytes of the Bowman's capsule which also deposit a layer of a matrix (Figure 7) (Abrahamson, 2009).

This dual secretion means that the early GBM is a layered structure which then fuses to form one membrane, an idea backed up by studies demonstrating that podocytes and endothelial cells both actively secrete extra cellular matrix proteins during GBM development (Abrahamson, 1987, Lelongt et al., 1987, Reeves et al., 1980, Abrahamson, 1985, Kanwar et al., 1984).

1.17.4 Maturation Processes of Podocytes are not fully Understood

Progress has been made in elucidating the mechanisms that regulate the early stages of podocyte development through the use of embryo explant models (Pavenstädt et al., 2003). While these studies have provided useful insights they cannot provide information regarding the later stages of podocyte maturation as the endothelial population dies and as such, a complete GBM cannot be formed (Loughna et al., 1998). Podocytes rely on interactions with the GBM to achieve the later stages of maturation (Abrahamson, 2009) an idea further backed up by studying the 'capillary loop' stage of development. It is at this point that many processes associated with vasculogenesis occur and it is also the stage when podocytes begin to form structures associated with a mature morphology e.g. foot processes (Abrahamson, 2009). It is possible that an explant culture in which endothelial cells were able to survive could be used to study these later stages of podocyte maturation.

1.17.5 Neonatal Mouse Kidneys

Previous studies have indicated that under appropriate conditions, endothelial cells derived from adult mouse kidneys can survive in culture (Loughna et al., 1998); however, this tissue cannot be used to study nephrogenesis as it does not contain any MM or UB cells. It is not clear why endothelial cells within embryonic mouse kidneys undergo rapid cell death following explantation, whereas endothelial cells from adult kidneys can survive, but this

could possibly be due that the fact that the adult cells are more mature, and/or may have a different origin. The neonatal kidney provides a potential solution as it is more mature than the embryo kidney, and it is thus possible that the endothelial cells present within the neonatal tissue might have different properties than those of the embryo kidney that might enable them to survive in culture. An advantage of the neonatal kidney tissue over adult tissue is that the former is still undergoing nephrogenesis, and therefore contains MM and UB cell populations (Dickinson et al., 2005). The fate of endothelial cells in neonatal mouse kidney explant culture has not been previously investigated and will provide an interesting study as renal vasculogenesis was previously not though to occur *ex-vivo* (Bernstein et al., 1981).

1.17.6 Generating an Explant Model of Nephrogenesis Using Neonatal Mouse Kidneys

An ideal method for establishing an explant culture using Neonatal Mouse Kidneys is the disaggregation-reaggregation technique first published by Unbekandt and Davies 2010. This method has the advantage of simultaneously providing the opportunity to study the activity of endometrial epithelial cells when introduced to kidney explant culture as any cell population can be introduced into the culture after the disaggregation step to produce a chimeric explant (Unbekandt and Davies, 2010). Chimeras have previously been generated by a student in The University of Liverpool using endometrial cells and embryonic mouse kidneys confirming the technique is feasible (Simic, 2010).

1.17.7 Disaggregation and Reaggregation of Mouse Kidneys

In the Unbekandt and Davies study, E11.5 mouse kidneys were disaggregated, and then reaggregated in the presence of an exogenous cell type in order to produce fine grained chimeras where the fate of the exogenous cells could be investigated (Figure 8). The method also allows the investigation of siRNA-mediated knockdown of specific target genes in the introduced cells. One key innovation contributing to the success of the method was the use of a Rho-Kinase (Uehata et al., 1997) inhibitor for 24 hours at the start of culture, which reduced the degree of cell apoptosis previously observed within such ‘fine grained chimeras’ thought to be attributable to loss of tissue derived cell signalling (Coles et al., 1993), or anoikis resulting from the removal of the nascent epithelial cells from their basement membranes. The inhibitor is removed after 24 hours of culture because ROCK is known to be essential for nephron formation and by this point the cell-cell signalling associated with growth is sufficient to prevent apoptosis (Unbekandt and Davies, 2010).

Using this system the authors were able to culture fine grained chimeras demonstrating structures associated with normal renal development e.g. comma shaped bodies (Unbekandt and Davies, 2010).This method differs from that described by Auerbach and Grobstein as no 'whole' exogenous tissues are introduced to the kidney structure which would have the limiting factor of incomplete integration and perfusion of the cells of interest (Auerbach and Grobstein, 1958).

Figure 8: Disaggregation and Reaggregation of Neonatal Kidneys Allows Formation of Chimeras and Neonatal Explants

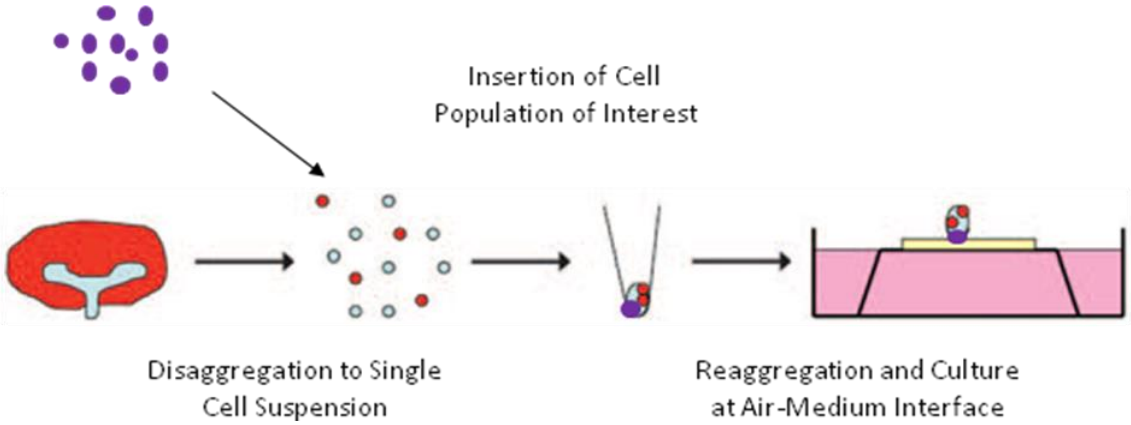


Fig8. Disaggregation-Reaggregation protocol developed by Unbekandt and Davies allows generation of fine grained chimeras in order to study developmental processes and the specific action of an introduced cell population of interest (Unbekandt 2012) For the present study the population of interest will consist of endometrial epithelial cells (Purple). Image reproduced from (Unbekandt 2012).

1.18 Specific Markers of Nephron Development and Endometrial Cells

In order to assess the development of potential nephron structures including endothelial cell populations within the mouse neonatal kidney explant cultures and to track the survival and proliferation of the human endometrial cells within the chimeras, specific markers were selected for analysis.

1.18.1 Kidney Explant Culture

1.18.2 Wilms Tumour Protein (Wt1)

Wt1 is a transcription factor well characterised in the mammalian kidney and first investigated due to its association with nephroblastoma, also known as Wilms Tumour (Armstrong et al., 1993). Other than a low degree of expression in the spinal cord, Wt1 is expressed in mesodermally derived tissues and is thought to play some role in mesenchymal to epithelial transition (MET). In the mouse, it is first expressed in the MM

(Armstrong et al., 1993). Expression then increases as the MM condenses and forms the epithelial cells of the nephron (Armstrong et al., 1993). Correspondingly, studies of mouse kidney development have found little expression at the mesonephros stage but strong expression in the metanephros, with Wt1 being confined to the presumptive podocytes by the s-shaped body stage (Armstrong et al., 1993). For this reason Wt1 will be used in this study to identify developing kidney structures in explant culture.

1.18.3 Synaptopodin

Synaptopodin is an actin-associated protein found to be expressed in neural dendrites and renal podocytes (Mundel et al., 1997). Development of synaptopodin expression occurs as the capillary loop stage and is associated with loss of mitotic activity meaning that it can be taken as a marker of more mature podocytes (Burrow, 2000). For this reason analysis and identification of mature podocytes in this study will be performed using this marker.

1.18.4 Laminin

Laminin is a matrix protein which can be found in the glomerular basement membrane and has been demonstrated to be important for glomerular maturation. While this protein is found in many tissues the characteristic staining in association with podocytes makes it a useful tool in identifying putative glomerular structures in the culture (Kikkawa et al., 2003).

1.18.5 Platelet Endothelial Cell Adhesion Molecule 1 (Pecam)

Pecam is a cell surface molecule which can be reliably used to identify endothelial cells (Baldwin et al., 1994). It is also present on reticuloendothelial cells, platelets, monocytes, neutrophils (GeneID, Accessed 2013) and in Kaposi's Sarcoma (Ganjei-Azar P, 2006). These characteristics make PECAM a useful marker in identifying endothelial structures as a characteristic pattern of expression will be observed.

1.18.6 Calbindin

Calbindin28k is a calcium binding protein used to identify ureteric bud structures in analysis of chimeric mouse embryo kidney explants (Michos, Michos, 2012). Previous studies have shown that it can be identified in tissues before nephrogenesis has begun (Davies, 1994), that expression does not decrease with branching of the UB and that it can be used to identify collecting duct structures of mature nephrons (Davies, 1994). This makes Calbindin

an ideal structure for UB tubulogenesis in the kidney explant culture and in aiding the identification of mature nephron structures.

1.19 Chimera Culture

1.19.1 Mucin1

Mucin 1 (MUC1) is a glycoprotein first proposed as a window of implantation marker in the endometrium, possibly playing a role in the first stage of embryo implantation (Aplin et al., 1998). MUC1 has been identified on the luminal epithelium with constant expression increasing in the secretory phase (Aplin et al., 1998).

The expression of MUC1 appears to vary in the endometrium as multiple splice variants give the molecule different functions depending on the location, a fact evidenced by the loss of MUC1 from uterine pinopodes in the mid-secretory phase (Horne et al., 2005). For this reason MUC1 cannot be a specific marker for a particular process within the human endometrial cells but can be used as a quantitative measure to assess their presence. If a specific process were to be identified the products from PCR analysis would need to be sequenced in order to identify the splice variant.

1.19.2 Progesterone Receptor

Progesterone receptor A and B arise from the same gene and expression is dependent on the cellular compartment of the endometrium with a general increase towards the secretory cells as oestrogen primes the endometrial cells (Isaac et al., 2001). Progesterone acts to prevent further growth of epithelial cells and stimulates decidualization. There is a documented drop in expression in epithelial glands of functionalis in the period of transition between proliferative and secretory phases. This contrasts with persistent expression in the stroma (Beier-Hellwig et al., 1994, Roberts et al., 1989). The pattern of expression in the basalis is different as and progesterone receptor is more persistently documented throughout the cycle in the glands and stroma of this area (Beier-Hellwig et al., 1994).

1.19.3 Human Specific Antibody

In order to locate the human endometrial cells within the chimera a human specific antibody will be used. Several potential antibodies are available including: anti-

Mitochondrial, anti-Cytoplasmic and anti-Nuclear, and determining which will be the most useful will form the basis of a pilot study.

1.19.4 Laminin

Laminin will be used in the chimeras to assess the proliferation of the human endometrial cells by their ability to form glandular structures within the culture. Glandular structures are surrounded by a laminin rich membrane and although the antibody used is not human specific a measure of glandular structure formation may be obtained. As polarised glandular cells typically are aligned along a basement membrane, co-staining for Laminin and a human specific antibody will allow investigators to assess the ability of endometrial cells to form glandular structures in the chimera (Abrahamson, 2009).

1.20 Aims and Objectives of the Study

1.20.1 Aims

Research into the endometrium and endometriosis is still in its early stages. Though the endometrium has long been a source of fascination to clinicians it is only in recent years that attempts have been made to deconstruct its workings through scientific investigation. Endometriosis represents a pathological endometrial condition where the high proliferative potential characteristic of the endometrium is observed. The aspects of this study focussing on the endometrium aim to increase understanding of these processes either in the laboratory setting or through *in silico* investigation. In the laboratory investigation it is hoped that characteristics of an ASC i.e. proliferation and multipotency may be observed in a kidney chimera model. For the bioinformatics investigation it is hoped that some of the cellular and molecular mechanisms governing processes in normal endometrium and patients with endometriosis may be elucidated via analysis of transcriptomic datasets.

A separate section of this study will investigate renal events in the neonatal kidney explant culture. It is hoped that an explant culture can be established in which the endothelial cell population is viable such that late maturation processes of podocytes and other nephron structure may be investigated further. This is an area in which progress would be useful as the podocyte represents the functional unit of the glomerulus and renal pathologies often affect this cell; understanding normal physiology may help to investigate and improve management of pathological conditions.

1.20.2 Objectives

Endometrial-Kidney Chimera

- To establish methods of effectively tracking a population of human endometrial cells when cultured in a chimeric model with neonatal mouse kidneys.

- To investigate possible phenotypic changes within a population of human endometrial cells when cultured in a chimeric model with neonatal mouse kidney cells using immunofluorescence and RT-qPCR. Specific assessment will be made of:
    - The endometrial cells' ability to survive and proliferate in culture.
    - The endometrial cells' ability to form glandular structures in culture

- To investigate possible phenotypic changes within a population of neonatal mouse kidney cells when cultured in a chimeric model with Human Endometrial cells using Immunofluorescence and RT-qPCR.

Kidney Explant Model

- To establish a neonatal kidney explant model using either partial or complete disaggregation of neonatal mouse kidney tissue.

- To investigate the ability of this neonatal explant model to form nephron structures in culture using Immunofluorescence and RT-qPCR for renal markers: WT1, Laminin and Calbindin

- To investigate the ability of this neonatal explant model to form mature podocytes and endothelial structures using Immunofluorescence and RT-qPCR for renal markers WT1, PECAM, Synaptopodin, Alkaline Phosphatase and Calbindin.

Bioinformatics

- To use published transcriptomic datasets to identify differentially regulated genes within the 'Normal' endometrium at different phases of the menstrual cycle.

- To analyse these differentially regulated genes using a 'Systems Biology' approach with a view to identifying molecular networks of interest and enabling further research.

- To compare data obtained from the 'Normal' endometrium with that obtained from patients with endometriosis with a view to identifying potential biomarkers and drug targets.

Chapter 2: Materials and Methods

2.1 Sample Collection and Processing

2.1.1 Sample Collection

Patient operating lists were routinely consulted at the Liverpool Womens Hospital and suitable patients for inclusion in ongoing research trials identified. Patients were approached and fully informed voluntary consent was obtained to collect an endometrial sample. Information provided by researchers included benefits and risks of taking part in the study including the fact that patients could choose to withdraw at any time without this affecting their standard of care. Examples of Consent forms and Patient Information provided are included in the appendix.

Samples were obtained under the study: "The role of the identified regulators of cell fate (RCF) and metastasis-inducing-proteins (MIP) in endometrial stem/progenitor cells (SPC) in endometriosis" LREC Ethics Reference Number: 09/H1005/55.

All Patients:

- Two blood samples obtained before induction of anaesthesia: one Full Blood sample and one Serum samples

Patients Undergoing Surgery for Benign Indications:

- Full thickness endometrial biopsy obtained using a sterile scalpel and forceps after making a midline incision at the anterior aspect of the uterus.

Once samples were obtained they were divided into several collection tubes depending on their future use:

- Neutral Buffered Formalin (NBF) (Sigma Aldrich)
- Phosphate Buffered Saline (PBS) (Sigma Aldrich)
- RNALater (Applied Biosystems)
- 'Sample Collection Medium' (Endometrial Scraping)

The composition of 'Sample Collection Medium' may be found in the Appendix Section 6.7.

2.1.2 Sample Processing

All sample processing was conducted in a class II cell culture hood, all waste coming into contact with human tissue was sterilised with 1% Virkon (Antec). Identifying information

was removed from samples to preserve patient anonymity and a unique laboratory sample number and code was assigned to each sample depending on the patient details.

Sample Categories

- SPCE – Endometriosis of any stage with no 'SPCEX' indicators applying
- SPCN – No documented Endometriosis or any of the 'SPCEX' indicators applying
- SPCEX – Hormone therapy, Nulliparous, Previous endometrial ablation, Previous Dilatation and Curettage. Patient may or may not have Endometriosis.
- EP- Post-menopausal patients.

This code is followed by a number depending on when the patient was recruited to create a unique sample ID e.g. 'SPCE 112'

Collection medium, Hanks solution and RNA Later samples were labeled with sample code and placed in the fridge in 'Tissue Culture' laboratory to be used for ongoing studies in the University of Liverpool. NBF samples were labeled, dated and placed in the fridge in the Histology laboratory.

Whole blood samples were separated into two 2ml cryotubes and stored in the appropriate box space in the -80°C freezer depending on the sample code after which the storage location was entered into a labeled box plan in the freezer log. 'Serum' blood samples were obtained by centrifugation at 3000rpm for 10 minutes and pipetted into two micro test tubes and stored at -80°C, PBS samples were snap frozen and stored at -80°C. Examples of paperwork required to be completed for sample processing are included in the appendix. Relevant patient demographic details were also recorded for samples used in the study. Information included: Sample ID, Sample Type, Patient Age, Patient BMI, Indication for surgery, Presence or absence of endometriosis, Menstrual Cycle Phase as well as any additional information e.g. Hormonal Contraception.

2.2 Primary Culture of Endometrial Cells

Once an endometrial scraping was obtained and stored in collection media, primary culture was employed to cultivate a population of endometrial epithelial cells which could then be placed in the chimera. Endometrial tissue was removed from 'Sample Collection Medium' and transferred to a 10cm culture dish where it was finely dissected using 2 sterile scalpel blades (Swann Morton) to increase surface area for enzymatic digestion until 1 mm^3 pieces

were achieved. The sample was then transferred to a 30ml universal tube with Dulbecco's Modified Eagle Medium (DMEM), washing the culture dish with DMEM ensure the entire sample was transferred. To collect the cells the sample was placed in a centrifuge (Sigma Aldrich 4K15) at 500 Relative Centrifugal Force (g) for 5 minutes. The supernatant was removed and the cells re-suspended in 4ml DMEM.

To achieve a single cell suspension enzymes and co-factors were added for an initial digestion:

- Collagenase (20 mg/ml, 500µl) (Sigma Aldrich)
- Dispase (10 mg/ml, 500µl) (Roche)
- DNase (4 mg/ml, 100µl) (Invitrogen)
- MgCl2 (50µl 100mM)

The cells were transferred to a shaking water bath at 37°C for 1.5 hours.

2.2.1 Crude Fractionation of Epithelial and Stroma Cell Populations

After initial incubation the resulting digest was filtered through a 40 µm cell strainer into a 50ml test tube. The flow-through was washed with DMEM and represented the stromal population. The retentate represented the epithelial population and was transferred to a second 50ml test tube by backwashing with 30-40ml DMEM. Gentle pipetting was used to dislodge adherent cells.

Epithelial Fraction Culture

The Epithelial Fraction (EF) was centrifuged at 500g for 5 minutes to collect the cells after which the supernatant was discarded and the EF re-suspended in 1ml DMEM with further enzyme added to digest the intact epithelial glands:

- Trypsin (1ml 0.25%) (Sigma Aldrich)
- DNase (4mg/ml, 100µl)

The EF was then incubated at 37°C for 20 minutes in the shaking water bath. After this final digestion the EF was gently pipetted for 3 to 5 minutes in order to liberate single cells after which the Trypsin was inactivated with 1 ml DMEM. To remove the Trypsin the EF was then centrifuged at 500g for 5 minutes to pellet cells with the supernatant being discarded. The EF was then re-suspended in 10ml 'Endometrial Culturing Medium' and plated onto a 10 cm

culture dish. The composition of 'Endometrial Culturing Medium' is included in the Appendix Section 6.7.

Stromal Fraction Culture

The stromal fraction did not require further enzymatic digestion and subsequently could be centrifuged to discard the Trypsin containing medium and re-suspended in 'Endometrial Culturing Medium' before plating onto a 10cm culturing dish.

Selective Adherence

The stromal population would adhere preferentially to the 10cm culturing dish over the epithelial population so after 15-20 minutes the fraction was re-plated to increase isolation of the two individual populations.

2.2.2 Vybrant Labeling of Human Endometrial Cells

In order to identify the human endometrial cells after incorporation into the neonatal mouse kidney chimera they were first labeled using the 'Vybrant CFDA SE Cell Tracer Kit' (Invitrogen) which consisted of Carboxy fluorescein diacetate, succinimidyl ester (CFDA SE) and Dimethylsulfoxide (DMSO). This kit marked the cells with green fluorescence, allowing investigators to validate the human specific antibodies used in the study. The protocol was followed according to the manufacturer's instructions.

The CFDA SE was allowed to warm to room temperature after which a 10mM stock solution was prepared by adding 90ul of DMSO to one vial of 500µg CFDA SE. This solution was then diluted with Phosphate Buffered Saline (PBS) (Sigma Aldrich) to give a final concentration of 20µM CFDA SE.

Labeling Human Epithelial Cells

To label the human cells with Vybrant, the 'Endometrial Culture Medium' was removed from the a 10cm culture dish containing adherent cells and replaced with 6ml of a 20µM CFDA SE solution pre-warmed to room temperature. The dishes were then returned to the incubator for 15 minutes at 37°C after which the CFDA SE solution was removed and replaced with pre-warmed Endometrial Culture Medium' and incubated for a further 30 minutes at 37°C. During this time the CFDA SE underwent acetate hydrolysis after which the fluorescent signal could be observed within the cells.

2.2.3 Trypsinisation of Human Endometrial Cells

In order to introduce the human endometrial cells into the chimera they first needed to be removed from the culture dishes to which they adhered. To achieve this, the cells were washed twice with 10ml pre-warmed PBS before 10ml 1× Trypsin was added. The plate was then incubated for 10 minutes at 37°C during which time the cells detached from the dish. After incubation the Trypsin was inactivated using 10ml DMEM with 10% Fetal Bovine Serum (FBS) and the cells in suspension were transferred to a 15ml conical tube.

A ratio of 1 in 5 human to mouse cells was used for the study. In order to calculate the volume of human endometrial cell suspension to introduce to the chimera the live cells were counted using an automated cell counter (Biorad) and Trypan Blue (Sigma Aldrich).To count the live cells 10µl of cell suspension was transferred to a cell counting plate and 10µl Trypan Blue added. The two solutions were mixed and 10µl transferred to a haemocytometer which was placed in an automated cell counter.

2.3 Disaggregation of Neonatal Mouse Kidneys

Neonatal Mice (2-3 day's old) were sacrificed according to Schedule 1 Home Office Protocol, CD1 and C57BL/*6* 'Black6' mice were used for the study. The kidneys from the neonatal mice were isolated using a dissecting plate, forceps, surgical scissors and stored on ice in Minimum Essential Medium Eagle (MEME) (Sigma Aldrich) in a 1.5ml microfuge tube. The kidneys were transferred to a cell culture hood and dissected in a 10cm culture dish until $1mm^3$ sections were achieved. The sections were transferred to a 20ml test tube and the culture dish washed thoroughly with MEME to ensure all kidney sections were transferred.

The kidney sections were allowed to settle at the bottom of the test tube and the medium removed and replaced with 20ml Ca^{2+} free PBS (Sigma Aldrich).

- **Trypsin Protocol**

After 1 minute the PBS was removed and the tissue re-suspended in 3ml 1×Trypsin solution with 20µl DNase (5mg/ml) and placed in a water bath for 20 minutes at 37°C. After incubation the sample was gently pipetted for 1 minute to aid disaggregation and incubated for a further 5 minutes at 37°C after which 10ml DMEM with 10% FBS was added to inactivate the Trypsin.

- **Collagenase Protocol**

After 1 minute the PBS was removed and the tissue re-suspended in 2ml Collagenase (Sigma Aldrich) (2mg/ml) with 20µl DNase (5mg/ml) and incubated for 45 minutes at 37°C. After incubation 10ml DMEM with 10% FBS was added to inactivate the collagenase.

After this initial digestion the tissue was centrifuged for 3 minutes at 1000g, the medium discarded and DMEM with 10% FBS used to wash the tissue. The tissue was centrifuged again for 3 minutes at 1000g and re-suspended in 6ml Kidney Culture Medium (KCM), the composition of which can be found in the Appendix Section 6.7. The live kidney cells in suspension were at this point counted using a cell counter with Trypan Blue. Based on the results appropriate calculations were made to ensure a ratio of 1 in 5 endometrial to kidney cells. The final volume was dependent upon the mass of kidney tissue obtained at the start of the experiment and the volume of medium used during the protocol.

2.4 Re-aggregation of Human Endometrial and Mouse Kidney Cells

The ratio of Human to Mouse cells used in this study was 1 in 5 and as the size of the cell pellets produced was $1.5x10^6$ this meant $1.2x10^6$ kidney cells would be transferred to a number of microfuge tubes to be mixed with $0.3x10^6$ human cells. The number of pellets able to be generated from each experiment was dependent on the number of neonatal mice available.

For each experiment, two conditions were generated.

1. Pellets with human and mouse cells termed Endometrial/Kidney (E/K)
2. Pellets containing only kidney cells termed Kidney/Kidney (K/K)

Once the cell suspensions were transferred to the microfuge tubes they were re-aggregated to form cell pellets by centrifuging for 2 minutes at 700g. The pellets were gently pipetted to dislodge them from the tube, gently expelling medium from the base upwards. The pellets were then removed from the microfuge tubes inside the cell culture hood and placed onto a triangular section of Millipore Isopore Membrane Filter (Millipore) on a metal grid in a 3cm culture dish with 1ml Kidney Culture Medium. To prevent apoptosis 1µl ROCK inhibitor (5mM) (Calbiochem) was added to the medium for the first 24 hours of culture after which it was removed by replacing the medium with Kidney Culture Medium.

Kidney Culture Medium was changed every 48 hours. Neonatal kidney explants and chimeras were fixed after 2 hours (Day 0), 3 Days and 7 Days of culture by placing in either Trizol (Invitrogen) or Spectophotometric Grade Methanol (Sigma Aldrich) and stored at -20°C.

2.5 Gelatine Fixing and Sectioning of Chimeras

2.5.1 Preparation of Gelatine Solution

In order to be sectioned for analysis the fixed cell pellets needed to be fixed in a gelatine sucrose solution. The solution consisted of 15g Sucrose (Sigma), 7.5% Gelatine from Porcine Skin (Sigma) made up to 100ml with PBS. The solution was mixed in a glass conical flask and placed on a heating plate with a magnetic stirrer. Once the solution was adequately mixed and melted it appeared clear. At this point it was transferred to a water bath at 37°C with foil placed over the top to prevent evaporation.

2.5.2 Preparing Fixed Rudiments

The fixed chimeras were stored in methanol at -20°C and were transferred through a series of methanol dilutions, and finally to PBS before gelatine embedding. To this end, the explant was removed from the 100% methanol fixing solution and transferred to a 1.5ml microfuge tube containing 1ml 75% methanol solution. The explant was allowed to rest for 2 minutes after which the 75% methanol solution was replaced with solutions of 50%, 25% and 10% methanol respectively at 2 minute intervals. After the explant was transferred to 10% methanol solution it was washed 3 times with 1ml 1xPBS. At this point the 1ml PBS was removed and 1ml sucrose/gelatine solution was added to the rudiment, the composition of gelatin sucrose solution may be found in Appendix Section 6.7. The microfuge tube was placed in the water bath for 30 minutes to allow the sucrose to permeate the tissue. Gelatine solution was also transferred to the bottom of two weighing boats to provide a surface upon which the cell pellet could be mounted after the sucrose had permeated the tissue.

2.5.3 Gelatine Embedding and Generation of Frozen Sections

The cell pellet was removed from the microfuge tube and placed with the Millipore Membrane at 90° to the gelatine surface of the weighing boat so it was 'standing up' and was held in place with tweezers. Molten gelatine was then dripped over the section to 'fix' it in this erect position. Once the gelatine was set the rudiment was dissected out using a scalpel blade and placed onto a labelled cork disk identifying the sample. The sample was then covered with 'Bright Cryo-M-Bed' compound (Bright Instrument Company) and placed on dry ice to set. Once the sample became opaque it was ready for sectioning. Samples were sectioned using a cryostat and mounted onto 'Superfrost' slides (Menzel Gläzer) and stored at -20°C.

2.6 Immunofluorescence

2.6.1 Slide Preparation

Once the conditions for the staining run were decided, slides with the appropriate sections were located from those stored at -20°C. The slides were then labelled with the experimental condition (e.g. particular antibody to be applied or designated as control) and sections were encircled with a hydrophobic marker pen to ensure applied solutions stayed on the section.

Blocking

To prevent nonspecific binding of applied antibodies, sections for immunostaining were first 'blocked' using a 'blocking solution' which consisted of 1% Normal Goat Serum (NGS) (Sigma Aldrich) made up with 1x Phosphate Buffered Saline (Sigma Aldrich). The blocking solution was centrifuged at 13400g for 5 minutes to minimise transfer of impurities to the samples and 100µl were applied per sample for 40 minutes at room temperature.

2.6.2 Primary Antibodies

When incubation was complete the blocking solution was removed and primary antibody solutions were applied. The primary antibody solution consisted of 1% NGS made up using 1xPBS and the primary antibody of interest at the appropriate concentration e.g. 1 in 200. Primary antibody solutions were centrifuged for 5 minutes at 13400g and 100µl applied per sample. Incubation was overnight at 4°C in a humidified chamber.

Table 1: Primary Antibodies Used in the Study

Target Molecule	**Animal Raised in and Clonality**	**Species Specificity**	**Concentration**	**Manufacturer**	**Reference/ID Number**
Pecam	Rat Polyclonal	Non Specific	1:200	BD Pharmingen	550274
Synaptopodin	Mouse Monoclonal IgG_1 (Clone G1D4)	Non Specific	1:2	Acris Gmbh	BM5086
WT1	Mouse Monoclonal IgG_1 (Clone 6F-H2)	Non Specific	1:250	Millipore	6FH2 05-753
Mitochondria	Mouse Monoclonal IgG_1 (MTC02)	Human	1:200	Abcam	Ab3298
Nuclear	Mouse Monoclonal IgG_1 (Clone 235-1)	Human	1:200	Millipore	MAB1281
Cytoplasm	Mouse Monoclonal IgG_1	Human	1:200	STEM 121	AB-121-U-050
Laminin	Rabbit Polyclonal	Non Specific	1:1000	Sigma Aldrich	L9393
Calbindin	Mouse Monoclonal IgG_1 (Clone CB-955)	Non Specific	1:200	Abcam	Ab 82812

2.6.3 Secondary Antibodies

After incubation with the primary antibody solution was complete, the primary antibody was removed and samples washed by applying and removing 100µl of PBS three times. After the third wash the Secondary Antibody solution was applied which consisted of 1% NGS made up using 1xPBS and the Secondary Antibody of interest at the appropriate concentration e.g. 1:1000.

The Secondary Antibody should correspond to the animal the Primary Antibody was raised in, for example:

Primary: Wt1 *Rabbit* Polyclonal, **Secondary:** Alexa Fluor-488 labelled Goat anti-*Rabbit*

After the Secondary Antibody was applied the samples were incubated for 2 hours at room temperature in a dark humidified chamber. All secondary antibodies were used at a concentration of 1:1000 and supplied by Invitrogen, Paisley UK.

Table 2: Secondary Antibodies

Antibody	**Conjugated Fluorophore**	**Reference/ID**
Goat anti Rat IgG	594 (Red)	Alexa fluor A11007
Goat anti Mouse IgG_1	488 (Green)	Alexa fluor A22121
Goat anti Mouse IgG1	594 (Red)	Alexa fluor A21125
Donkey anti Rabbit IgG	488 (Green)	Alexa fluor A21206

2.6.4 DAPI Staining and Mounting

After incubation was complete the secondary antibody solutions were removed and 3X 100µl PBS washes applied to the samples. After the third PBS wash, the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) was applied to the samples to stain cell nuclei. The DAPI was used at a 1 in 100,000 dilution of a 1mg/ml stock in PBS. This solution was added to

samples and incubated for 5 minutes in a darkened humidified chamber. The DAPI solution was removed after incubation and one PBS wash applied.

To mount the slides the PBS wash was removed and 1 drop of immunofluorescence mounting medium (Dako) applied, a cover slip (50x24mm) was then placed over the section. To make the mounting more permanent and prevent spoiling the edges of the slides were fixed with nail varnish.

2.7 Polymerase Chain Reaction

Cell Pellets were immersed in 500µl Trizol whilst attached to the Millipore membrane in 1.5ml microfuge tubes. Before RNA extraction was carried out the membrane was removed and the tissue homogenised; this was achieved using a hand-held homogeniser (Pellet Pestle, Sigma). Trizol is a potential carcinogen and was handled inside a cell culture hood. The homogeniser was then placed into the tube with the sample and activated in short bursts until the sample was no longer visible. After this the Millipore membrane was removed with tweezers and safely disposed of.

2.7.1 RNA Extraction and Quantification

To begin the extraction 200µl Chloroform (Sigma Aldrich) was added to the sample in Trizol, the lid of the microfuge tube tightly closed, and the sample shaken vigorously for 15 seconds. The samples were then centrifuged for 15 minutes at 11400g. After the centrifugation, the samples were observed to have separated into 3 visible layers. The topmost clear layer containing the RNA was transferred to a new microfuge tube, being careful not to transfer any of the middle or lower layers. 500µl isopropanol (Sigma Aldrich) was then added and the sampled were inverted 5-6 times and incubated at room temperature for 10 minutes. To increase the yield of RNA the samples were stored overnight in the freezer at -20°C.

To pellet the RNA, the samples were centrifuged for 10 minutes at 12000g after which an RNA pellet was visible at the bottom of the tube. After removing the isopropanol, the pellet was then washed in 75% ethanol (Sigma Aldrich) using nuclease free water for dilution. The sample was then centrifuged for 5 minutes at 7,500g. If the RNA was to be stored, the ethanol supernatant was discarded and replaced with a further 1000µl of 75% ethanol and stored at -20°C. Once the RNA was extracted it was quantified using the nanodrop spectrophotometer. In order to do this a 1µl sample of nuclease free water was initially

placed on the instrument and measured as a 'blank' to create a baseline measurement. Once this was performed each RNA sample was quantified using 1µl samples. Results are recorded in appendix.

2.7.2 DNase Treatment of RNA

DNase treatment is a necessary step in RNA extraction to remove potential genomic DNA which may affect PCR results. The ethanol supernatant was discarded and the RNA pellet allowed to dry. The pellet was then dissolved in 10-50µl nuclease free water. Gentle pipetting was used to avoid shearing the RNA. Once dissolved, 8µl of this RNA solution was transferred to a 200µl microfuge tube and 1µl DNAse buffer (Promega) added. To commence the reaction 1µl DNAse enzyme (Promega) was added and the sample incubated at 37°C for 30 minutes. After incubation the reaction was stopped by adding 1µl 'Stop' buffer (Promega). To denature any excess enzyme the contents of the microfuge tube were incubated for 15 minutes at 60°C. If cDNA synthesis followed immediately, the samples were placed on ice in preparation for this, if not they were stored at -20°C.

2.7.3 Complementary DNA Synthesis: Preparation Stage

Complementary DNA (cDNA) was synthesised from the DNase treated RNA. Reactants were assembled in a 200µl microfuge tube and consisted of:

- 8µl DNase Treated RNA
- 1µl Random Hexamers (100ng/µl) (Qiagen)
- 1µl Deoxyribonucleotide Triphosphate (dNTP) (10mM) (Invitrogen)
- 4µl Nuclease Free Water (Fisher Bioreagents)

The reaction volume at this point was 14µl and the sample was incubated at 65°C for 5 minutes and placed on ice for 1 minute.

2.7.4 Complimentary DNA Synthesis: Enzymatic Stage

Reactants for the enzymatic stage were added to the solution in the 200µl microfuge tube as follows:

- 1µl 5x Buffer (Invitrogen)
- 1µl 0.1M Dithiotheritol (DTT) (Invitrogen)
- 1µl Superscript III (Invitrogen)

The sample was incubated at 25°C for 5 minutes then transferred to the thermocycler (Applied Biosciences) where it was incubated at 50°C for 60 minutes followed by a step at 70°C for 15 minutes to denature the Superscript III. After the incubation was complete the cDNA was stored in a freezer at -20°C

2.7.5 PCR Primers

Table 3: Chimera Primers

Primer	**Sequence**	**Tm (°C)**	**Product Size (bp)**	**Specificity**	**Source**
M PGR	**F:** 5-CCTTGCTTGCACGCTTGG-3 **R:** 5-CTGGAATTTCTGCCTCCTTC-3	60.05 56.10	229	Mouse	In house
M Muc 1	**F:** 5-GAGGGTACTTTTAGTGCCTC-3 **R:** 5-CCAGGGCAAGGAAATAGACG-3	55.17 58.33	217	Mouse	In house
h PGR	**F:** 5-CAGTGGGCGTTGGAAATGA-3 **R:** 5-TGGTGGAATCAACTGTATGTCTTGA-3	58.36 60.22	83	Human	In house
Tata Box Protein (Human)	**F** 5-GACGAGTTCCAGCGCAAGGGTT-3 **R** 5-GCACCCTGAGGGGAGGCCAA-3	65.54 66.54	110	Human	In house
h MUC1	**F**: 5-CGATCGTAGCCCCTATGAGA-3 **R**: 5-TGAGCAGCCCACCTGAACTC-3	58.18 62.12	220	Human	(Shyu et al., 2011)
Tata Box Protein (Mouse)	**F:** 5-GTGCGTCAGGCGTTCGGTGG-3 **R:** 5-AATAGTGATGCTGGGCACTGCGGA-3	66.58 66.59	113	Mouse	In house

Table 4: Neonatal Kidney Explant Primers

Gene Name	Primer Sequence	Tm (°C)	Product Length (bp)	Source
WT1	**F** 5-CCAGTGTAAAACTTGTCAGCG-3 **R** 5-TGGGATGCTGGACTGTCT-3	57.75 57.43	234	In House
Synaptopodin	**F** 5-CGCCAGGGACCAGCCAGATAG-3 **R** 5-CTGCCCAAGCAGTCTCCTTTC-3	64.61 61.22	164	In House
PECAM	**F** 5-CAGGACCACGTGTTAGTGTT-3 **R** 5-ACTCCTGATGGGTTCTGACT-3	57.67 57.77	81	(Murad et al., 2003)
Alkaline Phosphatase	F 5-AGGGTACACCATGATCTCAC-3 R 5-TCTCCCAGGAACATGATGAC-3	56.32 56.62	189	In House
Calbindin	**F** 5-AGAACTTTCTAAAGGACCTA-3 **R**-AAGCCTTATTGAACTCTTTC-3	50.34 51.02	213	In House
Tata Box Protein (Mouse)	**F** 5-GTGCGTCAGGCGTTCGGTGG-3 **R** 5-AATAGTGATGCTGGGCACTGCGGA-3	66.58 66.59	113	In House

2.7.6 Polymerase Chain Reaction Conditions

Reactants were assembled in a 200µl tube with a final reaction volume of 20µl with the following constituents per reaction:

- 10µl SYBR Taq (Sigma Aldrich),
- 7µl Nuclease free water,
- 1µl Template e.g. cDNA or Nuclease Free Water for 'no template' controls
- 1µl Forward Primer (6.25pmol/µl)
- 1µl Reverse Primer (6.25pmol/µl)

A 'No Template Control' (NTC) was used in each experiment and a housekeeping gene employed to normalise gene expression. The samples were loaded into the PCR machine Rotorgene 3000 (Corbett) and the reaction started using the following parameters: Hold

95°C 6 minutes. Cycling 95°C 6 Seconds, 58°C 20 seconds, 72°C 30 seconds. For this study 45 cycles were used.

In each PCR series 3 technical replicates were obtained for housekeeping genes and 2 technical replicates obtained for genes of interest. For both the neonatal kidney explant and chimera investigations 3 biological replicates were analysed.

2.7.7 Gel Electrophoresis

Gel electrophoresis was performed using the products of a PCR reaction to determine the number of products produced and the approximate size of each product.

Gel Preparation

A 'double gloving' technique was used throughout this method as the procedure involves the use of ethidium bromide, a known carcinogen, and care was taken to avoid skin exposure. A 1% Agarose Gel was used for electrophoresis and this was produced by mixing 1g Agarose (Bioline) with 99ml Tris-Acetate EDTA (TAE) buffer, the composition of which may be found in the Appendix Section 6.7. This mixture was heated in an 800W microwave (Sanyo) for 1 minute 30 seconds to produce the gel solution. Once this had occurred a clear colourless solution was observed. The solution was allowed to cool and 3µl Ethidium Bromide (0.5µg/ml) (Sigma Aldrich) added. An appropriately sized electrophoresis cassette was prepared by taping the open ends to create a structure into which the gel could be poured and allowed to set. The electrophoresis comb was set in place to create the wells for the PCR products. The gel was allowed to set for 40 minutes then placed into an Electrophoresis Chamber (SCIE-PLAS) and covered adequately with TAE.

Product Solution Preparation, Analysis and Image Capture

A final volume of 15µl product solution was added to each well within the Agarose gel. The solution consisted:

- 3µl 5xDNA Loading Buffer (Bioline)
- 10µl PCR product
- 2µl Nuclease Free water

Product solutions were prepared in separate microfuge tubes and pipetted into a separate well once the gel had been placed into the Electrophoresis Chamber. To provide a

reference for the product size, 5µl of Hyperladder IV (Abcam) was pipetted into a separate well. The Electrophoresis Chamber was connected to the power supply (Consort EV231) and activated. The parameters used for this study were 100 volts for 40 minutes. After the gel had run the power supply was switched off and the gel examined under ultraviolet light, capturing the image with an appropriate camera (GENE FLASH).

2.7.8 Calculation of Relative Gene Expression

Once all the qPCR runs were complete the data necessary to calculate relative gene expression in the Kidney Explants and Chimeras was analysed. The Relative Gene Expression was calculated using the cycle at which the PCR reactions become exponential; in order to do this a constant 'Threshold' is set for each experimental condition (Figure 9). For Kidney Explants the Threshold was 0.020804 and for the Chimeras 0.012. These points were set by the user based on the observed characteristics of the reactions in the series.

Figure 9: Threshold Value for Quantification of Relative Gene Expression

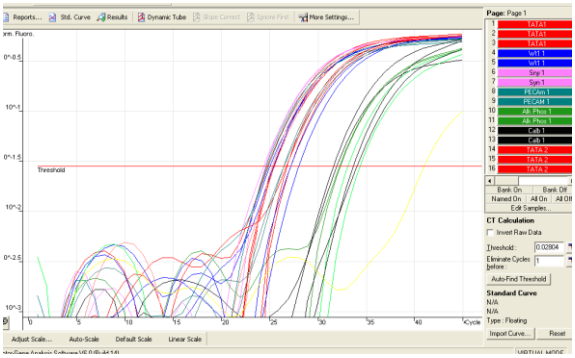


Fig9. A 'Threshold' value is required to generate Ct values for analysis of relative gene expression. Threshold is user defined and consistent for all RT-qPCR series.

For each reaction the number of cycles it takes for the reaction to become exponential or 'Cycles to Threshold' (Ct) for a gene of interest is compared to the Ct value for the housekeeping gene to determine relative expression. Biological and technical replicates were averaged to give an 'Average Ct' for each primer set on each day. Then Average Standard Deviation was also calculated for each primer set on each day.

2.7.9 Relative Quantity

Day 0 was taken as the baseline value for each gene of interest and the housekeeping genes, with the relative change in expression calculated at Day 3 and Day 7 using the formula:

$$\text{Relative Quantity} = 2^{(\text{Average Ct at Day 0} - \text{Average Ct at Day 3 or 7})}$$

2.7.10 Relative Gene Expression

The previous calculations give the relative quantity of each gene on Day 3 and Day 7 compared to Day 0 but to determine *relative expression* these values need to be compared to the relative quantity of the housekeeping gene, this is calculated using the formula:

$$\text{Relative Gene Expression } (\Delta\Delta Ct) = \frac{\text{(Relative Quantity (RQ) of Gene of Interest on Day x)}}{\text{(RQ of Housekeeping Gene on Day x)}}$$

2.7.11 Standard Deviation of relative Gene Expression and Standard Error

The Standard Deviation (SD) of the ΔΔCt values at Day x is calculated using the formula:

$$\text{SD } \Delta\Delta Ct = \Delta\Delta Ct \frac{\text{(RQ Housekeeping Gene)}}{\text{(SD RQ Housekeeping Gene)}}$$

Where: RQ=Relative Quantity

SD= Standard Deviation

The Standard Error is necessary to plot error bars for relative expression and gives information about the variability of results. It is calculated using the formula:

$$\text{Standard Error} = \frac{\text{SD } \Delta\Delta Ct}{\sqrt{3}}$$

In instances where the Standard Deviation of only 2 biological replicates of a gene of interest was available the Standard Error was calculated using the formula:

$$\text{Standard Error} = \frac{\text{SD } \Delta\Delta Ct}{\sqrt{2}}$$

In instances where the Standard Deviation of only one biological replicated of a gene of interest was available no calculation of Standard Error was performed.

2.8 Bioinformatics Methods

2.8.1 'Normal Endometrium' Literature search

To identify suitable microarray data for inclusion in the study the search term 'Normal Human Endometrium' was submitted to the 'GEODatasets' database on National Centre for Biotechnology Information (NCBI) returning 280 datasets grouped into 37 data series. These data series were individually assessed against a group of inclusion and exclusion criteria to determine their use in the study.

Inclusion Criteria

- Study of Human Endometrium
- Full thickness endometrial biopsy taken
- Premenopausal Patients
- Benign Endometrium free of pathology e.g. Endometriosis
- Patients free of hormonal treatment 3 months prior to biopsy

Exclusion Criteria

- Endometrial Pathology studied, Benign or Malignant
- Hormonal Treatment e.g. Combined Oral Contraceptive Pill
- Primary culture of human cells prior to analysis
- Isolation of Specific Cell Populations
- Study of endometrial cell lines
- Epigenetic Study

Further information of series not included in the study along with reason for exclusion may be found in the appendix.

This search identified the data series 'GSE4888' as suitable for inclusion in the study as it analysed biopsies collected from normally cycling patients undergoing hysterectomy/biopsy for benign indications between the ages of 23 and 50 who had not received hormonal therapy in the past 3 months (Talbi et al., 2006). This dataset has also been used extensively by previous publications to represent the 'normal endometrium' or as control data when studying gene expression in endometrial pathology (Savaris et al., 2011, Aghajanova and Giudice, 2011, Aghajanova et al., 2010).

2.8.2 Further 'Normal Endometrium' Dataset Refinement

The dataset GSE4888 was provisionally identified for use in the study as it best matched the inclusion/exclusion criteria; however it contained some samples that would not be appropriate for analysis of 'Normal' endometrium. For this reason further refinement was carried out.

- Pipelle biopsy samples were removed from the dataset as this form of biopsy may not represent full thickness endometrium.
- Biopsies obtained from patients with endometriosis or adenomyosis were removed from the dataset as their pathologies are associated with abnormal endometrium
- One biopsy obtained from a patient prescribed 'Thyroxine' was removed from the study as this medication may alter gene expression in the endometrium

This refinement resulted in a dataset of 15 biopsies suitable for inclusion in the study (Figure 10) consisting of:

1. Proliferative Phase Endometrium (n=1)
2. Early Secretory Phase Endometrium (n=3)
3. Mid Secretory Phase Endometrium (n=5)
4. Late Secretory Phase Endometrium (n=6)

Figure 10: Number of Patient Samples Within Each Phase of the Menstrual Cycle: Normal Endometrium

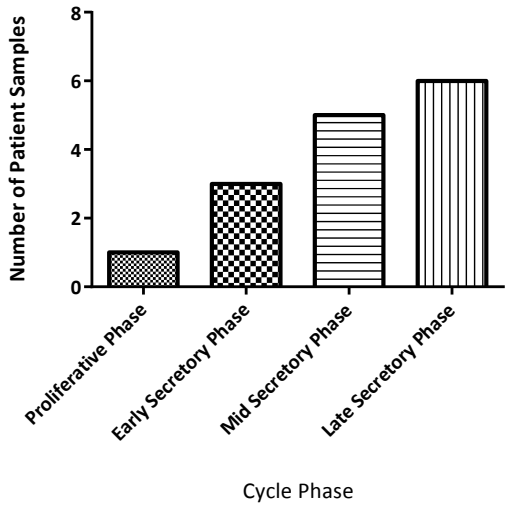


Fig10. Samples from the transcriptomic dataset GSE4888 were separated into menstrual cycle phase at which biopsies were obtained.

To carry out gene expression analysis one biopsy was required to be selected as a control value to compare gene expression across the dataset, 'Patient ID 598' which corresponds to the proliferative phase endometrial sample 1. This sample was used as a control in the original study from which the dataset is taken and represents the only proliferative phase sample.

2.8.3 Endometriosis Literature Search

To identify suitable microarray data for inclusion in the study the search term 'Human Endometriosis' was submitted to the 'GEODatasets' Database on NCBI which returned 334 datasets arranged into 26 data series. These series were individually assessed against a group of inclusion and exclusion criteria for their suitability for inclusion in the study.

Inclusion Criteria

- Human Endometrium Studied
- Patients Suffering from Endometriosis
- Premenopausal Patients
- Full thickness endometrial biopsy
- Patients free of hormonal treatment 3 months prior to biopsy

Exclusion Criteria

- Non Endometrial Tissue
- Primary cell culture of endometrial biopsies
- Isolation of specific cell populations
- Study of malignancy
- Study of non-human tissue
- Epigenetic study carried out
- Study of endometrial cell lines

A summary of the data series investigated and the reasons for exclusion are included in the appendix. After selection there remained 5 data series which could potentially be used in a study of gene expression in Human Endometriosis: GSE5108, GSE6364, GSE7305, GSE12768 and GSE37837.

These datasets were analysed with a set of further restriction criteria designed to ensure the data accurately reflected gene expression across different phases of the menstrual cycle and was comparable to the dataset gathered for 'Normal' endometrium.

Further Restriction Criteria

- Eutopic Endometrium Studied
- Secretory Phases Samples Predominantly studied
- Secretory Phase samples documented as 'Early', 'Mid' or 'Late'

Based on this selection the series 'GSE6364' was selected for analysis in the study, GSE5108, GSE7305, GSE12768 and GSE37837 were discarded and not used in any further analysis.

Detailed investigation of the data series is included in the appendix.

2.8.4 Further Endometriosis Dataset refinement

The Data Series 'GSE6364' had been provisionally identified as suitable for inclusion in the study however not all of the samples within the Data series were appropriate. For example of the 37 samples in the series only 21 represented eutopic endometrium of patients with endometriosis. These useful samples were extracted from the series and categorised according to which phase of the menstrual cycle they represented (Figure 11).

1. Proliferative Phase Endometrium (n=6)
2. Early Secretory Phase Endometrium (n=6)
3. Mid Secretory Phase Endometrium (n=9)

Figure 11: Number of Patient Samples Within Each Phase of the Menstrual Cycle: Endometriosis

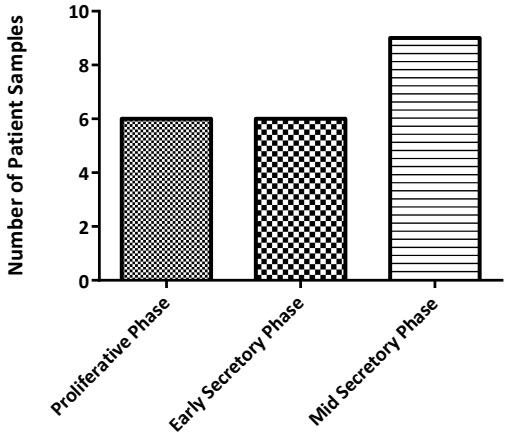


Fig11. Samples from the transcriptomic dataset GSE6364 were separated into menstrual cycle phase at which biopsies were obtained. No data was available for the late Secretory phase

The sample 'GSM150195' was selected as a control as it represents a proliferative phase eutopic endometriosis sample; this is in line with the previous analysis of 'Normal' endometrium. The 5 remaining 'Proliferative Phase Endometrium' samples were discarded from the analysis in order to make the analysis of 'Endometriosis' data comparable with that of 'Normal' endometrium which used 1 Proliferative phase sample as a control. It was hoped that by making these study designs identical data obtained from 'Normal' and 'Endometriosis' analysis would be comparable; however it is recognized that creating an average of these 6 Proliferative phase samples may be useful for future study when considering the 'Endometriosis' data alone.

2.8.5 Identification and Isolation of Differentially Regulated Genes

After identifying data suitable for inclusion in the study the relevant '.gz' files were obtained from NCBI. For each patient biopsy gene expression data and gene identifying information e.g. Gene Ontology terms were extracted from the dataset using the 'R' statistics package version 2.15.1.

These gene expression values were then compared to those of the control sample to identify up or down regulated genes within the 'Early', 'Mid', and 'Late' Secretory phase of the menstrual cycle. A mean log fold change of + or – 2 was considered statistically significant.

This transformation generated a set of up and down regulated genes for the Early, Mid and Late Secretory Phase in the 'Normal' endometrium and Early and Mid Secretory phase in the 'Endometriosis' endometrium. The use of a 'Z-score' was trialled for gene selection however the number of differentially regulated genes yielded by this approach was too small for further analysis and the Log_2 fold change used was considered sufficiently significant. The 'R' code used to generate both sets of differentially regulated genes is included in the appendix.

2.8.6 Analysis of Differentially Regulated Genes and Cellular Networks in 'Normal' and Endometriosis' Data Series

Differentially regulated genes identified for 'Normal' and 'Endometriosis' samples were submitted to Ingenuity Pathways Analysis (IPA) to identify up and down regulated functional networks of interest as well as individual up and down regulated genes. The

parameters for Ingenuity analysis included Human Species and limiting to 140 reactions in readout.

Other tools used for analysis include: Database for Annotation Visualisation and Integrated Discovery (DAVID), Cytoscape and STRING.

Chapter 3: Optimization and Validation of Methods

Before the main experiments could be carried out in this study several pilot investigations were performed. The purpose of these experiments was to optimise the explant protocol, as neonatal kidneys and human endometrium had not previously been grown in chimeric culture and to optimise the proposed RT-qPCR.

3.1 Optimisation of 'Vybrant' Labelling

In order to investigate the activity of the human cells in the chimera is was necessary to reliably identify them during the analysis. To achieve this, two different methods were used: first the cells were labeled with a CFDA-SE 'Vybrant' stain prior to inclusion in the chimera and secondly, human specific immunofluorescence detection was used after fixation and sectioning.

Optimisation of the 'Vybrant' labeling protocol was required as previous research in the Liverpool laboratory (Simic, 2010) indicated that when fixing chimeras cultured using the Davies technique (Unbekandt and Davies, 2010) methanol should be used, as fixing with Paraformaldehyde causes the explant to detach more easily from the Millipore membrane making both sectioning and staining more difficult. As it was not known if fixing with methanol would attenuate the 'Vybrant' labeling a pilot study was carried out that involved fixing 'H6' kidney cells (Fuente Mora et al., 2012) in either 100% methanol or 4% paraformaldehyde (Figure 12). Cells were observed at Day 4 and Day 7 after fixation to check for attenuation of fluorescence. The findings of this pilot study are summarised in Figure 12 and do not demonstrate a clear difference between the fixation methods. It should be noted that the Day 4 samples were imaged at a lower magnification than the Day 7 samples.

Figure 12: No difference can be demonstrated between Methanol and Paraformaldehyde Fixation

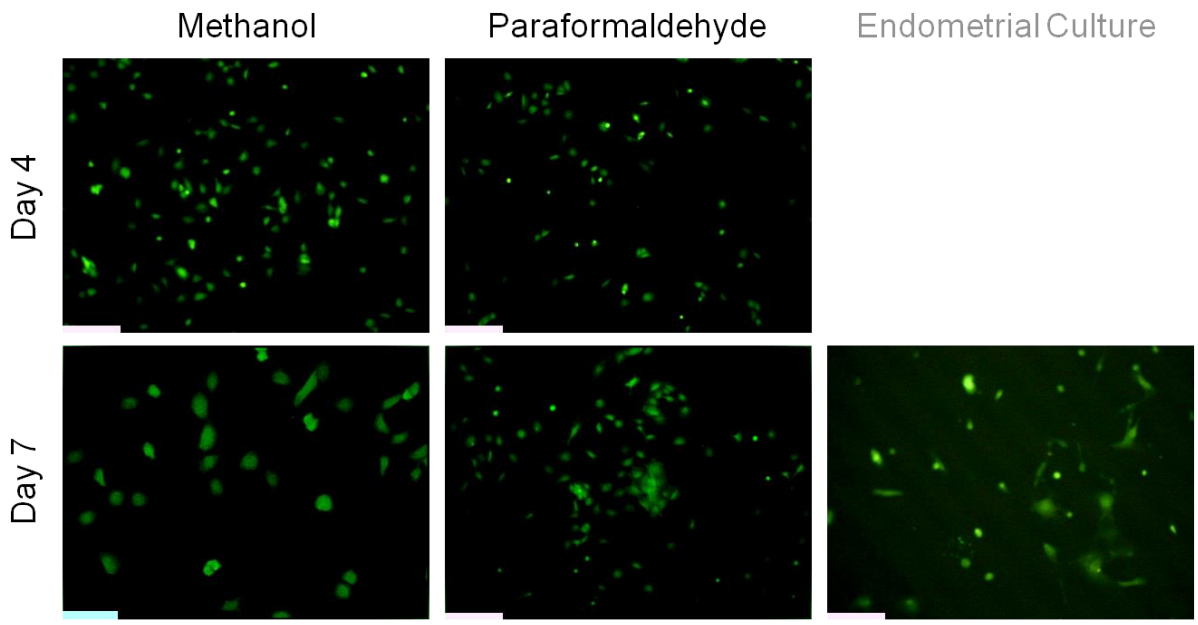


Fig12. Vybrant labelled cells from 'H6' kidney cell line were fixed with either 100% Methanol or 4% Paraformaldehyde. Cells were observed at Day 4 and Day 7 post fixation to detect attenuation of fluorescence. Human endometrial cells cultured for 7 days after Vybrant labeling were used as a control. Scale Bar day 7 methanol image 50µm, all other images 100µm

3.1 Optimisation of Human Specific Antibody Staining

3.2 Optimisation of Human Specific Antibodies

The second method of identifying human cells was via immunofluorescence using a human specific antibody. As a number of potential antibodies were available it was necessary to evaluate their use for inclusion in the main study. To achieve this, immunofluorescence staining of Day 0 Vybrant labelled cells in the CD1 chimera was performed. Human specific antibodies trialled were: Human anti -nuclear, Human anti-mitochondrial and Human anti-cytoplasmic antibodies (Figure 13). Human anti nuclear stain demonstrated poor localisation however good staining was achieved for both human anti mitochondrial and human anti cytoplasmic. As thin (10μm) sections were used for this study and the mitochondria were not always evenly distributed throughout the cell it was decided that the anti-cytoplasmic antibody would be used for further experiments.

Figure 13: Trialing Human Specific Antibodies

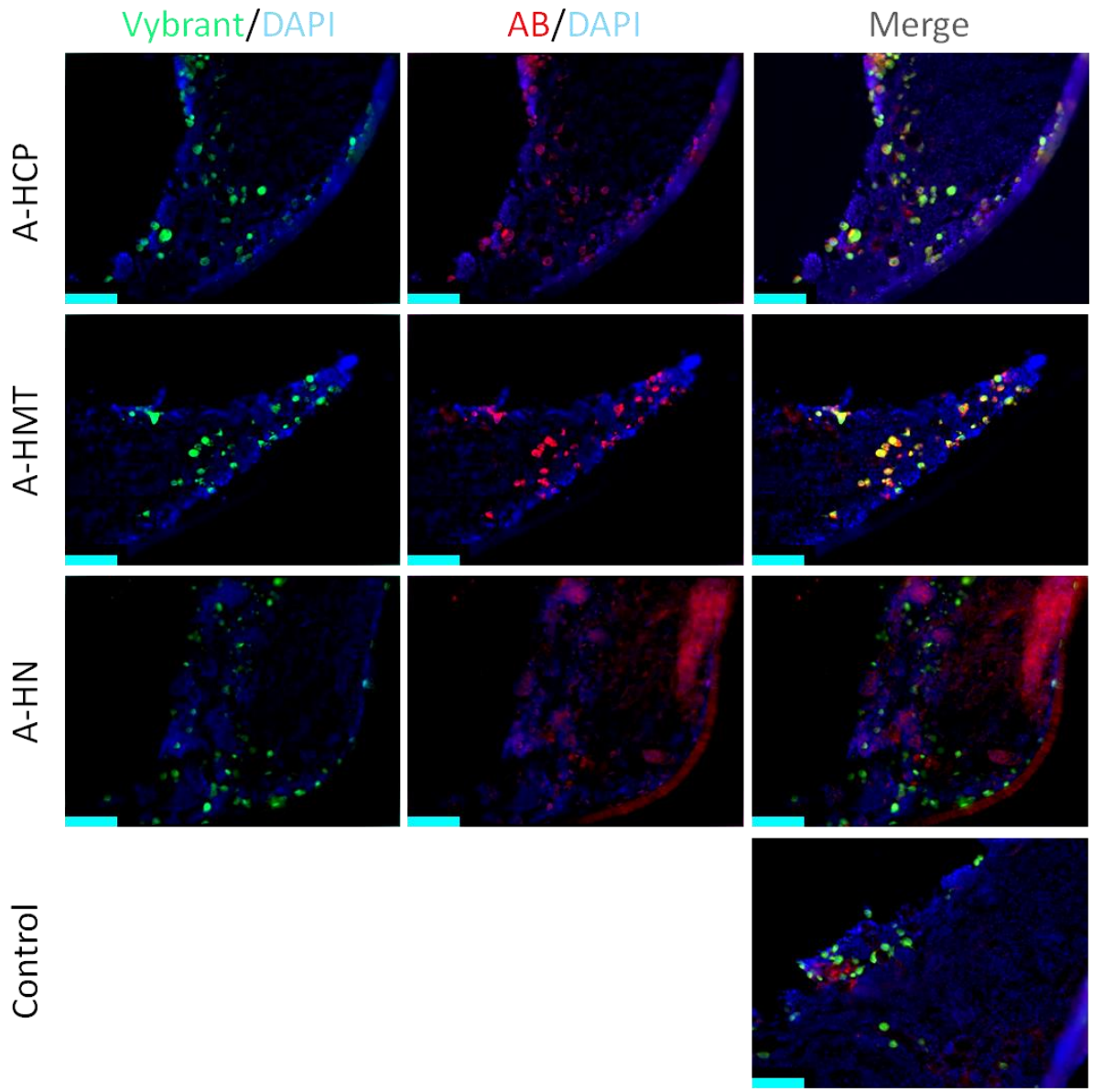


Fig13. Human endometrial epithelial cells were labeled with Vybrant (Green) and cultured for 2 hours in a chimeric explant with CD1 mouse kidneys before fixation, sectioning and immunostaining with human specific antibodies (Red) and DAPI (Blue). Human specific antibodies used included: Anti human cytoplasmic, anti human mitochondrial and anti human nuclear antibodies. Both anti cytoplasmic and anti mitochondrial localise human cells well, this is not demonstrated with anti nuclear staining. Scale Bar 100µm.

3.3 Optimisation of Kidney Explant Model: Complete or Partial Disaggregation

As neonatal kidneys had not been used in an explant culture of this type before it was necessary to consider the disaggregation process used. As the neonatal kidney is more mature than that of the embryo, it was thought that a complete disaggregation may result in widespread apoptosis. Two protocols for neonatal kidney disaggregation were trialled, one providing a partial disaggregation and another which produced a more complete disaggregation. Outcomes were assessed using immunofluorescence for PECAM and Synaptopodin as well as (Figure 14); the aim was to assess which method of disaggregation would provide the optimum environment for the human cells and allow nephrogenesis to occur. Immunofluorescence for PECAM and Synaptopodin demonstrates that vessel-like structures that stained for positively for PECAM, and clusters of podocytes were present in samples prepared using the 'trypsin' protocol. While this is only a qualitative assay the degree of specific staining observed in the trypsin protocol appeared to be greater than in the collagenase protocol it was for this reason that the trypsin protocol was selected for inclusion in the main study.

Figure 14: Pecam/Synaptopodin Immunofluorescence: Complete or Partial Disaggregation?

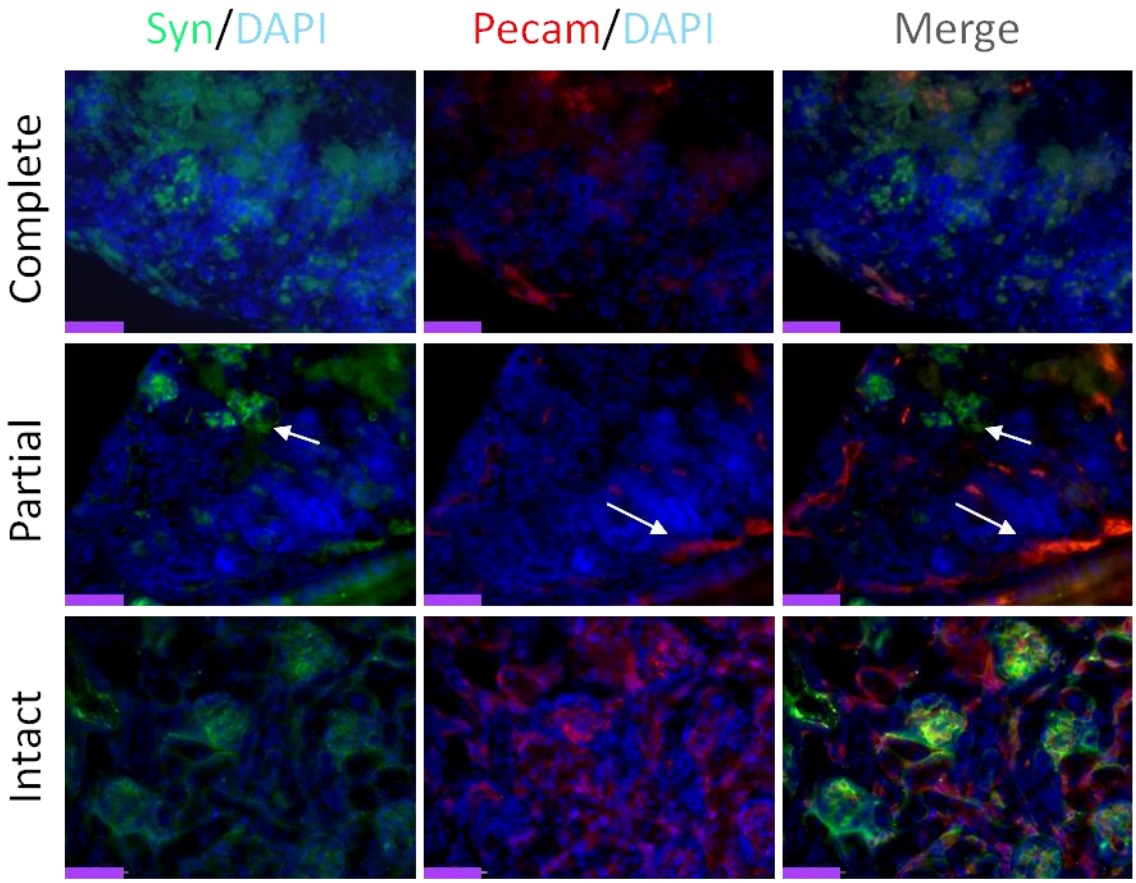


Fig14. Intact neonatal kidney and kidney explants cultured for 7 days at an air-medium interface were Immunostained for Pecam (Red), Synaptopodin (Green) and DAPI (Blue). Sections generated after partial disaggregation we compared with those generated after complete disaggregation. Partially disaggregated sections showed more evidence of Pecam and Synaptopodin positive structures (arrows). An intact neonatal kidney section was also stained as a control value. Scale bar 50μm.

3.3.1 Further Modifications to Disaggregation-Reaggregation Protocol

The kidney explants generated from this original experiment were fixed in methanol or treated with Trizol after 3, 5 or 7 days of culture. From the results of the experiment it would seem that a partial disaggregation was more favourable and this was selected for use in the main experiment. This decision meant it was necessary to modify the protocol and fix some explants after just 2 hours of culture in the main experiment in an attempt to identify whether structures visualised at Day 3 and Day 7 were forming in culture or were simply the result of incomplete disaggregation, Figure 15 demonstrates the difference in structures present between Day 0 and Day 3 of subsequent experiments. The fixation protocol for the main experiment was therefore set at: 2 Hours of Culture (Designated Day 0), 3 Days of Culture, and 7 Days of Culture.

Figure 15. Comparison between Day 0 and Day 3 Kidney Explant Immunofluorescence

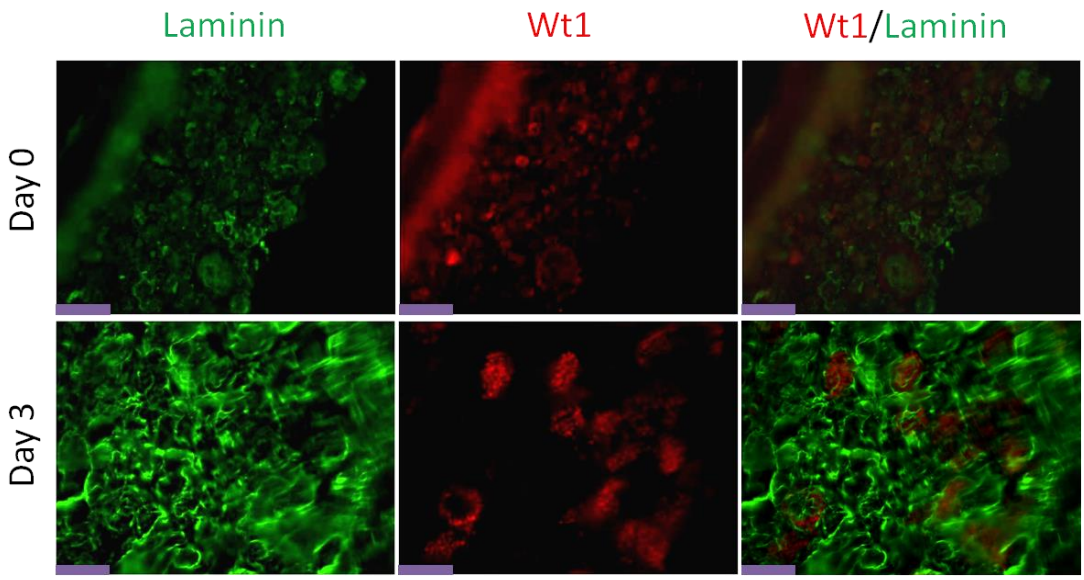


Fig15. Immunofluorescence images comparing WT1 (Red) and Laminin (Green) Staining from Day 3 of the pilot experiment are presented compared with images obtained from Day 0 of a subsequent experiment. It can be noted a difference in the number of WT1 positive organized structures between Day 0 and Day 3 highlighting the importance of analyzing images at Day 0 to identify whether structures are developing in vitro or are the result of incomplete disaggregation. Scale bar 50µm

3.4 RT-qPCR

3.4.1 Validation of RNA extraction and cDNA synthesis

In order to validate the efficacy of the DNase treatment step and cDNA synthesis step of the qPCR analysis, a PCR series was carried out on one of the samples from the kidney explants, in this case an explant fixed after 2 hours (0 Days) of culture. The PCR was run with the same parameters as the main study with 3 different templates: RNA extracted from the sample, DNase Treated RNA from the sample and cDNA synthesised from the DNase treated RNA. The primers used were the housekeeping genes (Figure 16). After RNA extraction was carried out genomic DNA is likely to be present in the sample; in this case a peak will be seen on the melt curve. The DNase step is intended to remove the genomic DNA and if it is successful, the sample will come to threshold much later and a template-specific peak will not be produced on melt curve analysis. Synthesis of cDNA is the final step in the process and the resulting template should come to threshold early and produce a large peak on melt analysis. Analysis was performed for both Kidney explant and Endometrial-Kidney Chimeras (Figure 16).

Figure 16: RT-qPCR Analysis of RNA, DNase Treated RNA and cDNA from Neonatal Kidney Explant

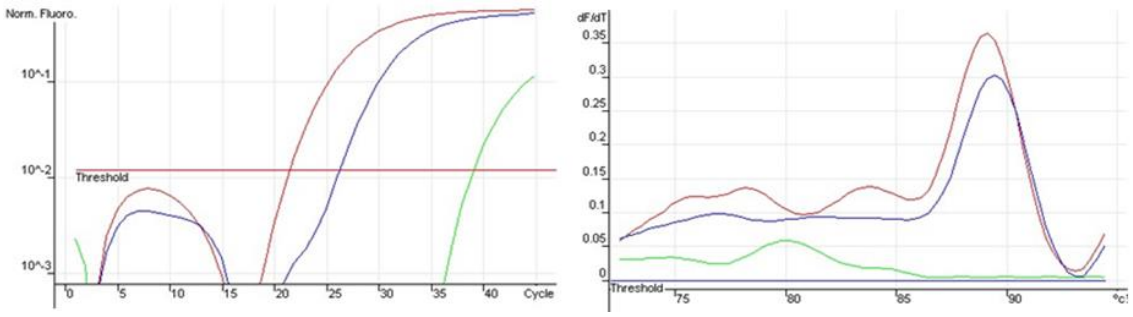


Fig16. Samples obtained from a neonatal kidney explant cultured for 7 days were tested at different stages of complimentary DNA synthesis to investigate the efficacy of the process using Real time Quantitative PCR. Samples included: RNA (Red), Dnase Treated RNA (Green), complimentary DNA (Blue). The RNA sample becomes exponential producing a melt curve peak. The DNase sample becomes exponential 15 cycles later and does not produce a melt curve. Complimentary DNA becomes exponential early in the reaction and forms a melt curve.

3.4.2 Identification of Human and Mouse Specific Primers

Human and Mouse specific Primers for: Tata Box Protein (Tbp) (Housekeeping Gene), Progesterone Receptor (PGR) and Mucin1 (Muc1) were identified in-house as they were used by a previous student undertaking analysis of chimeric explants using mouse embryos. It was necessary to verify that these stock aliquots had not become contaminated and were indeed species-specific. RT-qPCR was carried out for each primer set (6 in total) with a positive control for human cells, a positive control for mouse cells and an NTC.

3.4.3 Human and Mouse Specific Housekeeping Genes

The human and mouse specific housekeeping genes used in this study were validated at different times and as a result different positive controls were used. These conditions are summarised in Table 5 below.

Table 5: Positive Mouse and Human cDNA Controls

Primer Set	**Positive Mouse Control**	**Positive Human Control**
Human Specific Tbp	cDNA synthesised from a mouse uterus in the Sherrington Laboratory	cDNA synthesised from a 'Normal' endometrial sample at Liverpool Womens Hospital
Mouse Specific Tbp	cDNA synthesised from a mouse kidney in the Sherrington laboratory	cDNA synthesised from an 'Endometriosis' endometrial sample at Liverpool Womens Hospital

Both human and mouse specific Tbp produced an exponential curve on RT-qPCR for the relevant positive control and a band indicating an appropriate product size on Gel electrophoresis according to results of a 'Primer BLAST' assessment (Figure 17). The primers were also tested for species specificity by performing an RT-qPCR reaction with cDNA of a differing species e.g. Mouse Tbp with Human (H) cDNA. No product was detected in these lanes indicating that the primers were species specific.

Figure 17: Gel Electrophoresis of Housekeeping Genes

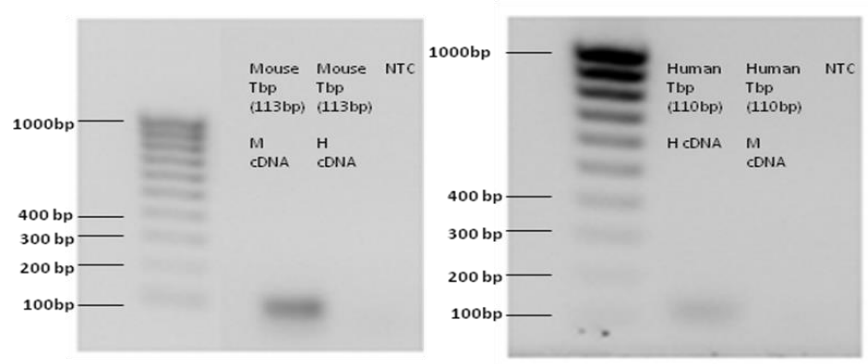


Fig17. Human and Mouse specific housekeeping genes (Tata Box Protein) were located from in-house stocks and tested for species specificity and contamination. Positive control for Human was 'Normal' endometrial cDNA synthesised from a patient sample obtained at Liverpool Womens Hospital. Positive control for Mouse was Mouse Kidney cDNA extracted in the Sherrington laboratory. Hyperladder IV was used to analyse the PCR products after gel electrophoresis to check against the predicted size and species specificity. No products were found in No Template Control lanes or lanes where a differing species cDNA was used e.g. Human Tbp with M cDNA. This indicated the primers were free from contamination and species specific.

3.4.4 Genes of Interest: Progesterone Receptor and Mucin1

Human and mouse specific primer sets for PGR and Muc1 (4 in total) were tested for contamination and species specificity with positive control for human cells being cDNA synthesised from a 'Normal' endometrial sample at Liverpool Womens Hospital and positive control for mouse cells being cDNA synthesised from a mouse uterus in the Sherrington Laboratory. Human specific PGR and Mouse specific PGR and Muc1 produced an exponential curve on RT-qPCR for the relevant positive control and a band indicating an appropriate product size on Gel electrophoresis according to results of a 'Primer BLAST' assessment (Figure 18).

Figure 18: Identification of Mouse Specific MUC1, Progesterone Receptor and Human Specific Progesterone Receptor primers

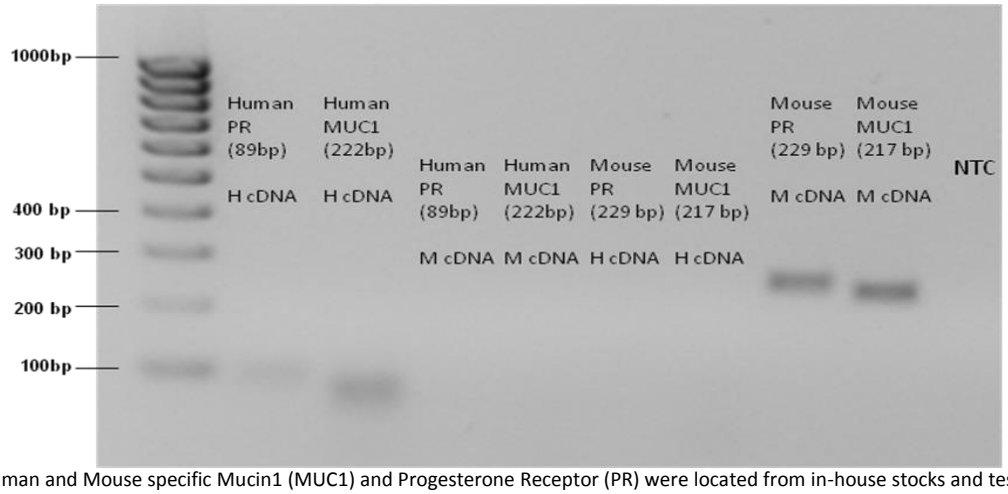


Fig18. Human and Mouse specific Mucin1 (MUC1) and Progesterone Receptor (PR) were located from in-house stocks and tested for species specificity and contamination using Real Time Quantitative PCR and Gel Electrophoresis. Positive control for Human was normal human endometrial cDNA extracted from a 'Normal' patient sample obtained at Liverpool Womens Hospital. Positive control for Mouse was cDNA synthesised from a mouse uterus at the Sherrington laboratory. Bands produced analysed against 'Hyperladder IV' correlate with expected size of products and NTC lane shows no signs of contamination as well as no signs of cross species reactivity indicated by no product in lanes where species specific primers were reacted with cDNA of a different species e.g. Human PR with Mouse (M) cDNA. Human MUC1 did not produce a satisfactory product.

It was observed that the product produced for human specific Muc1 was not the expected size indicating that the correct product had not been produced (Figure 18). This could either be due to non-specificity of the primers or, given the size of the product, a primer dimer. On repeat analysis a suitable product was not produced. A literature search was carried out and it was noted that human Muc1 exhibits multiple splice variants (more than 15) and a primer BLAST assessment indicated that in-house Muc1 primers would only identify 4 of these (Zhang et al., 2013). A literature search was undertaken to identify human specific Muc1 primers that would cover more splice variants (Shyu et al., 2011) was identified and the primer sequences assessed with 'Primer BLAST' indicating 20 splice variants may be produced. These primers were then assessed using RT-qPCR and Gel Electrophoresis using the same conditions as previously (Figure 19). The primers were free from contamination and produced a product of expected size observed on Gel Electrophoresis.

Figure 19: Identification of Human Specific MUC1 Primers

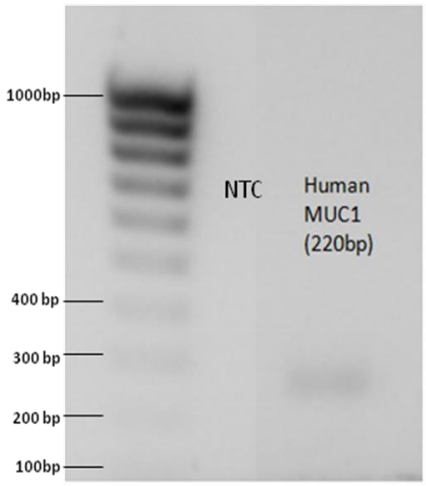


Fig19. New Human MUC1 primers obtained from a literature search were tested for species specificity and contamination using RT-qPCR and Gel Electrophoresis. Positive control for Human was 'Normal' cDNA synthesised from a patient sample obtained at Liverpool Womens Hospital. Positive control for Mouse was cDNA synthesized from a mouse uterus at the Sherrington laboratory. The primers were specific and free from contamination producing a product consistent with the expected size of 220 base pairs (bp)

3.4.5 Kidney Explant PCR, House Keeping Genes and Genes of Interest

Primers for: Tbp (Housekeeping Gene), Wt1, Synaptopodin, Pecam and Calbindin were identified in-house as these Genes of Interest are frequently assessed by researchers at the Sherrington laboratory. It was necessary to verify that these stock aliquots had not become contaminated and would produce appropriate products on RT-qPCR.

RT-qPCR was carried out for each primer set with cDNA synthesised from mouse kidney used as a positive control and an NTC (Figure 20).

Figure 20: Identification of Specific Primers for Kidney Explant Analysis

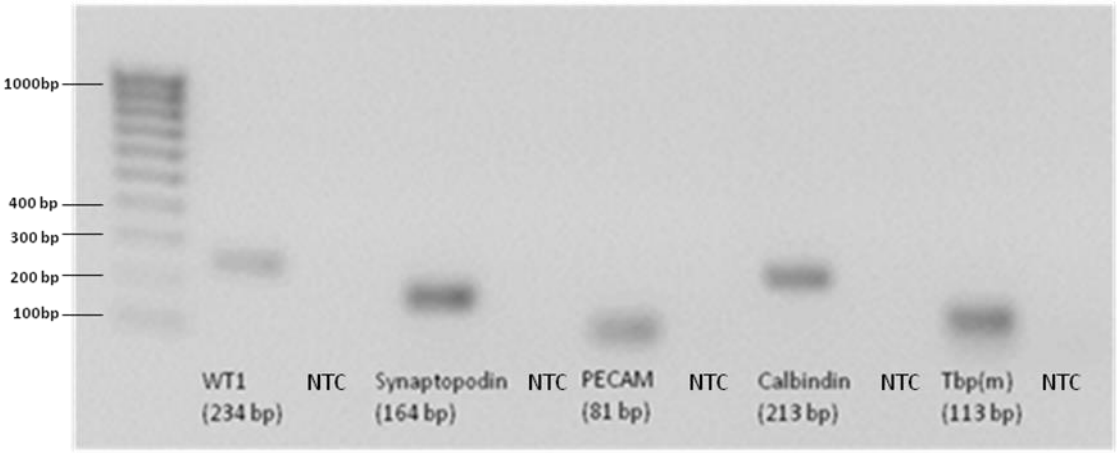


Fig20. Primers for genes of interest: Wilms Tumour 1 (WT1), Synaptopodin, PECAM, Calbindin and Tata box protein (Tbp) were located from in house stocks and tested for specificity and contamination using Real Time Quantitative PCR and Gel Electrophoresis. Positive control for Mouse was cDNA synthesised from a mouse kidney at the Sherrington laboratory. The bands produced correlate with expected size of products and No Template Control lanes show no signs of contamination.

Both the housekeeping gene and genes of interest produced an exponential curve on RT-qPCR for the positive control and a band indicating an appropriate product size on Gel Electrophoresis according to results of a 'Primer BLAST' assessment. There was no sign of a product in the NTC lane (Figure 20).

Chapter 4: Results

4.1 Chimera Results

4.1.1 Migration of Human Cells through the Chimera

Initial analysis was performed on unstained frozen sections made from chimeras containing Vybrant-labelled endometrial cells fixed at 2 hours (Day 0), 3 days and 7 days respectively (Figure 21), three of the explants generated from each time point (Day 0, Day 3, Day 7) were sectioned in preparation for analysis with two immunofluorescence runs performed per section. The results presented are representative samples. It can be observed that the distribution of the Vybrant labelling alters as time in culture increases. At Day 0, after only 2 hours of culture, cells displaying bright green fluorescence were located at the periphery of the section, where they could be seen at the base of the tissue pellet, attached to the membrane filter, and also on the pellet surface. By Day 3 the cells appeared to have permeated the tissue such that the full thickness of the section contained green fluorescent cells. At this time point, the fluorescent population comprised cells that displayed bright fluorescence, and cells that displayed weak fluorescence. By Day 7, fluorescence was still present throughout the full thickness of the section, but in comparison to Day 3, it appeared that there were fewer bright green fluorescent cells. Furthermore, it was difficult to tell if the weak fluorescent signal observed throughout the section was from human cells that had depleted their level of fluorescence due to cell division, or whether it was simply background autofluorescence.

Figure 21: Distribution of Vybrant Staining Changes With Increased Time in Culture

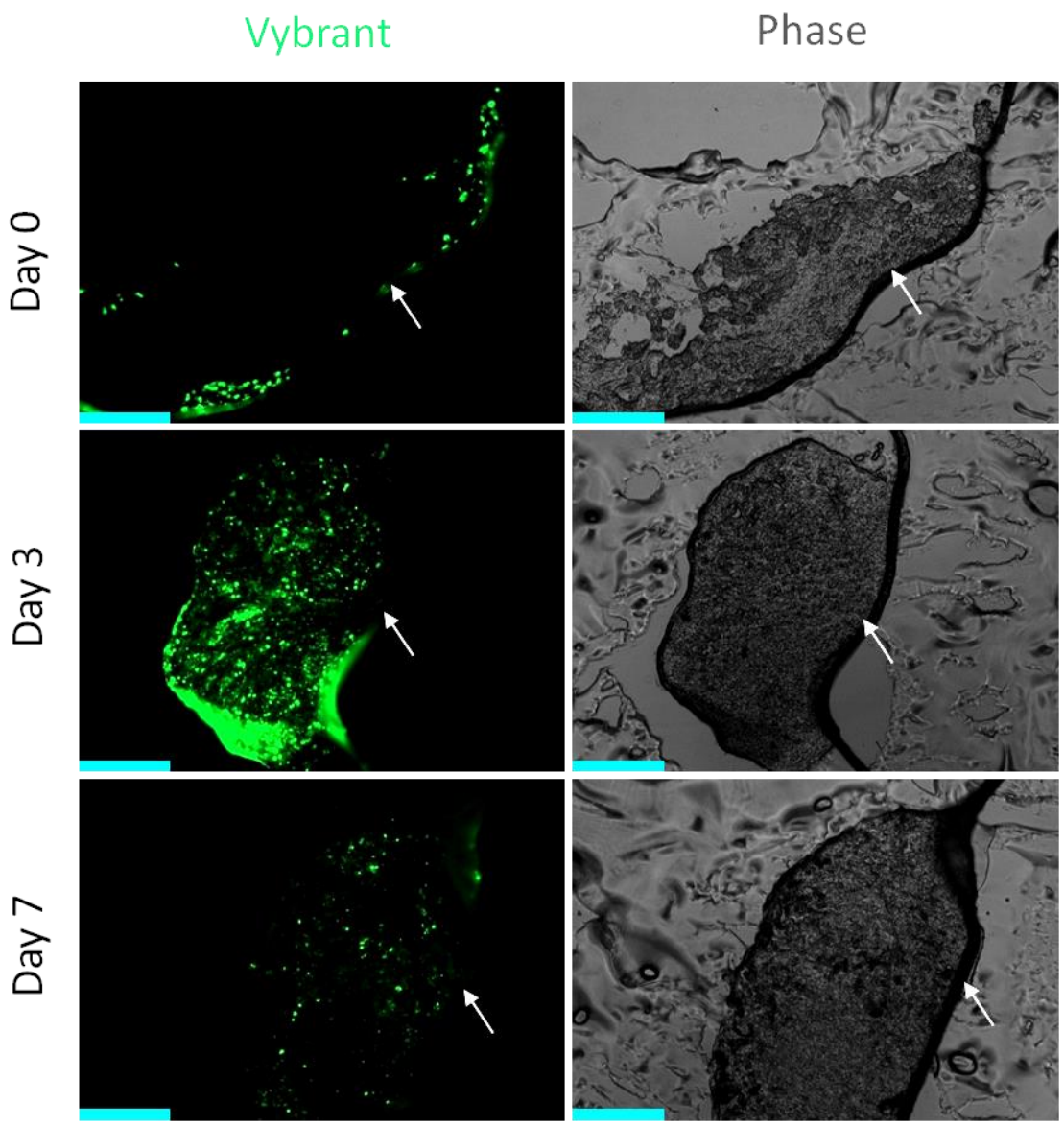


Fig21. Frozen sections of chimeric explants containing endometrial cells labelled with Vybrant prior to culture were analysed using fluorescence and phase microscopy. Sections demonstrate the distribution of Vybrant fluorescence as at the indicated time points. Arrows mark the membrane on which the chimeras were cultured. Scale bar 100µm.

4.1.2 Minimal Co-Localisation is Detected Between Vybrant and the Human-Specific Anti-Cytoplasmic Antibody at Day 7 of Culture

One of the observations from analysis of the Vybrant sections of endometrial epithelial-neonatal kidney chimeras was that fewer positive staining areas could be identified on Day 7 than on Day 0. There are a number of possible explanations for this, the most likely being that either the endometrial cells are losing the Vybrant label due to cell division or the Vybrant signal is decreased because the endometrial cells were dying. To distinguish between these two possibilities, frozen sections were immunostained with human specific anti-cytoplasmic antibody at Day 0, Day 3 and Day 7 with the aim of identifying cells co-staining for this antibody and Vybrant (Figure 22). As expected, at Day 0 most cells displayed both green and red fluorescence, indicating that all Vybrant-labelled cells expressed the human-specific cytoplasmic protein. By Day 3, many of the cells expressing the anti-cytoplasmic protein appeared to display weak green fluorescence. By Day 7, there were noticeably fewer cells expressing the anti-cytoplasmic protein and these tended to have little or no green fluorescence. These areas did not often co-localise with red fluorescence, suggesting that was most likely due to background autofluorescence. The observation that many cells expressing the human-specific cytoplasmic protein within the Day 3 chimeras displayed weak green fluorescence suggested that some of the endometrial cells had proliferated between Day 0 and Day 3. The observation that there were noticeably fewer cells expressing the human-specific cytoplasmic protein within the Day 7 chimera suggested that many endometrial cells died between Day 3 and Day 7. However, the fact that the few human cells present at Day 7 displayed lower levels of green florescence than those at Day 3, suggested that surviving human cells were likely to have gone through at least one cell division between the two time points.

Figure 22: Anti Cytoplasmic Immunofluorescence Staining of Vybrant Labelled Chimeras

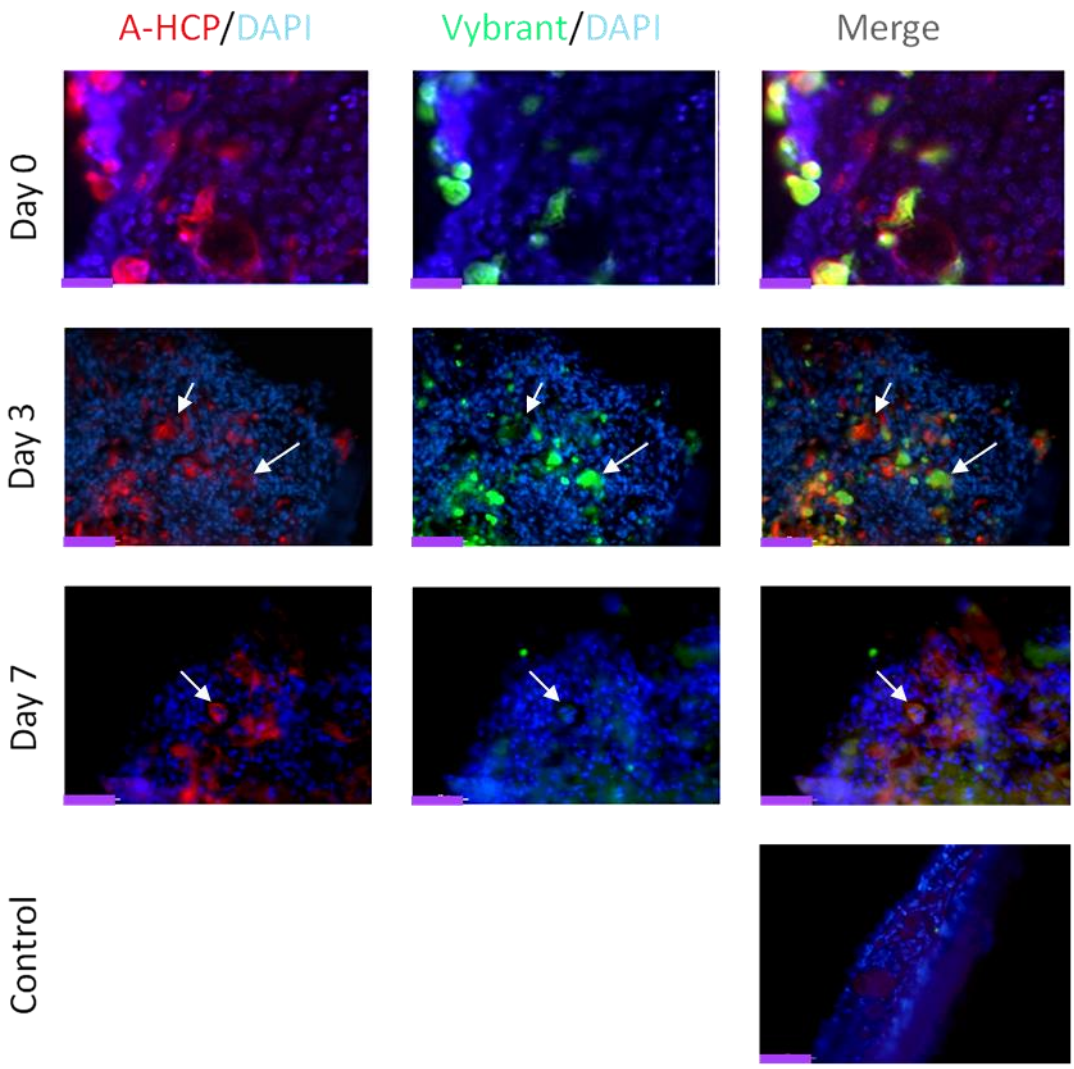


Fig22. Frozen sections of chimeric explants containing endometrial cells labelled with Vybrant fluorescence prior to culture were immunostained with anti-cytoplasmic antibody (A-HCP) (Red) and the nuclear stain DAPI (Blue). Co-localisation can be readily observed after 2 hours of culture (Day 0). At Day 3 and Day 7 co-localising cells are less readily identified and areas of staining for either Vybrant or anti-cytoplasmic antibody that do not co-localise are observed. Arrows identify potentially co-staining cells. Biological and technical repeats were conducted with the images shown being representative. Scale bar 50µm.

4.1.3 'Bright' Vybrant Staining Present at Day 7 Does Not Represent Viable Cells

Another observation from the sections of chimeras containing Vybrant-labelled human endometrial cells was that there were a number of intense green fluorescent spots present in the day 7 sample that did not co-stain for the human-specific cytoplasmic protein. Further analysis under higher power showed that these spots did not contain a nucleus, suggesting that they were not representing viable cells (Figure 23).

Figure 23: Day 3 and Day 7 Vybrant Labelled Chimera Frozen Sections Stained With Human Anti-Cytoplasmic Antibody

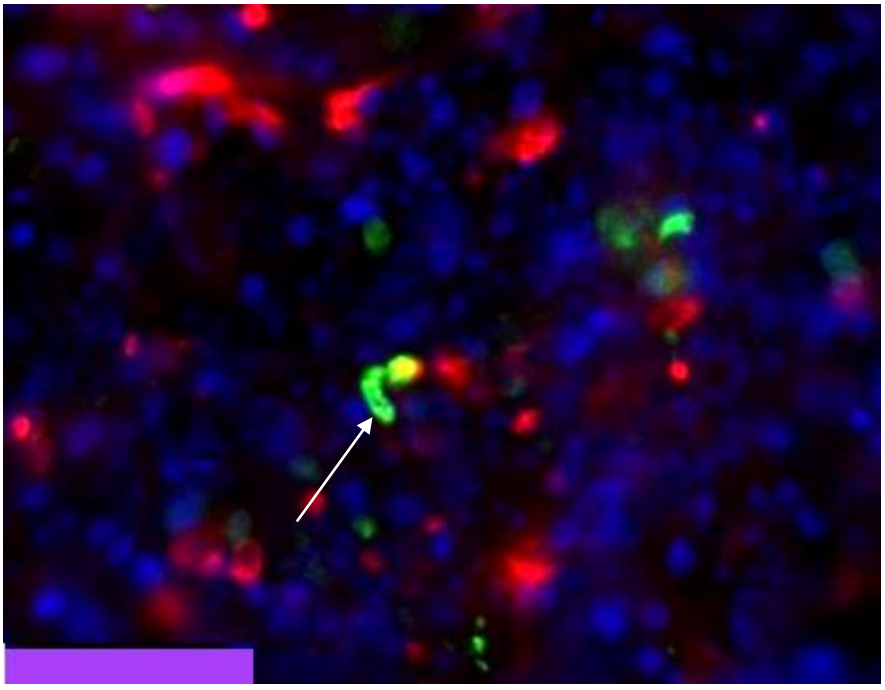


Fig 23. Frozen section from a Day 7 chimera containing Vybrant labelled endometrial cells (Green) was immunostained with anti-cytoplasmic antibody (Red) and the nuclear stain DAPI (Blue). Areas of non co- staining fluorescence may be identified which are not associated will a cell nucleus. It is possible that these areas represent cell fragments. Scale bar 50μm.

4.1.4 Endometrial Chimera Experiment Including the Stromal Fraction

It was observed from anti-cytoplasmic staining of chimeras including endometrial epithelial cells that few positively co-localising cells could be identified at Day 7 of culture.

This finding was not consistent with a previous study, where cells from disaggregated endometrial tissue containing the whole, non-fractionated population, were present beyond a 7 day period following transplantation into the kidneys of adult *SCID* mice (Masuda, 2012). To investigate if the demise of the endometrial epithelial cells in the neonatal kidney chimeras was due to an absence of endometrial stromal cells, both the epithelial and stromal fractions isolated from human endometrium were included in a subsequent chimeric culture (Figure 24). Due to their small size, chimeras from Day 7 of this experiment could not be sectioned so immunofluorescence from Day 0 and Day 3 is presented. On Day 0 only two cells positive for Vybrant can be identified and the cytoplasmic stain shows a more diffuse pattern than previously. At Day 3 the Vybrant signal was less discrete and distributed throughout the full thickness of the tissue section. The areas of positive co-staining at Day 3 appear to be few in number. Though this is a qualitative assay it can be asserted that increased numbers of human cells are were not observed when the stromal population was added to the chimera.

Figure 24: Anti-Cytoplasmic Immunostaining of Chimeras cultured with Both Stromal and Epithelial Population

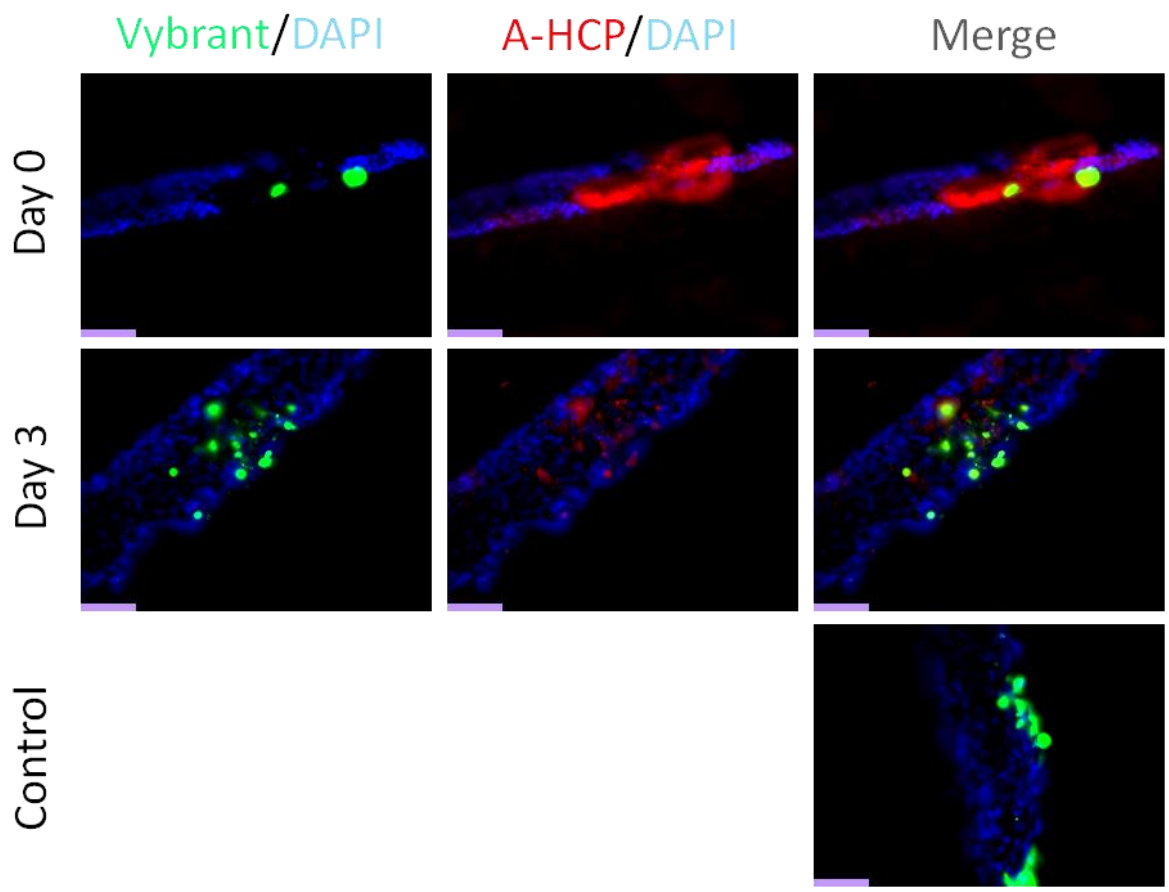


Fig24. Human endometrial epithelial cells were labelled with 'Vybrant' fluorescence (Green) and cultured for 2 hours, 3 days and 7 days in a chimeric explant with CD1 neonatal mouse kidneys. Frozen sections were stained with human specific anti-cytoplasmic antibody (Red) at the indicated time points. In this biological replicate no images were able to be obtained for Day 7 of Culture. Technical replicates were obtained with the images shown being representative. Scale bar 50µl.

4.1.5 Human Cells Do Not Form Glandular Structures within the Chimera

A previous study has shown that human endometrial cells appear to differentiate and form gland-like structures following their introduction into adult mouse kidney. A key aim of the current study was to investigate if human endometrial cells would behave similarly if exposed to the environment of mouse neonatal tissue. In order to test this, chimera sections from Day 0, Day 3 and Day 7 were stained with an anti-laminin antibody in order to identify basement membranes (Figure 25). A No Template Control was also stained. If human gland-like structures had formed, they would consist of polarised Vybrant-stained cells organised with their basal surface atop a basement membrane (Valentijn et al., 2013). At Day 0 of culture a well organised Laminin architecture was observed with Vybrant positive cell aggregates interspersed in an unorganised fashion, most of which were located in close proximity to the membrane with some more centrally located within the section (Figure 25A). By Day 3, the number of Vybrant stained cells was noticeably greater within the centre of the section, but there was no evidence that glandular-like structures had formed (Figure 25B). At Day 7, few positive aggregates remained and the organised Laminin architecture could not be readily identified (Figure 25C). Interestingly, considering the Laminin staining, it seemed to become more disrupted over the course of the culture. At Day 3 there still seem to be intact Basement Membranes (BMs) present though thinner than at Day 0. At Day 7, although there was plenty of laminin immunostaining, it did not appear to be organised into BMs.

Figure 25: Day 0, Day 3 and Day 7 Vybrant Labelled Chimera Frozen Sections Stained With Anti-Laminin Antibody

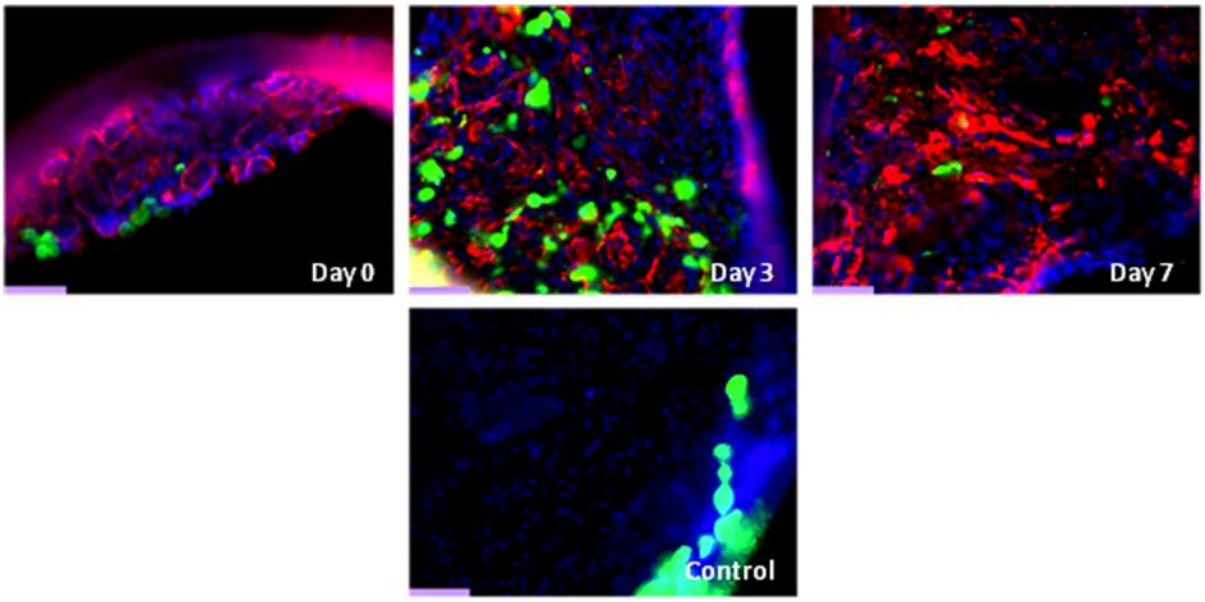


Fig25. Human endometrial epithelial cells were labelled with Vybrant fluorescence (Green) and cultured for 2 hours (A), 3 days (B) and 7 days (C) in a chimeric explant with CD1 neonatal mouse kidneys. Frozen sections were stained for Laminin (Red) and DAPi (Blue) at these specific time points as well as a No Template Control (D). No glandular-like structures are identifiable in these sections. Biological and technical repeats were conducted with the images shown being representative. Scale Bar 50μm.

4.1.6 Laminin Structures in Chimeras Appear Disorganised Compared With Kidney Explants

The unexpected finding of apparent disruption within the Laminin architecture associated with increased time in culture was investigated further by comparing the chimeras with the reaggregated kidney explants (Figure 26). Sections from Day 0 and Day 7 were assessed and in both the chimera and the neonatal kidney explants, laminin structures appeared different in morphology on Day 7 than Day 0. It was noted that the Laminin structures in the chimera seemed more disorganised than in the kidney explant. This observation is reminiscent of a previous study in which human and mouse mesenchymal cells were demonstrated to have a detrimental effect on the development of renal structures in embryonic mouse kidney rudiments (Kuzma-Kuzniarska et al., 2012), possibly through the secretion of growth factors such as Transforming Growth Factor Beta (TGFβ).

Figure 26: Comparison of Chimera and Kidney Explant Frozen Sections Stained With Anti-Laminin Antibody at Day 0 and Day 7 of Culture

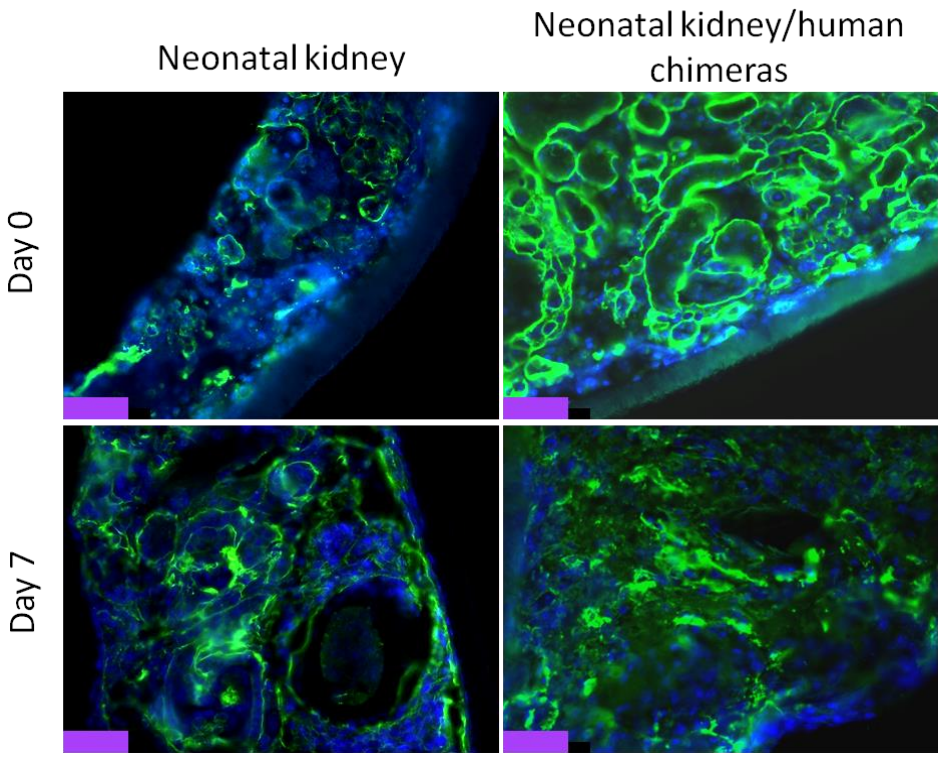


Fig26. Frozen sections from neonatal kidney explants and chimeras were immunostained for laminin (Green) and DAPI (Blue). Comparison of Day 0 and Day 7 sections identified a more organized laminin architecture at Day 7 in neonatal kidney explant compared to chimeras despite and organized laminin architecture observed in chimeras at day 0. This highlights the possibility that endometrial cells in the chimera disrupt basement membrane structure, possibly through the secretion of growth factors e.g. TGF-β. Biological and technical repeats were conducted with the images shown being representative. Scale bar 50μm.

4.1.7 Relative Gene Expression within the Chimera

To investigate for evidence of survival and differentiation of the endometrial epithelial cell population included in the chimeric culture, Real Time quantitative PCR (RT qPCR) was performed using species specific primers for PGR and Mucin1 (Muc1) (Figure 27). Progesterone Receptor is documented as a marker of a more differentiated phenotype in endometrial cells (Jabbour et al., 2006) and Muc1 is a marker of endometrial cells thought to be associated with the implantation window (Aplin, 2006). If the endometrial cells were surviving and differentiating within the chimera it would be expected that both of these markers will be upregulated. Progesterone Receptor and Muc1 were also assessed with mouse specific primers to investigate whether the endometrial cells were inducing expression of these markers in the kidney cells.

RT qPCR was carried out on cDNA extracted from 3 biological replicates of the chimera with one biological replicate consisting of Day 0, Day 3 and Day 7 explants. The housekeeping genes used were human-specific and mouse-specific Tbp which have been validated in human and mouse tissues (Wu et al., 2013, Wei et al., 2013, Pernot et al., 2010). Two technical replicates were carried on each gene of interest in each reaction series (Figure 26).

Interpretation of this data is limited, as with several of the primers (e.g. human progesterone receptor) there was insufficient data collected to perform statistical analysis. It was also the case that there was insufficient data to calculate the standard error; when this situation arose standard error bars were omitted from the graph. These missing data are due to the samples not reaching the threshold value in the RT-qPCR which in itself is a finding as it implies that there was very little RNA present in the samples at the time of fixation. This was particularly the case with the human-specific genes of interest.

Trends can be described in the remainder of these data, but with the caveat that the experiments have been performed only a few times and might not therefore be reproducible. Human progesterone receptor demonstrated an initial downregulation at Day 3 followed by a large upregulation at Day 7. Mouse progesterone receptor exhibited a sustained downregulation from Day 3 onwards. Human Muc1 was initially upregulated at Day 3 followed by downregulation at Day 7 and mouse Muc1 was substantially downregulated both at day 3 and day 7. It would seem that endometrial associated genes are not detected in the mouse cells after Day 0 and that Progesterone Receptor is upregulated in the endometrial cells at Day 7. It is not possible however to take any solid evidence from this PCR study as none of the findings were statistically significant. There are a number of possible reasons for this one of which may be the low number of both biological and technical repeats and it is possible that performing a greater number of technical repeats on a greater number of biological repeats would have helped more accurately identify what was happening in the chimeras.

Figure 27: Relative Gene Expression of Human and Mouse Genes of interest within the Endometrial – Neonatal Mouse Kidney Chimera

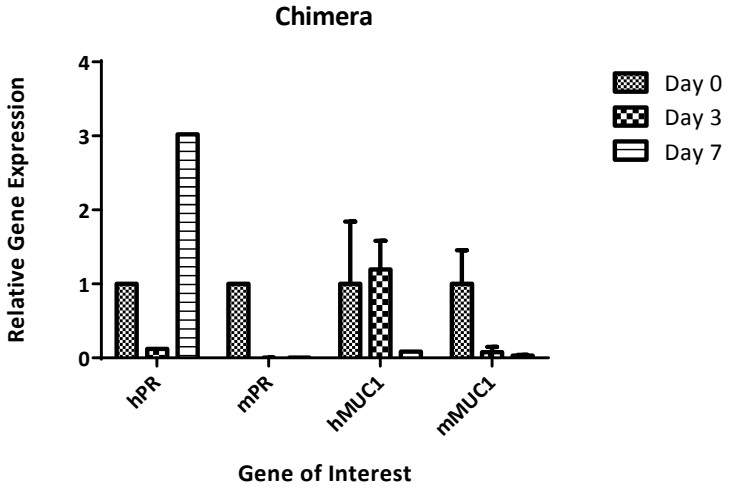


Fig27: Relative Expression of Genes of Interest in the Endometrial – Neonatal mouse kidney chimera. Error bars are not included for several samples as standard error was not able to be calculated due to missing data. Relative expression of mouse progesterone receptor and mouse Muc1 demonstrated a substantial sustained decrease in expression after Day 0. Human Progesterone Receptor demonstrates downregulation at Day 3 followed by upregulation at Day 7. Mouse Progesterone Receptor demonstrates a substantial sustained down regulation. Interpretation of these findings is limited by lack of statistical analysis

4.2 Immunofluoresence Analysis of Neonatal Kidney Explants

Frozen sections generated from neonatal kidney explants were immunostained for a variety of renal markers. Three of the explants generated for each time point (Day 0, Day 3, Day7) were sectioned for analysis with at least two immunofluorescence runs completed for each stain. The results presented are representative. The aim was to investigate the presence of kidney structures forming *ex vivo* following disaggregation and reaggregation and to determine the fate of endothelial cells in the explants. Frozen sections were stained from explants fixed after 0, 3 and 7 days of culture.

4.2.1 Wt1/Laminin

At Day 0, only a few Wt1 positive cells could be identified, and were typically localised within Wt1 positive cell clusters that were encircled by a well-defined basement membrane, as indicated by laminin immunoreactivity (Figure 28 A). By Day 3 it appeared that the number of Wt1 positive cell clusters had increased. These Day 3 clusters appeared to have a smaller diameter than those present at Day 0, and were not always encircled by a basement membrane. In the cases where a basement membrane was present it appeared to have decreased in thickness and regularity. At Day 7, Wt1 positive cell clusters were also identified, and in most cases, were encircled by a basement membrane (Figure 28 B). There appeared to be more of these aggregates than at Day 3 and the positive Laminin staining appears to have increased in intensity whilst maintaining a proximity to the Wt1 positive aggregates. The organization of these structures is decreased however. These findings may be suggestive of the demise of incompletely disaggregated glomeruli as culture progresses and development of new glomerular structures.

Figure 28 A: Wt1/ Laminin Immunofluorescence of Kidney explant Frozen Sections

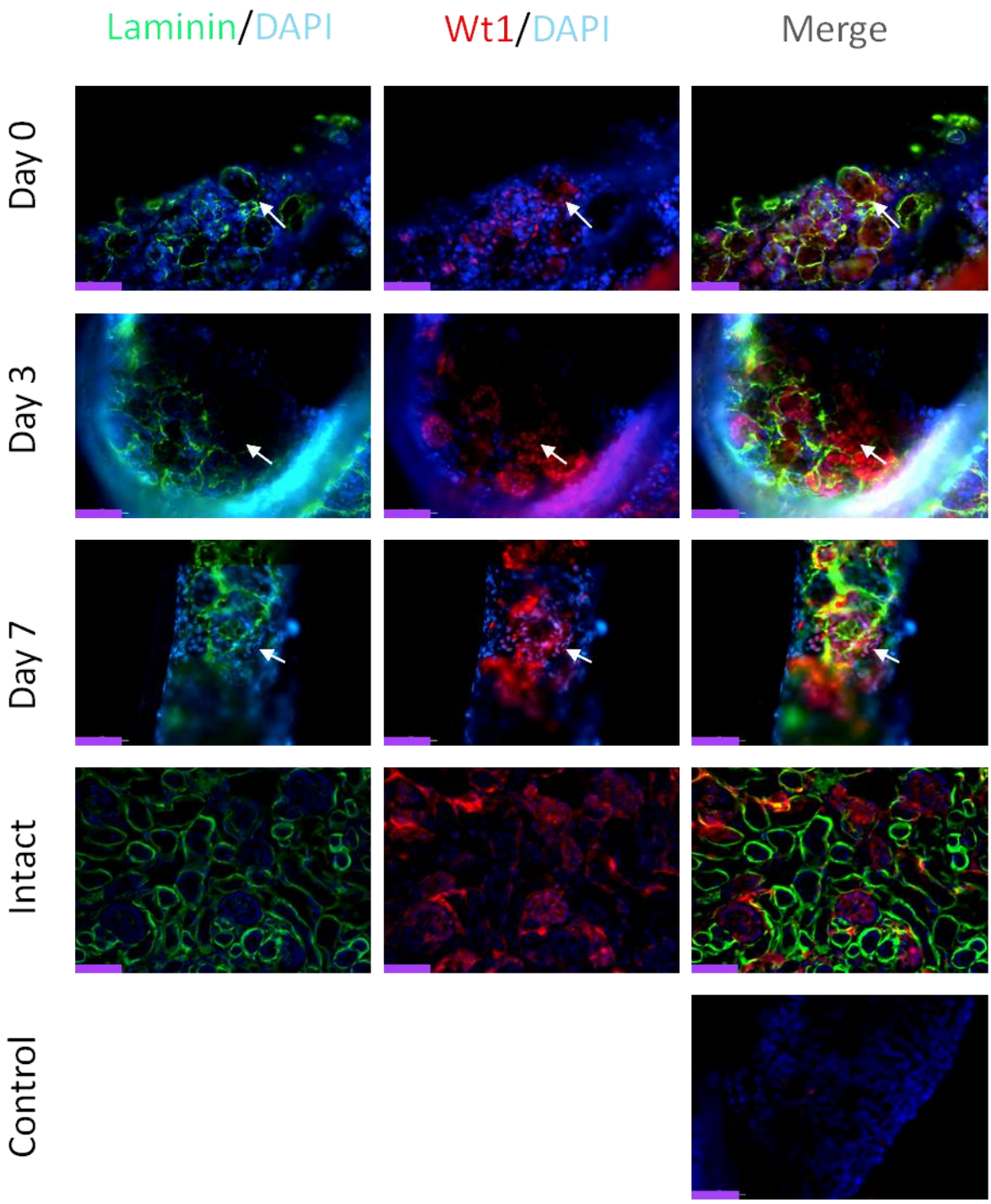


Fig 28 A: Frozen sections from neonatal explants were immunostained for Wt1 (Red), Laminin (Green) and DAPI (Blue). Variations in the morphology of Wt1 and laminin positive staining structures are observed as culture progresses. Biological and technical repeats were conducted with the images shown being representative. Scale bar 50µm.

Figure 28 B: Enlarged Images of Glomeruli from Wt1/Laminin Immunofluorescence

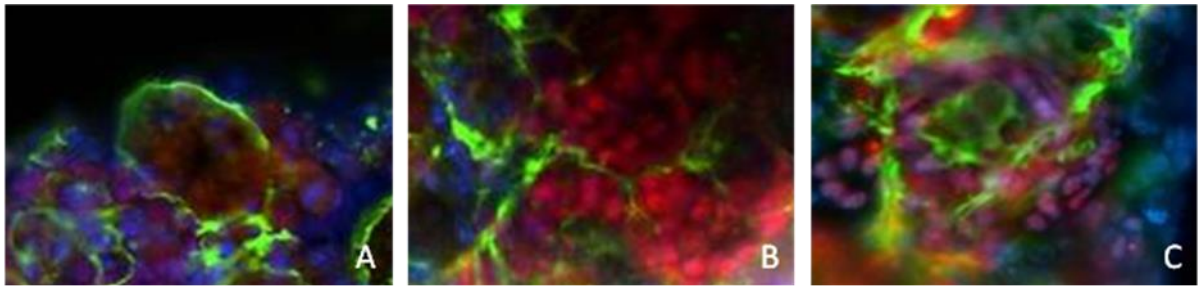


Fig28B. Enlarged views of neonatal explant frozen sections immunostained for: Wt1 (Red), Laminin (Green), and DAPI (Blue). Stained structures from different durations of culture are presented. **A:** Day 0, **B:** Day 3, **C:** Day 7.

4.2.2 Pecam/Synaptopodin

At Day 0, positive staining for the endothelial cell marker, Pecam, could be observed in the section; however, the Pecam positive cells appeared to be randomly dispersed throughout the section and were not organised into vessel-like structures (Figure 29 A). A few Synaptopodin positive cell clusters were also observed at this time point. By Day 3, Pecam positive cells appeared to be organised into vessel-like structures. Positive staining was also observed within clusters of Synaptopodin positive cell clusters, which was in contrast to the situation with Day 0 sections, where the synaptopodin positive clusters appeared to lack endothelial cells. At Day 7, the staining pattern for Pecam and Synaptopodin was similar to that at Day 3. The most striking difference in staining was consistently observed between Day 0 and Day 3 of culture (Figure 29 B). At Day 0, the Synaptopodin aggregates appear 'hollow' with a distinctly different morphology to those seen at Day 3, which appear to contain cells staining positively for Pecam. Pecam staining also appeared to change from single cell staining at Day 0 to the appearance of cord like structures at Day 3. These findings may suggest that structures observed at day 0 are the product of incomplete disaggregation and the aggregates observed on day 3 and day 7 are podocytes proliferating *ex vivo.*

Figure 29 A: PECAM/Synaptopodin Immunofluorescence of Kidney Explant Frozen Sections

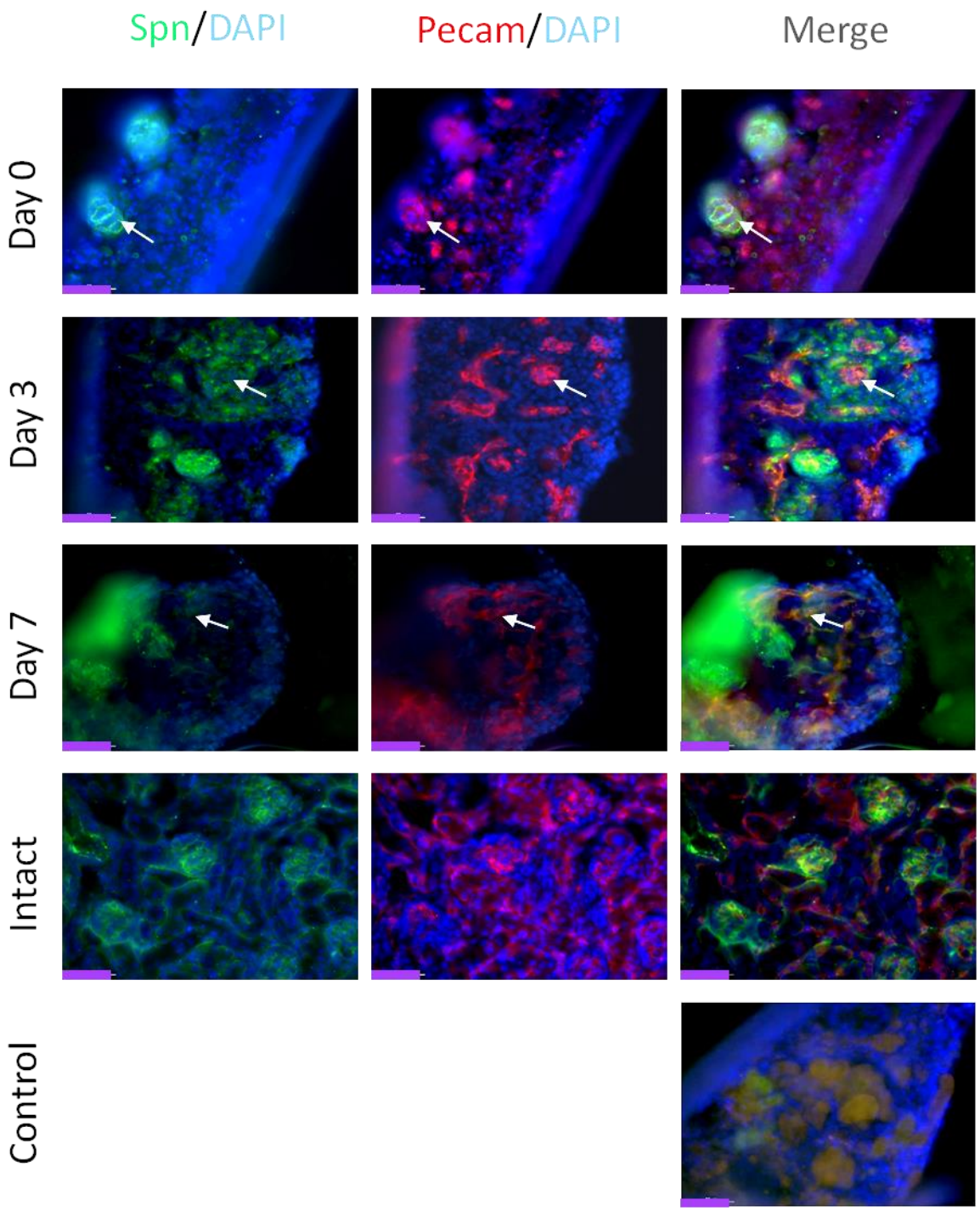


Fig29 A. Frozen sections from neonatal kidney explants were immunostained for Pecam (Red), Synaptopodin (Green) and DAPI (Blue). At day 0 positive staining for Pecam is identified but it does not appear to be organised into discernible structures. At day 3 and day 7 cord like structures positively staining for Pecam are readily identified. Synaptopodin positive aggregates at day 0 vary considerably in their morphology from day 0 compared to day 3 and day 7. Biological and technical replicates were obtained with these images being representative. Scale bar 50µm.

Figure 29 B: Enlarged Glomeruli Images from Day 0 and Day 3 Pecam/Synaptopodin Immunofluorescence.

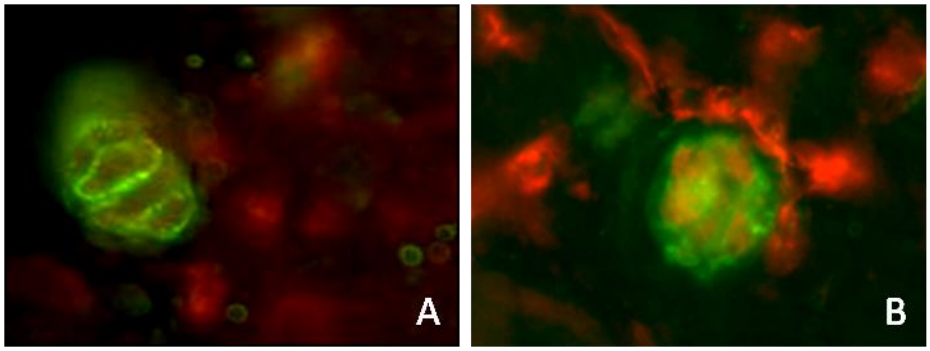


Fig 29 B. Enlarged views of neonatal explant frozen sections were immunostained for: Pecam (Red) and Synaptopodin (Green). Stained structures from different durations of culture are presented. A: Day 0, B: Day 3. At day 0 a structure staining positively for synaptopodin may be identified with morphology differing from synaptopodin positive aggregates observed at day 3. At day 3 Pecam aggregates are observed in close proximity to synaptopodin staining, the architecture of which appears more disorganised than day 0.

4.2.4 Calbindin/Laminin

The calbindin staining does not appear to change much during the time course (Figure 30). One notable exception to this was the staining on the 'black 6' strain mouse which produced a large lumen lined with Calbindin positive staining cells at Day 7. However this was not a consistent finding and was in the different 'black6' strain mouse; this result is found in the appendix.

Figure 30: Comparison of Day 0 and Day 3 Culture of Calbindin/Laminin Immunostaining

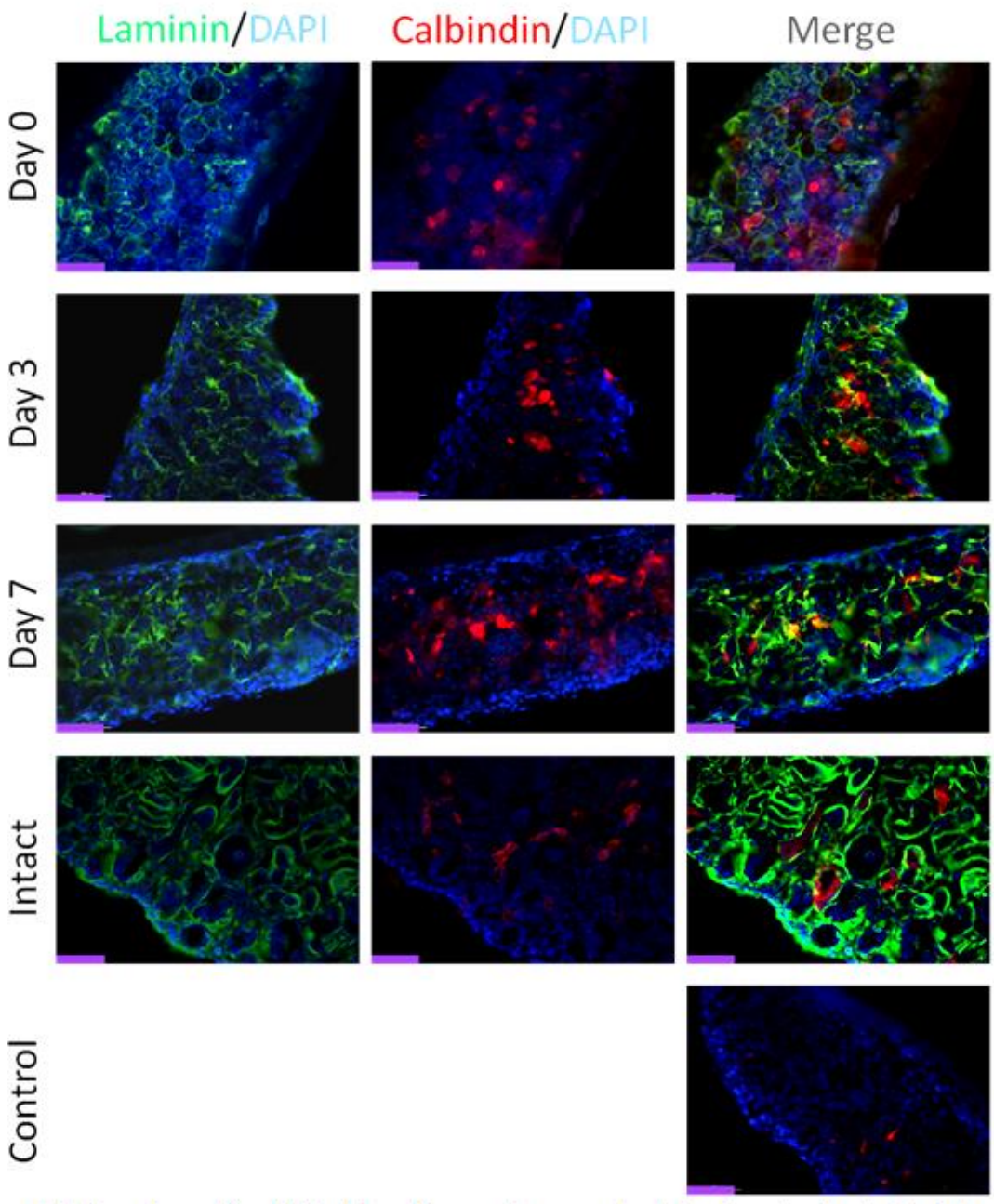


Fig 30. Neonatal mouse kidneys (CD1 Strain) were disaggregated, reaggregated and cultured at an air medium interface for: 3 hours, 3 days and 7Days. Frozen sections were stained for Calbindin (Red), Laminin (Green) and DAPI (Blue). Biological and technical replicates were obtained with these images being representative. Scale bar 50µm.

4.2.5 Relative Gene Expression in Kidney Explants

RT qPCR was carried out on cDNA extracted from 3 biological replicates of the kidney explants with one biological replicate consisting of Day 0, Day 3 and Day 7 explants. The housekeeping gene used was mouse-specific Tbp which has been validated in mouse tissues (Pernot et al., 2010). Two technical replicates were carried on each gene of interest in each reaction series (Figure 31).

Whilst trends in the relative expression of the genes of interest may be discussed, interpretation is limited due to the large standard error of gene expression in neonatal kidney explants (Figure 31). When analysing the data there seem to be two possible explanations for this. Firstly, variation was observed between many of the technical replicates in each reaction. A second source of variation was observed between the biological replicates for each gene of interest. It is likely that a combination of these factors have led to the large standard errors observed. Observing Calbindin some product is detected on Day 0 but is undetectable by Day 7. Both Wt1 and Pecam appear to decrease from Day 0 to Day 7, but both transcripts are detectable at all time points. Synaptopodin demonstrates a potentially interesting pattern of expression, initially decreasing from Day 0 to Day 3 but then increasing again from Day 3 to Day 7. However, it is not possible to conclude anything in regard to relative expression levels over the time course due to the large error bars and the small sample size and it is possible that carrying out more technical replicates on a larger number of explants would increase the likelihood of correctly identifying any trends which may exist in the data.

Figure 31: Analysis of Relative Gene Expression in Neonatal Mouse Kidney Explants

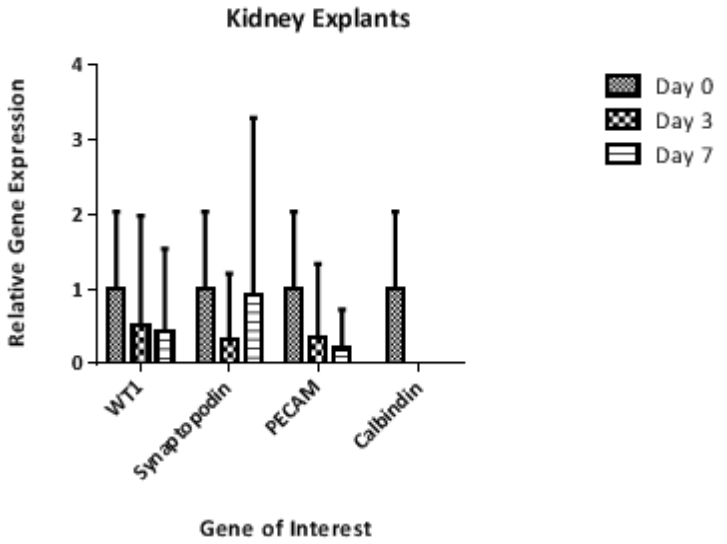


Fig31. Relate expression of genes of interest in the neonatal kidney explant. Further statistical analysis was not performed due to the large standard error associated with the experiment. *Wt1* and *Pecam* demonstrate a steady decrease in gene expression*, Calbindin* demonstrates a dramatic and sustained decline in gene expression. *Pecam* demonstrates an initial decrease at Day 3 followed by an increase in genes expression at Day 7.

4.3 Bioinformatics Results

4.3.1 Cycle Phase Specific Differentially regulated Genes: Normal Endometrium

Using the 'R' statistics package, differentially regulated genes were calculated in each of the study cycle phase relative to the proliferative phase for the 'Normal' endometrium dataset. As the secretory phase progresses it is observed that the number of differentially regulated genes increases relative to the proliferative phase (Figure 32).

Figure 32: Cycle Phase Specific Differentially Regulated Genes: Normal Endometrium

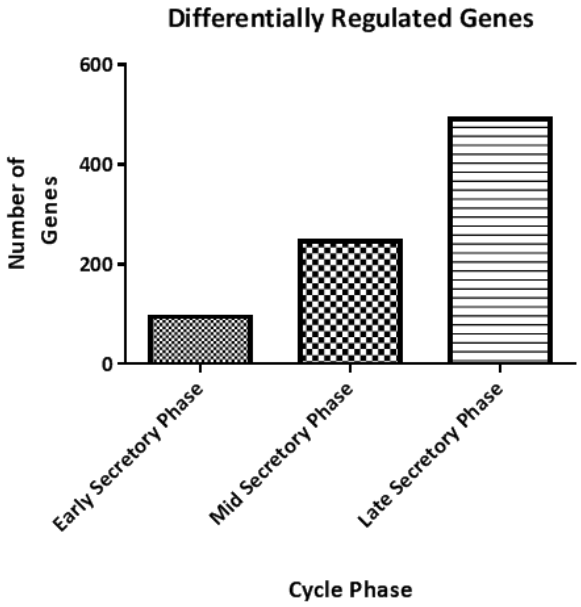


Fig32. Differential gene expression was calculated for different phases of the menstrual cycle compared to the proliferative phase after microarray analysis. The 'R' statistics package was used for analysis with a cut of +/- 2 log mean fold change considered significant. As the cycle progresses the number of differentially regulated genes increases.

4.3.2 Cycle Phase Specific Differentially Regulated Genes: Endometriosis

Using the 'R' statistics package, differentially regulated genes were calculated in each of the study cycle phases relative to the proliferative phase for the endometriosis data set (Figure 33). It is noted that in endometriosis the number of differentially regulated genes relative to the proliferative phase is substantially more in both the early and mid secretory than was observed in the normal endometrium. Similar to analysis of normal endometrium there is an increase observed as the cycle progresses.

Figure 33: Cycle Phase Specific Differentially Regulated Genes: Endometriosis

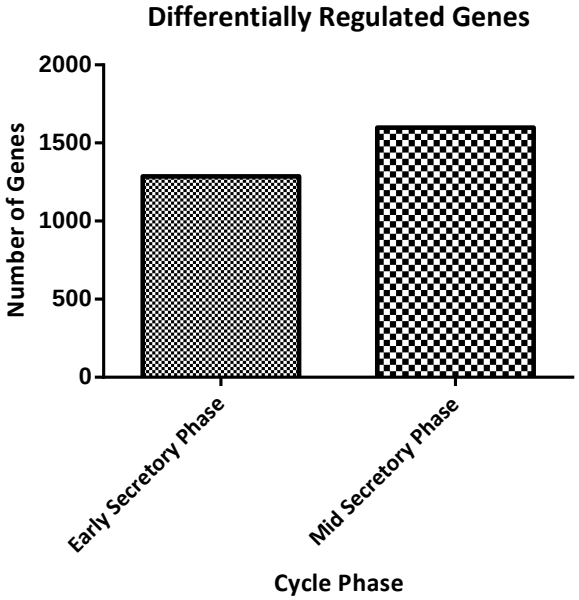


Fig33. Differential gene expression was calculated for different phases of the menstrual cycle compared to the proliferative phase after microarray analysis. The 'R' statistics package was used for analysis with a cut of +/- 2 log mean fold change considered significant. As the cycle progresses the number of differentially regulated genes increases, this is similar to endometriosis however there is an increase in the total number of differentially regulated genes.

4.3.3 Ingenuity Pathways Analysis Output

Ingenuity Pathways Analysis was used to investigate differentially regulated genes from the Early, Mid and Late Secretory Phase of the 'normal' endometrial transcriptomic datasets. A large amount of data was generated from this analysis and the greatest challenge of this bioinformatics study is to focus on clinically and physiologically relevant data in order to draw valid conclusions. For each cellular compartment of each dataset information is produced on a number of relevant processes e.g. 'Canonical pathways'. This is demonstrated in Table 6 using 'Early Secretory' phase as an example.

Table 6: Output Data from Ingenuity Pathways Analysis

Dataset Analysed	Cellular Compartment	Output Analysis
Early Secretory	Intracellular Compartment	Molecular Networks Biofunctions Canonical Pathways Molecules Upstream Regulators Toxicology Lists Toxicology Functions
	Extracellular Compartment	Molecular Networks Biofunctions Canonical Pathways Molecules Upstream Regulators Toxicology Lists Toxicology Functions
	Merged compartments	Molecular Networks Biofunctions Canonical Pathways Molecules Upstream Regulators Toxicology Lists Toxicology Functions

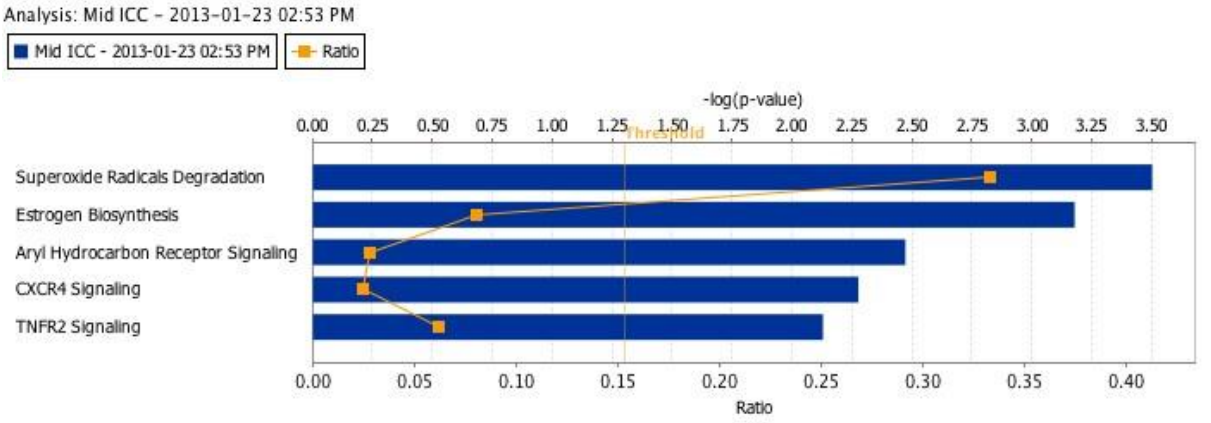
This output was thoroughly analysed and using background knowledge of molecules and pathways currently under investigation in endometrial disease with networks and specific molecules of interest being identified.

4.3.4 'Normal' Endometrium: Super Oxide Radicals Degradation

Within the Mid Secretory Intracellular dataset 'Superoxide Radicals Degradation' was identified as the top enriched canonical pathway (ratio = 2/6) (Figure 34). Individual molecules involved in this pathway were analysed and Superoxide Dismutase 2 (SOD2) was identified as a gene specifically upregulated in the endometrial dataset. SOD2 is an

oestrogen-mediated gene thought to prevent accumulation of Reactive Oxygen Species (ROS) which provides an avenue for further research regarding its function in the endometrium during the implantation window.

Figure 34: Top Enriched Canonical Pathways to the Mid Secretory Intracellular Dataset and Super Oxide Radicals Degradation Pathway



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Path Designer Superoxide Radicals Degradation

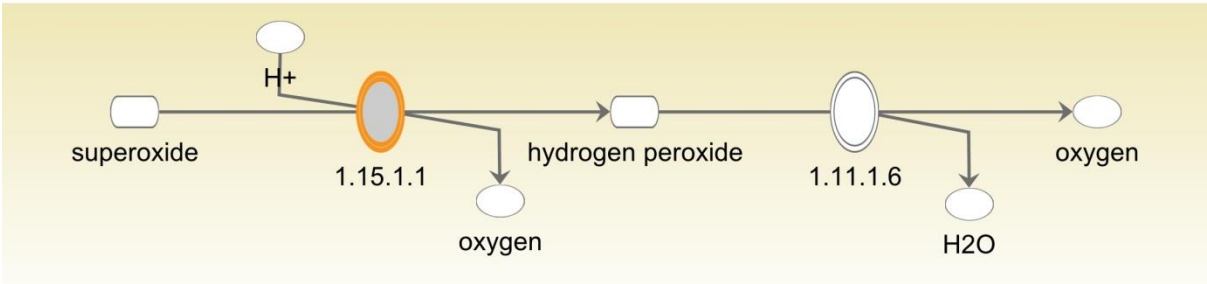

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Fig34. Ingenuity Pathways Analysis for Normal Endometrium. Top Canonical Pathways output from Mid-Secretory Intracellular Datset, 'Superoxide Radicals Degradation' is the top enriched pathway in this dataset. (B) Diagram of 'Superoxide Radicals Degradation' pathway

4.3.5 'Normal' Endometrium: Tryptophan Degradation to 2-amino-3-carboxymuconate Semi Aldehyde

Tryptophan Degradation to 2-amino-3-carboxymuconate Semi Aldehyde represents the top canonical pathway enriched to the Late Secretory intracellular transcriptomic dataset for normal endometrium (ratio = 3/7) (Figure 35). Further analysis of individual enzymes in this pathway using STRING software (Networks, 2013) identified the Indoleamine 2,3-dioxygenase (IDO) as being upregulated within the endometrial dataset. This gene has been previously identified within murine endometrium (Jeddi-Tehrani et al., 2009) and provides a potential further avenue in the study of normal endometrium.

Figure 35: Top Enriched Canonical Pathways to Late Secretory Intracellular Dataset and Tryptophan Degradation to 2-amino-3-carboxymuconate Semi Aldehyde Pathway

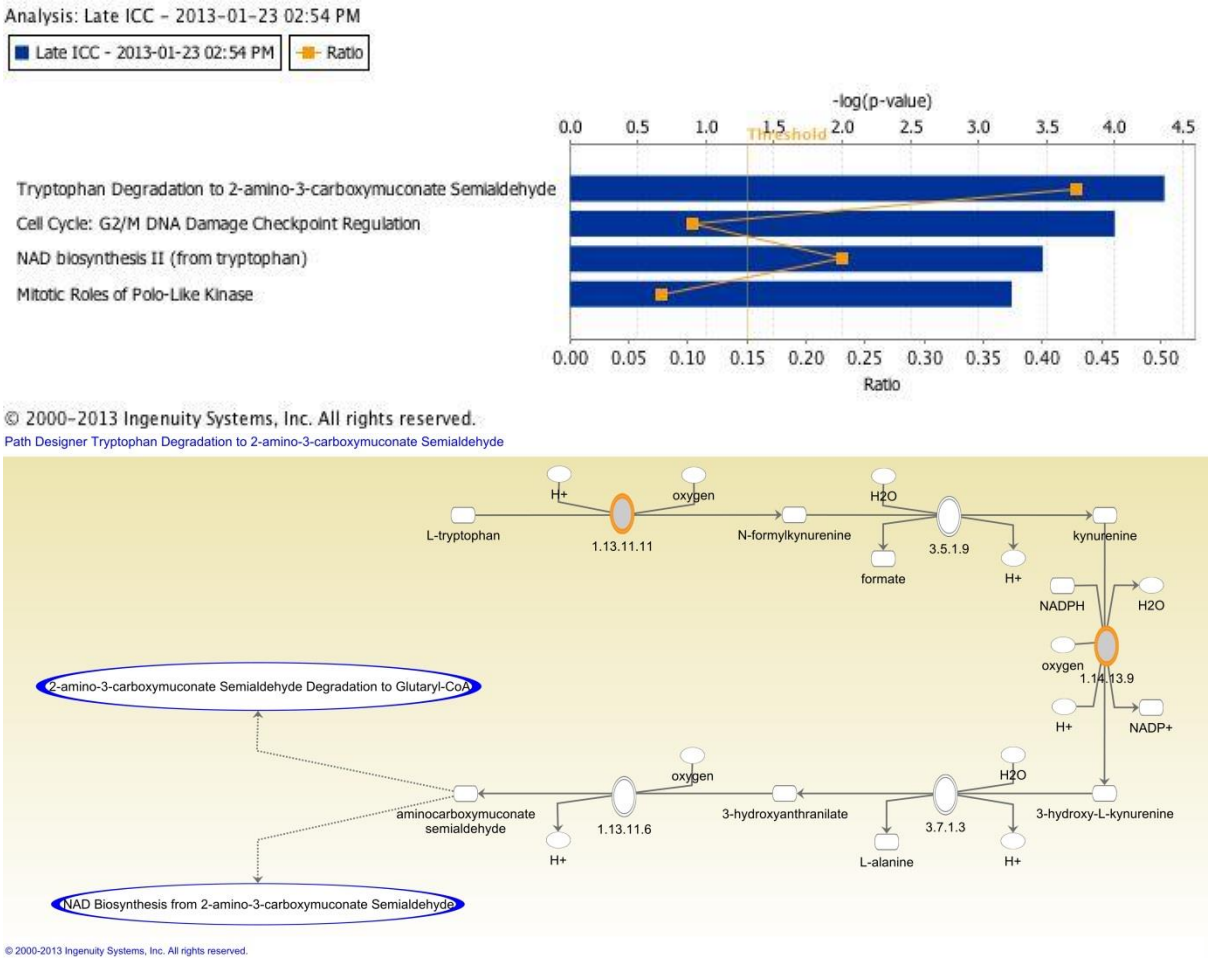

Fig35. Ingenuity Pathways Analysis for Endometriosis dataset. (A) Top Canonical Pathways output from Late-Secretory Intracellular Datset, 'Tryptophan Degradation to 2-amin-3-carboxymuconate Semialdehyde is the top enriched pathway in this dataset. (B) Diagram of Tryptophan Degradation to 2-amin-3-carboxymuconate pathway

4.3.6 Endometriosis Results

As with the normal endometrium the 'Endometriosis' datasets were submitted to IPA for analysis with a large amount of output data generated for analysis. As there were many more differentially regulated genes in Endometriosis, additional analysis into clusters of molecules was performed using Cytoscape. This analysis revealed several highly interconnected clusters within the differentially regulated genes, particularly in the mid-secretory phase. The top cluster is pictured below (Figure 36) and is representative of the level of connectivity of these clusters containing 28 nodes and 330 edges. Another cluster of interest from this output contained several molecules from the Fibroblast Growth Factor (FGF) family: FGF9, FGF18, FGF16, FGF20, FGF7, and FGF1.

Figure 36: Cytoscape Output Cluster from Mid-Secretory Dataset

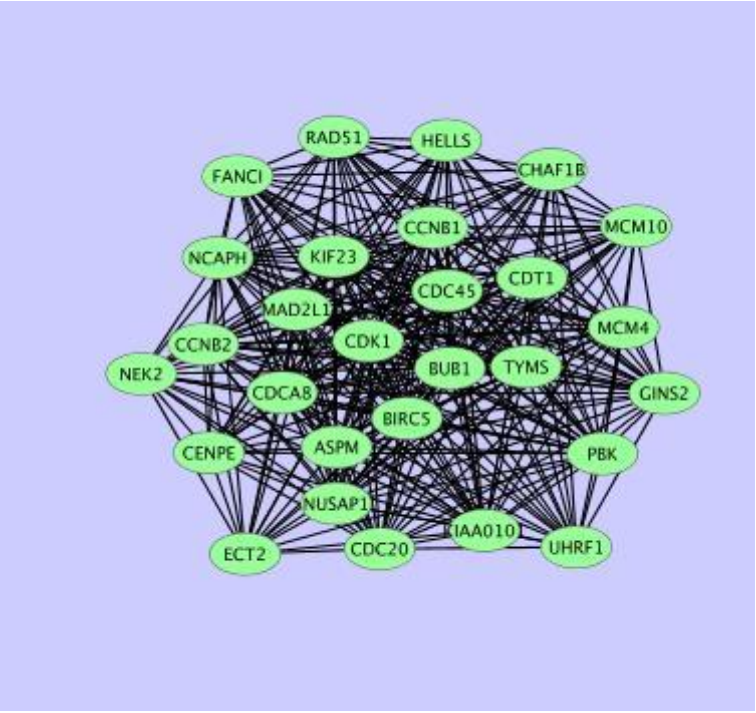

Fig36. Cytoscape output from mid secretory dataset for endometriosis microarray samples. The 'Top Cluster' is pictured and comprises 28 Nodes with 330 Edges. This highly connected cluster identifies molecules of interest for further research in an attempt to characterise the endometrium of endometriosis patients.

Chapter 5: Discussion

The main aim of this study was to investigate the potential of neonatal mouse renal tissue to provide a suitable micro-environment to support the growth and glandular differentiation of human endometrial cells *in vitro*, the long-term goal being to investigate behavioural differences between normal endometrial epithelial cells, and those isolated from patients with endometriosis. A second aim was to investigate if endothelial cells within the explanted neonatal mouse kidney tissue were able to survive and generate cord-like structures reminiscent of immature blood vessels; this is an important question because it is known that endothelial cells in embryo-derived kidney explants are unable to survive and die quite rapidly *in vitro* (Loughna et al., 1998). The final aim of the study was to use bioinformatics to investigate variation in gene expression throughout the menstrual cycle in eutopic normal endometrium and endometrium from patients with the endometriosis with the future aim of identifying potential biomarkers and drug targets.

The main findings were that human endometrial cells displayed little or no potential to grow and differentiate into glandular-like structures within the environment of the mouse neonatal kidney *in vitro*, and thus, the culture system appears to be unsuitable for investigating the behaviour of human endometrial cells. Interestingly, from analysis of non-chimeric mouse neonatal kidney explants, it was found that endothelial cells within the tissue were able to survive *in vitro,* and appeared to generate primitive vessel-like structures. Molecular networks of interest were identified in the bioinformatics investigation which warrant further study, these included: 'Super Oxide Radicals Degradation', 'Tryptophan Degradation to 2-amino-3 Semi Aldehyde' and Wnt/β Catenin Pathway.

The discussion will consider each of the 3 main topics investigated during the MPhil project in turn.

5.1 Impaired Survival of Endometrial Cells

When analysing immunofluorescence images from chimera experiments it was noted that the Vybrant intensity decreased throughout the 7 day time course and apart from some very intense spots, was barely detectable by Day 7. Analysis with a human-specific anti-cytoplasmic antibody showed that there were still human cells remaining at day 7, but they appeared to be fewer in number than at day 3. It may be inferred that the more intense 'bright' staining observed on Day 3 and Day 7 was actually cell debris. This is an idea backed up by the relative low intensity of positively co-staining cells identified on Day 7 and the

fact the previous work with Vybrant in endometrial cells shows that it cannot distinguish between live and dead cells (Defrère et al., 2006). While no quantitative analysis was performed on these images it would seem that there has not been an increase in endometrial cell number from Day 0 to Day 7 and in fact the population may have decreased in number. This observation also backs up the idea of the endometrial cells dying in the culture.

There are several potential explanations to account for the apparent death of endometrial cells in culture: one possibility is that the cells were the subject of attack from the neonatal mouse immune system. This outcome has not been considered in previous assays using mouse embryonic kidney as mouse embryos do not exhibit immune activity in the same way as adult mice, and *SCID* mice are used in xenotransplantation studies. There is little evidence in the literature regarding the immune activity of neonatal mice though some have suggested it is reduced compared to adult mice (Toth, 1968). It may be reasonable to suggest though, that for the neonatal mice to survive in the extra-uterine environment a degree of immune-competency must be established. However, it is possible that many of the immune cells were lost from the kidney during the disaggregation process and no literature regarding immune activity using the current reaggregation protocol is available. This suggests that, while neonatal mice have not been previously used, immune attack remains a possible but unlikely explanation for the death of the endometrial cells in culture.

An alternative explanation for the apparent decrease in number of the endometrial population is that the Vybrant cell tracking stain could cause cell death through toxicity. A literature search identified a previous publication using a mouse model of endometriosis induction which demonstrated that this method was not toxic to human endometrial cells (Defrère et al., 2006). This would seem to demonstrate that the Vybrant labelling was not responsible for cell death; however, the concentration used in the 2006 study was 8µM whereas the concentration used in the present study was 20µM. This is more than double the concentration previously used and it is possible that this could affect viability; however, it is also the concentration recommended by the manufacturer (Invitrogen) and no obvious evidence of cell death was observed in preliminary studies labeling ‘H6’ or endometrial cells.

Another possibility is that the primary endometrial epithelial cells employed in this study were mainly differentiated cells, which do not usually survive in long term culture. There

are many challenges associated with the culture of primary human endometrial epithelial cells and relatively little is known about this population compared with the stroma despite the fact that it is thought to be instrumental to endometrial function. This deficiency is highlighted by performing a literature search for 'Human Endometrial Epithelial Culture' on the Pubmed search engine which retrieves only 94 publications compared to 1088 publications for the term 'Human Endometrial Cell Culture'. Within the 94 publications identified in this search many utilised endometrial epithelial cancer cell lines rather than benign primary endometrial cells, as was used in this study. Given the relative difficulty of studying this population *in vitro*, evidenced by the fact that only a few laboratories in the world are able to establish growth of short-term cultured primary endometrial cells, it would have been useful to establish a parallel model to study the survival of these cells after Vybrant labelling at Day 0, Day 3 and Day 7. The lack of comparison this control condition would have offered represents a limitation of this study.

A fourth explanation is that this co-culture environment lacks the essential support needed for endometrial cell survival. As mentioned above, primary endometrial epithelial cells are known to be challenging to isolate and culture and it is possible that the endometrial population underwent apoptosis due to loss of cell-cell contact. The addition of the ROCK inhibitor for 24 hours to the culture was designed to prevent this (Unbekandt and Davies, 2010) but has not been optimised for the endometrial cells and may not be sufficient to encourage endometrial cell survival. Apoptosis may be triggered by disruption of the endometrial tissue necessary to select the epithelial population removes the other important cell types e.g. stromal cells as well as extracellular factors such as basement membrane proteins which in themselves may be essential for cell survival. Previous studies have documented unspecified 'human endometrium deposits' surviving for up to 4 weeks in a Heterologous mouse model (Grümmer et al., 2001), however this model used the peritoneum as a microenvironment and it is possible that the kidney is not conducive to endometrial cell survival. Interestingly the 'human endometrial deposits' used in this study were intact endometrial fragments, meaning that tissue architecture was preserved. This may serve to highlight the importance of cell-cell contact within the endometrial cells suggesting that singly dispersed cells, as used in this study, may not be the optimal culture condition.

It is also possible that production of various substances by neonatal kidney cells during culture may produce a hostile environment for endometrial growth. Another indication that

this environment may not be optimal for the survival of endometrial cells is that endometriotic deposits are not reported in the kidney in women despite being widely found throughout the abdominal cavity and beyond (Impey, 2012).

While a large proportion of the endometrial population appears to have died, a small number of positively co-staining cells were identified at Day 7 of culture. This finding indicates that some cells survived and offers another explanation for the apparent decrease in endometrial population. As the only selection performed was crude fractionation for the epithelial population and no cell sorting was performed in this experiment it is likely that the cells included in the chimera represented a mixed population. As it is known that ASCs are likely to be rare cells in the endometrium and it is possible that the terminally differentiated population of cells died and the surviving, small, fraction are a progenitor population (Snippert and Clevers, 2011).

5.2 Invasion and Formation of Glandular Structures

Observations of unstained chimera sections on Day 0, Day 3 and Day 7 have demonstrated that Vybrant positive cells appeared to migrate, eventually becoming distributed throughout the full thickness of this tissue after 3 days in culture. It is known that the culture medium used in this experiment contains a Phenol Red compound which exhibits some oestrogenic effects (Croxtall et al., 1990). It is possible that this migration and invasion seemingly demonstrated by the epithelial cells in culture is similar to the migration observed *in vivo* by the luminal epithelium in the proliferative phase of the menstrual cycle. While this behaviour is interesting and may demonstrate some of the properties of epithelial cells it does not demonstrate a high proliferation potential within the endometrial population.

As well as assessment of cell survival, investigation was undertaken into the ability of the endometrial cells to form glandular structures in the chimera using an anti-laminin antibody. When assessing the Day 7 immunofluorescence images a ‘glandular structure’ would have been identified as a low intensity of green Vybrant staining surrounded by laminin immunoreactivity. It was observed that Vybrant positive cells were few and sparsely distributed throughout the tissue and did not seem to be associated with laminin positive basement membranes. Taken together with the human specific antibody studies it may be asserted that the endometrial cells have not demonstrated a capacity to survive and differentiate to form glands in the neonatal mouse kidney chimera.

It is interesting to note a difference in Laminin immunoreactivity when comparing the chimeras and the neonatal explant tissue. Structures in the day 7 chimeras positively staining for laminin appeared disorganised when compared with structures in the neonatal kidney explants at day 7.This is consistent with previous reports that introduction of a human cell population in a chimeric model of this type can affect nephrogenesis (Kuzma-Kuzniarska et al., 2012). In this particular case human mesenchymal stem cells were used and the authors hypothesised the disruption of nephron formation may occur via secretion of growth factors e.g. TGF-β (Kuzma-Kuzniarska et al., 2012). This raises the possibility that the presence of the endometrial cells in the neonatal kidney impairs the ability of this tissue to form organised structures and backs up the idea that interaction is taking place between the two cell populations.

An alternative explanation for the lack of proliferation observed by the endometrial cells is that even if the micro environment were optimal the chimera explants may be too small to demonstrate this. The progenitor population in the endometrium is postulated to constitute a rare group of cells with ASC characteristics. The chimeras contained 150000 cells at a ratio of 1 human cell to 5 neonatal kidney cells. This means a population of approximately 25000 endometrial cells was present in each chimera. A combination of a rare progenitor population and impaired survival of the endometrial cells may have substantially affected proliferative potential any progenitor cells.

5.3 Experimental Refinements and RT-qPCR Analysis

While only a crude fractionation was performed the experiments up to this point had used what was primarily an epithelial population. The possibility was considered that in order to proliferate the epithelial cells required interaction with the stromal population. In an attempt to more accurately reconstitute this ‘niche’ a further experiment was performed in which both the epithelial and stromal fraction were cultured in the chimera. This is consistent with work from xenotransplantation studies which have demonstrated an increased proliferative potential in a mixed population of endometrial cells compared with a sorted population (Masuda et al., 2007). Immunofluorescence images of the mixed epithelial and stromal chimeras failed to demonstrate an obvious improvement in the capacity for cell survival and proliferative potential in the endometrial cell population. As progenitor cells are thought to reside within both the epithelium and the stroma this is unlikely to be due to a decreased concentration of progenitor cells within the endometrial

fraction; one explanation for these findings is experimental error. This is evidenced by the fact that frozen tissue sections on Day 0 appear smaller than in previous experiments on Day 0 even though they supposedly contain the same number of cells. There also appear to be fewer Vybrant staining cells in these sections. It was noted during this experiment the cell pellets produced were more liable to fragment resulting in incomplete transferral to the Millipore membrane.

Analysis of relative expression of human and mouse Progesterone Receptor and Mucin 1 was also performed at Day 0, Day 3 and Day 7 of culture. One factor limiting statistical analysis is the large quantities of missing data, particularly for the human specific genes. These data are missing because the exponential signal in the PCR channel did not reach the user defined threshold in many cases. This finding could be indicative of a small amount of human gene expression possibly due to a lack of viable human cells in the chimera at Day 3 and Day 7. This being said there are trends in the data which may be worth describing, albeit in this limited context. The expression of human Progesterone Receptor appears to decrease between Day 0 and Day 3 of culture before then increasing dramatically at Day 7. Progesterone receptor is known to be a marker of more differentiated cells in the endometrium (Jabbour et al., 2006). It is possible then that between Day 0 and Day 3 of culture there is widespread death of the more differentiated human cell populations within the culture leaving only the more undifferentiated populations remaining e.g. ASCs. As the culture progresses from Day 3 to Day 7 these cells become more differentiated and start expressing progesterone receptor. It has previously been documented that Phenolsulfanphthalein, which is present in DMEM F12 culture medium has an oestrogenic action which in this case might serve as the differentiation stimulus (Sigma Aldrich) (Croxtall et al., 1990). Mouse progesterone receptor demonstrates a sustained downregulation from Day 0 of culture indicating that the endometrial cells have not induced expression of this marker in the mouse progenitor population, a finding which contradicts previous work from the University of Liverpool studying endometrial cells in a mouse embryo chimera (Simic, 2010). What is interesting is that there appears to be some expression on Day 0 in the mouse, given the small amount of data this can actually be attributed to a single result and as such experimental error cannot be ruled out in generating this trend.

Human Mucin 1 demonstrated an initial upregulation at Day 3 followed by substantial downregulation at Day 7. Muc1 was originally proposed as a marker of implantation in the human endometrium and when implantation occurs cell-cell contact mechanisms stimulate

the downregulation of Muc1 from luminal epithelium. It could be argued that these same cell-cell signalling mechanisms are the reason down regulation can be observed in the chimeras at Day 7. Mucin1 is also a progesterone mediated gene and the lack of progesterone in the culture medium may also have contributed to its downregulation; however these hypotheses would not explain the initial upregulation at Day 3 (Aplin, 2006). Mouse MUC1 demonstrates a sustained downregulation from Day 0 and it is also possible that cell-cell contact mechanisms are responsible for this.

5.4 Neonatal Kidney Explant

Investigation of the immunofluorescence images generated from the neonatal explant experiments produced interesting observations. Several trends of gene expression can be observed in the RT-qPCR analysis; however the large standard error of the samples means that statistical analysis was not possible, limiting the interpretation of these findings.

The RT-qPCR data was investigated in order to find the source of the large standard error and two possible explanations identified. Firstly variation is observed between technical replicates and as only 2 were performed per gene in each PCR series increasing this number would allow the gene expression within each biological replicate to be more accurately assessed. Another source of standard error is the observed variation between the biological replicates. As the standard error is calculated based on the number of biological replicates, in this case 3, an increase of this number would increase accuracy of findings. There are also experimental factors that could explain the variation between these replicates. As each replicate is cultured separately it is possible that slight and unavoidable variations in the experimental procedure, e.g. variation in culture medium change intervals, will affect gene expression. Another potential source of error relates to the size of the explants; while 150000 cells were included there was difficulty associated with transferring the explants from the micro test tube to the section of Millipore filter. This resulted in some explants fragmenting and being incompletely transferred which may have affected gene expression.

Wt1/Laminin staining appeared to demonstrate an increase in the number of Wt1 positive cells which may be an indication that active nephrogenesis was occurring in the tissue. In the Day 0 sections few sparse glomeruli were visualised with morphology different to those seen on Day 3 and Day 7. It is suggested that these structures represent intact glomeruli present due to impartial disaggregation which may die as the culture progresses, to be replaced by ‘new’ developing glomeruli. This would be expected from a tissue such as the

rodent neonatal kidney which still has nephrogenic potential (Dickinson et al., 2005). This finding was not backed up by RT-qPCR findings though which demonstrated a steady decrease in *Wt1* expression throughout the culture, although some expression was still present at Day 7. However, the differences in *Wt1* expression levels were not statistically significant.

Calbindin and Laminin staining was included in the analysis as a marker of ureteric bud. Immunofluorescence did not demonstrate a discernible trend in expression of Calbindin. RT-qPCR demonstrates a substantial and sustained downregulation of Calbindin. One explanation for this downregulation could be that, as Calbindin is a ureteric bud marker 'used up' by nephrogenesis, the decreasing quantity of ureteric bud and consequently Calbindin expression would be expected of a nephrogenic culture (Gilbert, 2010).

One of the key aims of the investigation of neonatal mouse kidney explants was to identify if endothelial cells were able to survive and if so, whether they could be found in close proximity to podocytes such that a double layered glomerular basement membrane might be formed *in vitro*. The markers selected for this analysis were Pecam and Synaptopodin. Analysis of immunofluorescence revealed that Pecam staining could be observed on Day 7 of culture and gene expression was detected on Day 7 using RT-qPCR. These findings suggest that a proportion of the endothelial population have survived in culture. The pattern of Pecam expression was also analysed with a change noted from Day 0 to Day 3 suggestive of re-organisation of the endothelial cells. At Day 0 positive staining is observed but it is confined to single cells whereas at Day 3 'cord like' structures were consistently observed, possibly representing the formation of capillaries. Additionally, positive Pecam staining was identified in close proximity to Synaptopodin positive cell aggregates. This is an interesting observation because it is known that endothelial cells within embryonic kidneys die shortly following explantation (Loughna et al., 1998).

There are a number of possible explanations for the survival of this population. Previous studies where the fate of endothelial cells in explant culture has been investigated have not used the disaggregation-reaggregation method employed in this study (Loughna et al., 1998) and it is possible that this factor contributed to survival of this population. It is also possible that cells in the neonatal kidney are more terminally differentiated than in the embryo which would increase the likelihood of the endothelial cells surviving in the explant environment. A third possibility is that endothelial cells in the neonatal kidney have a

different origin to endothelial cells in the embryo. The origin of glomerular endothelial cells has been extensively investigated and the relative contribution of endogenous cells and those migrating from the vasculature is currently unclear (Abrahamson, 2009). If the endothelial population differs in its origin at these time points it may affect the likelihood of this population surviving in explant culture.

As was identified in the Wt1/Laminin stained sections, few structures resembling glomeruli were identified at Day 0 with an increase in Synaptopodin positive cell aggregates resembling immature glomeruli at Day 3. The change in morphology observed suggests that the structures at Day 0 are the result of incomplete disaggregation and actually decompose before progenitor cells give rise to the new structures. An interesting observation that can be made with the Synaptopodin staining is that these 'glomeruli' appear to be hollow structures at Day 0 but by Day 3 cells staining positive for Pecam can be identified, a finding suggestive of nephrogenesis. These findings are not corroborated by RT-qPCR investigation however; the relative PECAM gene expression, even with the large standard error, suggests that there is downward trend in expression levels by Day 7. Some gene expression is still detected however indicating that while proliferation may not be occurring the endothelial population has survived in culture. The trend in synaptopodin expression indicates an initial downregulation, possibly due to the disaggregation process, followed by upregulation which may indicate that this population is proliferating and that some nephrogenesis is occurring. However this value has the largest standard error and is not statistically significant.

When combined the analysis of the neonatal kidney explants provides conflicting evidence with regards to nephrogenesis occurring in the culture. The immunofluorescence from WT1/Laminin and PECAM/Synaptopodin staining would seem to suggest this process is occurring but this is not backed up by RT-qPCR. Immunofluorescence findings relating to Calbindin/Laminin do not demonstrate a discernible trend however RT-qPCR demonstrates a sustained downregulation. Analysis of PECAM/Synaptopodin seems to indicate that endothelial cells have survived in culture and that these cells are in close proximity with podocytes.

5.5 Bioinformatics

This bioinformatics study analysed the most suitable datasets available from microarray experiments of normal eutopic endometrium and eutopic endometrium from patients with endometriosis. The defined selection criteria were as explicit and exclusive as possible in order to fully consider the multiple pitfalls in the large number of existing published datasets.

Preliminary analysis of the selected datasets has revealed some interesting findings. When identifying differentially regulated genes it was noted that the number of genes increased as the cycle progressed both in the 'Normal' endometrium and patients with endometriosis. This is plausible as different stages of the secretory phase are known to be associated with different cellular functions and processes (Talbi et al., 2006). For example, the main function of the human endometrium is to accept an embryo for implantation and facilitate further progression of pregnancy. This being the case, the endometrium does not allow implantation of the best quality embryo except for a few days in the mid secretory phase of the cycle (Talbi et al., 2006). The changing cellular processes responsible for this function in normal endometrium are of interest as well as in endometriosis where many authors have reported aberrations in the expression of a variety of proteins, genes and resulting abnormal cellular functions (Aghajanova and Giudice, 2011, Burney et al., 2007). In keeping with those reports, it was also noted that the number of differentially regulated genes in endometriosis relative to the control was substantially more than in normal endometrium, an almost tenfold increase. This may reflect the different cellular processes involved in endometriosis. Given that this study was performed on eutopic endometrium, this finding is consistent with the clinical finding of associated infertility with endometriosis thought to be due to the prevention of embryo-implantation. It also supports the retrograde menstruation theory, as an abnormal eutopic endometrium that is shed and deposited in the pelvic cavity could have an increased propensity to establish lesions.

In analysis of the 'Normal' endometrium two pathways of interest were identified as being significantly upregulated in their respective phases and compartments. Super Oxide Radicals Dismutase identified and closer investigation identified the enzyme SOD2, a component of this pathway significantly upregulated in the dataset. SOD2 is thought to prevent the accumulation of intracellular Reactive Oxygen Species (ROS), a product of normal cellular metabolism (Bürkle, 2002). When ROS are allowed to accumulate they can

result in DNA damage and mutations. Cells have several mechanisms to prevent this accumulation and interestingly one of the well described mechanisms, Telomerase action, is thought to be downregulated in the mid secretory phase endometrium (Williams et al., 2001). The mid secretory phase represents the implantation window, a time when mutations would be extremely unfavourable as if an embryo were to implant a great deal of cellular proliferation would take place. If telomerase is downregulated then a compensatory mechanism would be expected and SOD2 represents an interesting candidate.

The second pathway of interest was 'Tryptophan Degradation to2-amino-3-carboxymuconate Semi Aldehyde. Within this pathway the enzyme IDO was significantly upregulated within the dataset. IDO has been previously studied within murine endometrium (Jeddi-Tehrani et al., 2009) and is important in tolerance of self, fetal and tumour antigens. It is logical that this gene should be upregulated in the secretory phase as this is a time when implantation may occur and tolerance of foreign antigens is important. The regulation of 'self tolerance' is interesting in relation to autoimmune disease and it is thought that one reason behind the higher prevalence of these diseases in females is due to the altered immune environment associated with pregnancy in which the maternal host must prevent rejection of the fetal 'allograft'.

In addition to these two 'Top Canonical Pathways' The Wnt/Beta Catenin pathway was noted to be upregulated in both the mid and late secretory 'whole' datasets. The Wnt/Beta Catenin pathway, when activated, is associated with cellular proliferation and the maintenance of an undifferentiated state (Valentijn et al., 2013). Activation of the Wnt pathway results in stabilisation of the Beta Catenin complex with localisation to the cell nucleus resulting in the activation of a group of downstream proteins. This pathway has been well characterised in intestinal epithelium (Fevr et al., 2007) and recently work has been commenced in the endometrium (Valentijn et al., 2013, Nguyen et al., 2012). An intact Wnt pathway has recently been demonstrated in pre- as well as post-menopausal endometrium and given that the postmenopausal maintains proliferative potential, the conservation of this pathways suggests that it may have a role in the regenerative capacity of the endometrium (Nguyen et al., 2012). Investigation is ongoing and work at the Liverpool Womens Hospital aims to characterise some of the downstream proteins of this pathway in the endometrium with one notable example being SOX9 (Valentijn et al., 2013). As endometriosis is a proliferative disease it is likely that the Wnt/Beta Catenin pathway is

involved in some way in its pathogenesis. It will be interesting then to perform further analysis on both the 'Normal' and 'Endometriosis' transcriptomic datasets as this has the potential to identify Wnt related molecules which may be differentially regulated between the two. Preliminary identification using this *in silico* approach may then provide the basis for further 'wet' laboratory investigation.

5.6 Bioinformatics: Endometriosis

Analysis of transcriptomic datasets from the eutopic endometrium of patients with endometriosis yielded a greatly increased number of differentially regulated genes compared with normal endometrium such that analysis could be performed using Cytoscape. Cytoscape is a software platform for visualizing molecular interaction networks and biological pathways. These networks are then integrated with annotations, gene expression profiles and other state data (Cytoscape, 2013). This program was used in this study in the context of investigating pathways within the Heparan Sulfate Interactome. Several highly interconnected network clusters of interest were identified within the dataset, one example is pictured in Fig33 with 28 nodes and 330 edges. One of these clusters contained several molecules belonging to the group 'Fibroblast Growth Factors' (FGFs). These molecules are associated with a wide variety of cellular processes including angiogenesis, wound healing and embryonic development (Goetz and Mohammadi, 2013) and are the subject of active investigation within the endometrium. In humans there are 22 described members of the FGF family (Ornitz and Itoh, 2001) and investigation into FGF function in the endometrium has been performed, e.g. basic FGF has been linked to the growth of stromal Colony Forming Units (CFU) (Gargett et al., 2008). Full characterisation of FGFs and FGF receptors has not yet been performed in the endometrium and represents an interesting potential for future investigation.

One particular area the FGFs have been considered in is embryo implantation, a process that is impaired in endometriosis (Sak et al., 2013). While FGF-1 was identified upregulated in this dataset it has previously been demonstrated downregulated in endometriosis (Sak et al., 2013). A previous study using PCR has also shown no difference in the expression of FGF2 in eutopic endometrium of patients with endometriosis and controls (Lee et al., 2007).

Clearly angiogenesis is an interesting process in endometriosis as it is aberrantly thought to be involved in lesion establishment. The identification of this highly interconnected cluster

provides an interesting avenue for further investigation of FGFs in the endometrium. One possible example would be to look at angiogenesis processes known to be associated with these FGFs and use immunohistochemistry to compare expression between women with and without endometriosis. It would also be useful to characterise FGF receptor expression within the endometrium using either immunohistochemistry or other methods such as Fluorescence in Situ Hybridisation (FISH). As many of the processes associated with FGFs are dysregulated in endometriosis it would also be interesting to investigate the expression both of FGFs and FGF receptors in ectopic endometriotic lesions.

5.7 Limitations of the Study

There are several limitations of this study outlined throughout the discussion section. The PCR analysis is limited by missing data and variation observed between both technical and biological replicates in the kidney explant analysis. As a result of this, statistical analysis on both the chimera and neonatal kidney explant RT-qPCR data was not possible which severely restricts interpretation of these findings. Further biological replicates for both the chimeras and neonatal explants would have been carried out but were limited by the availability of neonatal kidneys. As these specimens need to be 2-3 days of age, the window for preparing the endometrial cultures was relatively short and on several occasions the kidneys were not able to be obtained due to either pseudo pregnancy or infanticide.

One factor limiting the analysis of the chimeras was a lack of a control culture of endometrial epithelial cells labelled with the Vybrant fluorescent dye. This would have been useful in assessing the viability of the endometrial cells in culture after labelling, as it is currently not clear which factor causes the apparent decrease in cell number. A second benefit that would have been gained from this control would be immunofluorescence staining using the anti-laminin antibody used in the study. This would definitively establish whether the endometrial cells were producing Laminin structures or whether those structures observed originated entirely from the neonatal mouse kidney tissue.

When preparing the endometrial epithelial cells for inclusion in the chimera a 2D culture method was used. There are documented limitations of this approach as the 2D environment does not represent the *in vivo* conditions, particularly for epithelial cells which are known to act in a more physiologically relevant manner when cultured in 3D spheroid culture (Valentijn et al., 2013). It is possible however that culturing the cells in spheroids

with Matrigel before disaggregation and inclusion into the chimera may have placed too much stress upon the cells resulting in a change of morphology.

A limitation of the chimera experiment including both epithelial and stromal cells was the poor viability of Day 7 chimeras such that analysis was not able to be completed using immunofluorescence. This is likely to be due to experimental error as difficulty was experienced transferring these particular chimeras to the Millipore membrane as they exhibited an increased propensity to fragment meaning that not all tissue was transferred to the membrane.

In the bioinformatics analysis of endometrium from patients with endometriosis it would have been useful to analyse samples from the Late Secretory Phase. The absence of these samples limits the interpretation of the data however a comprehensive literature search was carried out and Late Secretory phase transcriptomic datasets obtained on a comparably microarray platform could not be identified. The absence of this data in the literature suggests that this limitation could not be accounted for by the investigators.

5.8 Further Work

Given the paucity of statistically significant findings in this study there is considerable potential for further work. Refinements could be made to the kidney chimera model to improve study of the human cell population. Vybrant staining could be optimised to improve visualisation of the cells as high intensity staining presented difficulty in image interpretation. Further investigation into the fate of the endometrial cells in the chimera could be performed by immunostaining for either markers of cell death e.g. Caspases or mouse specific leukocyte markers to attempt to identify if an immune response was occurring. If a neonatal mouse immune response were identified then further experimentation could include a chimera culture using neonatal kidney obtained from *SCID* mice as this would allow endometrial cell survival.

Refinements could be made to the chimera model of endometriosis by considering the culture medium used. It is known that the angiogenic activity of endometrial cells is increased if cultured in peritoneal fluid of women with endometriosis (Oosterlynck et al., 1993). It is possible then that this aspect of the microenvironment might be important in the cells proliferative potential. The model could be refined to include conditioned medium

from primary culture of endometrial cells from patients with endometriosis; however immune aspects would have to be considered and possibly embryos or *SCID* mice used.

While the findings from the chimera experiment seem to indicate a limited use for this model in the investigation of endometriosis one avenue for further work may be considered. Previous models have investigated the attachment of endometrial cells in the process of lesion formation and have demonstrated the in both epithelial and stromal populations attachment occurs within 1 hour with invasion a similarly quick process completed within 24 hours (Witz et al., 2001). This is consistent with the findings of this study which found endometrial cells throughout the full thickness of the chimera by day 3 of culture. Further characterisation of this model could be undertaken investigating these processes at time points within the first 24 hours of culture which may yield some findings before a large increase in the endometrial population occurs.

Analysis of neonatal kidney explants seemed to demonstrate the formation of glomerular structures on culture. To elucidate with greater accuracy the processes occurring, an increased number of biological and technical repeats would be useful in RT-qPCR analysis and quantitation of immunofluorescence images could be performed. As it is hypothesised that cell populations capable of forming mature glomerular basement membrane are surviving in culture, one further experiment would be to attempt to characterise these structures using both immunofluorescence and RT-qPCR for markers of mature GBM e.g. Laminin 5II and Type IV collagen (Takemoto et al., 2006).

When investigating the survival of endothelial cells in the culture one explanation is that the disaggregation-reaggregation protocol could be responsible for their survival, as previous investigation into this cell population in explant culture did not use this method (Loughna et al., 1998). A useful further investigation would be to compare endothelial cell survival in explants generated from embryonic kidneys with those from neonatal kidneys in order to help establish whether the neonatal environment in some way responsible for the survival of this population.

In this preliminary *in silico* analysis normal endometrium and endometrium from patients with endometriosis has been investigated. The next step in this work would be to combine these analyses, overlaying pathways of interest, in order to find upstream mediators which may be in some way responsible for the aberrant proliferation seen in endometriosis and

which could be targeted for wet laboratory investigation and eventually clinical intervention.

In analysis of 'Normal Endometrium' the canonical pathway 'Superoxide Radicals Degradation' was upregulated within which 'SOD2' was identified as a molecule of interest. A 'wet' laboratory experiment may be designed to investigate the role of SOD2 in preventing ROS accumulation in secretory phase endometrium. Tissue samples from 'SPCN' patients gathered at the Liverpool Womens Hospital could be immunostained for SOD2 and ROS to assess correlation of these two molecules. Telomerase could also be stained for and quantitative analysis may help to demonstrate a potential correlation.

In analysis of endometrium from patients with endometriosis transcriptomic datasets from comparable microarray platforms were not obtained for the Late Secretory Phase endometrium. This represents a deficiency in the literature and one possible further investigation would be to perform a microarray experiment on a Late Secretory phase endometrial sample from a patient with endometriosis fulfilling the inclusion/exclusion criteria outlined in the 'Methods' section. Obtaining this data would allow further investigation into the cellular and molecular mechanisms associated with this proliferative disease.

Endometriosis can be considered a benign disease showing metastatic behaviour as the eutopic endometrium spreads to distant sites (Impey, 2012). To investigate this further transcriptomic datasets from microarray experiments may be analysed to identify common molecular pathways between patients with endometriosis and patients with endometrial cancer. A literature search has already been carried out and a suitable dataset identified. Early limiting factors have been identified in this analysis as cancer samples are almost always collected from post-menopausal patients and as such cannot be separated according to menstrual cycle phase. This introduces a large confounding factor into any analysis. One possible way around this would be to compare both cancer and endometriosis datasets to a post menopausal dataset used as a control.

5.9 Conclusions

It is difficult to make final and solid conclusions from this study and a large amount of further work is required to confirm the preliminary findings outlined. However when analysing the endometrial cell activity in the neonatal mouse kidney chimeras it can be asserted that the cells did not proliferate or form glandular structures in culture and it is likely that widespread cell death occurred. Possible explanations for this include: a hostile niche provided by the neonatal kidney, Vybrant toxicity, cell death due to lack of cell-cell contact, apoptosis of more terminally differentiated cells leaving the progenitor population and immune attack by resident murine leucocytes. The analysis does seem to indicate that some endometrial cells survived and this was combined with an increase in progesterone receptor expression at Day 7, suggesting that these were the less well differentiated cells, possibly an ASC or TA population. When considering the neonatal kidney explant study examination of immunofluorescence images seems to indicate that a degree of nephrogenesis took place in the neonatal kidney explants, although RT-qPCR findings to not corroborate this. It appeared that a population of endothelial cells had survived to Day 7 of neonatal kidney explant culture and were in close proximity to podocytes, a finding which warrants further investigation. The bioinformatics approach to the endometrium and endometriosis has yielded several interesting results and leads for potential for future 'Wet' laboratory investigation. This approach to hypothesis generation is becoming more widespread and represents an exciting prospect for the future.

Chapter 6: Appendix

6.1 References

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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.

6.3 Patient Consent Form

Liverpool Women's

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

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

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

Please initial box

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

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

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

SDS0210M P.I.S. (February 2005)

6.4 Patient Clinical Data Collection Form

Endometriosis Studies Clinical Data Collection Form

Sample ID: _____

Date of sample: _____/_____/_____

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

Age: _____

Height (m): _____

Weight (kg): _____

BMI: _____

Smoker: yes/no (circle as appropriate)

Endometriosis: yes/no (circle as appropriate)

Endometriosis stage: _____

Adenomyosis: yes/no (circle as appropriate)

Menorrhagia: yes/no (circle as appropriate)

Fibroids: yes/no (circle as appropriate)

Reason for Surgery: _____

Previous gynae history/surgery: _____

Parity: _____ Infertility: _____

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

TOP: _____

Days of bleeding: _____

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

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

Contraceptive/hormone treatment: _____

Other information: _____

_____

_____

6.5 Patient Demographic Information

Sample ID	Sample Type	Patient Age	Patient BMI	Reason for Surgery	Endometriosis	Cycle phase	Additional information
SPCN125	Full Thickness	47	32.6	Pelvic Pain	No	Day 4 – Proliferative	
SNCN128	Full Thickness	47	23.3	Prolapse	No	Day – Proliferative	
SPCEX118	Full Thickness	42	34.8	Menorrhagia	No	Unknown	Provera

6.6 Additional Immunofluorescence Images

Figure 37: CD1 Chimera: Anti Cytoplasmic Staining Repeat 1

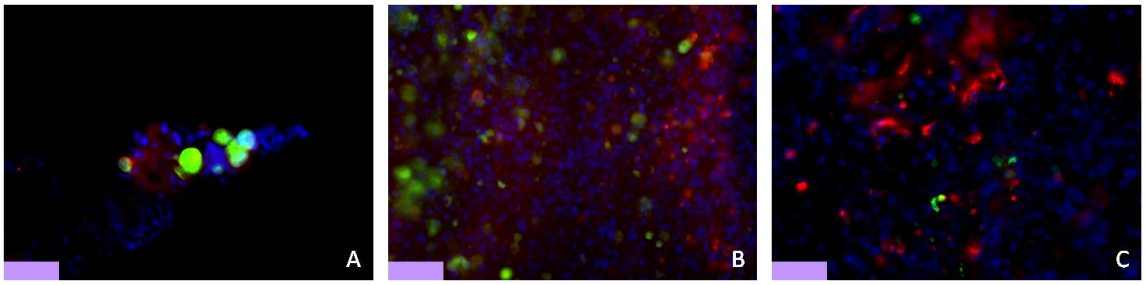

Fig37. Human endometrial epithelial cells were labelled with 'Vybrant' fluorescence (Green) and cultured for 2 hours, 3 days and 7 days in a chimeric explant generated with the Disaggregation-Reaggregation technique developed by Davies et al with CD1 neonatal mouse kidneys. Frozen sections were stained with human specific anti-cytoplasmic antibody (Red) and DAPI (Blue). **A**. 2 hours (0 days) of culture. **B**. 3 Days of Culture. **C**. 7 Days of Culture. Scale bar 50µl

Figure 38: CD1 Chimera: Laminin Staining Repeat

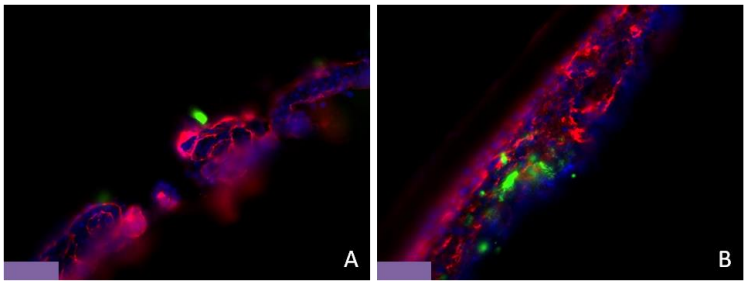

Fig38. Human endometrial epithelial cells were labelled with 'Vybrant' fluorescence (Green) and cultured for 2 hours (Day 0), 3 days and 7 days in a chimeric explant using the Disaggregation-Reaggregation technique developed by Davies et al with CD1 neonatal mouse kidneys. Frozen sections were stained for Laminin (Red) DAPI (Blue) at these specific time points. . **A.** Day 0, **B.** Day 3. Scale Bar 50µm

Figure 39: CD1 WT1 Laminin Repeat 1

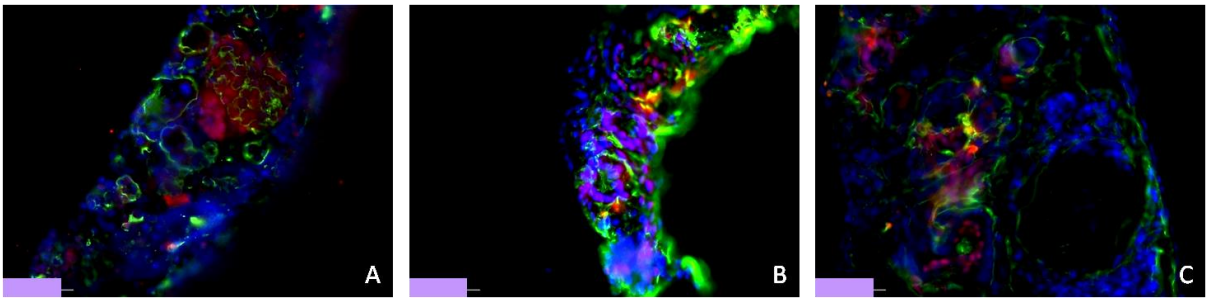

Fig39. Immunofluorescence stain of neonatal explant cultured from CD1 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with WT1 (Red), Laminin (Green) and the nuclear stain DAPI (Blue). **A** Sample fixed after 2 hours of culture (Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 40: CD1 WT1 Laminin Repeat 2

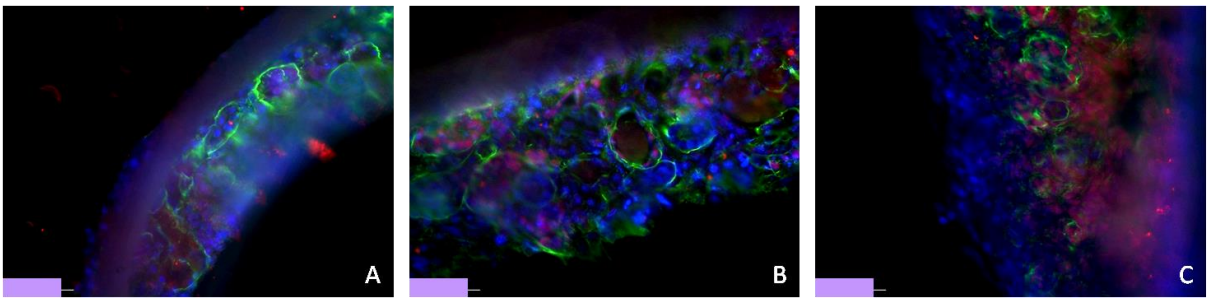

Fig40. Immunofluorescence stain of neonatal explant cultured from CD1 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with WT1 (Red), Laminin (Green) and the nuclear stain DAPI (Blue).. **A** Sample fixed after 2 hours of culture (Day 0). **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 41: Black 6 WT1 Laminin Repeat 1

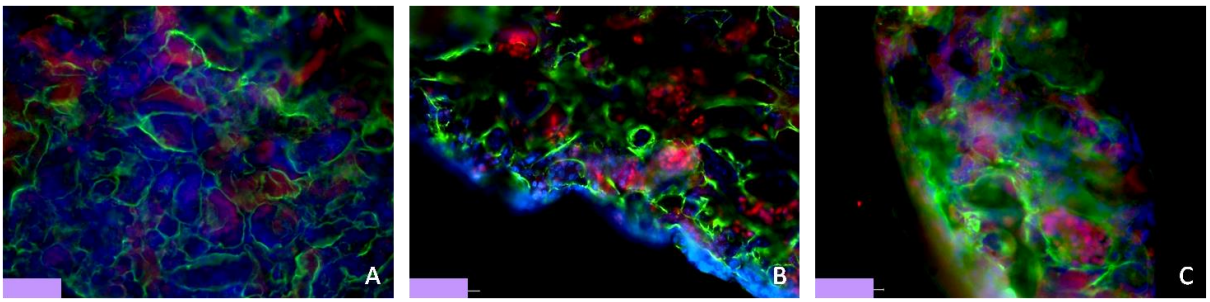

Fig41. Immunofluorescence stain of neonatal explant cultured from Black 6 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with WT1 (Red), Laminin (Green) and the nuclear stain DAPI (Blue).. **A** Sample fixed after 2 hours of culture (Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 42: CD1 PECAM Synaptopodin Repeat 1

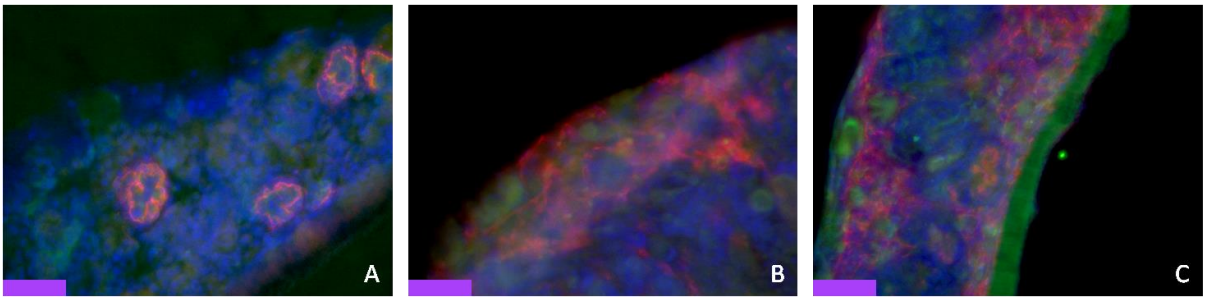

Fig42. Immunofluorescence stain of neonatal explant cultured from CD1 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with PECAM (Red), Synaptopodin (Green) and the nuclear stain DAPI (Blue). **A** Sample fixed after 2 hours of culture (Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 43: CD1 PECAM Synaptopodin Repeat 2

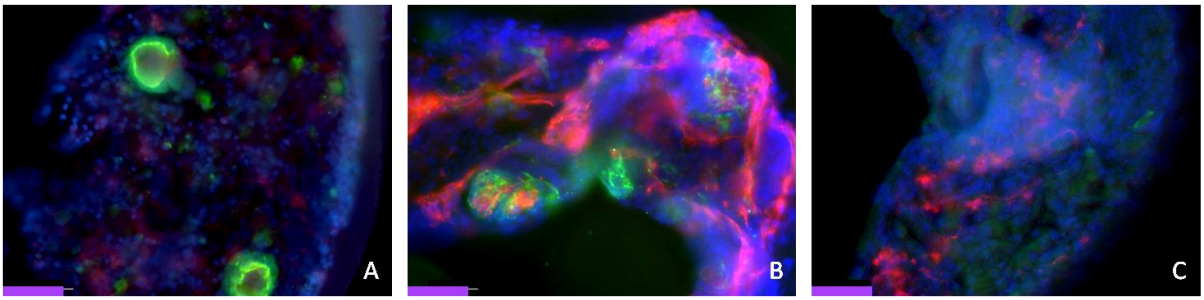

Fig43. Immunofluorescence stain of neonatal explant cultured from CD1 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with PECAM (Red), Synaptopodin (Green) and the nuclear stain DAPI (Blue).. **A** Sample fixed after 2 hours of culture (Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 44: CD1 PECAM Synaptopodin Repeat 3

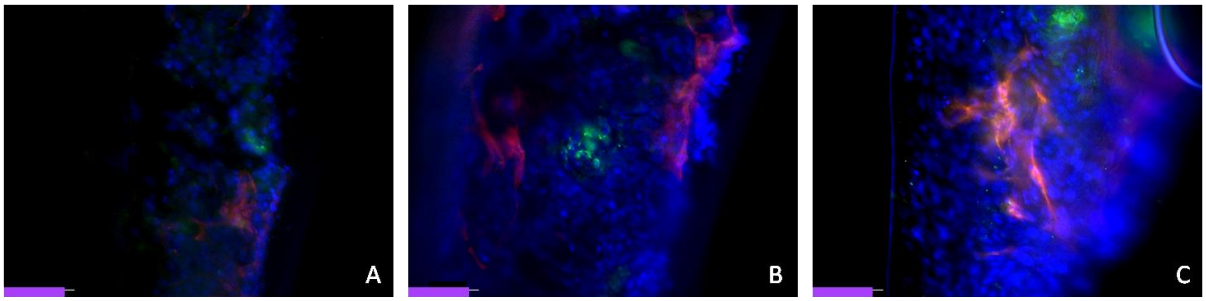

Fig44. Immunofluorescence stain of neonatal explant cultured from CD1 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with PECAM (Red), Synaptopodin (Green) and the nuclear stain DAPI (Blue).. **A** Sample fixed after 2 hours of culture (Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 45: CD1 Calbindin Laminin Repeat 1

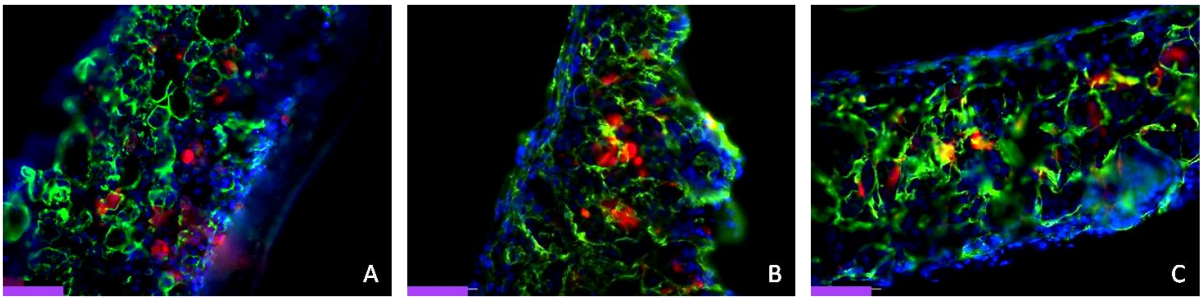

Fig 45. Immunofluorescence stain of neonatal explant cultured from CD1 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with Calbindin (Red), Laminin (Green) and the nuclear stain DAPI (Blue). **A** Sample fixed after 2 hours of culture Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 46: CD1 Calbindin Laminin Repeat 2

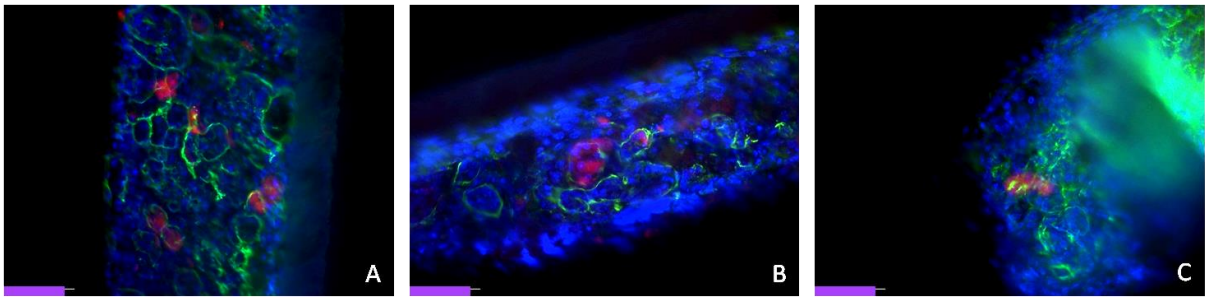

Fig46. Immunofluorescence stain of neonatal explant cultured from CD1 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with Calbindin (Red), Laminin (Green) and the nuclear stain DAPI (Blue).. **A** Sample fixed after 2 hours of culture (Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 47: Black 6 Calbindin Laminin Repeat 1

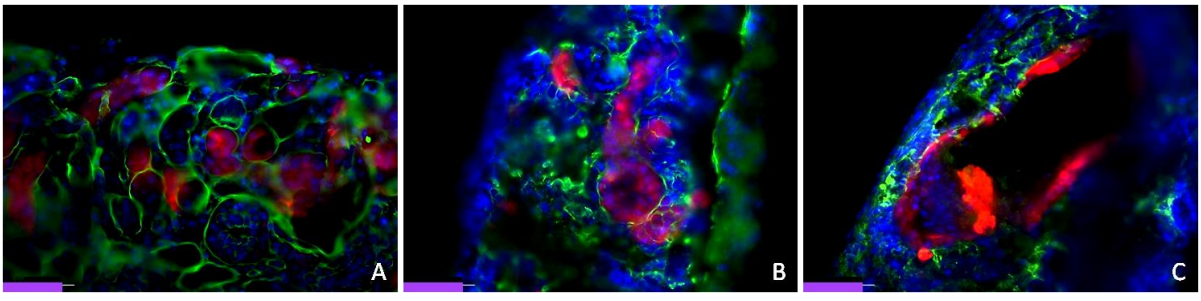

Fig47. Immunofluorescence stain of neonatal explant cultured from Black6 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with Calbindin (Red), Laminin (Green) and the nuclear stain DAPI (Blue).. **A** Sample fixed after 2 hours of culture (Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

Figure 48: Black 6 Calbindin Laminin Repeat 2

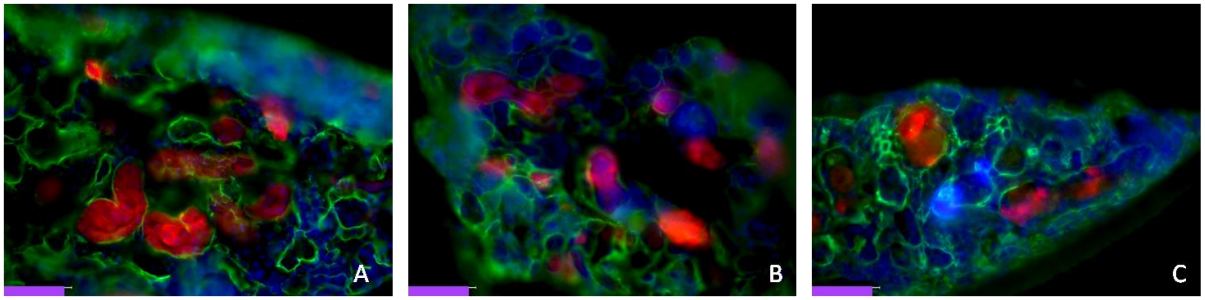

Fig48. Immunofluorescence stain of neonatal explant cultured from Black6 Strain mice after disaggregation and reaggregation using the technique developed by Davies et al. Explants were stained with Calbindin (Red), Laminin (Green) and the nuclear stain DAPI (Blue).. **A** Sample fixed after 2 hours of culture (Day 0. **B** Day 3. **C** Day 7. Scale bar 50µm

6.7 Reagent Formulations

Sample Collection Medium:

- Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich)
- 1% Fetal Bovine Serum (FBS) (Gibco)
- Primocin 1:500 Dilution in Medium (Invitrogen)

Endometrial Culturing Medium:

- 500 ml DMEM F12 Ham (Sigma Aldrich)
- 10 ml L-glutamine 2mM (Gibco)
- 1 ml Primocin
- 50 ml FBS
- 50 µl recombinant human EGF 10ng/ml (Sigma Aldrich)

Kidney Culture Medium

- 10% DMEM FBS
- L-Glutamine (2mM; 1:100)
- Penicillin Streptomycin (1:100) (Sigma Aldrich)

Gelatine Sucrose Solution

- 15g Sucrose, Reagent Grade (Sigma Aldrich)
- 7.5g Gelatine from Porcine Skin Type A (Sigma Aldrich)
- Made up to 100ml with PBS

Tris-Acetate EDTA (TAE)

- Tris (2M)
- EDTA (0.05M)
- Acetic Acid to pH 7.8

6.8 Reagent Specifications

Reagents	Company City Country	Catalogue number
Acetic Acid	AnalaR	UN175121
Agarose	Bioline, London, UK	BIO-41025
5x FS Buffer	Invitrogen, Paisley, UK	Y02321
5xDNA Loading Buffer	Bioline	BIO-37045
Bright Cryo-M-Bed	Bright Instrument Company Limited	N.A.
Chloroform	Sigma Aldrich, Dorset, UK	101146896
Collagenase	Invitrogen, Paisley, UK	17104-019
Collagenase	Sigma Aldrich, Dorset, UK	1001375121
DAPI	Sigma Aldrich, Dorset, UK	D9542-10mg
Dako Fluorescent Mounting Medium	Dako North America, Carpinteria USA	S3023
Dispase	Gibco	17104-041
DNase	Roche, Germany	11284932001
DNase1 from bovine Pancreas	Sigma Aldrich, Dorset, UK	D4263-IVL CAS 9003-98-9
DNase Reaction Buffer	Promega	M198A
DNase Stop Solution	Promega	M199A
dNTP Invitrogen	Carlsbad Ca	10297-018
DTT	Invitrogen, Paisley, UK	Y00147

Dulbecco's Modified Eagle Medium (DMEM)	Sigma Aldrich, Dorset, UK	D5546
Dulbecco's Modified Eagle Medium (DMEM)HAM F12	Sigma Aldrich, Dorset, UK	D6421
EDTA	Sigma-Aldrich, Dorset, UK	E-5134
Ethidium Bromide	Molecular Sigma Biology, Dorset, UK	E-7637
Ethanol	Sigma-Aldrich, Dorset, UK	E7023
Fast Red TR	Sigma Aldrich, Dorset, UK	F8764-1G
Fetal Bovine Serum	Gibco	10270-098
Gelatine from Porcine Skin Type A	Sigma Aldrich, Dorset, UK	G1890-500G
Goat Serum	Sigma Aldrich, Dorset, UK	1001390834
Hanks Balanced Salt Solution	Sigma Aldrich, Dorset, UK	H9269
Hyperladder IV	Abcam	50472
Isopropanol	Sigma Aldrich, Dorset, UK	I9516
L-Glutamine	Gibco	35050-038
Methanol	Sigma Aldrich, Dorset, UK	M-3641
MgCl2	BDH, Leicestershire, UK	
Minimum Essential Medium Eagle (MEME)	Sigma Aldrich, Dorset, UK	M5650

Napthol AS-MX phosphate	Sigma Aldrich, Dorset, UK	N4875-500MG
Neutral Buffered Formalin	Sigma-Aldrich, Dorset, UK	HT501320-95L
Nuclease Free Water	Fisher Bioreagents,	BP2470-1
Penicillin, Streptomycin	Sigma Aldrich, Dorset, UK	P0781 SLBB9307
Phosphate Buffered Saline	Sigma Aldrich, Dorset, UK	D8537
Phosphate Buffered Saline (Ca^{2+} free)	Sigma Aldrich, Dorset, UK	D8537
Primocin	Invitrogen, Paisley, UK	P4417-100TA
Random Hexamers	Qiagen, West Sussex, UK	79236
Recombinant human EGF	Sigma Aldrich, Dorset, UK	
RNALater	Applied Biosystems, Warrington, UK	79236
RNase Free DNase	Promega	M610A
ROCK Inhibitor	Calbiochem	688001
Sucrose, reagent grade 98%	Sigma Aldrich, Dorset, UK	179949
Superstript III Reverse Transcriptase	Invitrogen, Paisley, UK	56574
SYBR Green Jumpstart Taq	Sigma Aldrich, Dorset, UK	1001469234
Tris	Gibco	15504-020
Trizol	Invitrogen, Paisley, UK	15596-026
Trypan Blue	Sigma Aldrich, Dorset, UK	T8154
Trypsin EDTA 1X Solution	Sigma Aldrich, Dorset, UK	T3924

Virkon	Antec International	
Vybrant CFDA SE Cell Tracer Kit	Invitrogen, Paisley, UK	V12883

6.9 Equipment Specifications

Equipment	Company, City	Catalogue Number
40µm Cell strainer		
Electrophoresis Chamber	Consort	EV231
Centrifuge 4K15	Sigma Aldrich, Dorset, UK	
GENE FLASH	Syngene Bioprinting	
Hydrophobic Marker	ImmEdge	H-4000
Microtome	Microm	HM 505N
Millipore Isopore Membrane Filters	Millipore	RTTP02500
Pellet Pestle, disposable	Sigma Aldrich, Dorset, UK	2359971-1EA MW07345
Scalpel Blades (Sterile)	Swann Morton	N.A.
Thermo Scientific Glaser Plus	Menzel Superfrost	J1800AMNZ
Electrophoresis Casette	SCIE-PLAS	HV10
Hand-held homogenisor	Qiagen, West Sussex, UK	9001272
Nanodrop Spectrophotometer		Thermoscientific Nanodrop Lite
Weighing Scales	Acculab VICON	
Thermal Cycler	Applied Biosciences	2720
Automated Cell Counter	Biorad	TC20
Fluorescent Microscope	Leica	DM2500

6.10 Concentration of RNA Samples Used in Study

Sample ID	Type	Concentration (ng/µl)	Factor	A260 (10 mm)	A280 (10 mm)	A3rd (10 mm)	A260/A280
K0 (1)	RNA	1157.6	40	28.941	16.011	0.018	1.81
K3 (1)	RNA	152.5	40	3.812	2.009	0.006	1.9
K7 (1)	RNA	255.4	40	6.385	2.804	0.003	2.28
K0 (2)	RNA	587.5	40	14.689	7.655	0.009	1.92
K3 (2)	RNA	211.5	40	5.287	2.956	0.011	1.79
K7 (2)	RNA	409.8	40	10.244	4.327	0.005	2.37
EK0 (1)	RNA	1075.7	40	26.892	7.723	0.006	3.48
EK3 (1)	RNA	828	40	20.7	7.358	0.003	2.81
EK7 (1)	RNA	1545	40	38.625	20.227	0.008	1.91
EK0 (2)	RNA	516.1	40	12.902	6.146	0.005	2.1
EK3 (2)	RNA	901.3	40	22.533	7.628	0.005	2.95
EK7 (2)	RNA	1145	40	28.625	10.632	0.01	2.69
K0 (3)	RNA	580.5	40	14.512	6.288	0.152	2.31
K3 (3)	RNA	512.8	40	12.821	6.111	0.002	2.1
K7 (3)	RNA	432.9	40	10.824	4.606	0.002	2.35
K0 (4)	RNA	410.2	40	10.256	4.459	0.002	2.3
K3 (4)	RNA	398.3	40	9.957	4.742	0.003	2.1
K7 (4)	RNA	786.4	40	19.66	7.154	0.247	2.75
EK0 (3)	RNA	499.1	40	12.477	5.116	0.003	2.44

EK3 (3)	RNA	519.3	40	12.983	5.192	0.003	2.5
EK7 (3)	RNA	624.9	40	15.623	6.997	0.004	2.23

6.11 Appendices for Bioinformatics Study

6.11.1 Data Series from 'Normal Human Endometrium' Literature Search Excluded from the Study

Data Series	Series Title	Number of Samples	Reason for Exclusion
Gene expression profiling of human NK cells	GSE24268	3	Cells Isolated
Gene expression profiling of C/EBPβ- and STAT3-deficient human endometrial stromal cells (HESC)	GSE41473	8	Stromal cells Isolated
CpG Methylation by Methyl 450K Bead Arrays from ENCODE/HAIB	GSE40699	62	Epigenetic Study
microRNA expression profile in endometrioid endometrial carcinoma (EEC)	GSE35794	22	Epigenetic Study
Altered Gene Expression Profile of Microvascular Endothelium in Placentas from IUGR/Preeclamptic Pregnancies	GSE25861	10	Non endometrial tissue
Open Chromatin by FAIRE from ENCODE/OpenChrom (UNC Chapel Hill)	GSE35239	37	Cell Culture Study
Unique Transcriptome, Pathways, and Networks in the Human Endometrial	GSE35287	80	Cells Isolated

Fibroblast Response to Progesterone in Endometriosis			
Gene Expression Analysis of Stage I Endometrial Cancers	GSE17025	103	Malignancy Study
Perivascular Human Endometrial Mesenchymal Stem Cells Express Pathways Relevant to Self-Renewal, Lineage Specification, and Functional Phenotype	GSE31152	15	Cells Isolated
Cervical carcinoma-associated fibroblasts are DNA diploid and do not show evidence for somatic genetic alterations	GSE29143	121	Non endometrial Study
Open Chromatin by DNaseI HS from ENCODE/OpenChrom (Duke University)	GSE32970	97	Non Endometrial Study
Transcription Factor Binding Sites by ChIP-seq from ENCODE/HAIB	GSE32465	339	Non Endometrial Study
Expression data from patients with advanced ovarian cancer	GSE19161	61	Malignancy Study
A DNA Methylation Fingerprint of 1,628	GSE28094	1628	Non Endometrial Study

Human Samples			
Gene expression profiles of endometriosis	GSE23339	19	Normal endometrium not studied
DNA Methylation Dynamics in Human Induced Pluripotent Stem Cells over Time (Agilent)	GSE24677	16	Stem Cells Studied
DNA Methylation Dynamics in Human Induced Pluripotent Stem Cells over Time	GSE24676	47	Stem Cells Studied
DNA Methylation by Reduced Representation Bisulfite Seq from ENCODE/HudsonAlpha	GSE27584	202	Epigenetic Study
Discovery of microRNAs and other small RNAs in solid human tumors: expression	GSE20414	38	Epigenetic Study
Discovery of microRNAs and other small RNAs in solid human tumors	GSE20418	40	Epigenetic Study
Affymetrix 500K Mapping Array data from ovary tumors	GSE11960	114	Non Endometrial Study
Cancer Genome Anatomy Project (CGAP) Human SAGE	GSE15309	327	Malignancy Study

MOE430A Analysis of Dll3 mutant vs. wild-type 9.5 dpc somite-level tissue	GSE15153	6	Non Endometrial Tissue
MOE430A Analysis of Dll3 mutant vs. wild-type 9.5 dpc presomitic mesoderm	GSE15152	6	Non Endometrial Study
Endometrial profile of low-dose estradiol and tamoxifen combination therapy	GSE14518	16	Non Human Study
Uterine stromal cell_cultured with uNK conditioned Medium	GSE9718	14	Cell Culture
Comparison of ES cells with relevant compartments of early embryos	GSE8881	40	Stem Cell Study
Differentially expressed genes in HEECs of eutopic endometrium of patients with endometriosis compared with control	GSE7846	10	Cell Isolated
Gene Profiling of Endometrium Reveals Progesterone Resistance and Candidate Genetic Loci in Women with Endometriosis	GSE6364	37	Endometriosis Study. Useful samples included in the series are duplicated of those used Talbi et al 2006
Human Uterine	GSE5244	51	Epigenetic Study

Leiomyomata microRNA gene expression			
Human endometriosis vs normal endometrium study - transcriptional profiling	GSE7305	20	Ovarian tissue studied. 'Normal' endometrial samples from unknown source
Human body index - transcriptional profiling	GSE7307	677	Non Endometrial Study
Comparison of gene expression profiles across the normal human body	GSE3526	353	Non endometrial Study
Actions of tamoxifen in the uterus and its molecular effectors in endometrial carcinogenesis.	GSE3013	18	Malignancy Study
Expression Project for Oncology (expO)	GSE2109	2158	Non endometrial Study
Cancer Genome Anatomy Project (CGAP) SAGE Libraries	GSE14	765	Malignancy Study

6.11.2 Data Series from 'Human Endometriosis' Literature Search Excluded from the Study

Data Series	Series Title	Number of samples	Reason for Exclusion
GSE40186	miRNA expression profiles in endometriotic cyst stromal cells (ECSCs).	15	Cell isolation and Culture
GSE31515	Transcriptome analysis of endometriotic stromal cells treated with hydrogen peroxide	17	Cell Isolation
GSE35287	Unique Transcriptome, Pathways, and Networks in the Human Endometrial Fibroblast Response to Progesterone in Endometriosis	79	Cell isolation
GSE17504	The PKA Pathway of Endometrial Stromal Fibroblasts Reveals Differentiation and Proliferative Potential in Endometriosis	20	Cell isolation and Culture
GSE11960	Affymetrix 500K Mapping Array data from ovary tumors	2	Non endometrial tissue
GSE16079	Promoter methylation profiles for ectopic vs. eutopic endometrium in SUP, VES and OME	3	Epigenetic Study
GSE11663	Human cleavage stage embryos are chromosomally unstable	23	Cell Isolation
GSE14 GSE15309	Cancer Genome Anatomy Project (CGAP) Human SAGE	1	Malignancy Study
GSE11691 GSE11769	Analysis of ectopic human endometrium and peritoneal tissues in nude mice	8	Mouse Tissue

	Euctopic and ectopic human endometrium (endometriosis)		
GSE7846	Differentially expressed genes in human endometrial endothelial cells derived from eutopic endometrium of patients with endometriosis compared with those from patients without endometriosis.	10	Cell isolation and Culture
GSE7307	Human body index - transcriptional profiling	29	Study Design not Available
GSE4888	Molecular phenotyping of human endometrium	3	Normal Endometrium data
GSE26346	Differential expression of microRNAs between eutopic and ectopic endometrium in ovarian endometriosis	3	Epigenetic Study

6.11.3 Further analysis of datasets initially identified for inclusion in Endometriosis Study

Data Series	Series Title	Study Comments	Potential Samples for Inclusion in Study
GSE5108	Whole genome deoxyribonucleic acid microarray analysis of gene expression in ectopic versus eutopic endometrium	Tissue from different sources: Endometrioma, Peritoneal Explant and Endometriotic matched samples obtained at Hysteroscopy and dilatation and curettage. 1 patient on GnRH anologue.	11 eutopic and 11 ectopic samples
GSE6364	Gene Expression Analysis of Endometrium Reveals Progesterone Resistance and Candidate Susceptibility Genes in Women with Endometriosis	Patients surgically documented and histologically confirmed, moderate-severe endometriosis at laparoscopy. Biopsy specimens obtained by Pipelle or dilatation and curettage.	21 Eutopic endometriosis samples
GSE7305	Human endometriosis is associated with plasma cells and overexpression of B lymphocyte stimulator	Tissues obtained from Zoion Diagnostics (Hawthorne, NY)	20 Samples: 10 endometriomas and 10 matched eutopic samples

GSE12768	Gene Expression Profile for Ectopic *Versus* Eutopic Endometrium Provides New Insights into Endometriosis Oncogenic Potential	Eutopic and Endometrioma samples Samples were luteal phase from patients operated for stage IV endometriosis	4 eutopic samples
GSE37837	Genome-wide expressions in autologous eutopic and ectopic endometrium of fertile women with endometriosis	26 normally cycling fertile women (age: 24–45 y) with BMI (20–22 k/m2) diagnosed ovarian endometriosis. Eutopic, Ectopic, Endometrioma samples collected	36 samples endometrioma and eutopic

6.11.4 'R' Code used to generate differentially regulated gene list for 'Normal' Endometrium

Lines labelled '#' Serve to explain the associated section of code.

```
#read in data from selected patients
geodata<-read.csv(file="GDS2052_full.csv")#assume header has been removed
geodata<-geodata[-54676,]#last row is corrupted

data<-log2(geodata[,c(3:10,12:17)])#log selected data
ann<-geodata[,c(1:10,12:17,30:37)]#annotation data
cyclePhase<-
factor(c("Proliferative","Early","Early","Mid","Mid","Mid","Mid","Late","Late","Late","Early","Mid","Late","Secretory"))#factor
describing patient groups

#series of indicies for location of sample groups
ref<-which(cyclePhase=="Proliferative")
early<-which(cyclePhase=="Early")
mid<-which(cyclePhase=="Mid")
late<-which(cyclePhase=="Late")

#patient group data series
ref.data<-data[,ref]
early.data<-data[,early]
mid.data<-data[,mid]
late.data<-data[,late]

#function to subtract log ref data from log patient data
compRef<-function(x)(Talbi et al.)

#patient ratio compared to reference and mean of these values for each patient group
early.ratio<-apply(early.data,2,compRef)
mean.early.ratio<-apply(early.ratio,1,mean)
mid.ratio<-apply(mid.data,2,compRef)
mean.mid.ratio<-apply(mid.ratio,1,mean)
late.ratio<-apply(late.data,2,compRef)
mean.late.ratio<-apply(late.ratio,1,mean)
#selected up- and down-regulated probes from each patient group at log fold change +/-2 as defined by fc
fc<-(2)
up.early<-unique(which(mean.early.ratio>fc))
down.early<-unique(which(mean.early.ratio<(-fc)))
up.mid<-unique(which(mean.mid.ratio>fc))
down.mid<-unique(which(mean.mid.ratio<(-fc)))
up.late<-unique(which(mean.late.ratio>fc))
down.late<-unique(which(mean.late.ratio<(-fc)))
#percentage up-regulated drops as percetage down-regulated rise from early to late
```

```
#interprate up- and down-regulated probes together (could seperate up- and down- by changing sub-setting)
earlyDE<-
data.frame(ann[c(up.early,down.early),early+2],early.ratio[c(up.early,down.early),],mean.early.ratio[c(up.early,down.early)])
colnames(earlyDE)[4]<-paste("log ratio",names(data)[early[1]])
colnames(earlyDE)[5]<-paste("log ratio",names(data)[early[2]])
colnames(earlyDE)[6]<-paste("log ratio",names(data)[early[3]])
colnames(earlyDE)[7]<-"Mean Log Ratio"
midDE<-data.frame(ann[c(up.mid,down.mid),mid+2],mid.ratio[c(up.mid,down.mid),],mean.mid.ratio[c(up.mid,down.mid)])
colnames(midDE)[6]<-paste("log ratio",names(data)[mid[1]])
colnames(midDE)[7]<-paste("log ratio",names(data)[mid[2]])
colnames(midDE)[8]<-paste("log ratio",names(data)[mid[3]])
colnames(midDE)[9]<-paste("log ratio",names(data)[mid[4]])
colnames(midDE)[10]<-paste("log ratio",names(data)[mid[5]])
colnames(midDE)[11]<-"Mean Log Ratio"
lateDE<-data.frame(ann[c(up.late,down.late),late+2],late.ratio[c(up.late,down.late),],mean.late.ratio[c(up.late,down.late)])
colnames(lateDE)[5]<-paste("log ratio",names(data)[late[1]])
colnames(lateDE)[6]<-paste("log ratio",names(data)[late[2]])
colnames(lateDE)[7]<-paste("log ratio",names(data)[late[3]])
colnames(lateDE)[8]<-paste("log ratio",names(data)[late[4]])
colnames(lateDE)[9]<-"Mean Log Ratio"

#write files
write.csv(earlyDE, file="EndometriumEarlyDE.csv")
write.csv(midDE, file="EndometriumMidDE.csv")
write.csv(lateDE, file="EndometriumLateDE.csv")
```

R' Code used to generate differentially regulated gene list for 'Endometriosis' Endometrium

```
#geodata<-read.csv(file="GSE6364 modified.csv")
#data<-log2(geodata[,c(3:18)])
#ann<-geodata[,c(1:26)]

geodata<-read.csv(file="GDS2737_full.csv", skip=114)
ann<-geodata[,c(2,40:59)]
data<-log2(geodata[,c(24:39)])
rownames(data)<-geodata[,1]
samples<-colnames(geodata[,c(24:39)])

cyclePhase<-
factor(c("Proliferative","Early","Early","Early","Early","Early","Early","Mid","Mid","Mid","Mid","Mid","Mid","Mid","Mid","Mid
"))

ref<-which(cyclePhase=="Proliferative")
early<-which(cyclePhase=="Early")
mid<-which(cyclePhase=="Mid")
ref.data<-data[,ref]#note no row names
```

```
early.data<-data[,early]
mid.data<-data[,mid]

compRef<-function(x)
early.ratio<-apply(early.data,2,compRef)
mean.early.ratio<-apply(early.ratio,1,mean)
mid.ratio<-apply(mid.data,2,compRef)
mean.mid.ratio<-apply(mid.ratio,1,mean)

#selected up- and down-regulated probes from each patient group at log fold change +/-2 as defined by fc
fc<-(2)
up.early<-unique(which(mean.early.ratio>fc))
down.early<-unique(which(mean.early.ratio<(-fc)))
up.mid<-unique(which(mean.mid.ratio>fc))
down.mid<-unique(which(mean.mid.ratio<(-fc)))

#interprate up- and down-regulated probes together (could seperate up- and down- by changing sub-setting)
earlyDE<-
data.frame(ann[c(up.early,down.early),c(2,3,10)],early.ratio[c(up.early,down.early),],mean.early.ratio[c(up.early,down.early)])
colnames(earlyDE)[4]<-paste("log ratio",names(data)[early[1]])
colnames(earlyDE)[5]<-paste("log ratio",names(data)[early[2]])
colnames(earlyDE)[6]<-paste("log ratio",names(data)[early[3]])
colnames(earlyDE)[7]<-paste("log ratio",names(data)[early[4]])
colnames(earlyDE)[8]<-paste("log ratio",names(data)[early[5]])
colnames(earlyDE)[9]<-paste("log ratio",names(data)[early[6]])
colnames(earlyDE)[10]<-"Mean Log Ratio"
rownames(earlyDE)<-geodata[c(up.early,down.early),1]
midDE<-data.frame(ann[c(up.mid,down.mid),c(2,3,10)],mid.ratio[c(up.mid,down.mid),],mean.mid.ratio[c(up.mid,down.mid)])
colnames(midDE)[4]<-paste("log ratio",names(data)[mid[1]])
colnames(midDE)[5]<-paste("log ratio",names(data)[mid[2]])
colnames(midDE)[6]<-paste("log ratio",names(data)[mid[3]])
colnames(midDE)[7]<-paste("log ratio",names(data)[mid[4]])
colnames(midDE)[8]<-paste("log ratio",names(data)[mid[5]])
colnames(midDE)[9]<-paste("log ratio",names(data)[mid[6]])
colnames(midDE)[10]<-paste("log ratio",names(data)[mid[7]])
colnames(midDE)[11]<-paste("log ratio",names(data)[mid[8]])
colnames(midDE)[12]<-paste("log ratio",names(data)[mid[9]])
colnames(midDE)[13]<-"Mean Log Ratio"
rownames(midDE)<-geodata[c(up.mid,down.mid),1]

write.csv(earlyDE, file="Endometriosis2EarlyDE.csv")
write.csv(midDE, file="Endometriosis2MidDE.csv")

earlyGenes<-rownames(earlyDE)
midGenes<-rownames(midDE)
all<-union(earlyGenes,midGenes)
overlap<-intersect(earlyGenes,midGenes)
```